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Suppression of NF-κB Activation and Proinflammatory Cytokine Expression by Shiga Toxin-Producing Escherichia coli

Nadja Hauf and Trinad Chakraborty

The NF-κB family of transcription factors forms one of the first lines of defense against infectious disease by inducing the expression of genes involved in inflammatory and immune responses. In this study, we analyzed the impact of Shiga toxin-producing Escherichia coli (STEC) on the NF-κB DNA-binding activity in HeLa cells. After a period of weak initial activation, DNA binding of NF-κB was actively suppressed by viable, E. coli secreted protein B (EspB)-secreting STEC. Sustained NF-κB activity was observed either using an isogenic mutant lacking EspB or after gentamicin-based killing of STEC after allowing bacterial attachment. These observations indicate that the ability of STEC to cause NF-κB activation is suppressed by a translocated bacterial effector protein, which is either EspB itself or requires EspB for delivery into the host cell. We found that STEC, enterohemorrhagic E. coli, and enteropathogenic E. coli all interfere with NF-κB activation initiated by TNF-α, indicating that suppression of signal-induced NF-κB activity is a property common to several attaching and effacing bacteria. As a consequence of NF-κB suppression, wild-type STEC induces significantly lower mRNA levels of IL-8, IL-6, and IL-1α upon prolonged infection periods compared with bacteria lacking EspB. For IL-8 and IL-6, the suppressive effect was also reflected at the level of cytokine secretion. Suppression of both basal and signal-induced NF-κB DNA binding by attaching and effacing-inducing bacteria appears to be an active strategy to counteract host defense responses, thus favoring intestinal colonization by these pathogens. The Journal of Immunology, 2003, 170: 2074–2082.

Bacteria enteric pathogens evolved different strategies to circumvent or counteract host defense mechanisms, thus permitting their survival and multiplication within the host. Intracellular microbial pathogens such as Salmonella dublin and Shigella dysenteriae evade mucosal inflammatory and immune responses by entering various cell types, including intestinal epithelial cells and phagocytes (1–4). In contrast, Yersinia pseudotuberculosis and Y. enterocolitica are largely extracellular bacteria that inhibit their phagocytosis by macrophages and polymorphonuclear neutrophils, in which they could not survive. Indeed, these bacteria use active mechanisms to suppress innate host defenses (5).

The important role of intestinal epithelial cells in activating and regulating the mucosal inflammatory and immune response is well documented (6). Mucosal inflammation is characterized by coordinate expression and up-regulation of a specific array of epithelial gene products, including secreted cytokines with chemoattractant (e.g., IL-8, macrophage-inflammatory protein 1α, and monocyte chemoattractant protein-1) or proinflammatory (e.g., TNF-α and IL-1α) function. For S. dublin, it was clearly shown that most (if not all) genes up-regulated after infection were regulated by NF-κB (4). NF-κB comprises a family of closely related transcription factors that play a key role in the expression of genes involved in inflammation and immune responses (7–9). Usually, the term NF-κB is collectively used for homo- and heterodimeric complexes formed by the so-called Rel/NF-κB proteins. In mammals, five of such proteins are known, referred to as RelA (p65), RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB2). NF-κB’s most obvious characteristic is its rapid translocation from the cytoplasm—where it is sequestered in an inactive form, bound to one of several inhibitory proteins (IκBα, IκBβ, IκBe)—to the nucleus in response to a great variety of extracellular signals. This signal-induced NF-κB activation is mainly accomplished by phosphorylation of IκB at two specific serine residues followed by polyubiquitination and IκB degradation by the 26S proteasome (8, 10). Because degradation exposes the nuclear localization signal on NF-κB, the liberated active NF-κB translocates to the nucleus, where it modulates gene expression by binding to the κB motifs of its target genes.

Shiga toxin-producing Escherichia coli (STEC)3 are an important cause of serious human gastrointestinal disease (11, 12). Central to STEC pathogenesis is the intestinal colonization generally resulting in a striking histopathological feature known as the attaching and effacing (A/E) lesion. After initial adherence to the intestinal mucosa, STEC triggers the localized destruction (effacement) of brush border microvilli and intimately attaches to the epithelial surface. It thereby induces the formation of a pedestal-like actin structure (A/E lesion) directly beneath adherent bacteria, through which STEC remains attached extracellularly to the host cell. A/E lesions are also produced by several other enteric pathogens, including the closely related enteropathogenic E. coli.

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3 Abbreviations used in this paper: STEC, Shiga toxin-producing Escherichia coli; A/E, attaching and effacing; EPEC, enteropathogenic E. coli; LEE, locus of enterocyte effacement; Esp, E. coli secreted protein; EHEC, enterohemorrhagic E. coli.
(EPEC), the prototypic A/E organism. The genetic determinant for A/E lesion formation in both STEC and EPEC is a chromosomally encoded pathogenicity island, called the locus of enterocyte effacement (LEE) (15–15).

Many of the proteins encoded within the LEE locus are part of a type III secretion system comprising proteins required for both the formation of a secretion complex (E. coli secretion and secretion of EPEC proteins) and the secretion of the LEE-located E. coli secreted proteins (Esp proteins). The secretion complex is inserted into the inner and outer membranes of the bacterium and secretes the EspS across the bacterial envelope. It is generally assumed that after initial attachment of EPEC and STEC to the intestinal epithelium, a LEE-encoded type III-secreted protein translocation tube is formed, which connects the pathogen with its target cell (15, 16). EspA seems to be a major component of this tube (17, 18), whereas EspD (19, 20) and EspB (18, 21, 22) appear to be inserted into the host membrane, forming a pore structure. Thus, additional Esp proteins such as EspE/translocated intimin receptor (23, 24) and EspF (25) can be directly injected from the cytoplasm of the bacterium into the host cell to modulate host responses. EspB appears to have a dual function as translocator and effector protein (18, 21, 22, 26–28).

Previous studies with EPEC have revealed a role for the NF-κB family of transcription factors in infected T84 intestinal cells (29, 30). These studies indicate that EspB-dependent activation of NF-κB, which is required for the transcription of a number of proinflammatory mediators, is preceded by the activation of T84-induced IκB degradation, and is no longer elicited in the absence of EspB. NF-κB activation in HeLa cells.

Materials and Methods

Bacterial strains and growth conditions

E. coli strains used for cell culture infections are shown in Table I. Bacteria were normally grown in Luria-Bertani broth at 37°C with shaking. For infection experiments, bacterial suspension from overnight cultures grown in Luria-Bertani broth were diluted 1/50 in serum-free MEM (Life Technologies, Rockville, MD; STEC and enterohemorrhagic E. coli (EHEC) strains) or 1/40 in serum-free DMEM (Life Technologies; EPEC strain). The cultures were incubated without shaking at 37°C with 5% CO2 to an OD600 of 0.8.

Generation of isogenic deletion mutants of STEC 413/89-1 and their complementation

A nonpolar in-frame deletion mutation of espB in STEC strain 413/89-1 (ΔespB) was constructed by allelic exchange. Two fragments containing the 5' and 3' ends of espB and additional flanking regions were generated by two separate PCRs (Expand High Fidelity PCR Kit; Roche, Basel, Switzerland) using chromosomal DNA as template. Fragment B1 was 1069 bp long and was amplified with oligonucleotides ΔB1 (5'–CAG GTC ACT CAT ATG TGA GGC CCT CGT GTG CTG-3') and ΔB2 (5'–AAT TGG ATT GGA TCC GTG AGT ATT CTA CGA AAC-3'), which incorporate NdeI and BamHI restriction sites (underlined), respectively. Fragment B2 was 1011 bp long and was generated using oligonucleotides ΔB3 (5'–GAT ATG ACA GGA TCC GCC CAT CAC ACT GAT-3') and ΔB4 (5'–TTC TAT TAT TAC CGT GGA CCA GAC TGC TAA ATA AC-3') with the incorporated BamHI and MluI restriction sites (underlined), respectively. The corresponding restriction endonucleases were used to enable ligation of both amplification products and for subsequent cloning of the obtained fragment into the temperature-sensitive vector pMak7000orT (36). The ligation of fragments B1 and B2 at the BamHI site created an in-frame deletion in the espB gene, such that 816 bp were removed from the open reading frame, fusing the 5' region of espB encoding the first 22 N-terminal amino acid residues with the 3' region encoding the last 20 C-terminal amino acid residues of EspB. The resulting recombinant suicide plasmid was introduced by electroporation into the wild-type strain and used to construct the deletion mutant by allelic exchange as described previously (17). The resulting strain harboring a mutated espB allele were identified by PCR using primers ΔB1 and ΔB4 and were confirmed by sequencing.

For complementation of the STEC 413/89-1 ΔespB mutant, the complete espB gene of STEC strain 413/89-1 was amplified by PCR using oligonucleotides ΔD3 (5'–TCT CAG TTA GGA TCC GAC TCA GCG GTA AAT-3') and eaeB413 (5'–CAG AAT TCT TAC CCA GCT AAG CCA ACC G-3') with incorporated BamHI and EcoRI restriction sites (underlined), respectively. The resultant 1032-bp fragment was subsequently cloned under control of an arabinose-inducible promoter into the vector pBAD/Myc-HisB (Invitrogen, San Diego, CA), generating plasmid pBAD::espB. The plasmid was transformed into the deletion mutant STEC 413/89-1 ΔespB using electroporation, thereby creating strain STEC 413/89-1 ΔespB + pBAD::espB. DNA sequencing, Esp protein secretion profiles, and Western blot analysis confirmed the generation of the required strain.

The deletion strain STEC 413/89-1 ΔespB/Δe1 was generated analogously to the ΔespB strain using the primer combinations ΔE1/ΔE2 (5'–GAA TCA CCA GCT AGC TCA GCC CGT GCG CAA ACA-3'/5'–GGC TAA TGG CCG CGG AGG TGG AAT TAA AGC TCT-3') and ΔE3/ΔE4 (5'–GCC GCA CCG CCG GGA CCC GCC GTG TTT GCT GCA CTG-3'/5'–GCC GTC ATC GAT TCG CTT TCG GAA CTG TAT) with incorporated Nhel, XmaII, and Clal restriction sites (underlined), respectively. The resulting in-frame deletion in the espB gene eliminated 1536 bp from the open reading frame by fusing the 5' region of espB/Δe1 encoding the first 19 N-terminal amino acid residues with its 3' region (encoding the last seven C-terminal amino acid residues).

