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Shigella flexneri Invasion of HeLa Cells Induces NF-κB DNA-Binding Activity

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Although information about the genetic basis and mechanisms of *Shigella flexneri* cellular invasion is accumulating, little is known about changes in cell signaling and their consequences following bacterium-host cell interactions. A general result of signal transduction is alterations in the levels and/or activities of transcription factors. Alterations in transcription factor binding activities were observed following challenge with *S. flexneri*. Changes in the DNA-binding activities of cellular transcription factors to AP1, AP2, cyclic AMP response element, CTF1/NF1, NF-κB/*Rel*, OCT1, and SP1 DNA-binding sites were investigated by electrophoretic mobility shift assays. NF-κB/*Rel* DNA-binding activity was enhanced more than 11-fold by cellular invasion; noninvasive *S. flexneri* strains induced low levels of κB DNA binding. Both subunits of the NF-κB transcription factor, p50 and p65, but not c-*Rel* (p85), are components of the κB DNA-binding activity. These data suggest that changes in cellular transcription factor binding activity are a consequence of *S. flexneri* invasion, and these changes could play a role in the initial host response or in the pathogenesis of the disease.

Shigella species are gram-negative enteric bacteria which cause diarrheal disease. Essential to the disease process are interactions between the bacterium and the epithelial cell which lead to bacterial entry, intracellular residence, and multiplication (26, 28). *Shigella* invasion occurs through an induced uptake mechanism and has been shown to require active participation by both the host and bacterial cells (17, 18). Viable cells and active bacterial metabolism are prerequisites for cellular invasion (17, 18), as formalin-fixed or kanamycin-treated bacteria do not invade cell monolayers. *Shigella* genes responsible for epithelial cell invasion (*ipaBCD* [6]) have been cloned and characterized. Active participation of host cells in the invasion process is required, as cells treated with agents which disrupt the cell cytoskeleton (18) or lead to depolarization of the cell membrane (18) are not invaded by shigellae. Recently, actin polymerization and myosin aggregation at contact points between bacteria and host cells have been described (9, 26). While information about the genetic basis and mechanism of cellular invasion is accumulating, little is known about cell signaling and its consequences which occur following bacterium-host cell interactions.

A variety of factors (mitogens, cytokines, and growth factors) initiate cellular signaling by binding specific transmembrane receptors (22, 33). Many receptors are associated with intracellular tyrosine kinase activity and initiate a succession of phosphorylations which activate plasma membrane-associated enzymes, such as phospholipase C and adenylate cyclase. The second messengers produced by these enzymes (Ca^{2+}, diacylglycerol, and cyclic AMP) activate cytoplasmic serine-threonine kinases. The precise linkage between cytoplasmic kinases and transcription factor activation is unknown but probably involves second messengers and phosphorylation (22). Phosphorylation is known to modulate the activity of the cyclic AMP response element binding protein (CREB), members of the *fos/jun* family (AP1), and NF-κB (4, 7, 22, 34). Activation of these transcription factors (AP1 and NF-κB) occurs posttranslationally in the presence of protein synthesis inhibitors (4, 22, 29, 30). Such preformed or primary transcription factors activate downstream genes, some of which encode secondary transcription factors which affect the expression of other target genes (22).

NF-κB is one of the best-described transcription factors and a member of the *rel* multigene family (34). NF-κB is a heterodimer of 50- and 65-kDa subunits and was originally defined as a positive regulator of the immunoglobulin kappa light-chain gene (7). In the majority of cells studied, NF-κB is found in an inactive form in the cytoplasm complexed to inhibitory protein I-κB (7, 34). Phosphorylation of I-κB by cellular kinases releases NF-κB, allowing its nuclear translocation and its binding to specific decameric DNA sequences called κB motifs, which can subsequently affect gene expression (7, 34).

