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Salmonella Effector AvrA Regulation of Colonic Epithelial Cell Inflammation by Deubiquitination

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AvrA is a newly described bacterial effector existing in *Salmonella*. Here, we test the hypothesis that AvrA is a deubiquitinase that removes ubiquitin from two inhibitors of the nuclear factor- κ B (NF- κ B) pathway, I κ B α and β -catenin, thereby inhibiting the inflammatory responses of the host. The role of AvrA was assessed in intestinal epithelial cell models and in mouse models infected with AvrA-deficient and -sufficient *Salmonella* strains. We also purified AvrA and AvrA mutant proteins and characterized their deubiquitinase activity in a cell-free system. We investigated target gene and inflammatory cytokine expression, as well as effects on epithelial cell proliferation and apoptosis induced by AvrA-deficient and -sufficient bacterial strains *in vivo*. Our results show that AvrA blocks degradation of I κ B α and β -catenin in epithelial cells. AvrA deubiquitinates I κ B α , which blocks its degradation and leads to the inhibition of NF- κ B activation. Target genes of the NF- κ B pathway, such as interleukin-6, were correspondingly down-regulated during bacterial infection with *Salmonella* expressing AvrA. AvrA also deubiquitinates and thus blocks degradation of β -catenin. Target genes of the β -catenin pathway, such as *c-myc* and *cyclinD1*, were correspondingly up-regulated with AvrA expression. Increased β -catenin further negatively regulates the NF- κ B pathway. Our findings suggest an important role for AvrA in regulating host inflammatory responses through NF- κ B and β -catenin pathways. (*Am J Pathol* 2007, 171:882–892; DOI: 10.2353/ajpath.2007.070220)

Every year, approximately 40,000 cases of salmonellosis are reported in the United States. Most persons infected with *Salmonella* develop diarrhea, fever, and abdominal cramps 12 to 72 hours after infection. AvrA is a *Salmonella* effector translocated into host cells by a type 3 secretion system. Recently studies showed that the AvrA gene is present in 80% of *Salmonella enterica* serovar.¹ Although its exact function is not entirely clear, AvrA belongs to the family of cysteine proteases regulating diverse bacterial-host interactions.² Other family members related to AvrA include the adenovirus protease AVP, *Yersinia* virulence factor YopJ (*Yersinia* outer protein J), and the *Xanthomonas campestris* pv. *vesicatoria* protein AvrBsT.

Wild-type *Salmonella* activates the nuclear factor- κ B (NF- κ B) pathway, whereas nonvirulent *Salmonella* strains, such as PhoP^c, attenuate the host innate immune response by preventing the activation of the NF- κ B pathway.³ The AvrA protein from nonpathogenic *Salmonella typhimurium* has been shown to inhibit activation of NF- κ B in cultured human epithelial cells.⁴ Mutation of the conserved catalytic cysteine in AvrA abolishes its ability to inhibit the proinflammatory NF- κ B pathways that are activated within infected cells.⁴ However, the mechanism of AvrA regulation of NF- κ B pathways is not completely known.

NF- κ B controls the expression of many cytokines and chemokines involved in inflammation and immune responses. NF- κ B activity is inhibited by the inhibitor of κ B (I κ B α). I κ B α binds to NF- κ B to mask the nuclear localization signal so that the NF- κ B dimer (p50 and p65) is retained in the cytoplasm. Phosphorylation of I κ B α by I κ B kinase (IKK) leads to the ubiquitination and degradation of I κ B α , resulting in nuclear translocation and activation of NF- κ B.⁵ β -Catenin is another protein that has been

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shown to be a negative regulator of the proinflammatory NF- κ B pathway in epithelial cells.^{6–9} This function is in addition to its roles in embryonic development and neoplasia such as colon cancer¹⁰ via enhancement of epithelial cell proliferation.

AvrA expression in *Salmonella* is able to stabilize β -catenin by inhibition of ubiquitination.¹¹ Polyubiquitination targets proteins for recognition and processing by the 26S proteasome, which degrades the ubiquitinated proteins and recycles ubiquitin. The attachment of ubiquitin to the target proteins requires a series of ATP-dependent enzymatic steps by E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligating) enzymes. Interestingly, both I κ B α and β -catenin are targeted for ubiquitination by a similar E3 ligase complex.¹²

The role of AvrA in an *in vivo* system and the mechanism by which AvrA inhibits NF- κ B or activates β -catenin signaling remain unknown. It has been suggested that injection of *Salmonella* AvrA stabilizes both I κ B α and β -catenin.^{4,11} In this study, we test our hypothesis that AvrA is a deubiquitinase, which removes the ubiquitin from both I κ B α and β -catenin. We have tested the role of AvrA in nonvirulent PhoP^c with sufficient or deficient AvrA expression.^{4,11} PhoP^c is a PhoP-PhoQ constitutive mutation of a wild-type *S. typhimurium* strain that increases the expression of PhoP-activated genes, represses the synthesis of approximately 20 proteins encoded by the PhoP-repressed genes, and attenuates virulence.¹³ We demonstrate that *Salmonella* with AvrA overexpression stabilizes both I κ B α and β -catenin in mouse models. In addition, we purified AvrA and AvrA mutant proteins and characterized their deubiquitinase activity in a cell-free system. While examining changes in target gene expression, we also investigated inflammatory cytokine expression, as well as effects on epithelial cell proliferation and apoptosis induced by AvrA-deficient and -sufficient bacterial strains. Our findings strongly suggest that AvrA is a deubiquitinase that regulates both the NF- κ B and β -catenin signaling pathways in intestinal inflammation.

