Activation of NF-κB by *Toxoplasma gondii* correlates with increased expression of antiapoptotic genes and localization of phosphorylated IκB to the parasitophorous vacuole membrane

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Accepted 27 May 2003
Journal of Cell Science 116, 4359-4371 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00683

Summary

Mammalian cells infected with *Toxoplasma gondii* are resistant to apoptosis induced by a variety of stimuli. We have demonstrated that the host transcription factor NF-κB plays a pivotal role in the *T.-gondii*-mediated blockade of apoptosis because inhibition is lost in cells lacking the p65 (RelA) subunit of NF-κB ($p65^{-/-}$). In the present study, we examined the effects of *T. gondii* infection on NF-κB activation and the expression of genes involved in the apoptotic cascade. Infection of wild-type mouse embryonic fibroblasts (MEFs) with *T.-gondii*-induced nuclear translocation of the p50 and p65 subunits of NF-κB as examined by immunoblotting of nuclear extracts, immunofluorescence and electrophoretic mobility shift assays. A comparison of apoptotic gene expression profiles from wild-type and $p65^{-/-}$ MEFs revealed distinct patterns of induction in response to *T. gondii* infection. In particular, the differences seen in the Bcl-2 and IAP families are consistent with the antiapoptotic responses observed in the resistant wild-type cells compared with the sensitive $p65^{-/-}$ fibroblasts. Consistent with NF-κB activation, *T. gondii* infection promoted phosphorylation of the inhibitor IκB. Interestingly, phosphorylated IκB was concentrated on the parasitophorous vacuole membrane (PVM), suggesting a parasite-directed event. Results from this study suggest that activation of NF-κB plays an important role in stimulation of antiapoptotic gene expression by *T. gondii*. Furthermore, recruitment of phosphorylated IκB to the PVM implies the presence of intrinsic factor(s) in *T. gondii* that might be used to manipulate the NF-κB signaling pathway in the host to elicit a survival response during infection.

Key words: *Toxoplasma gondii*, Apoptosis, NF-κB, IκB, Bcl2, IAP

Introduction

Toxoplasma gondii is an obligate intracellular protozoan belonging to the phylum Apicomplexa that causes opportunistic disease in immunodeficient individuals (Tenter et al., 2000). Primary infection during pregnancy might also result in congenital transmission and disease in newborns (Petersen et al., 2001). Infection with *T. gondii* initiates after ingestion of oocysts that originate from definitive feline hosts or tissue cysts present in undercooked infected meat (Tenter et al., 2000). The rapidly growing form of the parasite, known as the tachyzoite, establishes acute infection. Development of tachyzoites occurs within specialized parasitophorous vacuoles (PVs), which resist phagolysosomal fusion and actively recruit host cell organelles (Sinai et al., 1997; Sinai and Joiner, 2001; Sinai and Joiner, 1997). In immunocompetent individuals, *T. gondii* can establish an asymptomatic lifelong chronic infection as a result of an effective immune response that promotes encystation of the parasite and formation of bradyzoites (Boothroyd et al., 1997; Carruthers, 2002; Dubey et al., 1998). At the molecular level, the complex events regulating tachyzoite-bradyzoite interconversion involve modulation of both host and parasite gene expression (Blader et al., 2001; Singh et al., 2002).

Intracellular pathogens have evolved diverse strategies to avoid destruction by the immune system of the host. One of the defense mechanisms from the infected cell to counteract the invading pathogen is the induction of apoptosis, a process of cellular self-destruction that performs crucial functions normally during immune selection, tissue development and regeneration. Extensive literature has described molecular mechanisms in viruses (Cahir-McFarland et al., 2000), bacteria (Gao and Abu Kwaik, 2000) and, recently, in protozoan parasites (Heussler et al., 2001a) that interfere with the apoptotic process in order to enhance their survival. Infection of different cell lines of human and mouse origin with *T. gondii* inhibits apoptosis induced by a variety of stimuli including tumor necrosis factor α (TNFα) treatment, Fas-dependent cytotoxicity, ultraviolet irradiation, actinomycin D and growth factor deprivation (Nash et al., 1998; Goebel et al., 1999; Goebel et al., 2001) [see also accompanying article in this issue (Payne et al., 2003)]. We have shown that the *T.-gondii*-mediated blockade of apoptosis is lost in $p65^{-/-}$ fibroblasts, implying a role for the host transcription factor NF-

κB during apoptosis inhibition in parasite-infected cells (Payne et al., 2003).

NF-κB comprises a family of transcription factors with five members, p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB and c-Rel (Ghosh and Karin, 2002). These proteins form homo- or heterodimers and are sequestered in the cytoplasm of resting cells by inhibitor κB (IκB) proteins. Upon stimulation with a wide range of signals including proinflammatory cytokines (e.g. TNF-α and IL-1), mitogens, lipopolysaccharide (LPS) and ultraviolet irradiation, activation of NF-κB occurs via phosphorylation of IκB by the IκB kinase (IKK) complex. This results in degradation of IκB and translocation of NF-κB to the nucleus, where it regulates the transcription of genes involved in immune function, cell proliferation and survival (Ghosh and Karin, 2002). In addition, NF-κB induces the expression of genes whose products can inhibit apoptosis, such as the cellular inhibitors of apoptosis (c-IAP), the Bcl-2 members Bfl-1 and Bcl-x_L, and TNF-receptor-associated factors 1 and 2 (TRAF1 and TRAF2) (Karin and Lin, 2002).

We have described the loss of protection from apoptosis by *T. gondii* in p65$^{-/-}$ fibroblasts (Payne et al., 2003), suggesting that the transcription of NF-κB-dependent genes might be essential for the antiapoptotic response observed in wild-type infected cells. In the present study, we examined the effects of *T. gondii* infection on NF-κB activation and compared apoptotic gene expression profiles between wild-type and p65$^{-/-}$ infected fibroblasts. In addition, we established a foundation upon which to define the molecular mechanisms underlying the subversion the of NF-κB signaling pathway by *T. gondii* and its potential role in pathogenesis.

Materials and Methods

Cell lines and parasite

3T3 MEFs (provided by M. Karin, University of California San Diego), 3T3 p65$^{-/-}$ fibroblasts (Beg et al., 1995) (provided by D. Baltimore, California Institute of Technology) and Vero cells (ATCC CCL-81) were maintained in α minimum essential medium supplemented with 7% fetal bovine serum (FBS), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM L-glutamine (Gibco, Rockville, MD). In light of the continuous culture of the 3T3 cell lines, they can be considered to be immortalized. The RH strain of *T. gondii* was used exclusively and maintained by serial passage in Vero cells as previously described (Sinai et al., 2000); this strain carries a deletion in the hypoxanthine-xanthine-guanine-phosphoribosyl-transferase (HXGPRT) gene (Donald et al., 1996) (NIH AIDS Research and Reference Reagent Program).

Immunoblot analysis

MEFs were seeded in six-well plates at 2×10^5 cells per well and allowed to adhere overnight. Cells were infected with freshly passaged parasites at increasing multiplicities of infection (m.o.i.) for 20 hours. Nuclear and cytoplasmic fractions were prepared according to the protocol of Dignam et al. (Dignam et al., 1983) with the single modification of substituting 2 mM β-mercaptoethanol for 1 mM DTT. As controls for NF-κB activation, uninfected cells were treated with 20 ng ml^{-1} of murine recombinant TNFα (R&D Systems, Minneapolis, MN) for 20 minutes. Cells were harvested by scraping and pelleted by centrifugation at 300 ***g*** for 5 minutes. Immunoblots were performed as described previously (Payne et al., 2003). The following antibodies were used: polyclonal rabbit anti-p65 (Santa Cruz Biologicals, Santa Cruz, CA; 1:600 dilution), polyclonal rabbit anti-p50 (provided by N. Rice, National Cancer Institute, Frederick, MD; 1:600 dilution), polyclonal rabbit anti-IκBα (Cell Signaling Technology, Beverly, MA; 1:1000 dilution) and polyclonal rabbit anti-actin (Santa Cruz Biologicals; 1:600 dilution). Analysis of phosphorylated IκBα was performed with mouse monoclonal and rabbit polyclonal antibodies purchased from two different sources (Cell Signaling Technology and Santa Cruz Biologicals; 1:500 dilutions). The effects of proteosome inhibitors on phosphorylated-IκBα expression were examined in cells pretreated for 2 hours with 50 μM MG132 (Peptide Institute) prior to immunoblot analysis.

Immunofluorescence

MEFs were seeded in 24-well plates containing sterile 12 mm coverslips at 2×10^4 cells per well and incubated overnight. Cells were infected at an m.o.i. of 5:1 with freshly passaged parasites for 1 hours, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours and 20 hours, as indicated. Treatment with TNFα, where applicable, was performed at a concentration of 20 ng ml^{-1} for 20 minutes. Monolayers were washed in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4, 1.8 mM KH_2PO_4, adjusted to pH 7.4) supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$, fixed in 3% paraformaldehyde, and permeabilized in 0.1% Triton X-100 in PBS. Double immunofluorescence labeling was performed with primary rabbit polyclonal antibodies against either NF-κB p50 or p65 at 1:600 dilutions and mouse monoclonal anti-*T. gondii*-SAG1 (Argene, North Massapequa, NY) at a 1:1000 dilution. For phosphorylated IκB (P-IκB) analysis, cell monolayers were stained with mouse monoclonal anti-P-IκBα (Santa Cruz Biologicals) at a 1:300 dilution and rabbit polyclonal anti-SAG1 (a gift from L. Kasper, Dartmouth College, Hanover, NH) at a 1:1000 dilution. Proteins were localized using species-specific Oregon-Green- or Texas-Red-conjugated secondary antibodies (Molecular Probes, Eugene, OR) diluted at 1:2000 and visualized on a Leica TCS confocal microscope system at 100× magnification. Adjustment of brightness and contrast on digitally acquired images were applied uniformly to the entire field. Specificity of the anti-P-IκBα antibody was determined by competition studies with blocking peptide sc-8404 P (Santa Cruz Biologicals) that was mixed with the primary antibody at a concentration of 2 μg ml^{-1} for 2 hours prior to immunostaining.

Immunoelectron microscopy

Procedures for immunoelectron microscopy were followed as described by Folsch et al. (Folsch et al., 2001). *Toxoplasma*-infected fibroblasts were fixed for 2 days at 4°C with 8% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 0.25 M HEPES (pH 7.4). Cells were infiltrated, frozen, sectioned, labeled with rabbit anti-P-IκBα antibodies (Cell Signaling Technology) at a 1:5 dilution in PBS with 1% fish skin gelatin and directly treated with 5 nm protein-A/gold particles (Department of Cell Biology, Medical School, Utrecht University, The Netherlands). Grids were examined with a Philips 410 electron microscope (Eindhoven, The Netherlands) at 80 kV.

Electrophoretic mobility shift assay

For the electrophoretic mobility shift assay (EMSA), a double-stranded oligonucleotide containing the NF-κB consensus sequence (underlined) (5′-AGTGA<u>GGGGACTTTCC</u>CAGGC-3′; Promega, Madison, WI) was end labeled with [γ-^{32}P]ATP (ICN, Costa Mesa, CA) as described previously (Molestina et al., 2000). Binding reactions were performed at room temperature for 30 minutes in 15 μl of a mixture containing 5 μg nuclear protein and 1 μl (~50,000 cpm) radiolabeled oligonucleotide in binding buffer [4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA (pH 8.0), 0.5 mM DTT, 50 mM NaCl, 10 mM Tris (pH 7.5) and 50 μg poly(dI-dC)]. Antibodies to NF-κB p65 (Santa Cruz), p50, p52, RelB and c-Rel (gifts from N. Rice) were used for supershift assays. Reaction products were separated in 4%

nondenaturing polyacrylamide gels and analysed with the Storm Phosphorimager System (Molecular Dynamics, Sunnyvale, CA).

Apoptotic gene arrays

Wild-type and p65$^{-/-}$ MEFs were cultured in 10 cm dishes and infected with *T. gondii* at a m.o.i. of 5:1 or mock-infected with lysates of Vero cells that were prepared by syringe passage. Total RNA was isolated after 20 hours of incubation using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA (7 μg) from mock-infected and infected cells was used as template for synthesis of [α-^{32}P]dCTP (ICN, Costa Mesa, CA) labeled cDNA probes with an apoptotic array-specific primer set (SuperArray, Bethesda, MD). Probes were hybridized at high stringency (60°C overnight, using a proprietary hybridization solution; SuperArray) to nylon membranes contained in the Mouse Apoptosis GEArray Q Series (SuperArray). Arrays consist of a panel of 96 cDNA fragments from genes associated with apoptosis. Following hybridization, membranes were washed twice in 2× SSC with 1% SDS and twice in 0.1× SSC with 0.5% SDS for 10 minutes each at 60°C. Signals corresponding to the different mRNAs were detected by phosphorimaging, and integrated densitometric values (IDVs) were calculated from each spot with the ImageQuant software (Molecular Dynamics). Numerical data was normalized to levels of actin expression, and comparisons of gene expression between infected and mock-infected cells were performed with the GEArray Analyzer software (SuperArray).

RT-PCR

Total RNA (1 μg) from mock-infected and infected MEFs was used in a 15 μl reaction mixture containing 1× reverse transcriptase reaction buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT], 1 mM each deoxynucleoside triphosphate, 25 U recombinant RNasin ribonuclease inhibitor, 200 U of Moloney murine leukemia virus reverse transcriptase, and 0.5 μg of oligo$(dT)_{15}$ (Promega). Reverse transcription was performed at 42°C for 30 minutes. Subsequently, 3 μl cDNA products were amplified in a 25 μl reaction mixture containing 1.6 mM $MgCl_2$, 1× thermophilic DNA polymerase buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100), 6 μCi [α-^{32}P]dCTP, 0.2 mM dATP, dGTP and dTTP, 1.5 U Taq DNA polymerase, and 0.2 μM oligonucleotide primers. Sequences of forward and reverse primers for selected genes were as follows: Apaf-1, forward, 5′-CTGCAGAAGCGCAGCAAGGTCCTTATAGCCCT-CGT-3′; Apaf-1, reverse, 5′-CGGGAGCCCCGGCTGCTTTCTGG-CAATCTAGTCTC-3′; Bfl-1, forward, 5′-TTTGCAGTCTTTGCC-TCCTT-3′; Bfl-1, reverse, 5′-ATCTTCCCAACCTCCATTCC-3′; cyclophilin A, forward, 5′-AGCAGAGATTACAGGACATTGCGA-GCAGATGGGGT-3′; cyclophilin A, reverse, 5′-CACAGAAT-TATTCCAGGATTCATGTGCCAGGGTGG-3′; IAP-2, forward, 5′-TGCCTGCATACATCCCTGCACACACTACAATGATG-3′; IAP-2, reverse, 5′-GTTGGACATCAGAAGCTGTCAGAACAAAGAATG-AA-3′; NAIP-1, forward, 5′-ACGACAGCGTCTTCGCTAAT-3′; NAIP-1, reverse, 5′-GCACACTTCTGGCAAGTTCA-3′; TNFR-1, forward, 5′-GCTAGGCCACACCCAGGAACAGTCCTTTCCAAT-TC-3′; TNFR-1, reverse, 5′-CAAGTGCATTGTCCGGGTTAGGCA-CTGTGAGTTGG-3′. Primers were purchased from IDT (Coralville, IA). PCR reactions were performed at 95°C for 5 minutes and 28 cycles of 30 seconds of denaturation at 95°C, 1 minute of annealing at 55°C and 1 minute of extension at 72°C. PCR product sizes for selected genes were as follows: Apaf-1, 456 bp; Bfl-1, 487 bp; cyclophilin A, 436 bp; IAP-2, 459 bp; NAIP-1, 464 bp; and TNFR-1, 453 bp. Signals corresponding to the different PCR products were detected by phosphorimaging.

Data analysis

Where indicated, statistical analysis was done by collecting numerical data from a minimum of three experiments and performing a Student's *t* test. A P of <0.05 was used to determine statistical significance for all analyses.

Results

T. gondii induces nuclear translocation of NF-κB in infected MEFs

To determine whether *T. gondii* infection resulted in nuclear translocation of NF-κB, the intracellular localization of p50 (NF-κB1) and p65 (RelA) heterodimers was examined in infected MEFs by immunofluorescence staining and confocal microscopy (Fig. 1). Expression of p50 and p65 in uninfected cells was primarily restricted to the cytoplasm (Fig. 1A,B, top, respectively). Stimulation of MEFs with 20 ng ml^{-1} of TNFα for 20 minutes, as a positive control, caused translocation of both subunits to the nucleus. Upon infection with *T. gondii* for 20 hours, nuclear translocation of p50 and p65 occurred in cells containing intracellular parasites stained with SAG1 antibodies (Fig. 1A,B, middle, respectively). Notably, uninfected cells in the field failed to exhibit nuclear localization of NF-κB but were competent for TNFα-induced NF-κB translocation, because treatment of the mixed population with the cytokine resulted in translocation of p50 and p65 in all cells (Fig. 1A,B, bottom, respectively).

Induction of NF-κB nuclear translocation by *T. gondii* was confirmed by immunoblotting in nuclear fractions of infected MEFs. As shown in Fig. 1C, a dose-dependent increase in host cell nuclear p50 and p65 expression was observed following 20 hours of infection. Treatment of uninfected MEFs with TNFα resulted in a marked increase in p50 and p65 localization in nuclear extracts.

To determine whether NF-κB activation occurred early in infection, confocal microscopy experiments were performed in cells infected for 1-6 hours. As shown in Fig. 2, translocation of p50 and p65 occurred as early as 1 hour post-infection (p.i.) (Fig. 2A,B, top, respectively). The proportions of cells displaying p50 and p65 nuclear staining within the mixed population of uninfected and infected MEFs were also measured. Interestingly, the proportion of parasite-infected cells exhibiting p50 or p65 in the nucleus was ~80% regardless of the time of infection (P<0.05; Fig. 2A,B, bottom, respectively). By contrast, uninfected cells in the population exhibited only a 20% rate of translocation of p50 and p65. As expected, the proportion of infected cells increased over time (50% at 1 hour p.i. and 80% at 6 hour p.i.). These data indicate that activation of NF-κB translocation is a rapid event and specific to *T. gondii* infection.

T.-gondii-induced NF-κB is competent for binding to NF-κB promoter elements

The effect of *T. gondii* infection on NF-κB DNA binding activity was examined by EMSA. As shown in Fig. 3A, an increase in NF-κB binding activity of nuclear protein extracts from infected MEFs and TNFα-treated cells was observed in comparison to uninfected controls. The components of the NF-κB complex in *T.-gondii*-infected cells were identified by supershift assays with antibodies to different members of the NF-κB family. Supershifts of NF-κB complexes confirmed the presence of p50 and p65 heterodimers in infected cells. To a lesser extent, supershifts were also observed with antibodies to

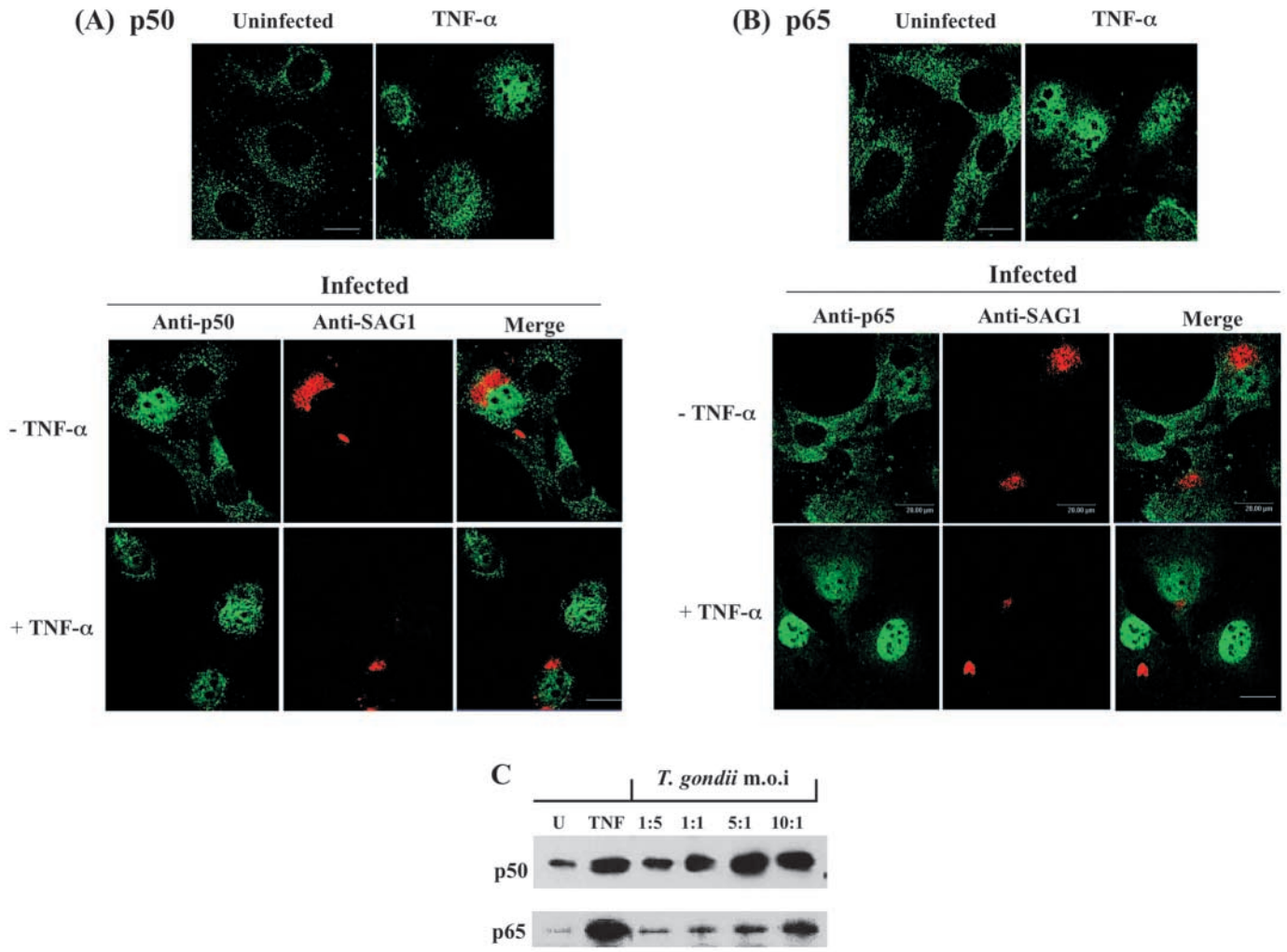

Fig. 1. *T. gondii* infection induces nuclear translocation of NF-κB. MEFs were incubated in medium alone (uninfected), treated with 20 ng ml^{-1} of TNFα for 20 minutes or infected with *T. gondii* at an m.o.i. of 5:1 for 20 hours. Confocal immunofluorescence microscopy was performed with primary rabbit polyclonal antibodies against NF-κB p50 (A) and p65 (B), and mouse monoclonal anti-*T.-gondii* SAG1. Proteins were localized using species-specific Oregon Green (NF-κB) or Texas Red (SAG1) conjugated secondary antibodies. Uninfected cells displayed mainly cytoplasmic staining of p50 and p65 (A,B, top, respectively). Stimulation with TNFα caused translocation of both subunits to the nucleus. Infection with *T. gondii* resulted in nuclear localization of p50 and p65 (A,B, middle, respectively). Uninfected cells in the same field failed to exhibit NF-κB translocation. Treatment of the mixed population of uninfected and infected MEFs resulted in translocation of p50 and p65 in all cells (A,B, bottom, respectively). Scale bar, 20 μm. (C) NF-κB translocation induced by *T. gondii* was confirmed in nuclear fractions of infected MEFs by immunoblotting. Cells were infected at increasing m.o.i. for 20 hours. Treatment of uninfected cells with TNFα (20 ng ml^{-1}, 20 minutes) was used as a positive control. Nuclear fractions were prepared as described in the Methods section and immunoblots were performed with polyclonal rabbit antibodies to p50 and p65. A dose-dependent increase in host cell nuclear p50 and p65 localization was observed in response to *T. gondii* infection.

the RelB and p52 members of NF-κB. Analysis of complexes from TNFα-stimulated MEFs mainly indicated the presence of p50 and p65 heterodimers only.

Analysis of NF-κB nuclear translocation in p65$^{-/-}$ MEFs infected with *T. gondii*

Distribution of NF-κB subunits was also examined in p65$^{-/-}$ MEFs in response to *T. gondii* infection. Results from confocal microscopy and immunoblot analysis revealed localization of p50 to the nucleus of uninfected, infected and TNFα-treated cells (data not shown). Accordingly, analysis of nuclear extracts by EMSA also showed similar levels of NF-κB binding activities among the three experimental conditions (Fig. 3B). Supershift assays of NF-κB complexes confirmed the presence of p50-p50 homodimers, but presence of p52 was only observed in *T.-gondii*-infected p65$^{-/-}$ cells. As expected, no supershift was detected with addition of antibodies to p65, indicative of the absence of this protein in the p65$^{-/-}$ cell line (Beg et al., 1995), which was confirmed by immunofluorescence and immunoblot analysis (data not shown).

T. gondii stimulates transcription of NF-κB-dependent genes with antiapoptotic properties

In addition to regulating the transcription of genes involved in

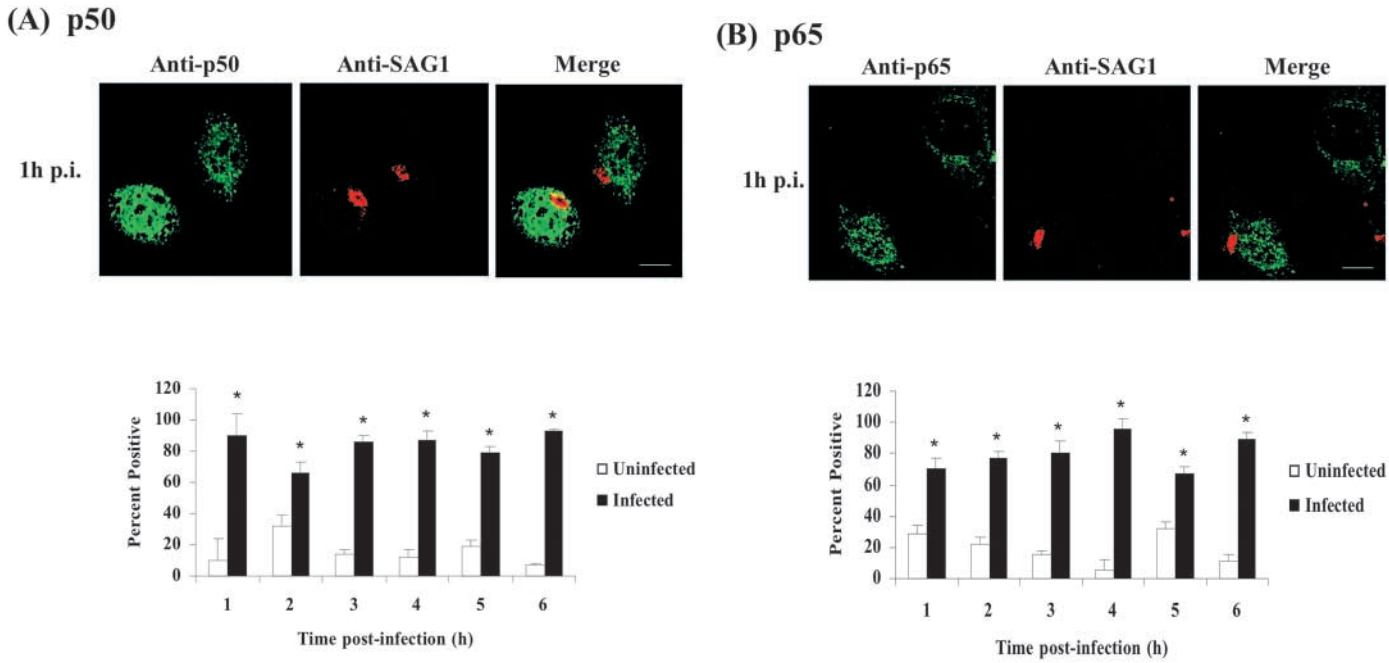

Fig. 2. The *T.-gondii*-mediated translocation of NF-κB occurs early in infection. MEFs were infected at a m.o.i. of 5:1 for 1 hours, 2 hours, 3 hours, 4 hours, 5 hours and 6 hours. Double immunofluorescence was performed with primary rabbit polyclonal antibodies against NF-κB p50 (A) and p65 (B) and mouse monoclonal anti-*T.-gondii* SAG1. Translocation of p50 and p65 occurred as early as 1 hour p.i. (A,B, top, respectively). Scale bar, 20 μm. Single-cell analysis of NF-κB translocation during the 6 hour incubation period showed significant differences in the proportions of infected cells displaying p50 and p65 nuclear staining compared with uninfected cells (A,B, bottom, respectively). Cells were counted in a minimum of 20 microscopic fields under 100× and numerical data was collected from three separate experiments. *, $P<0.05$.

immune function and cell proliferation, NF-κB plays a major role in regulating expression of antiapoptotic proteins (Karin and Lin, 2002). We sought to establish whether the activation of NF-κB by *T. gondii* correlated with an upregulation of antiapoptotic genes. DNA hybridization arrays (Superarray) probed with radiolabeled cDNA synthesized from total RNA of mock-infected and infected MEFs were used to monitor the expression of 96 genes implicated in the regulation of cell death or survival. Experiments were performed after 20 hours of infection to avoid effects of host cell lysis caused by parasite egress. As shown in Fig. 4A, a comparison of wild-type and $p65^{-/-}$ MEFs revealed distinct patterns of gene expression in response to *T. gondii* infection. The identities of all of the genes and their levels of expression as a result of infection are shown in Table 1. Overall, greater levels of induction were observed in wild-type cells among genes reported to be dependent on NF-κB activation compared with $p65^{-/-}$ (Table 1, bold). These differences were most notable in the expression of apoptosis-related genes belonging to the Bcl-2 and IAP families. Bfl-1 and Bcl-x_L, two Bcl-2 members with antiapoptotic properties that are regulated by NF-κB (Chen et al., 2000; Lee et al., 1999), were increased eightfold on average in wild-type MEFs, compared with a twofold increase in $p65^{-/-}$ cells (Fig. 4B, top). Levels of expression of Bcl-2 proapoptotic proteins such as Bad, Bax, Bid and Bim were increased by at least twofold in both cell lines (Fig. 4B, middle). Of note, expression of the pro-apoptotic caspase recruitment domain (CARD) member Apaf-1 was not increased in wild-type MEFs, but a significant (13-fold) upregulation was seen upon infection in $p65^{-/-}$ cells (Table 1).

Other genes previously shown to be targets of NF-κB include the inhibitors of apoptosis (IAP) (Wang et al., 1998). Expression of IAP1 and IAP2 increased threefold in infected wild-type MEF compared with twofold in $p65^{-/-}$ cells, whereas levels of X-IAP were comparable between the two cell lines (Fig. 4B, bottom). Strikingly, neuronal apoptosis inhibitory protein (NAIP1), which has been shown to inhibit apoptosis in neurons and several mammalian cell lines (Liston et al., 1996), was found to be upregulated 22-fold in wild-type MEFs versus twofold in $p65^{-/-}$ cells.

NF-κB has also been shown to regulate the expression of members of the TNF-receptor (TNFR) (Santee and Owen-Schaub, 1996) and TNFR-associated factor (TRAF) (Schwenzer et al., 1999) families. Six- and threefold increases in TNFR1 and TNFR2 expression, respectively, were observed in *T.-gondii*-infected wild-type MEFs compared with twofold increases in $p65^{-/-}$ cells (Table 1). Levels of TRAF-2, TRAF-3, TRAF-4 and TRAF-5 showed an apparent slight increase in $p65^{-/-}$ cells, whereas the expression of TRAF-1 and TRAF-6 was not increased in response to infection in either cell line.

In addition to host cell RNA, total RNA preparations of infected samples contain parasite RNA, which could conceivably become an artefact in the apparent gene modulation of MEFs by *T. gondii*. Our hybridization procedure was similar to that described by the previous microarray study of Blader et al., where it was reported that cDNA made from total *T. gondii* RNA had no significant cross hybridization to a human microarray spotted with ~22,000 cDNAs (Blader et al., 2001). The Blader study and the fact that the cDNA probes used in our study were generated using hybridization array

specific primers (Superarray) provide a high level of confidence that the differences in host gene expression are dependent on *T. gondii* infection and not cross-reactivity from parasite RNA contamination.

RT-PCR analysis of selected genes was performed as an additional approach to monitor changes in gene expression induced by *T. gondii*. As shown in Fig. 5, expression of *IAP-2*, *NAIP-1* and *TNFR-1* was markedly increased in response to infection, that of *Bfl-1* was slightly elevated, and that of *Apaf-1* was unaffected in wild-type MEFs. In infected p65$^{-/-}$ cells, expression of *Bfl-1*, *NAIP-1* and *Apaf-1* was increased, that of *IAP-2* remained unchanged, and that of *TNFR-1* apparently decreased. The housekeeping gene *cyclophilin A* (*ppiA*) was used as a control for equal loading of RNA samples during RT-PCR analysis.

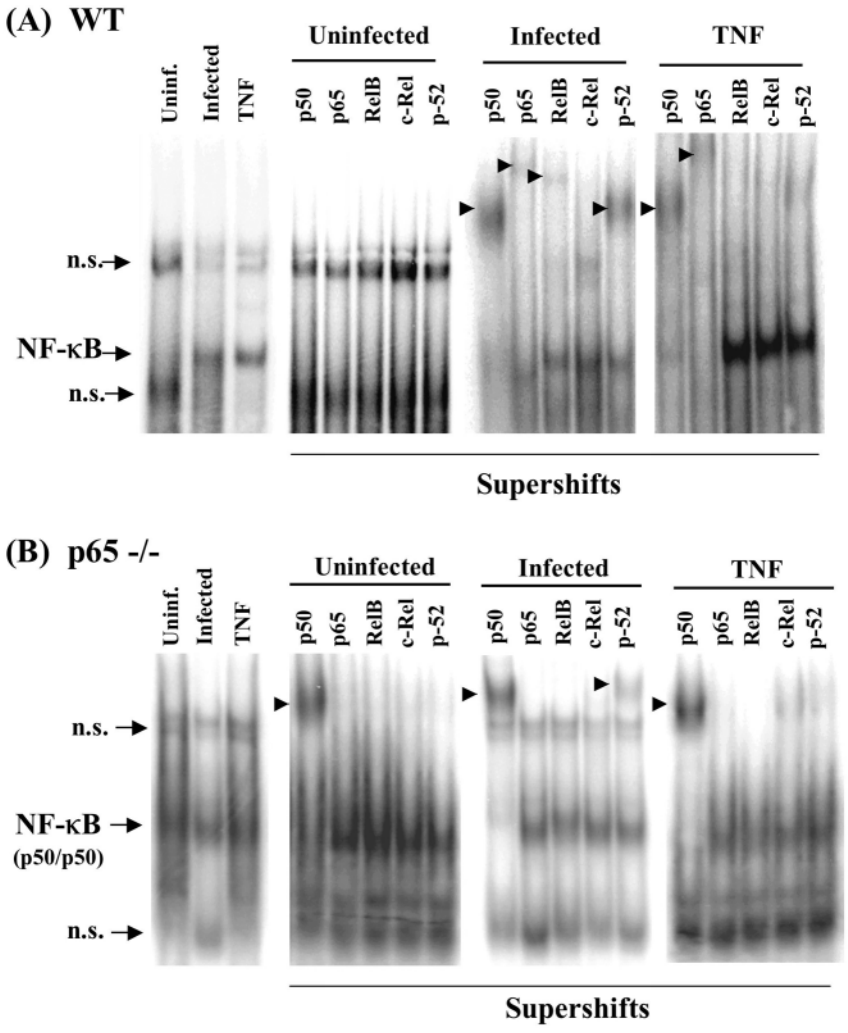

Fig. 3. *T. gondii* infection results in an increase in NF-κB DNA binding activity. (A) