Paneth Cell Cryptdins Act in Vitro as Apical Paracrine Regulators of the Innate Inflammatory Response*

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Patricia W. Lin‡§§, Peter O. Simon, Jr.‡**, Andrew T. Gewirtz**, Andrew S. Neish**, Andre J. Ouellette‡‡, James L. Madara**§§, and Wayne I. Lencer‡

From the ‡Gastrointestinal Cell Biology Department, Combined Program in Pediatric Gastroenterology and Nutrition, §Division of Newborn Medicine, and the **Departments of Pediatrics and ¶¶Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia 30322, ††Department of Pathology, College of Medicine, University of California, Irvine, California 92697-4800, §§Department of Pathology, University of Chicago, Chicago, Illinois 60637, and ¶¶Harvard Digestive Diseases Center, Boston, Massachusetts 02115

Intestinal-specific antimicrobial α-defensins, termed cryptdins, are secreted into the intestinal lumen by mouse Paneth cells in response to microbial pathogens. Cryptdins kill microbes by forming pores in their limiting membranes. The cryptdin isoforms 2 and 3 also can form anion-conducting pores in eukaryotic cell membranes, thus affecting cell physiology. Here, we find that when applied to apical membranes of the human intestinal cell line T84, cryptdin 3 (Cr3) induces secretion of the proinflammatory cytokine interleukin 8 (IL-8) in a dose-dependent manner. The induction of IL-8 secretion is specific to the cryptdins that form channels in mammalian cell membranes because cryptdin 4, which does not form pores in T84 cells, does not induce IL-8 secretion. Cr3 induces inflammatory cytokite secretion by activating NF-κB and p38 mitogen-activated protein kinase in a Ca2+-dependent manner, but influx by extracellular Ca2+ is not involved. Unlike other known inflammatory agonists, signal transduction by Cr3 occurs slowly, suggesting a novel mechanism of action. These results show that selective cryptdins may amplify their roles in innate immunity by acting as novel paracrine agonists to coordinate an inflammatory response with the antimicrobial secretions of Paneth cells.

Paneth cells, located at the base of small intestinal crypts, participate in innate immunity against invading pathogens by secretion of lysozyme and the microbial α-defensins termed cryptdins (1). At least 20 cryptdin isoforms have been described in the mouse (cryptdins 1–20) and two, HD-5 and HD-6, in humans (2–5). Cryptdins act by forming anion-conductive channels in microbial cell membranes that depolarize and kill the microbe (6–9). We also find that certain cryptdins, cryptdins 2 and 3, can form apical anion-conductive channels in eukaryotic cell membranes. The formation of such channels in the apical membrane of crypt epithelial cells causes a salt and water secretory response that flushes the intestinal crypt of noxious agents (10). Crypt epithelial cells may further contribute to innate intestinal host defense by orchestrating the recruitment of immune cells via the secretion of chemokines into the lamina propria, such as the neutrophil chemokine interleukin 8 (IL-8). IL-8 secretion initiates the first step in neutrophil recruitment into the mucosa and ultimately in the formation of the crypt abscess, which represents the hallmark of acute and chronic intestinal inflammation. Although Paneth cells respond to microbial pathogens by discharging their granule contents, it is not known whether the Paneth cell or the secreted cryptdins function as intrinsic components of the intestinal inflammatory response.

Pore formation in eukaryotic cells by a variety of agents causes many biological effects including cytokine release (11, 12). The complement membrane attack complex (C5–9), for example, forms pores on endothelial cells that induce IL-8 secretion via NF-κB activation (13). The pore-forming bacterial toxins such as staphylococcal α-toxin, streptolysin O, and Escherichia coli α-hemolysin activate NF-κB and cause IL-8 secretion from mammalian cells when applied at non-cytotoxic doses (12, 14–16). In most cell types, E. coli α-hemolysin and staphylococcal α-toxin act by conducting extracellular Ca2+ influx into the cell (11, 14). In some cell types, however, staphylococcal α-toxin forms a pore that conducts only monovalent ions, but it still can induce release of the cytokine IL-1β or the induction of apoptosis (11, 17).

Here, we report that the Paneth cell α-defensin cryptdin 3 (Cr3) induces IL-8 secretion from intestinal T84 cells via the Ca2+-dependent activation of the p38 mitogen-activated protein kinase (MAPK) and NF-κB signaling cascades. The activity is specific to the pore-forming activity of Cr3 in mammalian cell membranes because cryptdin 4 (Cr4) that does not form ion-conducting pores in T84 cells fails to induce an inflammatory response. Unlike most other pore-forming agonists, however, Cr3 does not act by inducing an influx of extracellular Ca2+. Signal transduction occurs through activation of NF-κB and MAPK, but the time course of activation and transcription is very slow. These results suggest that certain cryptdins may form anion-conductive channels in the apical membrane of crypt epithelial cells.