Materials and Methods

Bacterial Strains and Growth Conditions

Bacterial strains *S. typhimurium* mutant PhoP^c, PhoP^c AvrA[−], and PhoP^c AvrA[−]/AvrA⁺ were provided by Dr. Andrew Neish and Dr. Lauren Collier-Hyams from Emory University (Atlanta, GA). Bacterial growth conditions were as follows: nonagitated microaerophilic bacterial cultures were prepared by inoculation of 10 ml of Luria-Bertani broth with 0.01 ml of a stationary phase culture, followed by overnight incubation (~18 hours) at 37°C, as previously described.¹⁴ Bacterial overnight cultures were concentrated 33-fold in Hanks' balanced salt solution (HBSS) supplemented with 10 mmol/L HEPES, pH 7.4.

Streptomycin-Pretreated Mouse Model

Animal experiments were performed using specific-pathogen-free female C57BL/6 mice (Taconic Farms,

Germantown, NY) that were 6 to 7 weeks old. Water and food were withdrawn 4 hours before oral gavage with 7.5 mg/mouse of streptomycin (75 μ l of sterile solution or 75 μ l of sterile water as control). Afterward, animals were supplied with water and food. Twenty hours after streptomycin treatment, water and food were withdrawn again for 4 hours before the mice were infected with 1×10^7 colony-forming units of *S. typhimurium* (50- μ l suspension in HBSS) or treated with sterile HBSS (control) by oral gavage as previously described.^{9,14} At 6, 18, or 24 hours after infection, mice were sacrificed, and tissue samples from the intestinal tracts were removed for analysis. The histological studies, such as studies with hematoxylin and eosin staining, were performed on intestinal segments isolated from mice treated with AvrA-sufficient or -deficient bacterial strains.

Immunoprecipitation

Cells were rinsed twice in ice-cold HBSS and lysed in ice-cold immunoprecipitation buffer [1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris, pH 7.4, 1 mmol/L ethylenediamine tetraacetic acid (EDTA), 1 mmol/L ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, pH 8.0, 0.2 mmol/L sodium orthovanadate, and protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland)]. Samples were prepared as previously described.⁸ Blots were probed with anti- β -catenin antibody (BD Biosciences Transduction Laboratories, Lexington, KY).

Immunoblotting

Mouse epithelial cells were scraped and lysed in lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris, pH 7.4, 1 mmol/L EDTA, 1 mmol/L ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, pH 8.0, 0.2 mmol/L sodium orthovanadate, and protease inhibitor cocktail) and protein concentration measured. Cultured cells were rinsed twice in ice-cold HBSS, lysed in protein-loading buffer [50 mmol/L Tris, pH 6.8, 100 mmol/L dithiothreitol, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, and 10% glycerol], and sonicated. Equal amounts of proteins or equal volumes of total cultured cell lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti- β -catenin (BD Biosciences Transduction Laboratories), anti-I κ B α , anti-c-myc, anti-cyclinD1 (Santa Cruz Biotechnology, Santa Cruz, CA), or β -actin (Sigma, St. Louis, MO) primary antibodies (1:500 to 1:1000 dilution) and visualized by enhanced chemiluminescence. Chemiluminescent signals were collected and scanned from Hyperfilm ECL (GE Healthcare, Little Chalfont, Buckinghamshire, UK) with a Scanjet 7400c backlit flatbed scanner (Hewlett-Packard Co., Palo Alto, CA). For figures, the contrast of images was adjusted, arranged, and labeled in Adobe Photoshop and Adobe Illustrator (Adobe Systems Incor-

porated, San Jose, CA). Bands were quantified using NIH Image software (Bethesda, MD). The digital images are representative of the original data.

Real-Time Quantitative Polymerase Chain Reaction Analysis of Interleukin-6, c-Myc, and Cyclin D1

Total mRNA was extracted from scraping mouse colonic epithelial cells using TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and then subjected to real-time quantitative polymerase chain reaction (PCR) (SYBR Green PCR kit; Bio-Rad) with primers for mouse interleukin (IL)-6 (forward 5'-CAATTCCAGAAACCGCTATGA-3', reverse 5'-ACCACAGTGAGGAATGTCCA-3'), c-Myc (forward 5'-TGAGCCCCCTAGTGCTGCAT-3', reverse 5'-AGCCCGACTCCGACCTCTT-3'), and cyclin D1 (forward 5'-AGGTAATTTGCACACCTCTG-3', reverse 5'-ACAAAGCAATGAGAATCTGG-3'). All expression levels were normalized to the glyceraldehyde-3-phosphate dehydrogenase levels of the same sample, using forward (5'-CTTCACCACCATGGAGAAGGC-3') and reverse (5'-GGCATGGACTGTGGTCATGAG-3') primers. Percent expression was calculated as the ratio of the normalized value of each sample to that of the corresponding untreated control cells. All real-time PCRs were performed in triplicate. All PCR primers were designed using LaserGene software (DNASar, Inc., Madison, WI).

Salmonella-Induced Mouse IL-6 Secretion

Before terminating the animal experiment, mouse blood samples were collected by cardiac puncture and placed in tubes containing EDTA (10 mg/ml). Serum was obtained, and mouse IL-6 was measured using the Titer-Zyme Enzyme Immunometric Assay kit (Assay Designs, Inc., Ann Arbor, MI) according to the manufacturer's instructions.

AvrA Clone

The *avrA* gene is from wild-type *S. typhimurium* strain SL3201. Sequence analysis revealed that the *avrA* allele used in our study is identical to the allele from *S. typhimurium* LT2 (GenBank accession no. AE008830).