Table I. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristic(s)</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPEC E2348/69</td>
<td>Wild type (serotype O127:H6); human isolate</td>
<td>31</td>
</tr>
<tr>
<td>EHEC EDL933</td>
<td>Wild type (serotype O157:H7); human isolate</td>
<td>32</td>
</tr>
<tr>
<td>STEC 413/89-1</td>
<td>Wild type (serotype O26:H1); isolated from a calf suffering from diarrheaa</td>
<td>33</td>
</tr>
<tr>
<td>STEC 413/89-1 ΔespB</td>
<td>In-frame deletion in espB</td>
<td>34</td>
</tr>
<tr>
<td>STEC 413/89-1 ΔespB + pBAD/Myc-HisB:espB</td>
<td>Complementation of the deletion mutant STEC 413/89-1 ΔespB</td>
<td>34</td>
</tr>
<tr>
<td>STEC 413/89-1 ΔespE/tir</td>
<td>In-frame deletion in espE/tir</td>
<td>35</td>
</tr>
</tbody>
</table>
Preparation and analysis of STEC-secreted proteins by SDS-PAGE were performed as described previously (17, 24).

**Stimulation or infection of HeLa epithelial cells**

For stimulation, the cells were treated with human TNF-α (Sigma-Aldrich; 10 ng/ml for 15 min), PMA (Sigma-Aldrich; 50 ng/ml for 30 min), or IL-1α (Sigma-Aldrich; 2 ng/ml for 15 min). For infection, the cells were incubated with *E. coli* (listed in Table I) to give a multiplicity of infection of 50 bacteria per cell for a period of time ranging from 30 min to 8 h. At prolonged infection periods (2–8 h), washing steps with HBSS (Biochrom) were included to remove nonadherent bacteria. For the preincubation experiments, cells were first infected with pathogenic *E. coli* for different time periods and subsequently were stimulated with 10 ng/ml TNF-α for 15 min.

**Gentamicin assay**

After infection of HeLa cells with STEC (413/89-1) for 1.5 h as described above, STEC-infected monolayers were washed three times with HBSS and exposed to gentamicin (100 μg/ml) for the residual infection periods.

**EMSA**

Nuclear extracts from cells treated as indicated in the figure legends were prepared according to the method of Schreiber et al. (37) and analyzed by EMSA essentially as described previously (38, 39). In brief, the complementary oligonucleotides 5'-AGC TTC AGA G-3' and 3'-AGT CTC CAG CT-5' (Sigma-Aldrich) were annealed to generate a double-stranded xB probe containing the consensus binding site for NF-κB dimeric complexes (shown in bold; Ref. 40) and overhanging ends, which were 32P-labeled using Klenow enzyme. To separate unincorporated radioactive nucleotides from the radiolabeled DNA fragment, NuTrap probe purification columns (Stratagene, La Jolla, CA) were used according to the instruction manual. DNA binding reaction mixtures (15 μl) contained 3–4 μg of nuclear extract, 5 μl of 3X binding buffer (60 mM HEPES, pH 7.9; 3 mM DTT; 3 mM EDTA; 150 mM KCl; and 12% Ficoll), 1.6 μg of poly (dI-dC) (Roche), and 20,000 cpm of radiolabeled xB probe. Unless specifically indicated, the binding reaction also contained 6 ng of an unlabeled dsDNA fragment encompassing a mutated NF-κB DNA-binding site (38). This was added to reduce the formation of two nonspecific protein-DNA complexes, which migrate directly below the p50–p50-xB complex (compare Fig. 1 with Figs. 2 and 3; indicated by an arrowhead). The generation of both of these nonspecific complexes could not be prevented by addition of the nonspecific competitor poly (dI-dC) alone. After 25 min of incubation on ice, resultant nucleoprotein complexes were resolved from unbound DNA on a native 5% polyacrylamide gel and were detected by autoradiography of the dried gel.

For competition studies, a 1- to 100-fold molar excess of unlabeled xB probe or DNA fragment mutated in five bases of the xB consensus motif (38) was included in the binding reaction mixture. The mixture was incubated on ice for 10 min before the radiolabeled xB probe was added. For supershift experiments, 2 μg of a specific antisem were added to the binding reaction mixture before addition of the radiolabeled xB probe and preincubated with the nuclear proteins for 20 min on ice. An Ab that binds specifically to a particular DNA-binding protein can either prevent formation of a specific protein-DNA complex or further retard (supershift) its electrophoretic mobility (41). All Abs used for supershift experiments were rabbit polyclonal IgGs supplied from Santa Cruz Biotechnology (Santa Cruz, CA).

