Protective Role of Raf-1 in *Salmonella*-induced Macrophage Apoptosis

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Abstract

Invasive *Salmonella* induces macrophage apoptosis via the activation of caspase-1 by the bacterial protein SipB. Here we show that infection of macrophages with *Salmonella* causes the activation and degradation of Raf-1, an important intermediate in macrophage proliferation and activation. Raf-1 degradation is SipB- and caspase-1–dependent, and is prevented by proteasome inhibitors. To study the functional significance of Raf-1 in this process, the *c-raf-1* gene was inactivated by Cre-*loxP*–mediated recombination in vivo. Macrophages lacking *c-raf-1* are hypersensitive towards pathogen-induced apoptosis. Surprisingly, activation of the antiapoptotic mitogen-activated protein kinase kinase (MEK)/extracellular signal–regulated kinase (ERK) and nuclear factor κB pathways is normal in Raf-1–deficient macrophages, and mitochondrial fragility is not increased. Instead, pathogen-mediated activation of caspase-1 is enhanced selectively, implying that Raf-1 antagonizes stimulus-induced caspase-1 activation and apoptosis.

Key words: serine/threonine kinase • cell death • bacteria • proteases • monocytes/macrophages

Introduction

Salmonellae are facultative intracellular pathogens that cause a variety of enteric diseases, ranging from self-limiting gastroenteritis (mainly due to *Salmonella typhimurium*) to the more severe systemic typhoid fever (caused by *Salmonella typhi*). After consumption of contaminated food or water, *Salmonellae* reach the intestine and adhere to specialized epithelial cells (M cells). *Salmonella* exploits the host signal transduction cascades to induce the formation of membrane ruffles at the contact point between the bacterium and host cell, and is ultimately taken up in large vacuoles (1). Once the infected M cells are destroyed, the bacteria reach the mesenteric lymph follicles and are confronted by the host's macrophages.

For *Salmonella*, and for many other facultative intracellular pathogens, surviving this encounter is the key to a successful infection. Invasive *Salmonella* can persist within the macrophages in spacious vacuoles, which do not acquire lysosomal markers and may represent a relatively safe intracellular site where the bacteria can survive and multiply (2–4). Furthermore, invasive *Salmonella* induces phagocyte apoptosis in vitro (5–8) and in vivo (9). Apoptosis is mediated by a cell-intrinsic suicide program, the activation of which is regulated by different signals originating from both the intracellular and extracellular milieu. *Salmonella* shares the ability of inducing macrophage apoptosis with *Yersinia* (10, 11) and *Shigella* (12), suggesting that this may represent a hallmark of, and perhaps a selective advantage for, the establishment of enterobacterial infections.

Both epithelial cell invasion and the induction of macrophage apoptosis depend on a functional type III secretion system. Type III secretion systems translocate bacterial proteins directly into host cells and play a pivotal role in the interaction between pathogenic bacteria and their hosts (13). The type III secretion genes essential for epithelial cell invasion and apoptosis induction are clustered at centisome 63 of the *Salmonella* chromosome, in a region denominated *Salmonella* pathogenicity island (SPI)[1]-1. The SPI-1–encoded

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[1]*Abbreviations used in this paper:* ERK, extracellular signal–regulated kinase; ES, embryonic stem; floxed, flanked by *loxP* sites; HMF, heavy membrane fraction; IAP, inhibitor of apoptosis; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK or ERK kinase; moi, multiplicity of infection; NF, nuclear factor; poly I:C, poly inosinic:cytidyllic acid; Q/W, quenching/washing solution; RT, room temperature; SPI, *Salmonella* pathogenicity island; wt, wild-type.

bacterial proteins interact directly with eukaryotic signal transducers to activate signaling pathways of host epithelial cells (14). A functional type III secretion system is a prerequisite for the activation of the mitogen-activated protein kinase (MAPK) subgroups extracellular signal–regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38, and for the production of proinflammatory cytokines by epithelial cells infected with *Salmonella* (15).

The molecular mechanisms operating during the interaction of *S. typhimurium* with macrophages are less well characterized than those accompanying epithelial cell invasion. The SPI-1–encoded protein SipB has been shown to bind to and activates caspase-1, thereby causing apoptosis (16). Furthermore, we have previously addressed the question of *Salmonella*-mediated ERK (17) and JNK stimulation (18). In both cases, the mechanisms of activation used by the pathogen differed radically from those operating in epithelial cells and were not dependent on the function of the SPI-1–encoded type III secretion system (17, 18).

The Raf-1 kinase plays a key role in relaying proliferation signals but has been also implicated in inflammatory signaling induced by LPS (19, 20). Furthermore, depending on the cell type and the stimulus used, Raf-1 can exert a proapoptotic (21–23) or an antiapoptotic function (24–29). In this study, we show that Raf-1 is activated and degraded during infection with apoptosis-inducing *Salmonella*. Raf-1 degradation is a consequence of apoptosis induction, but kinase activation is not. Remarkably, Raf-1–deficient macrophages are hypersensitive towards pathogen-induced apoptosis, and this hypersensitivity correlates with enhanced caspase-1 activation. These data imply that Raf-1 activation upon infection with invasive *Salmonella* is part of the defensive response of the cells to the pathogen, and demonstrate for the first time that the Raf-1 kinase plays a role in antagonizing caspase-1 activation during pathogen-induced apoptosis.

Materials and Methods

Bacteria. *S. typhimurium* strains SR11, SL1344, LT2 (wild-type [wt]), SB111 (*invA*$^-$), and SB169 (*sipB*$^-$) were grown in 5 ml Luria-Bertani broth (1% Bacto Tryptone, 0.5% yeast extract, and 1% sodium chloride) at 37°C overnight for 16–20 h under agitation. To obtain highly invasive bacteria, overnight cultures were diluted to an OD_{600} of 0.02 in 50 ml TYP broth (1.6% Bacto Tryptone, 1.6% yeast extract, 0.5% sodium chloride, 0.25% dipotassium phosphate) and incubated for 5 h under agitation (8).

Conditional Inactivation of the c-raf-1 Gene. A genomic DNA clone corresponding to *c-raf-1* was isolated from a 129/Sv mouse genomic λ fix library. A 8.5-kb 5′-XbaI/BglII-3′ fragment containing exon 3 and surrounding sequences was used to assemble the targeting construct in pBSIISK$^-$. *loxP* sites were inserted as double-stranded oligonucleotides in the HindIII site 3′ of exon 3 and in the BamHI site 5′ of exon 3. The *loxP* oligonucleotide 3′ of exon 3 contains a PstI site that serves as a marker for the floxed (flanked by *loxP* site) allele. A *Neo/TK* cassette containing an upstream *lox*P site was excised from plasmid pGH1 and cloned into an XbaI-HindIII site contained in the *loxP* site 5′ of exon 3. E14.1 embryonic stem (ES) cells grown on γ-irradiated embryonic fibroblasts were transfected with AscI-linearized targeting vector and selected with 0.2 mg/ml G418. Homologous recombinants (*c-raf-1*$^{+/\text{flox}Neo/TK}$) were obtained with a frequency of 1 in 35, as detected by nested PCR and Southern blot analysis. Positive clones were transfected with a plasmid expressing the Cre recombinase (30). Cre expression led to the deletion of either the floxed exon 3 or the floxed *Neo/TK* cassette, or both. The latter two were enriched by negative selection with gancyclovir. Two clones harboring the floxed exon 3 (*c-raf-1*$^{flox/+}$) were injected into C57BL/6 blastocyst-stage embryos and transferred to pseudopregnant B/6CBAF1 mice for further development. Chimeric mice were mated to C57BL/6 animals and agouti offspring were genotyped. Germline transmission of the floxed allele was detected either by Southern blot or PCR analysis of tail DNA. *c-raf-1*$^{flox/flox}$ mice were mated to mice expressing Cre under the control of the inducible Mx1 promoter (31).

Cell Culture and Infection. Bone marrow–derived macrophages were isolated from Mx-Cre; Raf$^{flox/flox}$ mice and Raf$^{flox/flox}$ mice after induction of Cre expression by poly inosinic:cytidyllic acid (poly I:C) treatment in vivo (400 μg intraperitoneally, every other day, three injections total) or from caspase-1–deficient (32) and wt C57BL/6 mice. 2 d after the last injection, the bone marrow cells were collected and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 20% L-conditioned medium as a source of CSF-1 for 1 wk. Confluent cells (~5×10^6 cells per 100-mm-diameter tissue culture dish) were cultured for 16–20 h in medium without CSF-1 and then infected with bacterial cultures. A multiplicity of infection (moi; bacteria per macrophage) of 25 was used unless indicated otherwise. MAPK or ERK kinase (MEK) activation was inhibited by overnight pretreatment with 50 μM PD098059 (Calbiochem). Proteasome function was inhibited by pretreatment with 10 μM MG-115 and MG-132 for 90 min (Calbiochem).

Cell Lysis, Immunoprecipitation, and Western Blot Analysis. Cells from one 100-mm-diameter cell culture dish were washed twice with PBS and lysed in 300 μl solubilization buffer (10 mM Tris-HCl, pH 7.0, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 1% Triton X-100). Insoluble material was removed by centrifugation at 20,000 *g* for 30 min before immunoprecipitation. A rabbit polyclonal antiserum raised against a COOH-terminal peptide of *v-raf* (SP63, CTLTTSPRLPVF) was used to immunoprecipitate Raf-1. Immunocomplexes were collected after incubation with Protein A–Sepharose beads (Sigma-Aldrich). For Western blot analysis, cell lysates (30 μg/lane) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. After 1 h blocking in TTBS (10 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 0.1% Tween 20) supplemented with 5% milk powder, the membranes were probed with the appropriate primary antibodies (CSF-1R, rabbit serum generated against GST-CSF-1R [1-313] fusion protein; actin, caspase-1, -2, -3, and IκB, all from Santa Cruz Biotechnology, Inc.; caspase-8, Chemicon; cytochrome-c, BD PharMingen; cytochrome-c-oxidase-subunit IV, Molecular Probes; MEK-1 and panErk, Transduction Laboratories; pMAPK, New England Biolabs, Inc.; Raf-1 [NH_2-terminal], Transduction Laboratories; and Raf-1 kinase domain [33] diluted in 1% BSA [fraction V, Sigma-Aldrich]) in TTBS before incubation with peroxidase-conjugated secondary antibodies and detection by an enhanced chemiluminescence system (Pierce Chemical Co.).

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared as described previously (34). In brief, 2×10^6 cells, either untreated or infected with *S. typhimurium*, were washed twice with PBS and resuspended in

400 μl of buffer A (10 mM Hepes, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 1 mM PMSF). After incubation on ice for 5 min, NP-40 was added to a final concentration of 0.6%. Nuclei were pelleted and the cytoplasmic proteins were carefully removed. The nuclei were then resuspended in buffer C (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM PMSF). After 30 min at 4°C, the samples were centrifuged and the nuclear proteins in the supernatant were transferred to a fresh vial. 10 μg of nuclear extract was incubated with an end-labeled, double-stranded NF-κB–specific oligonucleotide probe (5-AATTCGGCTTGGAAATTCCCCGAGCG-3). As specificity controls, extracts were incubated with unlabeled wt or mutated (5-AGCTTAGATTTTACTTTCCGAGAGGA-3) probe before the addition of labeled oligo. The binding reaction was performed in a total of 20 μl of binding buffer (5 mM Hepes, pH 7.9, 50 mM KCl, 0.5 mM dithiothreitol, 1 μg poly [dI:dC], and 10% glycerol) for 20 min at room temperature (RT). After incubation, samples were fractionated on a 5% polyacrylamide gel and visualized by autoradiography.

Subcellular Fractionation. Cells were scraped in Mito buffer (250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10 μM PMSF, 1× protease inhibitor cocktail [Boehringer]). After incubation on ice for 30 min, cells were disrupted at 4°C in a 1-ml syringe fitted with a 25G hypodermic needle (15 strokes). Nuclei and unbroken cells were removed by centrifugation at 700 *g* for 5 min at 4°C. Supernatants were then further centrifuged at 13,000 *g* for 20 min at 4°C. The resulting pellet was defined as the heavy membrane fraction (HMF).

Chromatin Staining. 3×10^5 macrophages were seeded on a coverslip in a well of a 6-well cell culture dish. Chromatin condensation in infected macrophages was determined by staining with 0.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 1 min. Cells were washed twice with PBS and fixed with 3% formaldehyde in PBS (10 min at RT). Coverslips were mounted in 20% Mowiol (Sigma-Aldrich) in PBS. Chromatin condensation was assessed in randomly chosen areas of the sample by independent experimenters (300–500 cells/sample).

Mitochondrial and Raf-1 Staining. 3×10^5 macrophages were seeded on a coverslip in a well of a 6-well cell culture dish. Mitochondria were stained with 50 nM Chloromethyl-X-Rosamine (CM-X-ROS, Mitotracker red; Molecular Probes) for 10 min, a potential-sensitive fluorochrome that withstands fixation and permeabilization of cells (35). After infection, cells were permeabilized with 0.01% Triton X-100 (Pierce Chemical Co.) for 1 min and fixed in 4% paraformaldehyde. Fixed cells were washed with quenching/washing solution (Q/W: 50 mM NH_4Cl, 10 mM Pipes, pH 6.8, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, 5 mM $MgCl_2$) and incubated with primary Raf-1 antibodies (anti-

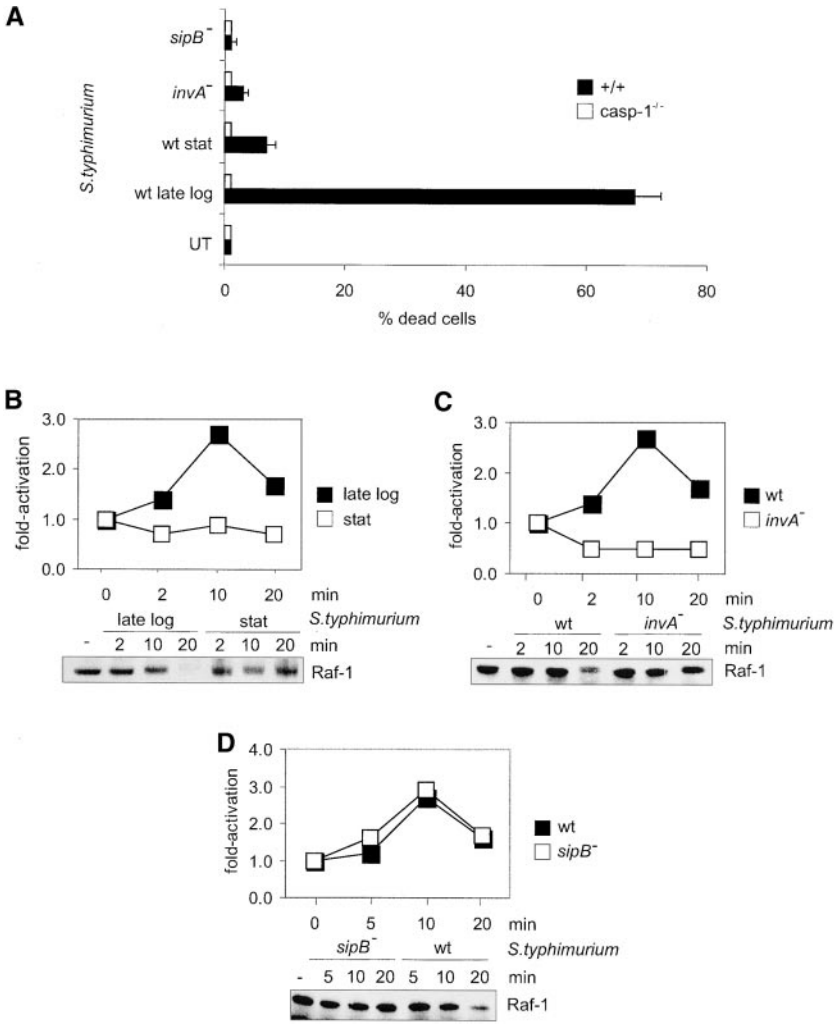

Figure 1. Activation of Raf-1 by *Salmonella* requires the type III secretion system, but is not a consequence of apoptosis. (A) Quiescent control (+/+, black bars) or caspase-1–deficient bone marrow-derived macrophages (casp-$1^{-/-}$, white bars) were infected with wt *Salmonella* in the late logarithmic (wt late log) or in the stationary phase (wt stat) of growth, or with the invasion-defective *invA*⁻ and *sipB*⁻ strains. Different wt strains perform identically in this assay; therefore, only one representative wt has been included. 25 min after infection, cells were stained with DAPI to reveal chromatin condensation. The percentage of cells containing condensed chromatin was determined by microscopical examination of triplicate samples. (B) Quiescent macrophages were infected with late logarithmic phase (late log, ■) or stationary phase (stat, □) wt bacteria. (C and D) Macrophages were infected with *invA*⁻ (□ in C) or *sipB*⁻ (□ in D) mutants and with the corresponding wt strains, all in late logarithmic phase. The kinase activity of Raf-1 immunoprecipitates prepared at different times after infection (moi 25) was measured in a coupled assay. The results are expressed as cpm incorporated into the substrate. The standard deviation was <5% in all cases and has therefore been omitted. The amount of Raf-1 contained in the immunoprecipitates was determined by immunoblotting.

SP63, anti–NH_2-terminal, and anti-kinase domain, respectively) in blocking solution (2% gelatin, 0.3% Triton X-100 in Q/W) for 60 min at RT. Cells were then washed in Q/W solution and incubated with fluorescein-conjugated secondary antibody (Alexa 488) in blocking solution for 60 min at RT. Cells were washed with Q/W solution, mounted (ProLong Antifade kit; Molecular Probes), and examined by confocal microscopy.

Assay of Raf Kinase Activity. Raf-1 kinase activity was measured as the ability of immunoisolated Raf-1 to activate recombinant MEK-1 in coupled assay using myelin basic protein as the endpoint of the assay (36).

Results

Raf-1 Activation by Salmonella Involves the Type III Secretion System, but Is Not a Consequence of Apoptosis. A functional SPI-1–encoded type III secretion system is essential for the ability of *Salmonella* to induce apoptosis. *Salmonella* grown to stationary phase (8), *invA*$^-$ (lacking an essential membrane component of the type III secretion apparatus) and *sipB*$^-$ mutants are incapable of doing so (Fig. 1 A). SipB causes apoptosis by binding to caspase-1 (16). Consistently, primary bone marrow–derived macrophages from caspase-1–deficient mice fail to undergo apoptosis within the 30 min of infection with wt *Salmonella* (Fig. 1 A).

To explore the connection between Raf-1 activation and apoptosis induction, we infected primary macrophages with invasive and noninvasive *Salmonellae* and compared their ability to activate Raf-1. Infection of quiescent primary bone marrow–derived macrophages with wt *Salmonella* in the late logarithmic phase of growth (invasive) caused moderate Raf-1 activation. However, wt bacteria in the stationary phase (noninvasive) were incapable to do so. Several different wt strains (SR11, SL1344, and LT2) behaved in identical manner (data not shown). Consistent with an involvement of the SPI-1–encoded type III secretion system in *Salmonella*-induced Raf-1 activation, the *invA*$^-$ bacteria in the late logarithmic phase did not stimulate the Raf-1 kinase, and caused a slight, but reproducible decrease in its basal activity (Fig. 1 B). In contrast, the *sipB*$^-$ mutant activated Raf-1 with identical kinetics and to identical extents as wt *Salmonella* (Fig. 1 C). The amount of immunoprecipitated Raf-1 decreased during the late phase of infection with invasive wt, but not with noninvasive wt or with the *invA*$^-$ or *sipB*$^-$ strain. From these results, we can conclude that SipB is not required for early Raf-1 activation, and that the decline in Raf-1 activation 20 min after infection is not attributable to the degradation of the protein. Therefore, Raf-1 activation is not a consequence of apoptosis.

Raf-1 Is Degraded in a Caspase-1–dependent Manner during Salmonella-induced Apoptosis. Next we investigated if the decrease in the amount of Raf-1 observed during the late phases of infection was dependent on caspase-1, and whether it was a reflection of the general demise of the cell undergoing apoptosis or rather a specific phenomenon involving this kinase selectively. Triton X-100 extracts from control (+/+) or caspase-1–deficient macrophages infected with *Salmonella* were immunoblotted with a Raf-1 antiserum. The amount of kinase was progressively reduced in +/+ macrophages starting from 5 min after infection with invasive *Salmonella*, but remained constant throughout infection with a *sipB*$^-$ mutant (Fig. 2 A) or of caspase-1–deficient macrophages with wt bacteria (Fig. 2 B). In contrast to Raf-1, the amount of MEK (data not shown) or ERK remained constant throughout infection. Thus, *Salmonella* induced caspase-1–dependent degradation of Raf-1, but not of MEK or ERK (Fig. 2, A and B).

Salmonella-mediated Raf-1 Degradation Can Be Prevented by Proteasome Inhibitors. Loss of Raf-1 from the Triton X-100 soluble fraction was paralleled initially by an increase

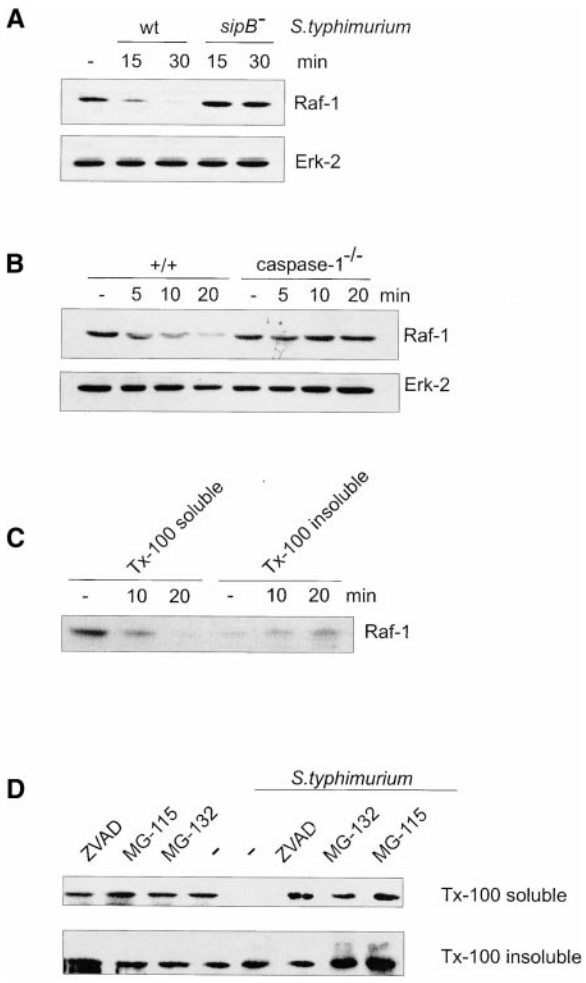

Figure 2. Raf-1 degradation is a consequence of macrophage apoptosis and is inhibited by proteasome inhibitors. (A) Bone marrow–derived macrophages were infected with either wt or *sipB*$^-$ *Salmonella*. (B) Primary macrophages were isolated from control (+/+) or caspase-1–deficient mice (caspase-1$^{-/-}$) and infected with invasive *Salmonella*. The amount of Raf-1 in Triton X-100 extracts of whole cells was determined by immunoblotting. (C) Macrophages were infected with invasive bacteria. The cells were lysed at the indicated times after infection, and the Triton X-100 (Tx-100) soluble fractions (30 μg, 1/10 of total) and the whole insoluble fractions were analyzed by immunoblotting with a Raf-1 antiserum. (D) Macrophages were treated with a caspase inhibitor (200 μM ZVAD-fmk) or with proteasome inhibitors (10 μM MG-132 and 10 μM MG-115) for 90 min before infection with *Salmonella* for 15 min. The amount of Raf-1 in the detergent-soluble and -insoluble fractions of whole cell lysates was determined by immunoblotting.

in Triton X-100 insoluble Raf-1 (Fig. 2 C). Accumulation in the Triton X-100 insoluble fraction is a general feature associated with protein ubiquitinylation, which normally precedes proteasome-mediated degradation. To gain some insight into the mechanism of Raf-1 degradation, we treated macrophages with the proteasome inhibitors MG-115 and MG-132 before infection with invasive *Salmonella*. Both inhibitors efficiently stabilized Raf-1, as did the caspase inhibitor Z-VAD-fmk. The proteasome inhibitors, but not the caspase inhibitor, caused the appearance of higher molecular forms of Raf-1, particularly in the Triton X-100 insoluble fraction of lysates, probably because of Raf-1 ubiquitinylation (Fig. 2 D). None of the inhibitors had any effect on the amount or solubility of Raf-1 in uninfected cells.

Conditional Inactivation of Raf-1 in Bone Marrow Cells. Disruption of the *c-raf-1* gene is embryonic lethal at midgestation and is accompanied by fetal liver apoptosis (unpublished observations). To obtain Raf-1–deficient macrophages, conditional inactivation of the *c-raf-1* gene was achieved by the insertion of *loxP* sites cloned 5′ and 3′ of exon 3. A selection cassette (a neomycin resistance gene for positive selection and the thymidine kinase gene of herpes virus for negative selection) was positioned between two *loxP* sites upstream of the floxed exon 3 (Fig. 3 A). The mutation was introduced into ES cells by homologous recombination. After transient Cre expression, ES cell clones in which the *Neo/TK* gene cassette, but not exon 3, had been excised were identified by Southern blot analysis (Fig. 3 B). Germline-transmitting chimeras were obtained and bred to C57Bl/6 mice. Mice homozygous for the *c-raf-1*^{flox} allele were phenotypically indistinguishable from wt animals. To allow inducible inactivation of Raf-1, *c-raf-1*$^{flox/flox}$ animals were crossed to mice expressing the Cre recombinase under the control of the Mx1 promoter (31). Injection of mice with poly I:C resulted in the efficient deletion of the floxed exon 3 in liver (data not shown) and bone marrow (Fig. 3 C, *c-raf-1*$^{\Delta/\Delta}$). Macrophages derived from these

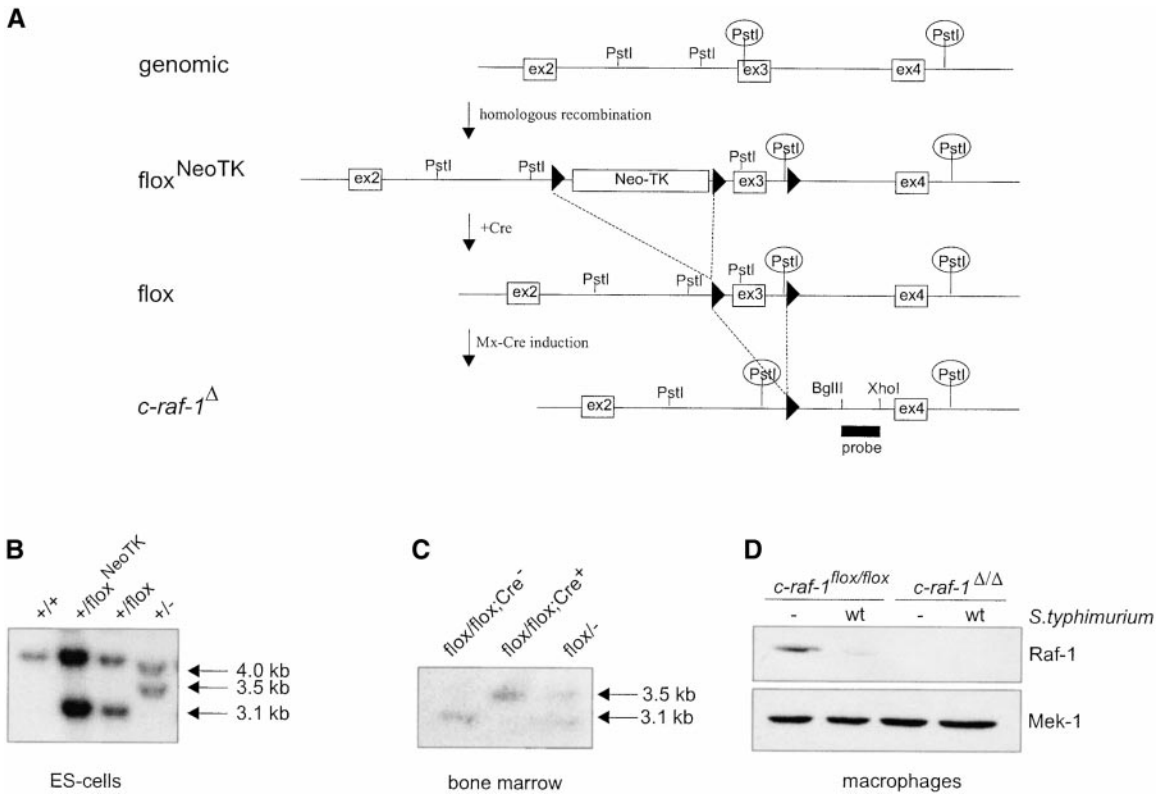

Figure 3. Conditional targeting of the mouse *c-raf-1* gene. (A) Schematic representation of the conditional targeting of the *c-raf-1* gene. Genomic, genomic locus before recombination; flox^{NeoTK}, homologously recombined targeting vector; flox, targeted locus after Cre-mediated removal of the *NeoTK* cassette; and *c-raf-1*$^{\Delta}$, *c-raf-1* locus deleted in bone marrow cells after in vivo induction of Mx-Cre. *loxP* sites (▶) were inserted 5′ and 3′ of exon 3 of the *c-raf-1* gene. Selection markers (a neomycin resistance gene for positive selection and the thymidine kinase gene of herpes virus for negative selection) were positioned between two *loxP* sites upstream of the floxed exon 3. The PstI sites delimiting the fragments obtained by digesting the genomic and mutated *c-raf-1* alleles are marked. The PstI site upstream of the 3′ *loxP* site was introduced with the targeting vector and serves as a marker for the floxed allele. (B) Southern blot analysis of PstI-digested genomic DNA from targeted ES cell clones after transient Cre expression. The probe used is shown in black in A. The *Neo/TK* gene cassette was excised by transiently expressing Cre. Excision was confirmed by Southern blot analysis with a *Neo/TK* probe (data not shown). (C) Southern blot analysis of PstI-digested genomic DNA isolated from bone marrow cells of *c-raf-1*$^{flox/flox}$;MxCre^{-} and *c-raf-1*$^{\Delta/\Delta}$; MxCre^{+} littermates after induction of Mx-Cre in vivo. Genomic DNA from a *c-raf-1*$^{flox/-}$ animal was used to mark the position of the *c-raf-1* alleles. (D) Western blot analysis of whole cell lysates from bone marrow–derived macrophages. Macrophages derived from *c-raf-1*$^{flox/flox}$*;MxCre*$^{-}$ mice and from *c-raf-1*$^{flox/flox}$*;Cre*$^{+}$ littermates treated in vivo with poly I:C were infected with invasive *Salmonella* (moi 25) for 20 min. The amount of Raf-1 in whole cell lysates was determined by immunoblotting.

bone marrow cells were devoid of Raf-1 protein, as shown by Western blot analysis, while poly I:C treatment of *c-raf-1*$^{flox/flox}$ animals that did not carry the Mx-Cre transgene did not have any effect on Raf-1 expression (Fig. 3 D).

Raf-1-deficient Macrophages Are More Sensitive Than WT Cells to Salmonella-induced Apoptosis. To determine whether activated Raf-1 functions as a pro- or antiapoptotic molecule in the context of *Salmonella*-induced cell death, we infected *c-raf-1*$^{\Delta/\Delta}$ and *c-raf-1*$^{flox/flox}$ macrophages with different amounts of bacteria and compared the number of cells undergoing apoptosis. The *c-raf-1*$^{\Delta/\Delta}$ macrophages proved more sensitive to pathogen-mediated apoptosis than *c-raf-1*$^{flox/flox}$ cells at all moi investigated (Fig. 4, A and B). In addition, apoptosis proceeded with faster kinetics in *c-raf-1*$^{\Delta/\Delta}$ macrophages than in *c-raf-1*$^{flox/flox}$ cells (Fig. 4 C). However, infection with wt *Salmonella* in stationary phase, with *invA*$^-$ or with *sipB*$^-$ bacteria, failed to induce apoptosis in *c-raf-1*$^{\Delta/\Delta}$ macrophages, and these cells were not more susceptible than *c-raf-1*$^{flox/flox}$ cells to cell death induced by *Listeria monocytogenes*, a process resembling delayed necrosis (reference 37, data not shown). Thus, Raf-1 plays a specific protective role in *Salmonella*-induced macrophage apoptosis.

The ERK Cascade Is Not a Downstream Target of Raf-1 in Salmonella-infected Macrophages and Does Not Play a Protective Role in Pathogen-mediated Apoptosis. Unlike Raf-1, ERK was activated equally well and with identical kinetics upon infection with invasive *Salmonella* and with an *invA*$^-$ mutant (Fig. 5 A). This suggests that Raf-1 is not involved in the activation of the ERK cascade by *Salmonella*. However, it is still formally possible that wt and invasion-deficient bacteria use distinct signal transduction pathways to stimulate ERKs, and that Raf-1 is involved in ERK activation by invasive bacteria selectively. To investigate this, we monitored ERK activation in *c-raf-1*$^{flox/flox}$ and *c-raf-1*$^{\Delta/\Delta}$ bone marrow–derived macrophages infected with wt or *invA*$^-$ *Salmonella*. ERK activation occurred normally in the Raf-1–deficient cells (Fig. 5 B). These results show that Raf-1 is not essential for ERK activation in *Salmonella*-infected macrophages. Consequently, the protective effect of Raf-1 against pathogen-mediated apoptosis is not mediated via this pathway.

Antiapoptotic effects ascribed directly or indirectly to ERK activation have been described (38, 39). To ascertain whether or not the ERK cascade had any protective effect on pathogen-mediated apoptosis independently of Raf-1, we treated *c-raf-1*$^{flox/flox}$ macrophages with the MEK-1 inhibitor PD98059 before infection with invasive bacteria. Although it successfully abrogated ERK activation by *Salmonella* (Fig. 5 C), the MEK inhibitor had no effect on pathogen-mediated macrophage apoptosis (Fig. 5 D).

NF-κB Is Not a Downstream Target of Raf-1 in Salmonella-infected Macrophages. A further downstream target of Raf-1 implicated in protection from apoptosis is the transcription factor NF-κB (40). Raf-1 activates NF-κB by inducing IκB phosphorylation and degradation. This pathway is distinct from MEK and ERK activation but involves MEKK-1 upstream of the IκB kinase complex (41). Infection of macrophages with invasive *Salmonella* caused rapid IκB degradation, whose extent and kinetics were identical in *c-raf-1*$^{flox/flox}$ and *c-raf-1*$^{\Delta/\Delta}$ macrophages (Fig. 6 A). In addition, infection with *Salmonell*a resulted in the rapid stimulation of NF-κB binding activity in nuclear extracts from cells of both genotypes (Fig. 6 B).

Thus, Raf-1 is not essential for phosphorylation-induced IκB degradation or NF-κB binding in *Salmonella*-infected macrophages.

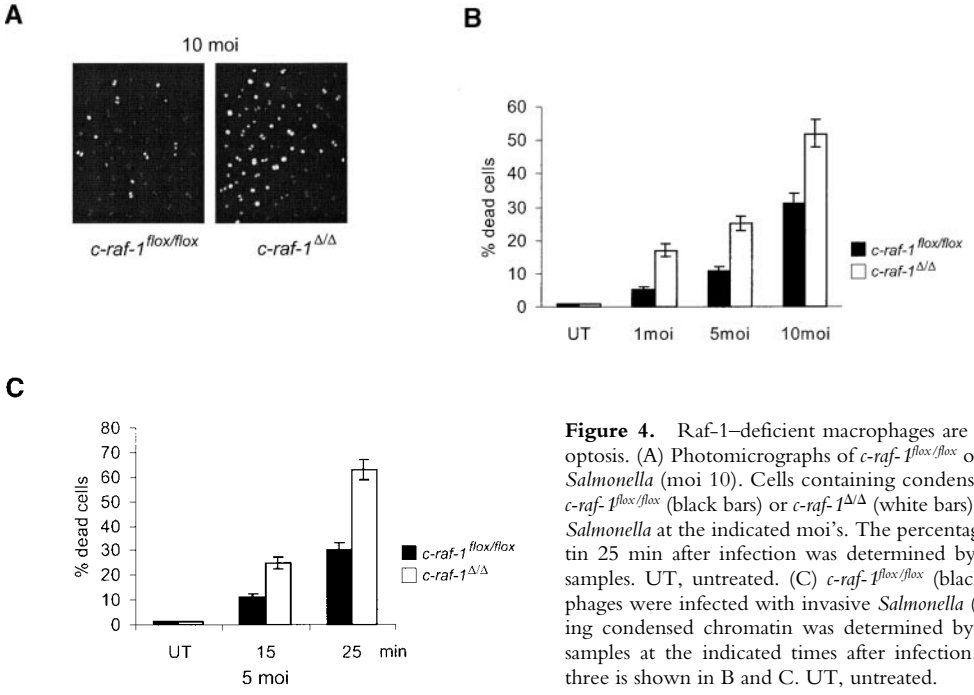

Figure 4. Raf-1–deficient macrophages are hypersensitive to *Salmonella*-induced apoptosis. (A) Photomicrographs of *c-raf-1*$^{flox/flox}$ or *c-raf-1*$^{\Delta/\Delta}$ macrophages infected with wt *Salmonella* (moi 10). Cells containing condensed chromatin appear as bright spots. (B) *c-raf-1*$^{flox/flox}$ (black bars) or *c-raf-1*$^{\Delta/\Delta}$ (white bars) macrophages were infected with invasive *Salmonella* at the indicated moi's. The percentage of cells containing condensed chromatin 25 min after infection was determined by microscopical examination of triplicate samples. UT, untreated. (C) *c-raf-1*$^{flox/flox}$ (black bars) or *c-raf-1*$^{\Delta/\Delta}$ (white bars) macrophages were infected with invasive *Salmonella* (moi 5). The percentage of cells containing condensed chromatin was determined by microscopical examination of triplicate samples at the indicated times after infection. One representative experiment out of three is shown in B and C. UT, untreated.

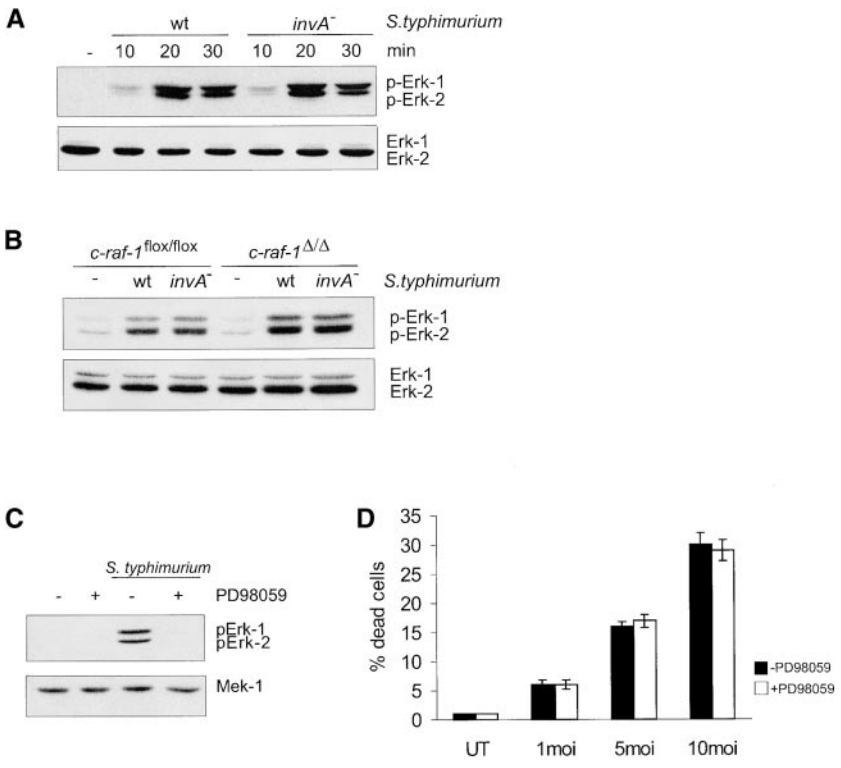

Figure 5. ERKs are activated independently of Raf-1 in *Salmonella*-infected macrophages, and ERK inhibition does not affect *Salmonella*-induced apoptosis. (A) Primary macrophages were infected with invasive *Salmonella* or with an *invA*⁻ mutant. At different times after infection, the presence of the phosphorylated, active forms of ERK (p-ERK) was detected by immunoblotting. An anti-ERK immunoblot is shown as a loading control. (B) *c-raf-1*$^{flox/flox}$ and *c-raf-1*$^{\Delta/\Delta}$ macrophages were infected with invasive *Salmonella* or with an *invA*⁻ mutant. The amount of phosphorylated ERK in whole cell lysates was determined after 20 min of infection. (C and D) *c-raf-1*$^{flox/flox}$ macrophages were either left untreated or treated with 50 μM MEK inhibitor PD98059 overnight before infection with invasive *Salmonella*. (C) The amount of phosphorylated ERK in whole cell lysates was determined after 20 min of infection. An anti-MEK immunoblot is shown as a loading control. (D) The percentage of cells containing condensed chromatin 25 min after infection with invasive *Salmonella* at the indicated moi's was determined by microscopical examination of triplicate samples. Black bars, untreated macrophages; white bars, macrophages treated with the MEK inhibitor PD98059 before infection. UT, untreated.

Lack of Involvement of the Mitochondria in the Antiapoptotic Effect of Raf-1. The Raf kinases have been proposed to modulate mitochondrial integrity by regulating the activity of Bcl-2 family members (26, 27, 29). Stimulus-induced translocation of the Raf-1 kinase to the mitochondrial compartment, which would bring it in the proximity of the putative substrates, has been postulated based on biochemical fractionation experiments (27, 42). We addressed the question of mitochondrial translocation of Raf-1 in *Salmonella*-infected macrophages by biochemical fractionation and confocal microscopy. Biochemical fractionation showed that a significant portion (5–10% in different experiments) of Raf-1 could be recovered from the HMF of macrophages, commonly referred to as "mitochondria-enriched." In addition, the amount of Raf-1 in this fraction increased after infection with invasive *Salmonella* (Fig. 7 A). The presence of Raf-1 in the HMF has been previously taken as an indication of the mitochondrial localization of this protein. This fraction contained mitochondrial proteins such as cox-IV and cytochrome c, and was completely devoid of cytosolic proteins such as caspase-3. However, it also contained the glycosylated form of the CSF-1 receptor, and was therefore contaminated with plasma membrane proteins (possibly from portions of the membrane as-

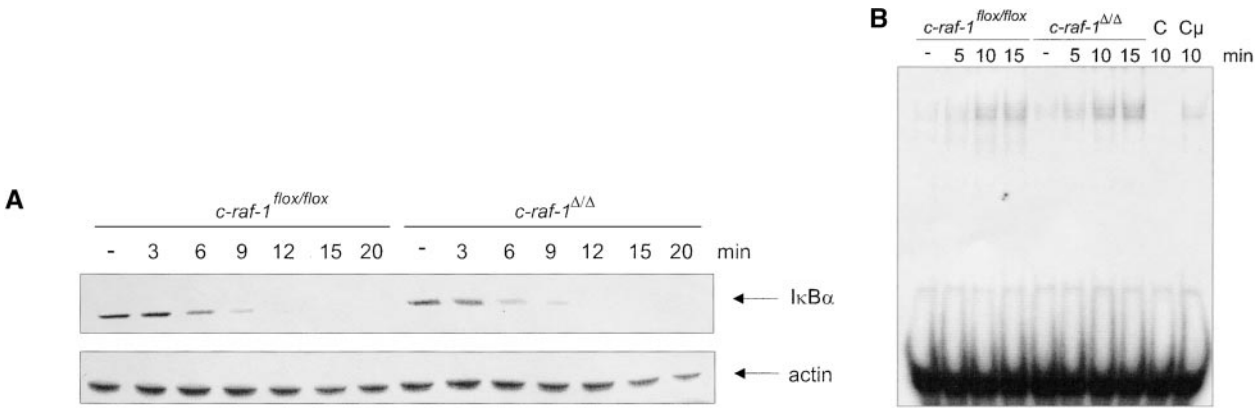

Figure 6. IκB degradation and NF-κB binding activity are normal in Raf-1–deficient, *Salmonella*-infected macrophages. *c-raf-1*$^{flox/flox}$ and *c-raf-1*$^{\Delta/\Delta}$ macrophages were infected with invasive *Salmonella*. (A) The amount of IκB or the loading control actin in whole cell lysates was determined by immunoblotting at different times after infection. (B) NF-κB binding activity in nuclear extract was determined by EMSA; in lanes C and Cμ, NF-κB binding of nuclear extracts from *Salmonella*-infected *c-raf-1*$^{flox/flox}$ macrophages for 10 min was competed with an excess unlabeled wt (C) or mutated oligos (Cμ).

sociated with the cytoskeleton). Thus, in spite of the enrichment for mitochondria, the HMF obtained by this method is not pure (Fig. 7 B). We used confocal microscopy to confirm or dispute Raf-1 mitochondrial localization by an independent method. Confocal analysis of macrophages stained with a Raf-1 antiserum showed a punctuate Raf-1 pattern, but did not reveal any colocalization with living mitochondria (visualized with Mitotracker red). Treatment with invasive *Salmonella* for 10 (data not shown) and 15 min did not alter Raf-1 localization (Fig. 7 C). The same results were obtained with antibodies directed against three different domains of the molecule. Raf-1 knockout macrophages were used to control for specificity.

Failure to detect mitochondrial localization of Raf-1 might be because of technical reasons; alternatively, localization might be extremely transient and therefore elusive. In an attempt to assess whether mitochondrial function was altered in knockout cells, we assayed cytochrome c release from wt and Raf-1–deficient macrophages. Rapid *Salmonella*-mediated apoptosis did not lead to any appreciable cytochrome c release in *c-raf-1*$^{flox/flox}$ or in *c-raf-1*$^{\Delta/\Delta}$ macrophages (data not shown). Thus, the apoptotic phenotype of Raf-1–deficient macrophages cannot be ascribed to mitochondrial fragility.

Salmonella-induced Caspase-1 Activation Is Increased Raf-1–deficient Macrophages. To investigate whether the ablation of Raf-1 had any effect on caspase-1, *c-raf-1*$^{flox/flox}$ and *c-raf-1*$^{\Delta/\Delta}$ macrophages were infected with invasive *Salmonella*. Caspase-1 activation was monitored by immunoblotting with an antiserum that recognizes the long subunit of the active enzyme (p20). p20 appeared with faster kinetics and in larger amounts in Raf-1–deficient macrophages than in *c-raf-1*$^{flox/flox}$ controls. In contrast, the kinetics and strength of caspase-2 activation were indistinguishable. Cleavage (and therefore activation) of the zymogens procaspase-3 and -8 did not occur during rapid *Salmonella*-induced apoptosis in c-*raf-1*$^{flox/flox}$ or *c-raf-1*$^{\Delta/\Delta}$ macrophages (Fig. 8).

Discussion

The interaction of *Salmonella* with the host's macrophages is an important event in the early phases of infection. Still, the signaling steps taking place during this interaction are largely unknown. In this paper, we show that Raf-1 is the only kinase of the MAPK pathway to be selectively acti-

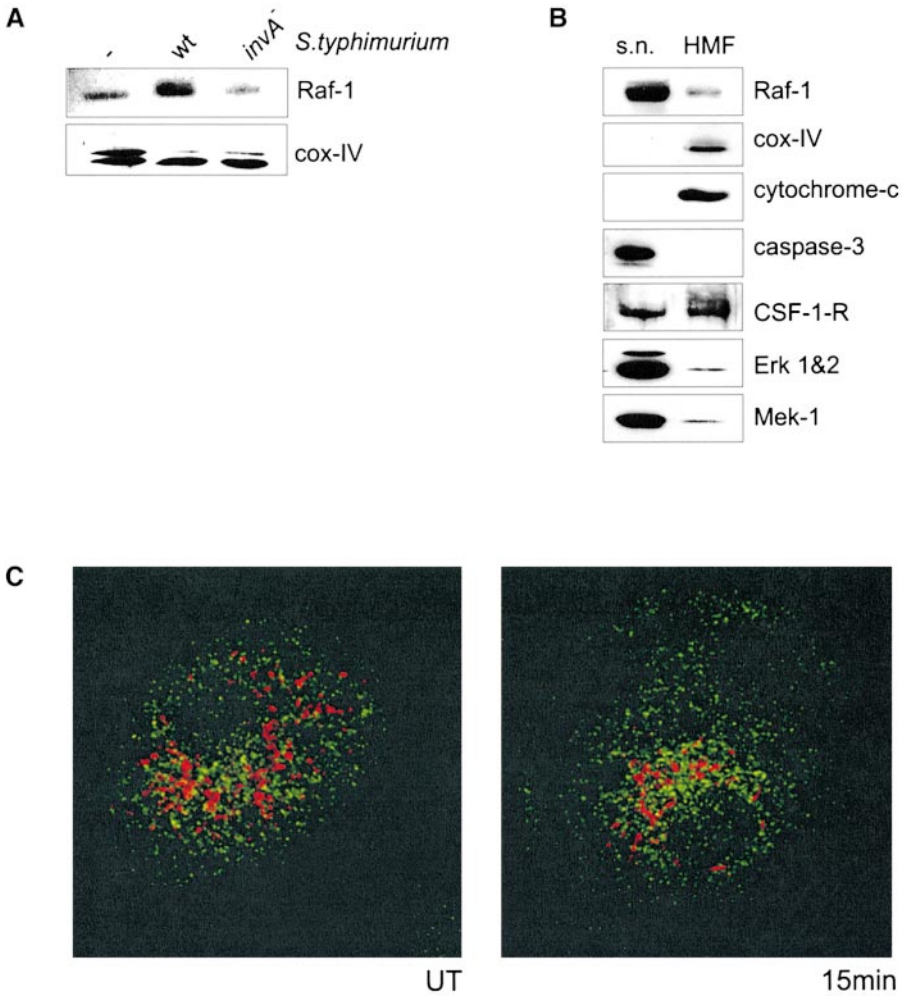

Figure 7. Enhanced recovery of Raf-1 in the HMF of macrophages infected with invasive *Salmonella*. (A) Primary macrophages were infected with invasive *Salmonella* or with an *invA*$^{-}$ mutant in the late log phase. 15 min after infections, the cells were homogenized and the HMF was obtained by differential centrifugation. The amount of Raf-1 and cox-IV present in 5 μg of the HMF was determined by immunoblotting. (B) Distribution of cellular proteins between the HMF and the supernatant of untreated macrophages. HMF and supernatant derived from 2×10^6 cells were immunoblotted with antisera directed against mitochondrial (cox-IV, cytochrome c), cytosolic (caspase-3, ERK, MEK, and Raf-1), and plasma membrane proteins (CSF-1 receptor). (C) Lack of colocalization of Raf-1 immunostaining with mitochondria in confocal microscopy. The mitochondria were stained with Mitotracker Red before infection of the macrophages with invasive *Salmonella*. At the indicated times, cells were preextracted, fixed, and stained with a monoclonal antibody against the NH_2 terminus of Raf-1 (green).

vated and degraded by apoptosis-inducing *Salmonella*. Furthermore, conditional inactivation of Raf-1 in macrophages showed that these cells are more sensitive towards *Salmonella*-mediated apoptosis, implying that Raf-1 has a protective function in this process. This hypersensitivity correlates with, and is probably attributable to, an increase in caspase-1 activation.

Raf-1 Is the Only Kinase of the MAPK Pathway Whose Activation Requires the Type III Secretion System. Macrophages activate Raf-1 in response to several different stimuli, notably LPS (19, 20, 43). It was therefore somewhat surprising that infection with *invA*$^-$ *Salmonella* actually decreased Raf-1 activity. Distinct reactions to soluble and particulate LPS have been observed before; notably, soluble LPS is more efficient in eliciting cellular responses (44). In the specific context of MAPK cascades, our own work has shown that LPS is likely to be the major determinant of ERK activation (17). In contrast, *Salmonella* stimulates JNKs by a mechanism distinct from LPS, but in this case the type III secretion apparatus is not involved (18, 45). Thus, Raf-1 is the first example of a signal transducer whose activation by *Salmonella* in macrophages requires the type III system. Interestingly, a *sipB* mutant was still able to cause Raf-1 activation. Consistent with this, activation of Raf-1 is normal in caspase-1–deficient macrophages that lack the only target of SipB identified so far in macrophages (data not shown). *sipB* mutants are noninvasive (46). They are able to secrete proteins in culture media (46), but cannot transfer them into cultured epithelial cells (47). Therefore, SipB may function as a translocase. If SipB does perform this function in macrophages, it is possible that Raf-1 activation does not need translocation of a *Salmonella* protein into the cell; activation may be engendered either by the recognition of a structural component of the secretion system (needle complex) or by a protein secreted into the medium. Alternatively, a yet unidentified protein whose translocation does not require SipB might be responsible for Raf-1 activation.

Raf-1 and Salmonella-mediated Apoptosis. The *sipB*$^-$ *Salmonella* mutants and the caspase-1–deficient macrophages allowed us to discriminate among three events that follow macrophage infection by *Salmonella*: Raf-1 activation, which is not affected by these mutations; and Raf-1 degradation and apoptosis, which require both SipB and caspase-1. Thus, it seems plausible that Raf-1 stimulation is a reaction of the cell to the encounter with potentially pathogenic *Salmonella*. Kinase activation is not an emergency response to apoptotic stress, since it occurs normally in *sipB*$^-$ infected macrophages, which do not undergo apoptosis. In contrast, Raf-1 degradation is a feature of apoptosis. Although Raf-1 is not cleaved in the absence of caspase activation, we failed to observe degradation products that would be diagnostic of a caspase-mediated cleavage. Besides the caspases, several other serine proteases have been implicated in the apoptotic process (48). In particular, the proteasome reportedly acts downstream of caspases (49–51). Among the targets of the proteasome are the transcription factor c-Fos (52), the antiapoptotic protein Bcl-2 (53), the proapoptotic protein Bid (54), and the natural caspase inhibitors of apoptosis (IAPs) (51). In *Salmonella*-infected cells, pretreatment with proteasome inhibitors protected Raf-1 from degradation. This was paralleled by the appearance of higher molecular forms of Raf-1, indicative of ubiquitinylation. Thus, Raf-1 degradation in the course of *Salmonella*-mediated apoptosis appears to be mediated by the proteasome.

Several signal transducers are degraded in apoptotic cells. In some cases, the proteins are activated by caspase cleavage and contribute to the apoptotic response (55–58); in others, it has been postulated that cleavage disables proteins that would counteract apoptosis (59). To determine the role of Raf-1 in pathogen-mediated apoptosis, we performed the conditional inactivation of the *c-raf-1* gene in bone marrow cells. The *c-raf-1*$^{\Delta/\Delta}$ macrophages from these bone marrow cells are significantly more prone to *Salmonella*-mediated apoptosis than *c-raf-1*$^{flox/flox}$ cells. Therefore, Raf-1 appears to play an antiapoptotic role in this process, and its degradation might be part of a bacterial strategy to weaken the eukaryotic cell.

The protective function of Raf-1 is not mediated via the NF-κB or the MEK/ERK pathway. In fact, Raf-1 is not essential for the activation of these pathways in this context. In the case of MEK/ERK, this finding was not totally unexpected, since we have shown before that the activation of these enzymes can be uncoupled from Raf-1 in macrophages by several criteria (43, 60). Furthermore, chemical inhibition of ERK stimulation does not have any impact on *Salmonella*-mediated apoptosis.

The mitochondria could have represented a likely target site of Raf-1 action during *Salmonella*-mediated apoptosis. We initially considered the possibility that in the absence of Raf-1 an increased fragility of these organelles might accelerate apoptosis. Biochemical fractionation experiments in other systems have indicated that Raf-1 is associated with mitochondria and that further translocation to this compartment accompanies stimulation with a growth factor (27) as well as with UV (42). The cell fractionation experiments performed here suggested a similar picture in *Salmonella*-infected macrophages. However, in confocal microscopy we did not observe any significant colocalization

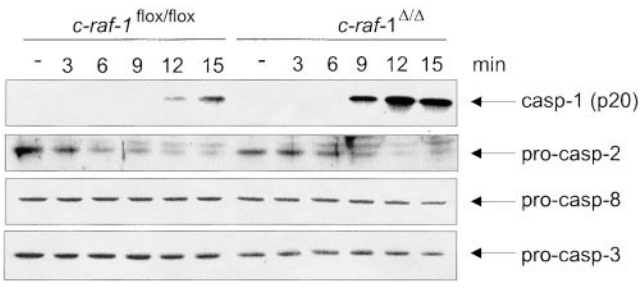

Figure 8. Increased caspase-1 activation by invasive *Salmonella* in Raf-1–deficient macrophages. *c-raf-1*$^{flox/flox}$ or *c-raf-1*$^{\Delta/\Delta}$ primary bone marrow–derived macrophages were infected with invasive *Salmonella* (moi 25). At the indicated times after infection, cells were lysed and the activation state of caspase-1, -2, -8, and -3 were analyzed by immunoblotting.

between Mitotracker red (used to stain living mitochondria) and the Raf-1 signal. Consistent with this result, A-Raf, but not Raf-1, can be visualized associated with mitochondria by immunogold staining followed by transmission electron microscopy (61). In addition, *c-raf-1*$^{\Delta/\Delta}$ cells did not show increased mitochondrial damage (as measured by cytochrome c release) after exposure to invasive *Salmonella*.

The experiments discussed above rule out MEK/ERK and NF-κB as downstream effectors, and mitochondrial integrity as the target of the antiapoptotic function of Raf-1 in *Salmonella*-infected macrophages. The only parameters altered in the Raf-1–deficient macrophages were the kinetics and strength of caspase-1 activation. This suggests that the kinase is somehow able to restrain the activation of this protease. Interestingly, lack of Raf-1 did not have any effect on the kinetics or extent of caspase-2 cleavage, which becomes activated slightly earlier than caspase-1 and participates in its activation (62). Cleavage of the zymogens procaspase-3 and -8, which are not activated in the course of rapid *Salmonella*-mediated apoptosis, did not occur in Raf-1–deficient cells. These data indicate that Raf-1 acts via a specific mechanism targeting caspase-1 selectively, and not by simply restraining caspase activation in general. This is remarkable in light of the fact that caspase-1 is needed to initiate proteasome-dependent Raf-1 degradation during apoptosis. Thus, the relationship between Raf-1 and caspase-1 is reciprocal, with the protease directing the degradation of the kinase that restrains its activation. At present, we do not know how Raf-1 controls caspase-1. It is possible that Raf-1, or one of its unknown downstream effectors, directly modifies this protease. Phosphorylation of human caspase-9 by the antiapoptotic kinase protein kinase B has been shown to inhibit protease activity (63). Alternatively, Raf-1 might directly or indirectly modulate the expression or activity of natural caspase inhibitors such as the IAPs (64). A specific caspase-1 inhibitor, ICEBERG, has been recently described (65). Also in this context, genetic evidence in *Drosophila* shows that activated D-Ras and D-Raf can inhibit apoptosis by antagonizing *Hid* (66, 67), whose recently cloned human orthologues have been shown to antagonize IAPs (68, 69). Raf-1–deficient cells generated by conditional gene inactivation will be an invaluable tool in future studies aimed at identifying the mechanism underlying the antiapoptotic function of Raf-1.

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