AvrA Expression and Purification

Salmonella full-length gene *AvrA* and one-point mutant C186A were cloned into N-terminal glutathione S-transferase (GST)-fused Vector pEGX-4T2 (Invitrogen) and transformed into *Escherichia coli* strain BL21(DE3). Bacteria were inoculated in 500 ml of Luria-Bertani broth media with 100 μ g/ml ampicillin; once the optical density reached 0.6, bacteria were induced with 0.1 to 1 mmol/L final concentration isopropyl β -D-thiogalactoside for another 3 hours. BL21 cells were spun down, washed with phosphate-buffered saline (PBS) twice, and lysed in 5 ml

of lysis buffer (50 mmol/L Tris, pH 7.7, 100 mmol/L NaCl, and 0.2 mmol/L EDTA) with a protease inhibitor tablet plus 200 μ l of lysozyme stock (20 μ g/ml). Samples were mixed well and followed by sonication for 5 seconds three times. Samples were spun down at high speed for 30 minutes at 4°C. Affinity purification was performed by using a glutathione-Sepharose resin (Amersham Bioscience, Piscataway, NJ) and further purified by ion exchange.

Cell Culture

Human embryonic kidney 293 cells and epithelial HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin-streptomycin, and L-glutamine. Human colonic epithelial HCT116 cells were cultured in McCoy's 5A medium supplemented with 10% (v/v) fetal bovine serum as previously described.⁸ The rat small intestinal IEC-18 cell line was grown in Dulbecco's modified Eagle's medium (high-glucose, 4.5 g/L) containing 5% (v/v) fetal bovine serum, 0.1 U/ml insulin, 50 μ g/ml streptomycin, and 50 U/ml penicillin.

T-Cell Factor Transcriptional Activity Assay

Cells were transiently cotransfected with 1 μ g of pGL3-OT [T-cell factor (TCF)-responsive reporter with wild-type TCF binding site] or pGL3-OF (mutant TCF binding site) using Lipofectin reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). pRL-TK vector (Promega, Madison, WI) was used as an internal control reporter. Cells were colonized with equal numbers of bacteria for 30 minutes, washed, and incubated in Dulbecco's modified Eagle's medium for 6 hours. Luciferase activity was monitored using the dual luciferase assay system (Promega).

Transfections and Immunoprecipitations

To generate the ubiquitinated I κ B α substrates for the reaction, we transfected T7-I κ B α into human embryonic kidney 293T cells with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Plasmids Flag-Fwd1, flag-IKK1, HA-Ub, and T7-I κ B α were gifts from Dr. B.E. Wadzinski (Vanderbilt University Medical Center, Nashville, TN¹⁵). All transfections were performed in 60-mm dishes with 8- μ g total DNA that included 1 μ g of HA-ubiquitin, 1 μ g of pcDNA3 Flag-FWD1, 1 μ g of flag-IKK1, and 5 μ g of pCMV4 T7-I κ B α . After 30 hours of transfection, cells were pretreated with 25 μ mol/L MG132 for 1 hour, followed by 20 ng/ml tumor necrosis factor- α for 30 minutes. Cells were washed with ice-cold PBS once, lysed in RIPA buffer [20 mmol/L Tris-HCl, pH 7.4, 137 mmol/L NaCl, 10% (v/v) glycerol, 0.1% (w/v) SDS, 0.5% (w/v) deoxycholate, 1% (v/v) Triton X-100, 2.0 mmol/L EDTA, 1.0 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L leupeptin, 10 mmol/L N-ethyl maleimide (NEM), and 1% protease inhibitors], sonicated, cleared by centrifugation (13,000 rpm per minute for 30 minutes

at 4°C), and immunoprecipitated with T7 tag antibody agarose (Novagen, Madison, WI). Immunoprecipitates were washed two times with RIPA buffer and two times with deubiquitination reaction buffers (50 mmol/L HEPES, pH 8.0, 0.1% Brij-35, and 3 mmol/L dithiothreitol). This yielded ubiquitinated I κ B α , used as a substrate for deubiquitinase. To generate the ubiquitinated β -catenin (ub- β -catenin) substrates for the reaction, we treated human embryonic kidney 293T cells with the 25 μ mol/L MG132 proteasome inhibitor for 2 hours, lysed cells with sonication in RIPA buffer, and immunoprecipitated β -catenin using anti- β -catenin antibodies.

In Vitro AvrA Deubiquitination Assays

For the cell-free AvrA deubiquitination assay, we used both ubiquitinated I κ B α (ub-I κ B α) and ub- β -catenin as the substrates. Immunoprecipitated ub-T7-I κ B α or ub- β -catenin was incubated with 1 μ g of recombinant bacterial AvrA protein or point mutant C186A protein at 37°C in 50 μ l of reaction buffer (50 mmol/L HEPES pH 8.0, 0.1% Brij-35, and 3 mmol/L dithiothreitol). Isopeptidase T (Boston Biochem, Cambridge, MA) served as positive control. Protease inhibitor *N*-ethyl maleimide (10 μ M) was added at the beginning of reaction. All reactions were terminated with 4 $\times$ SDS loading buffer at different time points and boiled for 5 minutes before immunoblot analysis. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Invitrogen) following standard procedures.¹⁶

We purified GST-AvrA and mutant AvrA C186A (mutated at the key cysteine required for its activity). We incubated ubiquitinated β -catenin with AvrA protein or AvrA C186A for 2 hours in reaction buffer at 37°C. Reactions were terminated by the addition of 6 $\times$ SDS loading buffer. We analyzed the samples by SDS-polyacrylamide gel electrophoresis and Western blotting using an anti-ubiquitin antibody (Affiniti, Exeter, UK) and reprobed with an anti- β -catenin antibody.