**RNA isolation and RT-PCR analysis**

Total RNA of infected HeLa cells was extracted with TRIzol reagent (Invitrogen) as recommended by the manufacturer’s instructions. RNA samples were quantified spectrophotometrically at 260/280 nm and treated with RNase-free DNase 1 (Ambion, Austin, TX) before reverse transcription to remove contaminating genomic DNA. One microgram of DNA-free total RNA was reverse transcribed into single-stranded cDNA according to protocols and reagents from Clontech Laboratories (Palo Alto, CA; Advantaq RT-for-PCR kit using oligo(dT)18 primer). One microtiter of the 20 μl reverse transcription reaction was amplified by PCR in 50 μl of a mixture containing 5 μl of 10X Titanium Taq PCR buffer (Clontech Laboratories), 1 μl of 10 mM dNTP mix (Amersham Pharmacia Biotech, Piscataway, NJ), 0.2 μmol each of the specific primer pairs listed in Table II, and 1 μl of 50X Titanium Taq DNA polymerase (Clontech Laboratories). For all PCRs, the following conditions were used: an initial denaturation step at 94°C for 1 min, two-step cycles of 30 s at 94°C and 1.5 min at 60°C, and a final extension step at 60°C for 3 min. The number of PCR cycles was adjusted as appropriate to maximize the differences between samples (GAPDH, 23 cycles; IL-6, 28 cycles; IL-1α, 30 cycles; IL-8, 33 cycles). To control for contamination with genomic DNA, experiments were performed omitting the enzyme during the reverse transcription step and subsequent PCR amplification using the GAPDH primer pair. PCR products (5–10 μl) were analyzed by 1% ethidium bromide-agarose gel electrophoresis. The level of amplified products were normalized to constant amounts of GAPDH mRNA. Each RT-PCR experiment was done in duplicate.

**ELISA**

Cytokine release from HeLa cells infected with STEC was examined at 6 h, 10 h, and 20 h. To prevent bacterial overgrowth of the epithelial cell cultures, the medium was depleted of nonadherent bacteria at 2 and 6 h. At the 6-h time point, adherent extracellular bacteria were killed by the addition of gentamicin (100 μg/ml). IL-1α, IL-6, and IL-8 concentrations were determined in cell supernatants using Biotrak human IL-1α, IL-6, and IL-8 ELISA kits (Amersham Pharmacia Biotech) following instructions of the vendor. The results presented are based on three independent experiments that were assayed in quadruplicate.

**Results**

**STEC transiently activates NF-κB** (RelA-p50 and RelB-p50) in HeLa epithelial cells

To investigate whether STEC (413/89-1) induces NF-κB DNA-binding activity as previously shown for EPEC (29), we first analyzed the kinetics of nuclear translocation of NF-κB in STEC-infected HeLa cells. Cells were first challenged with bacteria for specific infection periods ranging from 30 min to 6 h. Nuclear extracts were prepared and subjected to EMSA using a radiolabeled consensus oligonucleotide xB probe (see Materials and Methods). Noninfected cells served as negative control. The magnitude of migration of NF-κB DNA-binding activity was determined by autoradiography. The time course of the appearance of NF-κB DNA-binding activity is shown in Fig. 2. The results show that NF-κB DNA-binding activity is detectable 30 min postinfection and peaks at 1 h. At 1 h postinfection, NF-κB DNA-binding activity is maximal and remains unchanged up to 6 h postinfection.

**Table II. Oligonucleotide primers used for RT-PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Product Size (bp)</th>
<th>GenBank Accession Numbers (DNA/mRNA)</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5'-ACACACAGTCCATCCGATCCATCAC-3'</td>
<td>452</td>
<td>J04038/NM 002046</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'-CTCCCTGCCCTGTGCTGTA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5'-ATGACCTTCAAGCTGCGGCTGCT-3'</td>
<td>285</td>
<td>M28130/NM 000584</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'-CAAGCCACCTCGCTTCAACAC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5'-AGATGACCTGAGAACTCAAAC-3'</td>
<td>333</td>
<td>Y00081/M54894</td>
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<tr>
<td>Antisense</td>
<td>5'-AGTGACCTGAGAACTCAAAC-3'</td>
<td></td>
<td></td>
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<tr>
<td>IL-1α</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5'-CTGCTACCAGTCCACAAATG-3'</td>
<td>240</td>
<td>X03833/M28983</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'-CTGCTACCAGTCCACAAATG-3'</td>
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</table>
clearly detectable changes in protein DNA-binding activity were observed throughout the period of infection investigated (indicated by arrows in Fig. 1A). First, formation of the slower-migrating protein-DNA complex was markedly enhanced by STEC within 0.5–1.5 h after infection. At later time points (3–6 h), this complex disappeared almost completely. Second, the faster-migrating complex was either not induced or only poorly induced at early infection times (0.5–2 h), but unexpectedly at late infection periods, the amount of this complex decreased to below the baseline level of noninfected cells. As depicted in Fig. 1B, the upper band actually comprises two different complexes (C1a and C1b) migrating with nearly the same mobility.

To confirm the specificity of the detected protein-DNA complexes C1a, C1b, and C2 (Fig. 1B), a competition experiment was conducted (Fig. 2). Upon addition of increasing concentrations of the unlabeled κB oligonucleotide, a dose-dependent decrease of all three complexes was observed. As expected, an oligonucleotide containing a mutated NF-κB DNA-binding motif did not inhibit complex formation at any concentration tested.

To further characterize the composition of the complexes, nuclear extracts were preincubated with Abs directed against all known Rel/NF-κB proteins (RelA, RelB, c-Rel, p50, and p52) and were tested by EMSA (Fig. 3). Neither the anti-p52 nor the anti-cRel antiserum impaired the generation of any of the three complexes. Whenever the anti-p50 antiserum was added, the formation of C2 was blocked with concomitant appearance of a weak supershifted complex (S1; Fig. 3, lanes 3, 7–9, 13, 14, and 16). This indicates that C2 is likely to be formed by binding of p50-p50 homodimers to DNA. Moreover, preincubation with the anti-p50 antiserum resulted in a significant reduction of C1a and C1b, suggesting that p50 is also a component of these two complexes. Addition of the anti-RelA antiserum led to the complete disappearance of C1a and appearance of supershift 2 (S2; Fig. 3, lanes 4, 7, 10, 11, and 13–16). Thus, we concluded that C1a represents RelA–p50 heterodimers bound to the κB oligonucleotide. Analysis of the components of the C1b complex was found to be difficult because C1b migrates directly below the more prominent C1a complex (Figs. 1B and 3). Because the band comprising C1a and C1b was more reduced in the presence of anti-RelB in comparison with anti-cRel antiserum (Fig. 3, compare lanes 5 and 6 and lanes 8 and 9).

**FIGURE 1.** Kinetics of κB DNA-binding activity in STEC-infected HeLa cells. A, HeLa cells were noninfected (NI) or infected with STEC strain 413/89-1 for the time points indicated. Nuclear proteins were extracted and analyzed by EMSA using a radiolabeled oligonucleotide containing the consensus binding motif for NF-κB dimers. The binding reaction contained, in addition to the nonspecific competitor poly(dI-dC), 6 mg of an unlabeled DNA fragment, encompassing a mutated NF-κB DNA-binding site (for further explanation see also Fig. 2). This was added to reduce the formation of two nonspecific protein-DNA complexes (marked by an arrowhead), which were generated in great amounts in the presence of poly(dI-dC) alone (compare with Figs. 2 and 3). Protein-DNA complexes whose formation was affected by STEC are marked by arrows. B, The STEC-induced upper band (A) actually comprises two different protein-κB complexes, indicated as C1a and C1b, that migrate with nearly the same mobility. These two complexes could only be observed as separate bands in EMSA after prolonged electrophoresis.

**FIGURE 2.** Competition analysis of protein-κB complexes C1a, C1b, and C2. Nuclear extracts from HeLa cells infected for 1 h with STEC strain 413/89-1 were incubated with increasing concentrations (1- to 100-fold molar excess) of an unlabeled specific and nonspecific oligonucleotide, respectively, before the radiolabeled κB probe was added. The specific oligonucleotide encompasses the same consensus binding site for NF-κB dimeric complexes as the used radiolabeled κB probe, whereas the nonspecific oligonucleotide was mutated in five bases of this consensus sequence (38). Although the generation of the complexes C1a, C1b, and C2 in STEC-infected HeLa cells is due to specific protein-DNA interaction, the band marked by an arrowhead seems to result from nonspecific protein DNA binding to the radiolabeled κB probe. The intensity of this band declined by addition of a 100-fold molar excess of the unlabeled κB oligonucleotide, but was equally reduced by a 100-fold molar excess of the mutated oligonucleotide. Only the relevant portion of the EMSA is shown. NI, Noninfected control; −, without competitor.

**FIGURE 3.** STEC-induced protein-κB complexes contain Rel/NF-κB proteins. Supershift experiments were performed using nuclear extracts from HeLa cells infected for 1 h with STEC strain 413/89-1. The nuclear proteins were preincubated with Abs specific for the known Rel proteins p52, p50, RelA, c-Rel, and RelB either alone or in combination before addition of the radiolabeled κB oligonucleotide. The unlabeled mutated κB oligonucleotide, which was usually added to reduce the formation of the nonspecific protein-κB complexes (marked by an arrowhead), was omitted from the binding reaction. As control (lane 1), nuclear extract was incubated without (−) Abs. The Abs used resulted in reduced DNA binding of the respective Rel protein and partially caused the appearance of supershifts (S1 and S2). The predicted subunit composition of the complexes is shown by the bracket.
infection. A STEC mutant strain over, p50-p50 DNA-binding activity did not decrease as it did with higher at later times compared with early times (up to 1 h). More-
duced DNA-binding activity of RelA-p50 and RelB-p50 was even
increased in untreated cells. Hence, an EMSA was performed with nuclear extracts from HeLa cells infected for different time points with wild-type STEC (413/89-1) and with an isogenic mutant harboring an in-frame deletion in espB (ΔespB) (Fig. 4A). Surprisingly, the ΔespB mutant induced NF-κB DNA-binding activity with the same kinetic as the wild type in early infection periods (0.5–1 h). How-
ever, at prolonged infection times (2–6 h), ΔespB-infected cells revealed sustained increases of RelA-p50- and RelB-p50–DNA complexes as well as of p50–p50–κB complexes. The ΔespB-in-
duced DNA-binding activity of RelA-p50 and RelB-p50 was even higher at later times compared with early times (up to 1 h). Moreover, p50-p50 DNA-binding activity did not decrease as it did with the wild-type strain, but was slightly enhanced within 2–6 h after infection. A STEC mutant strain ΔespE/hr, which did not induce A/E lesions because of its inability to deliver the translocated intimin receptor EspE/translocated intimin receptor into host cells (Ref. 23 and unpublished data), behaves essentially like wild-type STEC with regard to transient NF-κB activation (Fig. 4B). This result clearly indicates that EspB from STEC actively inhibits sustained activation of these NF-κB dimers.