* The abbreviations used are: IL, interleukin; Cr3, cryptdin 3; Cr4, cryptdin 4; MAPK, mitogen-activated protein kinase; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N”-tetraacetic acid acetoxymethyl ester; HBSS, Hank’s buffered saline solution; TNF, tumor necrosis factor; MIP, macrophage inhibitory protein.

†† To whom correspondence should be addressed; GI Cell Biology, 300 Longwood Ave., Enders 1220, Boston, MA 02115. Tel.: 617-355-8599; Fax: 617-730-0498; E-mail: wayne.lencer@ch.harvard.edu.
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FIG. 1. Pore-forming Cr3 induces IL-8 secretion from T84 cells in a dose-dependent manner. a, IL-8 secretion induced in T84 cells treated with apical Cr3 at the indicated doses or apical S. typhimurium (mean ± S.D., n = 3). b, IL-8 secretion induced in T84 cells stimulated apically with pore-forming Cr3 versus non-pore-forming Cr4 (mean ± S.D., n = 3). Basolateral TNF-α (10 μg/ml) served as a positive control.

act in vivo via a novel mechanism of paracrine signal transduction to amplify their role in innate immunity by coordinating the antimicrobial activities of Paneth cell secretions with the induction of an intestinal inflammatory response.

EXPERIMENTAL PROCEDURES

Cell and Bacterial Culture—T84 cells, an intestinal cell line originally obtained from metastatic human colorectal carcinoma, were used to model intestinal epithelia. Confluent monolayers of T84 cells were grown on collagen-coated inserts (Costar Corning, Cambridge, MA) as described previously (18). Wild-type Salmonella typhimurium strain x306 was maintained and prepared for use via non-agonized microaerophilic conditions as described previously (19).

Cryptdin Purification and Synthesis—Synthetic Cr3 and recombinant Cr4 were purified to homogeneity as described previously (10, 20, 21).

IL-8 Secretion—Agonist-induced IL-8 secretion from T84 cells was measured by enzyme-linked immunosorbent assay as described previously (22, 23). Briefly, T84 cells on 0.33-cm² inserts were washed and incubated in 50 μl of apical and 300 μl of basolateral HBSS (unless otherwise indicated) for at least 10 min. Appropriate agonists were added for 5 h and then basolateral solutions were collected for IL-8 measurement.

Drug Treatments—To chelate intracellular Ca²⁺, T84 cells were treated with 30 μM BAPTA-AM (Molecular Probes Inc., Eugene, OR) for 45 min before agonist addition in HBSS with reduced Ca²⁺ as described previously (24). To chelate extracellular Ca²⁺, T84 cells were treated with 10 mM EGTA (Sigma) 1 h prior to agonist addition. Recombinant TNF-α (R&D Systems Inc., Minneapolis, MN), carbachol, and ionomycin (Sigma) were added as described in the figure legends.

Western Blot Analysis—After experimental treatment, T84 cells (0.33 cm²) were washed in ice-cold HBSS and RNA was extracted using TRIzol reagent (Invitrogen). Total RNA was reverse transcribed from random hexamer primers using MultiScribe reverse transcriptase (Applied Biosystems) as described previously (25).

Real-time Quantitative Reverse Transcriptase-PCR—After experimental treatment, T84 cells were fixed and immunostained for the p65 subunit of NF-κB as described previously (26).

IL-8 expression level was normalized to the 18 S RNA level of the same sample. Fold difference was the ratio of the normalized value of each sample to that of untreated control cells. (IL-8 primer sequences are: forward, 5'-aaaccaccggaaggaaccat; reverse, 5'-aaaccaccggaaggaaccat.)

RayBio™ Inflammation Antibody Array Analysis—After 5 h agonist treatment, the basolateral-conditioned media from stimulated T84 cells (0.33 cm², 4 inserts per condition) was isolated and assessed for cytokine secretion. Antibody array membranes were incubated in the conditioned media, and cytokines were detected per the manufacturer’s guidelines (inflammation antibody array III, Ray Biotech, Inc., Norcross, GA).

Statistical Methods—Data were analyzed for significance by Student’s t test.

RESULTS

Cr3 Induces IL-8 Secretion from T84 Cells—To test whether certain cryptdins may participate differentially in an in vitro intestinal inflammatory response, we utilized the human in-
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Fig. 3. Cr3 induces activation of p38 MAPK. Western blot analysis of phospho-p38 levels in T84 cells stimulated with apical Cr3 (100 µg/ml), TNF-α (10 ng/ml), or Cr4 (100 µg/ml) at the indicated time points. Equal loading was confirmed by assessment of total p38 on each Western blot.

Fig. 4. Cr3 induces transcription of IL-8 mRNA. IL-8 mRNA levels in T84 cells stimulated with apical Cr3 (100 µg/ml, light bars) or basolateral TNF-α (10 ng/ml, dark bars) as assessed by quantitative reverse transcription-PCR at the indicated time points.

Cr3 Induces IL-8 Secretion via Activation of the Transcription Factor NF-κB and MAPK—To examine the mechanism of signal transduction by Cr3, we tested for the induction of phospho-IκB-α by immunoblot (Fig. 2a). Phospho-IκB-α was first detected after 2 h of incubation with Cr3 (Fig. 2a, lane 3) and increased with time (compare lanes 1–4). In contrast, the inflammatory agonist TNF-α applied to basolateral membranes of T84 cells induced phosphorylation of IκB-α within 5 min (Fig. 2a, lane 5). This study shows that Cr3 acts via the apical membrane of intestinal cells to induce phosphorylation of IκB-α, but the time course of activation was slow compared with the classic inflammatory cytokine TNF-α.

Next we tested whether Cr3 induced the translocation of NF-κB from the cytoplasm to the nucleus as would be predicted for an inflammatory agonist acting via the phosphorylation of IκB-α. Translocation of NF-κB from the cytoplasm to the nucleus in T84 cells was assessed by immunofluorescence microscopy for the p65 subunit of NF-κB (Fig. 2, b–g). Unstimulated cells had cytoplasmic staining but no nuclear staining for the p65 subunit (Fig. 2b). In cells treated basolaterally with TNF-α, p65 was readily visualized inside the nucleus 1 h after application of the agonist (Fig. 2c). In cells treated apically with Cr3, the p65 subunit also was visualized inside the nucleus, but translocation to the nucleus was first visualized only 3–4 h after addition of Cr3 (Fig. 2, d–g). These data are consistent with the slower time course of IκB-α phosphorylation induced by Cr3 and confirm that Cr3 acts as an inflammatory agonist via the transcription factor NF-κB.

To determine whether Cr3 may also act via activation of p38 MAPK, we tested for agonist-induced phosphorylation of p38 MAPK by immunoblot (Fig. 3). Cr3 causes phosphorylation of p38 that was first observed 30 min after application to T84 cells and peaked at 2 h (Fig. 3, lanes 1–6). In contrast, Cr4, which does not form pores in T84 cells, had no effect (lanes 13–18). Like Cr3, TNF-α also induced an increase in phospho-p38, but the phosphorylation occurred more rapidly with peak levels observed at 30 min (lanes 7–12).

To confirm the slower time course of signal transduction and also the transcriptional regulation of IL-8 secretion by Cr3, we assessed agonist-induced IL-8 mRNA levels by quantitative

Fig. 5. Cr3-induced IL-8 secretion depends upon intracellular Ca2+. a, IL-8 secretion induced in T84 cells stimulated with 100 µg/ml apical Cr3, 100 µg/ml basolateral carbachol (CCh), or Cr3 with carbachol. Cr3 exhibited no synergy with carbachol (mean ± S.D., representative of three independent experiments performed in duplicate). b, IL-8 secretion induced in T84 cells stimulated with 100 µg/ml apical Cr3, 10 ng/ml basolateral TNF-α, or Cr3 with TNF-α. Cr3 and TNF-α acted synergistically to induce more IL-8 secretion than either agonist alone (mean ± S.D., representative of three independent experiments performed in duplicate). c, IL-8 secretion induced in T84 cells stimulated with 10 ng/ml basolateral TNF-α, 100 µg/ml basolateral carbachol, or 100 µg/ml apical Cr3 in the presence (gray bars) or absence (white bars) of the intracellular Ca2+ chelator BAPTA-AM (30 µM) (mean ± S.E., representative of three independent experiments performed in triplicate; *, p < 0.05).
real-time PCR (Fig. 4). T84 cells stimulated with TNF-α expressed increasing IL-8 mRNA levels over time with strong expression at 2 h and peak expression 3 h after the application of the agonist (Fig. 4, dark bars). IL-8 mRNA levels also increased over time after stimulation by Cr3 but with almost no expression until 3 h and peak expression at 4 h (Fig. 4, light bars), consistent with the slower time course of Cr3-induced activation of NF-κB and p38 MAPK. These results show that Cr3, like TNF-α, induces IL-8 secretion in intestinal T84 cells via signaling cascades that involve both NF-κB and p38 MAPK but with slower kinetics.

Proinflammatory Signal Transduction by the Pore-forming Cr3 Does Not Require Influx of Extracellular Ca2+—Because other pore-forming proteins may induce proinflammatory signaling cascades by permeabilizing the plasma membrane to extracellular Ca2+, we tested whether Cr3 acts on intestinal cells by this mechanism. First we asked whether Cr3 acts by increasing intracellular Ca2+. Cr3 was applied to T84 intestinal cells alone or together with the muscarinic agonist carbachol, which induced IL-8 secretion by elevating [Ca2+]i, or with TNF-α, which acted independently of [Ca2+]i (Fig. 5, a and b). Here, we found that Cr3 acts synergistically with TNF-α to induce a proinflammatory response (Fig. 5b, compare the fourth column with the second and third). In contrast, Cr3 did not act synergistically with the Ca2+-dependent agonist carbachol (Fig. 5a, compare the fourth column with the second and third). These results suggest that the signaling cascade induced by Cr3 overlaps with that induced by carbachol and may indeed involve the induction of intracellular Ca2+ transients.

To test this idea, we used the membrane-permeant Ca2+ chelator BAPTA-AM. BAPTA-AM had no effect on IL-8 secretion from T84 cells induced by the Ca2+-independent agonist TNF-α (Fig. 5c, compare the third and fourth columns) but inhibited the IL-8 secretory response induced by the Ca2+-dependent agonist carbachol (Fig. 5c, compare the fifth and sixth columns). BAPTA-AM also inhibited IL-8 secretion induced by Cr3 (Fig. 5c, the seventh and eighth columns). These results suggest that Cr3 causes an increase in intracellular Ca2+ in intestinal cells that activates the proinflammatory signaling cascade involving NF-κB and p38 MAPK.

To test whether Cr3 acts by permeabilizing the apical plasma membrane to Ca2+, we examined the IL-8 secretory response to inflammatory agonists in the absence of extracellular Ca2+. Ca2+ was removed from the apical buffer by using the non-membrane-permeant Cr2+-chelator EGTA. As predicted, apical EGTA strongly inhibited IL-8 secretion induced by application of the Ca2+-ionophore ionomycin to apical membranes of T84 cells (0.7 versus 0.1 ng/ml) (Fig. 6a). Unexpectedly, however, EGTA also caused a 50% reduction in the IL-8 secretory response to the basolateral application of the Ca2+-dependent agonist TNF-α. The reason for this effect on TNF-α signal transduction was not explained. Nonetheless, apical EGTA had no detectable effect on the IL-8 secretory response induced by apical Cr3. Thus, Cr3 cannot act by permeabilizing the apical membrane to Ca2+ and inducing extracellular Ca2+ influx.

To confirm these results, we measured IL-8 secretion induced by Cr3 in T84 cells bathed apically and basolaterally in nominally free Ca2+ buffer (Fig. 6b). Consistent with our results using EGTA, IL-8 secretion induced by the Ca2+-ionophore ionomycin (0.8 versus 0.2 ng/ml) was strongly inhibited by the removal of extracellular Ca2+. The removal of extracellular Ca2+, however, had no effect on IL-8 secretion induced by the Ca2+-dependent agonist TNF-α (1.9 versus 1.6 ng/ml) or by Cr3 (1.1 versus 0.8 ng/ml) (Fig. 6b). Thus, signal transduction by Cr3 does not require influx of extracellular Ca2+ into the cell.

Cr3 Induces Secretion of Multiple Proinflammatory Cytokines from T84 Cells—To determine whether the Paneth cell cryptdins act in a general pathway to initiate an inflammatory response, using a commercially available immunoblot array to 40 known proinflammatory proteins, we tested for the production and release of other cytokines. Apically applied Cr3 induced the basolateral secretion of 6 proinflammatory cytokines: macrophage inhibitory protein (MIP)-1α (Fig. 7, compare control and Cr3-treated monolayers, lane 1), MIP-1β (lane 2), MIP-1α (lane 3), IL-17 (lane 4), IL-12 p70 (lane 5), and IL-8 (lane 6). Basolateral TNF-α induced the same inflammatory cytokines and served as our positive control. These results show that Cr3 induces a general inflammatory response.