Immunohistochemistry

Tissues were fixed in 10% neutral buffered formaldehyde for 2 hours, transferred into 70% ethanol, and processed the next day by standard techniques.⁴ Immunohistochemistry for apoptotic nuclei [terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining] was performed on paraffin-embedded sections (1 μ m) of mouse colons. Paraffin sections were baked in an oven at 56°C for 20 minutes. The slides were deparaffinized and rehydrated in xylene, followed by graded ethanol washes at room temperature. Antigen retrieval was achieved by boiling the slides in a microwave oven in 0.01 mol/L sodium citrate buffer, pH 6.0. Slides were then incubated in hydrogen peroxide (3% H₂O₂ and 1% fetal bovine serum in PBS) for 20 minutes at room temperature to block endogenous peroxidase activity, followed by incubation for 20 minutes in 5% fetal bovine serum/PBS to reduce nonspecific background. TUNEL staining was

performed using the Apoptosis Detection Kit (ApopTag Plus Peroxidase *In Situ*; Chemicon International, Temecula, CA). A couple modifications were made to the kit's procedures: paraffin sections were baked (56°C for 20 minutes), and nuclei were counterstained with hematoxylin. The Automated Cellular Imaging System (ACIS; ChromaVision Medical Systems, San Juan Capistrano, CA) was used to quantitate TUNEL staining section according to the manufacturer's instructions.

Proliferation Determination: BrdU Staining

The number of proliferating cells was detected by immunoperoxidase staining for the thymidine analog bromodeoxyuridine (BrdU). 5-bromo-2'-deoxyuridine (100 mg/kg; Sigma) was injected i.p. 2 hours before sacrificing the mice. Specimens were fixed in 10% buffered formalin and handled as previously described in Materials and Methods. Slides were then incubated in 3% hydrogen peroxide for 20 minutes at room temperature to block endogenous peroxidase activity, followed by incubation for 20 minutes in a milk-peroxide solution (90 parts dH₂O, five parts skim milk, and five parts 3% hydrogen peroxide) to reduce nonspecific background. The slides were incubated with polyclonal BrdU antibody (1:2000; RDI Division of Fitzgerald Industries International, Concord, MA) for 1 hour at room temperature followed by biotinylated anti-sheep IgG (H+L) (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Antibody staining was visualized with diaminobenzidine (Envision⁺ System/HRP Kit; DakoCytomation California Inc., Carpinteria, CA) and counterstaining with hematoxylin.

Statistical Analysis

Data are expressed as mean $\pm$ SD. Differences were analyzed by Student's *t*-test. *P* values <0.05 were considered significant.

Results

AvrA Protein Expression Stabilized the Expression of I κ B α and Attenuated NF- κ B Activation in Vitro

Previous work indicates that nonvirulent *Salmonella* PhoP^c is able to attenuate NF- κ B signaling by inhibiting I κ B α degradation *in vitro*.³ To determine whether AvrA was responsible for the attenuation of NF- κ B signaling by PhoP^c, we focused on bacterial strains sufficient or deficient in AvrA: parental PhoP^c, PhoP^c AvrA mutant (AvrA⁻), or the AvrA complementary strain (PhoP^c AvrA⁺/AvrA⁺). This system allowed us to concentrate on the cellular function of AvrA and exclude other bacterially induced effects on the host. In the human colonic epithelial cell line HCT116, parental PhoP^c strain colonization inhibited I κ B α degradation, whereas PhoP^c AvrA⁻ bacteria induced significant I κ B α degradation. In cells colonized with PhoP^c AvrA⁻/AvrA⁺, the complementary AvrA

Figure 1. AvrA inhibited the NF-κB pathway in cultured epithelial cell models. **A:** Western blot of IκBα in intestinal epithelia colonized by *Salmonella* nonvirulent PhoP^c, PhoP^cAvrA⁻ (AvrA⁻), or AvrA⁻ transcomplemented with wild-type AvrA (AvrA⁻/AvrA⁺) *in vivo*. Equal amounts of total cell lysate were sequentially immunoblotted with antibodies against IκBα and β-actin (loading control). **B:** Location of NF-κBp65 in HCT116 cells by immunofluorescence. Immunostaining of NF-κB in cells colonized with *Salmonella* PhoP^c or AvrA⁻. Cells were fixed, permeabilized, and stained with anti-p65 antibody, followed by A594 anti-goat secondary antibody and 4,6-diamidino-2-phenylindole (DAPI). **Arrows** indicate cell nuclei. Images shown are from a single experiment and are representative of three separate repeats.

expression stabilized IκBα (Figure 1A, HCT116). The same response was also found in the rat small epithelial cell line IEC-18. Parental PhoP^c strain colonization inhibited IκBα degradation, whereas PhoP^c AvrA⁻ bacteria induced more IκBα degradation. The complementary AvrA expression in PhoP^cAvrA⁻/AvrA⁺ again stabilized IκBα (Figure 1A, IEC-18).

With IκBα degradation, NF-κB is free to translocate to the nucleus. PhoP^c AvrA⁻ bacteria-infected cells had strong nuclear staining of NF-κB p65 (Figure 1B), whereas when AvrA was complemented back in PhoP^c AvrA⁻/AvrA⁺, there was no NF-κB p65 translocation; this was comparable with the parental strain PhoP^c, in which colonization did not induce translocation (Figure 1B), indicating AvrA expression regulated IκBα degradation and NF-κB distribution. These data are consistent with previous studies that AvrA expression attenuates NF-κB activity using the NF-κB transcriptional activity and IL-8 real-time PCR methods.⁴ Taken together, our data suggest that AvrA expression in *Salmonella* strains is able to inhibit NF-κB activity *in vitro*.

Figure 2. AvrA inhibited the NF-κB pathway in a mouse model. **A:** Western blot of IκBα in mouse colonic epithelial cells colonized by PhoP^c, PhoP^c/AvrA⁻ (AvrA⁻), or AvrA⁻ transcomplemented with wild-type AvrA (AvrA⁻/AvrA⁺) *in vivo*. **B:** IL-6 levels in mouse serum 6 hours after bacterial infection. **C:** Real-time PCR of IL-6 mRNA in mouse colons. Data shown in **B** and **C** are mean ± SD for n = 3. Significance was at P ≤ 0.05.

AvrA Protein Expression Stabilized the Expression of IκBα and Attenuated Proinflammatory Signaling *In Vivo*

Using a streptomycin-pretreated mouse model, we further assessed IκBα expression and the NF-κB-dependent inflammatory cytokine IL-6 *in vivo*. As expected, infection with the parental PhoP^c strain inhibited IκBα degradation, whereas PhoP^c AvrA⁻ bacteria induced more IκBα degradation in mouse colonic epithelial cells (Figure 2A). In mice infected with PhoP^c AvrA⁻/AvrA⁺, AvrA expression stabilized IκBα (Figure 2A). Nonvirulent *Salmonella* parental PhoP^c did not induce a large amount of IL-6 secretion, whereas the AvrA-deficient strain

(AvrA⁻) increased IL-6 to 450 pg/ml. In contrast, AvrA overexpression (PhoP^c AvrA⁻/AvrA⁺) was able to bring IL-6 to control levels (Figure 2B). Likewise, real-time PCR demonstrated that the absence of AvrA expression in PhoP^c AvrA⁻ increased IL-6 mRNA, whereas complementation with AvrA in PhoP^c AvrA⁻/AvrA⁺ decreased IL-6 mRNA expression to the control levels similar to parental PhoP^c (Figure 2C). Thus, the AvrA protein from *S. typhimurium* inhibits activation of the NF- κ B pathway.

AvrA Acts As a Deubiquitinase to Attenuate I κ B Ubiquitination

The inhibitor of NF- κ B, I κ B α , is ubiquitinated before proteasomal degradation. Our data (Figures 1 and 2) and previous *in vitro* studies demonstrate that AvrA inhibits NF- κ B activity by stabilizing I κ B α .⁴ One possibility is that AvrA might block the ubiquitinating E3 ligase such as β -TrCP.¹¹ However, we did not detect any protein level change of β -TrCP with AvrA expression *in vitro* (data not shown). Furthermore, we found that AvrA expression in the bacterial strain did not change the general ubiquitination of proteins detecting by Western blot (data not shown).

Another possible mechanism of stabilizing I κ B α would be removing ubiquitin from I κ B α . It has been demonstrated that YopJ, a family member related to AvrA, acts as a deubiquitinase to inhibit the NF- κ B activity.¹⁷ We next tested the deubiquitinase activity of AvrA on I κ B α using a cell-free system.

Proteins of AvrA and the mutant AvrA C186A (mutated at the key cysteine required for its activity) were purified. Using a cell-free system to exclude the possible effect of other deubiquitinases, GST-AvrA was mixed with ubiquitinated I κ B α (ub-I κ B α). The ubiquitinated I κ B α decreased over 30 to 90 minutes (Figure 3A). The positive control, ubiquitin isopeptidase T, also reduced the ub-I κ B α over 90 minutes (Figure 3A, isopeptidase T). The deubiquitinase inhibitor NEM completely inhibited the deubiquitinase activity of AvrA (Figure 3A, NEM⁺). In contrast, the mixture of C186A and ub-I κ B α only slightly changed the amount of ubiquitinated I κ B α . As a control, the mixture of ub-I κ B α in buffer without adding AvrA protein did not change levels of ub-I κ B α after a reaction for 90 minutes (data not shown). These data excluded the possible contamination of deubiquitinase in the cell-free system. Coomassie blue staining showed equivalent levels of purified AvrA or C186A proteins in each reaction (Figure 3B).

In addition, the levels of ubiquitinated I κ B in the cell-free system were further confirmed by reprobing with an anti-ubiquitin antibody (Figure 3C). The ubiquitin was decreased (shown with less smear) in the reaction with AvrA proteins over 30 to 90 minutes. Compared with AvrA, the mixture of C186A and ub-I κ B α at over 30 to 60 minutes had more ubiquitinated proteins. By 90 minutes, some decrease in the amount of ubiquitinated I κ B was evident (Figure 3C). The deubiquitinase inhibitor NEM was able to block the deubiquitinase activity of AvrA and maintain the ubiquitinated protein (Figure 3C, NEM⁺).

Figure 3. AvrA is a deubiquitinase. **A:** AvrA deubiquitinated ubiquitinated I κ B α . The purified GST-AvrA or GST-C186A (mutated at the key cysteine required for its activity) proteins were mixed with ubiquitinated I κ B. The reaction was terminated at 30, 60, or 90 minutes. The ubiquitinated forms of I κ B were detected by immunoblot using anti-I κ B and anti-ubiquitin antibodies. Isopeptidase T is a deubiquitinase used as a positive control. **B:** Coomassie blue staining showed the total amount of AvrA or C186A in each reaction. **C:** AvrA reduced the ubiquitinated I κ B protein. The purified GST-AvrA or GST-C186A (mutated at the key cysteine required for its activity) proteins were mixed with ubiquitinated I κ B. The reaction was terminated in 30, 60, and 90 minutes. The ubiquitinated proteins were detected by immunoblot using anti-ubiquitin antibodies.