A number of viruses are known to induce the DNA-binding activity of NF-κB and other *rel* family members, as well as several other transcription factors (1, 3, 8, 14, 25). Cellular infection by human immunodeficiency virus, human T-cell leukemia virus type 1, and human cytomegalovirus induces NF-κB DNA-binding activity (1, 3, 8). Human cytomegalovirus also increases the DNA-binding activity of CREB and AP1 (8). Little information on cellular signaling alterations following bacterial infection exists. To better understand the pathogenesis of *Shigella flexneri*, we examined alterations in the cellular signal transduction pathway following invasion. We investigated changes in the activity of cellular transcription factor DNA binding to transcription factor DNA-binding sites (AP1, AP2, CREB, CTF1/NF1, NF-κB, OCT1, and SP1) following infection of HeLa cells with *S. flexneri*.

MATERIALS AND METHODS

Bacterial strains. Invasive and noninvasive strains of *S. flexneri* were used. *S. flexneri* SA100 and SA100NI have

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been previously described (37). SA100 is the wild-type strain, and SA100NI is a noninvasive variant which fails to bind the dye Congo red (27) and has a large deletion in the 220-kb virulence plasmid (27). Tn*phoA* mutagenesis was performed with Tn*phoA* and *S. flexneri* SA100 harboring pTroy9 (11) to generate a library of mutants which will be described elsewhere. *S. flexneri* 28 harbors Tn*phoA* in the *ipaBCD* operon within the *ipaB* gene (5). This strain is Sereny negative and not invasive for epithelial cells in culture. Strains with the ability to bind Congo red (R) and mutants which lack this ability are noted when strains are described. Strains were maintained in 20% glycerol–L broth (Difco Laboratories, Detroit, Mich.) at −70°C. For HeLa cell invasion studies, strains were grown overnight in L broth and split 1/1,000 into brain heart infusion broth (Difco). Bacterial strains were grown to the mid-logarithmic phase (optical density at 600 nm, 0.6), washed, and suspended in RPMI 1640 supplemented with 0.67 μg of $FeCl_3$ per ml and 0.45% glucose as described by Oak et al. (35) in preparation for HeLa cell challenge.

Cell culture methods. HeLa cells were maintained as monolayers in supplemented RPMI 1640 (5% fetal bovine serum, 2 mM glutamine, 50 U of penicillin G per ml, 50 μg of streptomycin per ml) in a 5% CO_2 humidified atmosphere. Cells were trypsinized and then seeded into 24-well plates at 2×10^5 per well. This cell number gave a nearly confluent monolayer of HeLa cells in 24 h. Antibiotic-free medium with the same composition was used to wash monolayers prior to invasion.

Invasion of HeLa cell monolayers. Bacterial strains were diluted into RPMI lacking antibiotics to a concentration of 8×10^6 CFU/ml. Medium was removed from HeLa cell monolayers and replaced with 500 μl of the bacterial suspension. The plates were then centrifuged for 10 min at 200 × *g* in a Beckman TJ-6 centrifuge and then incubated at 37°C for 0.5 h. Noninternalized bacteria were removed by aspiration, and monolayers were washed five times with wash medium (67% Hanks balanced saline solution, 33% RPMI 1640 supplemented with 5% fetal bovine serum, 50 μg of either kanamycin or gentamicin per ml). Plates were gently shaken for 1 min between washes.

Noninternalized bacteria were counterselected by replacing the final wash solution with RPMI–0.67 μg of $FeCl_3$ per ml–0.45% glucose supplemented with 50 μg of either gentamicin or kanamycin per ml and 5% fetal bovine serum. The plates were then incubated at 37°C; at 75 min postchallenge, cells were scraped from the wells and washed with phosphate-buffered saline (PBS); and nuclear extracts were prepared as described below.

Nuclear protein extraction. Nuclear proteins were extracted from HeLa cell cultures as described by Schreiber et al. (41). HeLa cell monolayers (2×10^5 cells per well) cultured in 24-well plates were washed once with PBS. Cells were collected by scraping in PBS and centrifuged at 430 × *g* for 5 min. Cells were resuspended in 1 ml of PBS, transferred to microcentrifuge tubes, and centrifuged for 15 s. Cell pellets were suspended in 400 μl of ice-cold buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-NaOH [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid [EGTA], 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and allowed to swell for 15 min on ice. The cells were then lysed by addition of 50 μl of 10% Nonidet P-40 with vigorous vortexing for 10 s. Nuclear pellets were collected by microcentrifugation for 30 s at 4°C and suspended in 100 μl of buffer B (20 mM HEPES-NaOH [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). The suspended nuclear pellet was vigorously shaken on a rotary platform for 15 min at 4°C. The supernatants containing nuclear proteins were separated from the pellet by microcentrifugation at 4°C for 5 min and stored in 25% glycerol at −20°C. Protein concentrations were determined by the Micro BCA protein assay kit (Pierce, Rockford, Ill.) against a bovine serum albumin standard curve.