Suppression of NF-κB DNA-binding activity is mediated by viable, metabolically active STEC bacteria

Given that the STEC-mediated NF-κB suppression is caused by a translocated bacterial effector protein, this effect would require ini-
tial bacterial attachment to the host cell, followed by formation of the protein translocation apparatus and subsequent injection of the effector into the host cell. As is shown in Fig. 1A, at 1.5 h postin-
fec
tion, enhanced NF-κB DNA-binding activity was seen, suggest-
ing that the postulated bacterial effector exerting the suppressive effect on NF-κB activation either has not yet been translocated or has only inefficiently been translocated into the host cell. More-
over, this time point was critical for initial attachment of STEC (413/89-1) because after washing the cells, adherent bacteria were still present. Therefore, the 1.5-h postinfec
tion point was chosen for the following experiment. HeLa cells were infected with live STEC that either were killed by gentamicin treatment at 1.5 h or were left untreated as control. At later infection times (4–6 h), gentamicin-treated cells showed considerably higher NF-κB DNA-binding activity than did untreated cells (Fig. 5). This result supports the idea that STEC actively causes NF-κB suppression by a translocated effector protein.

Infection of HeLa cells by STEC, EHEC, and EPEC interferes with NF-κB signaling initiated by TNF-α

To examine whether STEC and other A/E-inducing bacteria are able to interfere with NF-κB activation caused by a well-established NF-κB inducer such as TNF-α (7), the following set of experiments was performed. HeLa cells were preinfected with STEC (413/89-1), EHEC (EDL933), or EPEC (E2348/69) for dif-
terent times and subsequently stimulated with TNF-α for 15 min. Nuclear proteins from noninfected or infected cells, which were either stimulated with TNF-α or left unstimulated, were extracted and analyzed by EMSA (Fig. 6A). Preinfection of HeLa cells with each of the three strains used resulted in impairment of NF-κB activation triggered by TNF-α. Interestingly, the block in TNF-α-
induced NF-κB activation correlates with the level of bacterial attachment to their target cells. Thus, in the case of EPEC (E2348/69)—haboruing a bundle-forming pilus that allows rapid attach-
tment to HeLa cells (15)—complete inhibition of TNF-α-mediated NF-κB activation was already seen 4 h postinfection (Fig. 6A; EPEC, lane 8), whereas this effect was delayed in the more slowly adherent STEC and EHEC strains (STEC > EHEC). These results are consistent with the hypothesis that NF-κB suppression by STEC, EHEC, and EPEC is mediated by a translocated bacterial effector molecule, which then interferes at some level with NF-κB signaling pathways initiated by TNF-α.

To corroborate the role of EspB in NF-κB suppression, we com-
plemented the espB deletion strain with the pBAD::espB plasmid,

FIGURE 4. Infection of HeLa cells with an espB mutant from STEC causes long-lasting NF-κB activation. A, Time course of NF-κB activation upon infection with STEC (413/89-1) wild-type (WT), or an isogenic espB mutant (ΔespB). B, Time course of NF-κB activation after infection with an espE/hr mutant from STEC strain 413/89-1 (ΔespE/hr). Nuclear ex-
tracts were prepared at the time points specified and were analyzed for NF-κB DNA-binding activity by EMSA. Nuclear protein extracts from noninfected (NI) cells were used as negative control. The NF-κB dimeric complexes are indicated. pi, postinfection.

FIGURE 5. Treatment of STEC-infected cells with gentamicin results in prolonged NF-κB activation. HeLa cells were infected with live STEC (413/89-1) bacteria, which were killed by addition of gentamicin (100 μg/ ml) at 1.5 h postinfection (pi) or were not killed as control. Nuclear pro-
teins were extracted at the time points indicated and subjected to EMSA. The NF-κB dimeric complexes are indicated.
which expresses the espB gene from STEC strain 413/89-1 in an arabinose-inducible fashion (Fig. 6C). The experiment was repeated as above using the ΔespB mutant and the complemented strain. As expected, preinfection with the ΔespB mutant did not result in impairment of TNF-α-induced NF-κB activation. In contrast, in cells preinfected with the complemented ΔespB mutant (in the presence of 0.2% arabinose), the inhibitory effect on TNF-α-induced NF-κB activation was even more rapid than that observed with the wild-type STEC strain (Fig. 6, A and B).