AvrA Prevented β -Catenin Ubiquitination in Vitro

Recent studies have shown that β -catenin is a negative regulator of the proinflammatory NF- κ B pathway in epithelial cells.⁶⁻⁹ Interestingly, both I κ B α and β -catenin are targeted for ubiquitination by a similar pathway.¹² Thus, we next explored a possible mechanism of AvrA stabilization of β -catenin at the ubiquitination level. Cells were colonized with a parental PhoP^c or the PhoP^c mutant strain lacking AvrA (PhoP^c AvrA⁻), followed by the addition of the proteasome inhibitor MG262 to prevent proteasomal degradation of ubiquitinated proteins (Figure 4A). When model epithelial cells were colonized by PhoP^c AvrA⁻, increased levels of ubiquitinated β -catenin were observed, whereas the ubiquitination of β -catenin decreased in the presence of PhoP^c with intact AvrA expression. This suggests that the expression of the *Salmonella* AvrA effector may stabilize β -catenin by decreasing the ubiquitination of β -catenin.

Figure 4. The *Salmonella* AvrA protein modulated β -catenin ubiquitination and activity. HeLa cells were incubated for 1 hour with PhoP^c, AvrA^{-/-}, or PhoP^cAvrA^{-/-}/AvrA⁺, washed, and incubated for the times indicated with the proteasome inhibitor MG262. Total cell lysates were analyzed for ubiquitinated β -catenin and β -actin (loading control) by immunoblot. Higher-molecular weight ubiquitinated β -catenin is indicated by arrows. Data shown are from a single experiment and are representative of three separate experiments. **B:** AvrA deubiquitinated ubiquitinated β -catenin. In the cell-free system, purified GST-AvrA or GST-C186A proteins were mixed with ubiquitinated β -catenin. The reaction was terminated in 60 minutes. The ubiquitinated forms of β -catenin were detected by immunoblot using anti- β -catenin antibody. The total amount of AvrA or C186A in each reaction was shown by immunoblot using anti-GST antibody. **C:** Location of β -catenin in HCT116 cells by immunofluorescence. Immunostaining of β -catenin in cells colonized with *Salmonella* PhoP^c, AvrA^{-/-}, or AvrA^{-/-}/AvrA⁺. Cells were fixed, permeabilized, and stained with anti- β -catenin antibody, followed by A488 anti-mouse secondary antibody and 4,6-diamidino-2-phenylindole (DAPI). Arrows indicate cell nuclei. Images shown are from a single experiment and are representative of three separate repeats. **D:** AvrA regulates activation of the β -catenin-TCF signaling pathway. Cells were transiently transfected with a TCF-responsive luciferase reporter plasmid containing either a wild-type TCF-binding site (pGL3-OT) or a defective TCF binding site (pGL3-OF). Cells were then colonized with PhoP^c or AvrA^{-/-} for 30 minutes, washed, and incubated in medium for 6 hours. TCF-dependent transcription was measured by luciferase activity. Data are the means $\pm$ SD of a single experiment assayed in triplicate and are representative of three separate experiments.

One mechanism of stabilizing β -catenin would be blocking or removing ubiquitin from β -catenin. Therefore, we tested whether AvrA might inhibit ubiquitination of β -catenin by acting as a deubiquitinating protease. Again using the cell-free system, purified GST-AvrA was mixed with ubiquitinated β -catenin. The ubiquitinated forms of β -catenin disappeared after reaction for 60 minutes. In contrast, the mixture of the mutant AvrA C186A proteins and ub- β -catenin did not change the amount of ub- β -catenin (Figure 4B). GST Western blots showed an equal amount of AvrA or C186A in each reaction. These data indicate that AvrA is able to remove the ubiquitin moieties from ub- β -catenin.

Without degradation, β -catenin accumulates and translocates to the nucleus. Once in the nucleus, β -catenin can interact with the TCF and activate transcription.¹⁸ Our previous studies demonstrated that PhoP^c activates β -catenin pathways.¹¹ As shown in Figure 4C, nuclear β -catenin was observed in human colonic epithelial cells colonized by PhoP^c for 6 hours (Figure 4C, PhoP^c). On the contrary, AvrA^{-/-} infection did not induce β -catenin translocation (Figure 4C, AvrA^{-/-}). If AvrA was complemented in cells colonized with AvrA^{-/-}/AvrA⁺, nuclear β -catenin was again observed. To test whether AvrA is involved in activating β -catenin transcription activity, a luciferase reporter assay of TCF activation was used. The plasmid with wild-type TCF binding site (pGL3-OT) and luciferase reporter gene was used for transfection. A plasmid with the defective TCF-binding site (pGL3-OF) was used as a negative control. Cells were transfected with wild-type pGL3-OT or a defective TCF binding site pGL3-OF and then challenged with PhoP^c or AvrA^{-/-} bacterial colonization. In cells transfected with the defective TCF construct, TCF-induced luciferase activity was not significantly different from cells with no treatment (Figure 4D, pGL3-OF). In cells transfected with wild-type TCF construct, the luciferase activity was substantially higher in cells colonized by PhoP^c (with AvrA expression) when compared with those colonized by PhoP^c AvrA^{-/-} (Figure 4D, pGL3-OT), further demonstrating that AvrA expression activates β -catenin transcription by stabilizing β -catenin at the ubiquitination level, increasing β -catenin available for nuclear translocation, and thus increasing TCF-based transcription.