**DISCUSSION**

The results of this study show that Paneth cells may play a critical role in coordinating the innate immune response to invading pathogens in the intestine. Somewhere, Paneth cells respond to the presence of microbial pathogens by discharging their granule contents (1). The exocytosis of Paneth cell granules delivers high concentrations of antimicrobial α-defensins, termed cryptdins, into the intestinal crypt lumen. At these concentrations, the cryptdins disrupt microbial cell membranes, thus exerting their anti-microbial activities (1). Cryptdins 2 and 3 also can form pores in eukaryotic cell membranes in vitro (10). Such activity may be cytolytic in vivo. In our first studies on these peptides, however, we discovered that the cryptdins have their own cytoprotective mechanism that could prevent such potential damage to the intestine precisely be-
cause they can insert anion-conducting pores into the apical membrane of intestinal epithelial cells in vitro and presumably in epithelial cells lining the intestinal crypt in vivo (10). Such pore formation that causes a Cl\(^{-}\) secretory response would flush the crypt lumen and reduce the concentration of cryptdins below cytotoxic levels. We now find that this same activity allows the cryptdins to act as novel apical paracrine factors that induce an inflammatory response.

The proinflammatory activity of the cryptdins depends on their pore-forming activities. The cryptdins that cannot form pores in eukaryotic cell membranes do not induce an inflammatory response. Here, it is interesting to point out that Cr4, which cannot form pores in T84 cells and does not induce an inflammatory response, is nonetheless the most bactericidal of the cryptdin peptides and is highly membrane disruptive in bacteria (20). Although the structural differences between Cr3 and Cr4 are known, still we do not understand why these potent pore-forming peptides in bacteria display such strict specificity in their action on eukaryotic cell membranes.

We also find that signal transduction by Cr3 occurs through Ca\(^{2+}\)-dependent activation of NF-\(\kappa\)B and p38 MAPK, but formation of the cryptdin pore does not induce the influx of extracellular Ca\(^{2+}\), which is not required for the inflammatory response. These results are consistent with our previous studies on the ion-conducting properties of the Cr3 channel in intestinal T84 cells and by patch clamp technique in 293 HEK cells (10, 27).

There are other pore-forming proteins such as complement and certain bacterial toxins that induce IL-8 secretion from mammalian cells. Most of these act by permeabilizing the target cell membrane to Ca\(^{2+}\) (11, 14), but the cryptdins do not act in this way. Presumably, the cryptdins induce an increase in Ca\(^{2+}\) by release of intracellular stores. We also find that the cryptdins induce a proinflammatory response very slowly even when compared with other Ca\(^{2+}\)-dependent agonists. S. typhimurium, for example, induces a Ca\(^{2+}\)-dependent inflammatory response from T84 cells by ligand binding to TL5 on the basolateral membrane. Like Cr3, Salmonella also acts by activation of NF-\(\kappa\)B and p38 MAPK, but signal transduction by Salmonella is faster, causing phosphorylation of I\(\beta\)B-\(\alpha\) within 30 min and phosphorylation of p38 within 1 h (24, 28). Salmonella, also unlike Cr3, causes an influx of extracellular Ca\(^{2+}\) as well as the release of Ca\(^{2+}\) from intracellular stores (24). Thus, the mechanism of signal transduction by Cr3, although similar to other proinflammatory agonists in activating NF-\(\kappa\)B and p38 MAPK, is in other ways unique.

In certain cell types, some bacterial toxins, when applied at low doses, form pores in target cell membranes that do not conduct Ca\(^{2+}\) and yet, like Cr3, induce an inflammatory response. The induction of an inflammatory response correlates with efflux of K\(^{+}\) through the toxin pore and presumably on depolarization of the target cell membrane (11, 17). Perhaps Cr3 acts by a similar mechanism but via the induction of an anion rather than cation conductance.

Finally, the ability of Cr3 to act as a paracrine regulator of the intestinal inflammatory response raises the possibility that dysregulation of Paneth cell function may contribute to the development of disease. Although normal Paneth cell function may act in host defense, the secreted Paneth cell products are intrinsically cytotoxic and, as we show in this study, potentially proinflammatory. Thus, the regulation of Paneth cell secretions may run a fine line between health and disease. In patients with inflammatory bowel disease, for example, high colonic levels of the proinflammatory cytokines IL-8, IL-6, IL-1\(\beta\), and TNF-\(\alpha\) (29) induced increased neutrophil activity that correlates with disease severity (30). It also is possible that the developmentally normal deficiency of Paneth cells and their secretory products in the neonatal period of the human predisposes some premature infants to necrotizing enterocolitis, an inflammatory disease of the intestine related to deficiency in innate immunity (31–33). Here again, the complex interplay among cell types of the intestinal mucosa in the inflammatory response may critically involve the antimicrobial and proinflammatory activities of the pore-forming Paneth cell α-defensins.

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