EspB is needed for inhibition of IL-6 and IL-8 secretion by STEC-infected HeLa cells

NF-κB is an important transcriptional regulator of inducible expression of numerous genes involved in inflammatory and immune responses (7). To determine the effect of STEC-mediated NF-κB suppression on NF-κB-dependent gene expression, we first investigated the mRNA levels of IL-8, IL-6, and IL-1α in response to bacterial infection. The changes in cytokine expression of cells infected with wild-type STEC and the ΔespB mutant were analyzed by RT-PCR. For each experiment, mRNA level was normalized to GAPDH expression, which is unaffected by STEC infection. Using IL-8 and IL-6 gene expression as a readout, a time
course of infection was performed in which HeLa cells were infected with wild-type STEC and the ΔespB mutant for 6 h. As shown in Fig. 8A, the ΔespB mutant induced a significantly higher amount of IL-8–mRNA than did the wild type at all time points investigated. In addition, this mutant also exhibited increased IL-6 mRNA levels at 4 and 7 h compared with the wild type (Fig. 8A). However, at 3 h postinfection, both strains induced similar levels of IL-6 mRNA. For subsequent experiments addressing the expression level of the IL-1α gene, cells were only infected for 6 h, when NF-κB suppression by wild-type STEC was maximal. At this specific time point, the amount of IL-1α mRNA in wild-type STEC-infected cells is comparable to that expressed in noninfected cells, whereas ΔespB infection clearly induced IL-1α mRNA synthesis (Fig. 8B). Taken together, transcription of different NF-κB–regulated inflammatory cytokine genes was substantially reduced upon prolonged infection periods with wild-type STEC compared with the ΔespB mutant.

As expected, IL-8 and IL-6 release was suppressed at later time points in HeLa cells infected with EspB–producing wild-type STEC (Table III). This is consistent with the interpretation that a 6-h infection period is sufficient for translocation of the putative bacterial effector protein(s) mediating the maximum inhibitory effect on NF-κB activation (Fig. 6, A and B). Under the experimental conditions described, at 6 h no significant differences were observed for both IL-8 and IL-6 secretion (Table III). However, 20 h after infection with wild-type STEC, a 10-fold reduction of IL-8 release and a 6-fold inhibition of IL-6 secretion was detected (Table III). IL-1α secretion by noninfected or infected HeLa cells was below the threshold sensitivity (10 ng/ml) of the assay used and thus could not be quantified at any time point investigated. These results clearly show that wild-type STEC inhibits production of IL-8 and IL-6 by HeLa cells in an EspB–dependent manner.

### Discussion

The data presented in this study show that STEC, EHEC, and EPEC actively suppress bacteria- or TNF-α–induced NF-κB–DNA-binding activity in HeLa cells after an initial induction period. Evidence for suppression of NF-κB activation by these bacteria was derived from the following observations. First, wild-type STEC bacteria only transiently induce DNA-binding activity of RelA-p50 and RelB-p50, whereas DNA binding of p50 homodimers was reduced to even below the baseline level of noninfected cells after prolonged infection periods (3–6 h). Second, the STEC ΔespB mutant revealed persistent activation of RelA-p50 and RelB-p50 dimers with slight elevation of DNA binding of p50 homodimers at late infection times (2–6 h). This effect (persistent NF-κB activation) was specific, because it was not observed using an isogenic ΔespE/tir mutant strain that behaved essentially like the wild-type strain. Third, gentamicin-based killing of wild-type STEC at 1.5 h postinfection abrogated NF-κB suppression, suggesting that this effect was mediated by a translocated bacterial effector protein. Fourth, prior exposure of HeLa cells to STEC, EHEC, EPEC, or the complemented STEC ΔespB mutant interfered with subsequent NF-κB activation by TNF-α, a well-known NF-κB inducer. Collectively, these data indicate that EspB is required for suppression of both basal or signal-induced NF-κB DNA-binding activity. In addition, this suppressive effect is a common property of several A/E–inducing bacteria.

These results are reminiscent of suppression of RelA-p50 DNA-binding activity seen with Y. pseudotuberculosis and Y. enterocolitica. In that case, suppression is due to the intracytoplasmic presence of a secreted prokaryotic effector protein (YopJ or YopP, respectively), which is injected into the target cell via a type III secretion system (42–44). YopJ was shown to bind directly to the superfamily of mitogen-activated protein kinase kinases, thus inhibiting their kinase activity (44). One member of this superfamily is the IκB kinase β, which is a component of a large cytoplasmic multisubunit complex phosphorylating IκBα as well as IκBβ. Because phosphorylation of these IκBαs is blocked, subsequent IκB degradation and NF-κB activation is inhibited (42, 43). A search for proteins homologous to the Yersinia YopJ/P proteins in the sequenced genomes of two serotype O157 EHEC strains (including the EDL933 strain examined in this study) yielded no results, indicating that the effect observed here is mediated by a hitherto unknown effector protein in these strains.

Our data are also consistent with the notion that a translocated bacterial effector protein common to STEC, EHEC, and EPEC mediates suppression of both basal and signal-induced NF-κB activity by interfering at some level with NF-κB signaling. The delayed suppressive activity seen with these strains is in agreement with a sequence of events requiring initial bacterial attachment to

### Table III. STEC inhibits IL-8 and IL-6 secretion by HeLa cells in an EspB–dependent manner

<table>
<thead>
<tr>
<th>Infection</th>
<th>IL-8 (pg/ml) 6 h</th>
<th>IL-8 (pg/ml) 10 h</th>
<th>IL-8 (pg/ml) 20 h</th>
<th>IL-6 (pg/ml) 6 h</th>
<th>IL-6 (pg/ml) 10 h</th>
<th>IL-6 (pg/ml) 20 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>STEC 413/89-1</td>
<td>388 ± 28</td>
<td>1,184 ± 37</td>
<td>1,053 ± 31</td>
<td>101 ± 23</td>
<td>333 ± 34</td>
<td>315 ± 32</td>
</tr>
<tr>
<td>wild type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>STEC 413/89-1</td>
<td>386 ± 36</td>
<td>1,962 ± 40</td>
<td>10,815 ± 135</td>
<td>68 ± 18</td>
<td>384 ± 25</td>
<td>1,880 ± 51</td>
</tr>
<tr>
<td>ΔespB mutant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noninfected</td>
<td>50 ± 4</td>
<td>58 ± 4</td>
<td>164 ± 10</td>
<td>22 ± 4</td>
<td>24 ± 4</td>
<td>33 ± 4</td>
</tr>
</tbody>
</table>

*HeLa cells were infected with wild-type STEC or the ΔespB mutant. After 6 h of infection, gentamicin (100 μg/ml) was added to kill extracellular bacteria. The amount of IL-8 and IL-6 secreted was assayed by ELISA using cell culture supernatants collected at the indicated time points. Noninfected cells were used as control. Results are expressed as means ± SD from three independent experiments, which were analyzed in quadruplicate.
the host cell, followed by formation of the type III protein translocation apparatus with subsequent delivery of the putative effector protein into the host cell. Indeed, the rate and level of adhesion of STEC, EHEC, and EPEC is consistent with the respective appearance of NF-κB suppression caused by these bacteria. The observation that the STEC mutant lacking EspB, unlike the complemented STEC ΔespB mutant, is unable to suppress TNF-α-mediated NF-κB activation indicates that EspB is required for this activity. EspB appears to have a dual function, acting both as an effector protein in the cytoplasm of the host cell and as a part of the translocation machinery (18, 21, 22, 26–28).

In a previous study, Savkovic et al. (29) showed induction of NF-κB DNA-binding activity in T84 intestinal epithelial cells infected with wild-type EPEC (E2348/69) using EMSA. In this study, we found a similar time course of NF-κB activation using HeLa cells with enhanced DNA-binding activity of NF-κB dimers detectable at 1 h postinfection and reduction in DNA binding at 3 h postinfection. In the report by Savkovic et al. (29), it was concluded that the low level of NF-κB-DNA complexes observed at 3 h simply reflects the termination of the EPEC-induced NF-κB activation. However, using prolonged infection periods (3–6 h), we found that the low level of NF-κB DNA-binding activity seen at these later time periods is a result of active suppression of NF-κB activation. This suppression is a property common to many bacteria producing A/E lesions, because prior exposure of HeLa cells to STEC, EHEC, or EPEC interfered with subsequent NF-κB activation by TNF-α, a well-known NF-κB inducer. The data obtained indicate a crucial role of EspB in NF-κB suppression, rather than in NF-κB activation as it was previously reported (29).

The NF-κB family of transcription factors form one of the first lines of defense against infectious disease and cellular stress by inducing the transcription of numerous genes involved in inflammatory and immune responses. Thus, we monitored the kinetics of expression and secretion of IL-8, IL-6, and IL-1α after infection of HeLa cells using wild-type STEC and mutant bacteria lacking EspB. The transcription of these ILs is thought to involve regulation by NF-κB-binding sites in the promoters of the respective genes (7, 29, 45–47). The chemokine IL-8 is a potent chemoattractant for polymorphonuclear cells and directs recruitment of these cells into the infected epithelium (6, 48). The proinflammatory cytokines IL-1α and IL-6 play a well-documented role in activation of the mucosal inflammatory response and are induced and secreted by epithelial cells in response to different enteric pathogens such as Salmonella spp. and Shigella spp. (3, 6, 49). However, unlike these pathogens, many A/E bacteria inhibit the release of proinflammatory cytokines by suppressing NF-κB activation. Thus, A/E-induced bacteria, such as STEC, behave like Yersinia spp. and comprise a family of pathogens with strong anti-inflammatory properties.

The results of this study are also in agreement with a recent study (50) examining the ability of a diverse group of STEC clinical isolates to induce polymorphonuclear cell transmigration across and IL-8 secretion from T84 intestinal cells. Of the 10 STEC strains investigated, three strains lacking EspB (espB) and the outer membrane adenine intimin (eae) significantly induced more neutrophil transmigration and higher IL-8 secretion than did their eae- and espB-positive STEC and EPEC counterparts (50). Our finding that STEC lacking EspB exhibits prolonged NF-κB DNA-binding compared with wild-type bacteria and consequently induced increased IL-8 expression and secretion could clearly account for these observations.

In summary, our data indicate that STEC, EHEC, and EPEC actively suppress DNA binding of NF-κB dimers. Because NF-κB plays an important role in the generation of inflammation and immune responses, it is likely that the bacteria-mediated suppression of NF-κB activation represents a strategy of holding host defense mechanisms at bay while a beachhead for colonization is established. Put another way, suppression of NF-κB DNA-binding activity appears to have evolved as a pathogenic mechanism permitting bacteria to both escape and counteract the ensuing host inflammatory and immune responses, while remaining extracellular to the infected host.

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