AvrA Protein Increased *c-myc* and *Cyclin D1* Expression in Intestinal Epithelial Cells in Vivo

The β -catenin pathway regulates genes involved in cellular proliferation including *c-myc*.¹⁹ It was demonstrated that PhoP^c colonization activated the β -catenin pathway.⁷ A previous study *in vitro* showed that AvrA-deficient bacterial colonization decreased *c-myc* expression.¹¹ Using the streptomycin-pretreated mouse model infected with AvrA-sufficient or -deficient bacterial strains, the colonic *c-myc* level was determined from intestinal epithelial cell lysates. As shown in Figure 5A, β -catenin expression was increased by parental PhoP^c infection, whereas β -catenin expression was decreased by bacteria lacking AvrA expression. Complementary AvrA bacterial infection was

Figure 5. β -Catenin and its target gene expression are affected by AvrA *in vivo*. **A:** Western blot of β -catenin in intestinal epithelia colonized by *Salmonella* nonvirulent PhoP^c, PhoP^cAvrA⁻ (AvrA⁻), or AvrA⁻ transcomplemented with wild-type AvrA (AvrA⁻/AvrA⁺) *in vivo*. Equal amounts of total cell lysate were sequentially immunoblotted with antibodies against β -catenin and β -actin (loading control). Bands were quantified using NIH Image software (Bethesda, MD). **B:** Western blot of *c-myc* in intestinal epithelia colonized by *Salmonella* nonvirulent parental PhoP^c, PhoP^cAvrA⁻ (AvrA⁻), or AvrA⁻ transcomplemented with wild-type AvrA (PhoP^cAvrA⁻/AvrA⁺) *in vivo*. Bands were quantified using NIH Image software.

able to bring the protein expression of β -catenin to the level comparable with that in the parental PhoP^c infection. The *c-myc* expression decreased to approximately 40% of the normal control 18 hours after infection with bacteria lacking AvrA (AvrA⁻) but did not decrease when infected with parental PhoP^c (Figure 5B). In contrast, complementary AvrA bacterial infection increased the protein expression of *c-myc*. Likewise, real-time PCR of *c-myc* and *cyclin D1*¹⁰ indicated that *c-myc* and *cyclin D1* expression was increased in AvrA-sufficient bacterial infection (data not shown). These data suggest that the AvrA effector is able to elevate the expression of target genes in the β -catenin pathway.

AvrA Protein Affects Intestinal Epithelial Proliferation *In Vivo*

Next, we examined the biological effect of AvrA expression on epithelial proliferation, which is regulated by the β -catenin pathway. BrdU staining was performed to mea-

Figure 6. Murine large intestinal epithelial cell proliferation affected by AvrA expression. **A:** BrdU labeling of large intestine epithelial cells was performed 24 hours after infection with PhoP^c with or without AvrA. BrdU staining showed that the AvrA mutant strain induced dramatic decrease of epithelial cell proliferation, whereas PhoP^cAvrA⁻/AvrA⁺ with AvrA overexpression increased cell proliferation. **B:** Proliferation index in intestinal epithelial cells. BrdU-positive cells per three high-powered fields were counted from both proximal and distal colons. *n* = 3 in each experimental group. **P* < 0.0001.

sure the BrdU incorporation into newly synthesized DNA. BrdU-positive staining (brown) showed that the AvrA mutant strain (AvrA⁻) infection for 24 hours induced a dramatic decrease in epithelial cell proliferation, whereas PhoP^cAvrA⁻/AvrA⁺ with AvrA expression increased cell proliferation (Figure 6A). The number of the proliferating cells per intestinal glands further (Figure 6B) showed that AvrA⁻ infection induced less than one proliferative cell per intestinal gland, whereas PhoP^cAvrA⁻/AvrA⁺ increased IEC proliferation to 12 proliferative cells per intestinal gland (Figure 6B, PhoP^cAvrA⁻/AvrA⁺). Our data suggested that AvrA expression increased epithelial cell proliferation.

Apoptosis Regulated by AvrA during Bacterial Infection

It is known that both β -catenin²⁰ and NF- κ B pathways^{21,22} are involved in apoptosis of the intestinal epi-

thelial cells. Mucosal erosions, frequently found in the infected colon, are associated with apoptosis of surface epithelial cells in mice. We next assessed the cell apoptosis in mice infected with AvrA-sufficient or -deficient bacterial strains. Marked epithelial apoptosis was identified visually by TUNEL staining of mouse colon 24 hours after infection with wild-type *Salmonella* (Figure 7A, WT). Fewer apoptotic cells were found in parental PhoP^c-infected mouse colons; the apoptotic index was similar to that observed in control mice (Figure 7B). In contrast, with PhoP^c AvrA⁻ infection (without AvrA expression), the cell apoptosis was similar to that in the wild-type-infected mice *in vivo*. Although AvrA is complemented in PhoP^c AvrA⁻/AvrA⁺, the percentage of apoptotic cells decreased (Figure 7, A and B). The TUNEL staining data were consistent with the proliferation data shown in Figure 6: there was less epithelial cell proliferation and more apoptosis in colons infected by AvrA⁻ *Salmonella* and greater cell proliferation and no effect on increased apoptosis in colons infected with PhoP^c AvrA⁻/AvrA⁺.

Discussion

The goal of this study was to determine how the *Salmonella* effector AvrA attenuates the NF- κ B pathway and stabilizes β -catenin, thus inhibiting proinflammatory response in the host. This was achieved by examining the function of AvrA using a streptomycin-pretreated mouse model and epithelial cell models as well as by measuring the deubiquitinase activity in a cell-free system. We identify AvrA as a deubiquitinase that cleaves ubiquitin moieties from I κ B α and β -catenin. We found that *Salmonella* strains with sufficient AvrA expression stabilized I κ B α and β -catenin during bacterial-host cell interaction, leading to inhibition of NF- κ B signaling and activation of β -catenin as evidenced by decreased secretion of IL-6 and increased *c-myc* and cyclin D1 expression. Conse-

quently, AvrA expression in the bacterial strain inhibits the inflammatory response, increases β -catenin transcriptional activity and cell proliferation, and inhibits cell apoptosis. In contrast, *Salmonella* strains with deficient AvrA increase I κ B α degradation, NF- κ B activity, and IL-6 secretion. In addition, *Salmonella* strains with deficient AvrA induced β -catenin degradation, decreased *c-myc* and cyclin D1 expression, increased cell apoptosis, and inhibited cell proliferation.

Previous studies suggested that AvrA might block a ubiquitinating E3 ligase such as β -TrCP.¹¹ However, we did not detect any protein level change of β -TrCP with AvrA expression *in vitro* (data not shown). Instead, we found that AvrA actually acted as a deubiquitinase to remove ubiquitin moieties from the critical proteins I κ B α and β -catenin. Recently, YopJ, a *Yersinia* virulence factor, was also reported as a deubiquitinating enzyme that negatively regulates NF- κ B signaling by removing ubiquitin moieties from TRAF2, TRAF6, and I κ B.¹⁷ Our data demonstrate a similar function of *Salmonella* AvrA. It has also been demonstrated that Epstein-Barr virus is able to activate β -catenin by deubiquitination.²³ This suggests that this deubiquitinating function of microbes may be a global strategy by which microbes overcome the inflammatory response and survive in the host.

We demonstrate that the AvrA C186A mutant protein had reduced deubiquitinase activity as evidenced by cleaving less ubiquitin moieties from I κ B α (Figure 4) and β -catenin (Figure 5) compared with wild-type AvrA, but C186A was eventually able to decrease ub-I κ B α over 60 to 90 minutes. Perhaps the AvrA protein has multiple protease domains, and one amino acid point mutation is not enough to abolish completely the deubiquitinase activity. It will be interesting to assess further the protease domain of AvrA.

For a bacterium to be a successful vertebrate pathogen, it must overcome or alter many very effective host

defense mechanisms.²⁴ Both I κ B α and β -catenin are first phosphorylated and then ubiquitinated by the ubiquitin ligase complex, ultimately resulting in proteasomal degradation of the ubiquitinated proteins. Our studies demonstrate that AvrA modulates ubiquitination as a deubiquitinase but do not exclude an additional effect on phosphorylation. YopJ, a family member of AvrA protein, has recently been shown to mediate effects by selective acetylation of mitogen-activated protein kinases (MAPKs).²⁵ YopJ inhibits MAPK and the NF- κ B signaling pathways used in innate immune response by preventing activation of the family of MAPK kinases (MAPKKs).²⁵ In this study, YopJ acted as an acetyltransferase, using acetyl-coenzyme A to modify the critical serine and threonine residues in the activation loop of MAPKK6 and thereby blocking phosphorylation. The acetylation on MAPKK6 directly competed with phosphorylation, preventing activation of the modified protein. This covalent modification may be used as a general regulatory mechanism in biological signaling. It will be highly interesting to investigate the function of AvrA on phosphorylation and acetylation.

Previous studies using a HeLa cell model demonstrated that AvrA attenuated NF- κ B activity and augmented apoptosis in epithelial cells *in vitro*.⁴ However, in our mouse model infected with the AvrA-sufficient bacterial strain, there were fewer apoptotic epithelial cells compared with mice infected by the AvrA-deficient strain, suggesting that AvrA inhibits epithelial cell apoptosis *in vivo*. These apparently conflicting data may be due to the limitation of the cultured cell system: i) HeLa cells are a transformed cell line and therefore do not necessarily reflect behavior of normal epithelial cells, ii) for apoptosis and proliferation assays, one cannot measure beyond 48 hours because cells become confluent, and iii) the potential uncontrolled bacterial growth over 24 hours may damage cells nonspecifically even if cells were washed with PBS three times and gentamicin added to the media to inhibit extracellular bacterial growth. On the contrary, our *in vivo* system allows us to observe a relative long-term outcome and overcome the limitations of *in vitro* studies. We demonstrate that AvrA attenuates NF- κ B activity and promotes β -catenin activity, thus inhibiting apoptosis and augmenting proliferation in epithelial cells.

A recent study in enteropathogenic *E. coli* indicated that enteropathogenic *E. coli*-induced inflammation involves a balance between pro- and anti-inflammatory proteins. Extracellular factors, including flagellin and an unidentified type 3 secretion system-independent >50-kD protein, trigger inflammation, whereas intracellular type 3 secretion system-dependent factors attenuate this response.²⁶ *Salmonella* may also use a similar mechanism to keep a balance between pro- and anti-inflammatory proteins. Whereas *Salmonella* flagellin^{27,28} and lipopolysaccharide^{29,30} stimulate inflammation in the host, AvrA may play an essential role to attenuate the inflammatory response.

Taken together, our data provide strong evidence that the *Salmonella* effector AvrA modulates the proinflammatory NF- κ B pathway *in vivo*. AvrA acts as a deubiquitinase to inhibit ubiquitination, which in turn influences the ubiquitin-mediated proteolysis of I κ B α and β -catenin.

These data suggest the novel mechanism that bacteria modulate the NF- κ B and β -catenin pathways to affect consequently the inflammatory response and cell fate.

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