Electrophoretic mobility shift assay (EMSA). Seven oligonucleotides representing different transcription factor-binding sites (AP1, AP2, CREB, CTF/NF1, NF-κB, OCT1, and SP1) were radiolabeled with [γ-^{32}P]ATP by T4 polynucleotide kinase in accordance with the manufacturer's (Promega, Madison, Wis.) directions. The gel shift reactions contained 10 μg of nuclear protein extract, 4.0 μl of 5× binding buffer [20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.25 μg of poly(dI-dC) per μl, and 35 fmol of radiolabeled oligonucleotide in distilled water to a total volume of 20 μl. Extracts from mock- or *S. flexneri*-challenged HeLa cells were preincubated for 10 min in binding buffer, after which radiolabeled oligonucleotide was added and incubation was continued for another 20 min. For competition assays, a 25-fold molar excess of unlabeled CTF1, NF-κB, OCT1, and SP1 or a 50-fold molar excess of unlabeled AP1, AP2, and CREB oligonucleotide was added to the reaction mixture and incubated for 20 (CTF1, NF-κB, OCT1, and SP1) or 30 (AP1, AP2, and CREB) min with the nuclear extracts in binding buffer prior to addition of the corresponding radiolabeled oligonucleotide. For supershift assays, 500 ng of a specific NF-κB p65 or p50 or c-*Rel* affinity-purified rabbit polyclonal antibody was incubated with 10 μg of the protein extract for 30 min prior to incubation in binding buffer with radiolabeled NF-κB oligonucleotide. For blocking, 500 ng of the antibody (p65, p50, or c-*Rel*) and 1 μg of the cognate peptide were incubated at room temperature for 30 min and then incubated with the nuclear extracts in binding buffer for 20 min before addition of the κB probe and continued incubation for 20 min. Binding reactions were resolved on 4% high-ionic-strength nondenaturing polyacrylamide gels by using 1× Tris-borate-EDTA (TBE) buffer. Gels were dried at 75°C and exposed to Kodak XAR film with intensifying screens.

Antibodies to p50, p65, and c-*Rel* were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). The NF-κB p65 antibody is directed against a peptide within the amino-terminal *Rel*-related region of human NF-κB p65. The NF-κB p50(NLS) antibody is directed against a peptide encompassing part of the basic nuclear localization sequence and the N-terminal adjacent 11 amino acids of the p105 precursor of human NF-κB p50. The p50(NLS) antibody does not react with the p105 precursor protein by immunoprecipitation or immunoblotting. The c-*Rel* antibody was raised against a synthetic peptide corresponding to amino acids 498 to 517 within the divergent carboxyl-terminal domain of the predicted sequence of the murine c-*Rel* protein. The murine c-*Rel* antibody also reacts with the human c-*Rel* protein (Santa Cruz Biotechnology).

Densitometry. Autoradiograms were scanned with an Applied Imaging Systems Densitometer (Santa Clara, Calif.). Values are recorded as optical densities (density per unit area). Several scans were performed on different exposures to ensure linearity of the exposed film.

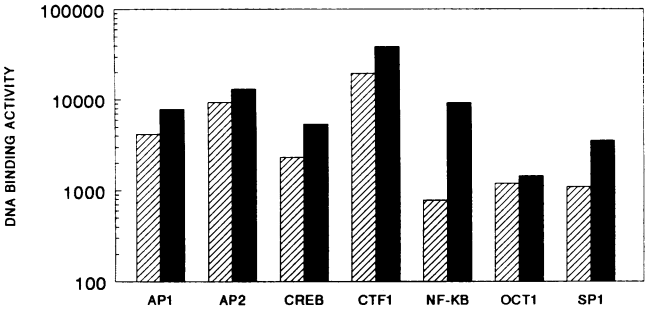

FIG. 1. Summary of the DNA-binding activities of seven transcription factors. Radiolabeled oligonucleotides corresponding to the DNA-binding sites for seven transcription factors (AP1, AP2, CREB, CTF1/NF1, NF-κB, OCT1, and SP1) were combined with 10 μg of crude nuclear protein extracts from mock- or *S. flexneri* SA100-challenged HeLa cells taken at 75 min postchallenge in separate gel shift assays. The amount of DNA-binding activity was determined by densitometric scanning of EMSA autoradiograms. Binding activities of mock-infected controls and infected cells are indicated by hatched and solid bars, respectively. Binding activity is given in optical density units per square millimeter.

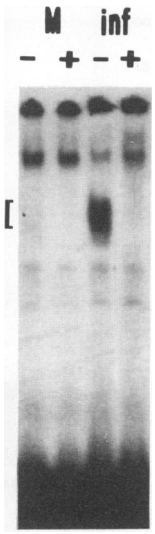

FIG. 2. Gel shift analysis of κB DNA-binding activity. A radiolabeled oligonucleotide corresponding to the κB DNA-binding element of the murine kappa light-chain gene enhancer (5′-AGTT GAGGGGACTTTCCCAGGC-3′) was combined with 10 μg of crude nuclear protein extract from mock- or *S. flexneri* SA100-challenged HeLa cells taken at 75 min postchallenge. The results of densitometric scanning and analysis of these data are shown in graphic form in Fig. 1. The specificity of the DNA-protein complexes formed was verified by competition with a 25-fold molar excess of unlabeled κB oligonucleotide. M, nuclear extracts from mock-challenged cultures; Inf, nuclear extracts from *S. flexneri* SA100-challenged cultures; competition within the EMSA with (+) or without (−) the unlabeled competitor κB oligonucleotide is indicated. The bracket to the left indicates the specific κB shifted complexes consisting of labeled κB oligonucleotide and κB-binding proteins.

RESULTS

***S. flexneri* infection alters the DNA-binding activities of several transcription factors.** We investigated whether *S. flexneri* invasion induces changes in the activities of transcription factors which could induce alterations in HeLa cell gene expression. HeLa cell monolayers were either mock infected or infected with *S. flexneri* SA100. At 75 min postchallenge, nuclear protein extracts were prepared and analyzed for the levels of cellular transcription factor DNA-binding activity by using a panel of radiolabeled oligonucleotides corresponding to the DNA-binding sites of seven transcription factors. Analysis was done by EMSA.

The results of EMSA analyses are presented in Fig. 1 as the amount of specific DNA-binding activity in *S. flexneri*-challenged cells (solid bars) compared with that in the mock-challenged control (hatched bars) at 75 min postchallenge. Little DNA-binding activity was observed at times earlier than 35 min (data not shown). Formation of these DNA-transcription factor complexes was inhibited by excess unlabeled cognate oligonucleotide (Fig. 2 and data not shown). *S. flexneri* SA100 enhanced the transcription factor DNA-binding activity to each of the seven transcription factor DNA-binding sites: that of AP1 was increased 1.9-fold; that of AP2 was increased 1.4-fold; that of CREB was increased 2.3-fold; that of CTF1 was increased 2-fold; that of OCT1 was increased 1.2-fold; and that of SP1 was increased 3.3-fold (Fig. 1). The most dramatic change in transcription factor DNA-binding activity occurred with the κB-binding site. As observed in previous studies with unactivated cells (4), a low level of nuclear κB DNA-binding activity was observed in mock-challenged control cultures (Fig. 2). The amount of κB-bound probe increased to greater than 11-fold over the mock-challenged control at 75 min postchallenge. In other experiments, this increase ranged from 7- to 11-fold. Specificity of protein binding to the oligonucleotide was demonstrated by using unlabeled κB competitor oligonucleotide at a 25-fold molar excess to compete for binding (Fig. 2).

Invasion is necessary for the *S. flexneri*-induced increase in NF-κB DNA binding. Both noninvasive and invasive strains of *S. flexneri* were used to determine whether cellular invasion by the bacteria was a prerequisite for the increase in κB DNA-binding activity. HeLa cell monolayers were challenged with the *S. flexneri* strains, and nuclear protein extracts were prepared 75 min after challenge. Extracts were assayed for κB-binding activity in an EMSA (Fig. 3A). The ability to induce κB-binding activity correlated with invasion (Fig. 3). Noninvasive *S. flexneri* SA100NI and 28R (noninvasive *ipaB* mutant) induced levels of κB binding activity slightly greater than that of mock-challenged cells. SA100NI induced 1.5-fold more κB binding activity in HeLa cells than in mock-challenged cells, and 28R induced 1.8-fold more activity (Fig. 3B). However, invasive *S. flexneri* SA100 induced κB binding activity sevenfold higher than that of mock-challenged cells and almost fourfold than that induced by the noninvasive 28R mutant (Fig. 3B).

The κB-binding complex contains the p65 and p50 subunits of NF-κB but not c-*Rel*. A variety of cellular transcription factor proteins are capable of binding to the κB DNA element (4). Furthermore, members of the *Rel* family of proteins can form homodimers and heterodimers (4). To characterize the protein composition of the κB-binding complexes, we utilized three affinity-purified antibodies raised against different *Rel* family member proteins in EMSAs. The nuclear extracts from HeLa cell monolayers that were mock challenged or challenged with *S. flexneri* SA100 were incubated with a specific polyclonal antibody to either p65 or c-*Rel* prior to addition of the radiolabeled κB oligonucleotide. Figure 4 shows that infected cells had enhanced levels of κB DNA-binding activity. The p65 antibody, but not the c-*Rel* antibody, reacted with a component of the κB complex

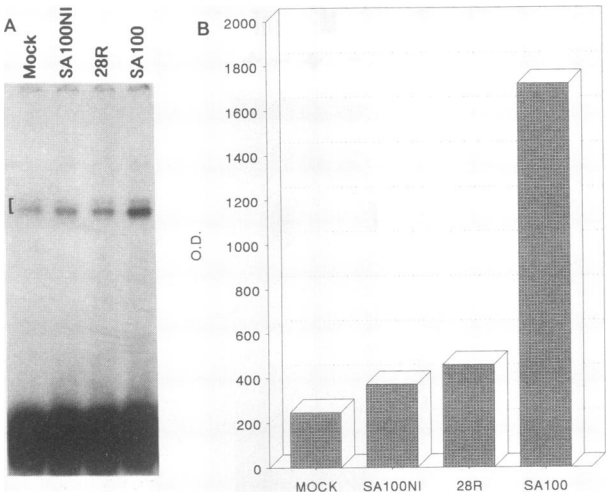

FIG. 3. *S. flexneri* invasion of HeLa cells is a prerequisite for increases in κB DNA-binding activity. Nuclear extracts from mock-challenged HeLa cells or HeLa cells challenged with *S. flexneri* SA100, SA100NI, and 28R were collected 75 min postchallenge and analyzed for κB DNA-binding activity by gel shift assays with the κB probe. The autoradiogram of the gel shift assay is shown in panel A, and the data are presented in graphic form in panel B. The bracket in panel A indicates the specific shifted κB oligonucleotide with bound κB DNA-binding proteins. O.D., optical density of the shifted oligonucleotide band in the autoradiogram.

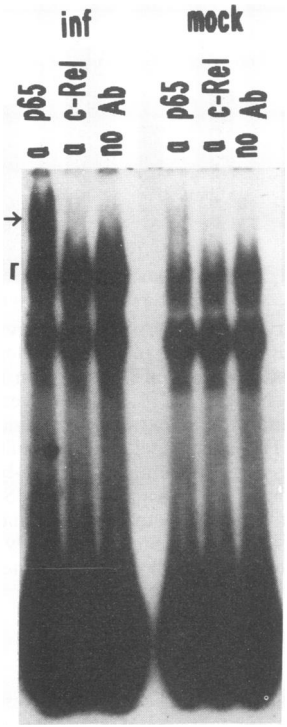

FIG. 4. NF-κB p65, but not c-*Rel*, is part of the κB DNA-protein complex. Nuclear protein extracts from mock- or *S. flexneri* SA100-challenged HeLa cells were used in a gel shift assay containing the κB probe. Each extract was also preincubated for 20 min with antibodies (Ab) against peptide components of either p65 or c-*Rel* before probe addition. Inf, extracts from SA100-challenged HeLa cells; Mock, extracts from mock-challenged HeLa cells. The arrow indicates the supershifted bands consisting of the κB oligonucleotide, κB DNA-binding proteins, and antibodies, and the bracket indicates shifted κB complexes consisting of κB oligonucleotide and κB DNA-binding proteins.

to produce a larger and more slowly migrating (supershift) species. The c-*Rel* antibody has been shown to produce a supershift in κB DNA-protein complexes derived from mouse pre-B-cell line 70Z/3 (11a). A detectable supershift that reflects the low amount of κB-binding activity was observed with anti-p65 in mock-challenged cultures. Figure 5 shows the specificity of anti-p65 binding. Extracts from infected or mock-infected cells incubated with anti-p65 prior to addition of the κB probe produced a supershift (compare lanes 1 and 2 with lanes 3 and 4). Anti-p65 blocked with the cognate peptide prior to addition of the extracts and the κB probe prevented the supershifts (lanes 7 and 8). The antibodies to p65 and c-*Rel* did not bind the free oligonucleotide in the absence of extract (lanes 5 and 6, respectively); therefore, NF-κB p65 must be present in the κB DNA-protein complex. To confirm that the κB-binding activity detected in HeLa cells was NF-κB, we investigated whether the p50 protein was also present in the κB DNA-binding complexes (Fig. 6). Anti-p50-specific antibody induced shifts of the κB complexes in both mock- and SA100-challenged nuclear extracts. Formation of the supershift was inhibited by preincubation of anti-p50 with the cognate peptide. Therefore, both p65 and p50 were shown to be present in the κB DNA-binding complexes. These data establish NF-κB as a major component of the κB DNA-binding activity present in mock- or *S. flexneri*-challenged HeLa cells.

DISCUSSION

In this study, we showed alterations of cellular transcription factor DNA-binding activity as a consequence of *S. flexneri* challenge of HeLa cells. These alterations appear to require cellular invasion, as mutant *Shigella* strains defective in epithelial cell invasion fail to alter transcription factor DNA-binding activity. The inability of *S. flexneri* to bind Congo red has been correlated with loss of epithelial cell invasiveness and with a large deletion in the 220-kb virulence plasmid (27). Noninvasive *Shigella* strain SA100NI does not bind Congo red and did not dramatically alter cellular transcription factor DNA-binding activity. Virulence has been shown to require the plasmid-encoded *ipaBCD* operon (6). The products of *ipaB* and *ipaC* are required for epithelial cell invasion (6). Strain 28R harbors Tn*phoA* in *ipaB* (5), fails to invade epithelial cells in culture, and also fails to dramatically alter transcription factor DNA-binding activity. Thus, cellular invasion by *S. flexneri* appears to be a prerequisite for considerable changes in transcription factor DNA-binding activity.

It is well established that the primary mode of gene regulation in eukaryotic cells is at the level of transcription (30). Numerous transcription factors and their binding sites have been identified and characterized (30). Eukaryotic promoters and enhancers consist of multiple binding sites for different cellular transcription factors. A number of stimuli have been shown to alter transcription factor DNA-binding activity through activation of cell signaling pathways (22, 33). Infection of cells by a variety of viruses (cytomegalovirus, herpes simplex virus type 1, human immunodeficiency virus type 1, human T-cell leukemia virus type 1, hepatitis B virus, adenovirus, and simian virus 40) results in activation of cellular transcription factors (1, 3, 4, 8, 14, 19, 25). Cellular transcription factors are key regulators of viral gene

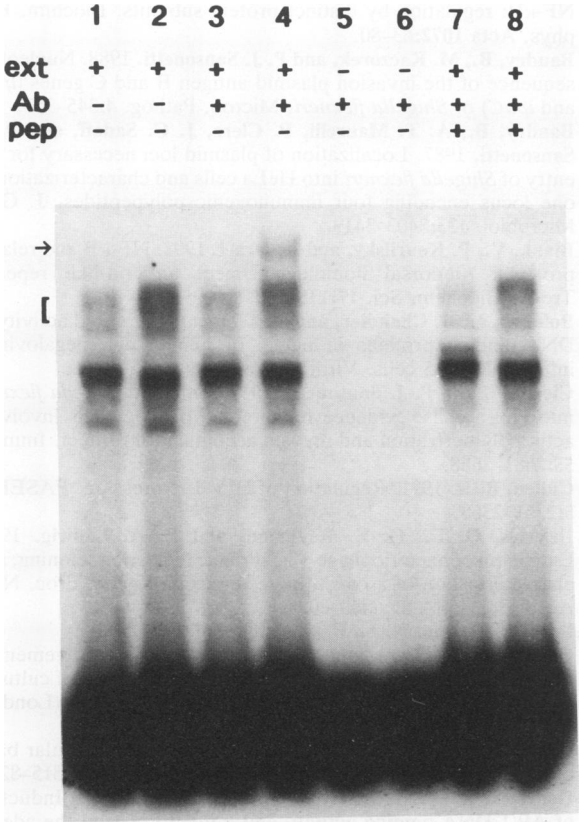

FIG. 5. The anti-p65–κB DNA-protein interaction is blocked by a cognate peptide. Nuclear extracts isolated from mock- or *S. flexneri* SA100-challenged HeLa cells were used in gel shift assays with the κB probe. The κB probe was incubated with extracts alone (lanes 1 and 2), p65 or c-*Rel* antibodies alone (lanes 5 and 6, respectively), anti-p65 and extracts (lanes 3 and 4), or anti-p65 blocked with its cognate peptide and extracts (lanes 7 and 8). Lanes 1, 3, and 7 contained extract from mock-challenged HeLa cells, and lanes 2, 4, and 8 contained extract from *S. flexneri* SA100-challenged HeLa cells. ext, extract; Ab, specific antibodies (lane 5, anti c-*Rel*; lanes 3, 4, 6, 7, and 8, anti-p65); pep, incubation of the p65 antibody with its cognate peptide prior to extract and probe addition. The arrow and bracket indicate the supershifted and shifted oligonucleotide complexes as previously described.

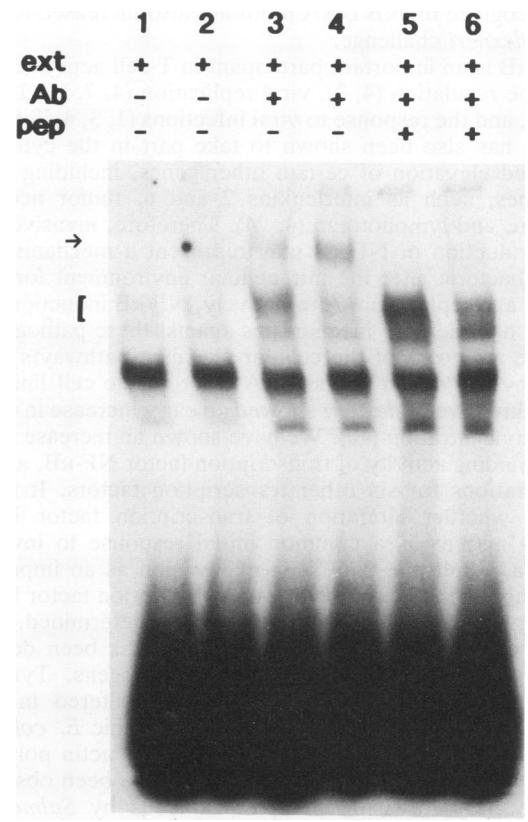

FIG. 6. NF-κB p50 is part of the κB DNA-protein complex. Nuclear protein extracts from mock- and *S. flexneri* SA100-challenged HeLa cells (75 min postchallenge) were used in a gel shift assay containing the κB probe. Lanes 1, 3, and 5 contained nuclear extract from mock-challenged HeLa cells. Lanes 2, 4, and 6 contained nuclear extract from *S. flexneri* SA100-challenged HeLa cells. Ext, extract; Ab, peptide antibody against the p50 subunit of NF-κB; pep, incubation of the p50 antibody with its cognate peptide prior to extract and probe addition. The arrow and bracket indicate the supershifted and shifted oligonucleotide complexes as previously described.

expression which can influence viral replication (10, 13, 16, 19, 21, 23, 31, 42). Thus, changes in transcription factor activity can lead to cellular changes which permit or enhance viral growth and replication. Alternatively, cellular transcription factors are participants in the host response to infection. The promoters of genes important in the immune response, like tumor necrosis factors α and β, interleukins 2 and 6, and interferon β, contain binding domains for a number of transcription factors, including NF-κB (32, 38).

Alterations in the DNA-binding activities of all seven cellular transcription factors were observed following bacterial invasion. It is unknown whether these alterations influence bacterial invasion, intracellular growth, and/or replication. Recent studies have documented the induction of tumor necrosis factor α following *Salmonella* invasion (2) and the induction of interferon production by fibroblasts infected with salmonellae or shigellae (20). It is intriguing to speculate that cytokine induction results from bacterium-induced transcription factor activity. Thus, transcription factor activation may play an important role in cellular responses to facultative intracellular bacteria.

Among the transcription factor DNA-binding activities examined, κB DNA-binding activity was increased to the highest levels following *S. flexneri* challenge. NF-κB was originally described as a B-cell-specific transcription factor that bound to the immunoglobulin kappa light-chain gene enhancer (7, 24). However, increases in κB DNA-binding activity have been demonstrated in most cell types in response to cytokines, phorbol esters, double-stranded RNA, UV irradiation, and viral infection (4). NF-κB is a heterodimer composed of 50- and 65-kDa protein subunits (4, 7, 24, 34). The increase in κB DNA-binding activity could be due to an increase in the amount or activity of any member of the *Rel* gene family (e.g., c-*Rel*, NF-κB [p50-p65], *relB*, KBF1 [p50-p50]) or transcription factors (EBP-1, MBP-2, PRDII-BF1, or KBF2), all of which recognize similar DNA motifs (4). By using antibodies specific for NF-κB p50 or p65 and c-*Rel*, we established that the increase in κB DNA-binding activity following *S. flexneri* challenge was mediated, at least in part, by p50 and p65 but not by $p85^{c\text{-}Rel}$. It remains to be determined whether other transcription factors

that recognize the κB DNA motif are also increased following *S. flexneri* challenge.

NF-κB is an important participant in T-cell activation (4), cytokine regulation (4, 7), viral replication (4, 7, 10, 19, 23, 31, 42), and the response to viral infections (1, 3, 4, 7, 8, 19). NF-κB has also been shown to take part in the cytokine-mediated elevation of certain other genes, including other cytokines, such as interleukins 2 and 6, tumor necrosis factor α, and lymphotoxin (4, 24). Therefore, invasive bacterial induction of NF-κB may represent a mechanism by which bacteria alter the intracellular environment for their growth and replication. Alternatively, NF-κB induction may initiate host defense mechanisms against these pathogens.

Little is known of the cellular signaling pathway(s) activated by *S. flexneri* invasion. A macrophage cell line harboring invasive *S. flexneri* showed an early increase in cyclic AMP concentration (40). We have shown an increase in the DNA-binding activity of transcription factor NF-κB, as well as alterations for six other transcription factors. It is not known whether alteration of transcription factor DNA-binding activity is a common initial response to invasive bacteria. While the data support invasion as an important prerequisite, the mechanism(s) of transcription factor DNA-binding activity alterations remains to be determined. Activation of cellular signalling components has been demonstrated for other invasive bacterial pathogens. Tyrosine phosphorylation has been shown to be altered in cells infected by salmonellae and enteropathogenic *E. coli* (12, 39). Intracellular calcium mobilization (15), actin polymerization, and myosin accumulation have also been observed (9). Recently, invasion of epithelial cells by *Salmonella typhimurium* was shown to require activation of phospholipase A_2 and subsequent production of leukotriene D_4 (36). Addition of leukotriene D_4 stimulated the internalization of an invasion-defective mutant of *S. typhimurium* (36). We are investigating the molecular mechanisms and the components of the signal transduction pathways induced by invasion by *S. flexneri* and other invasive bacteria.

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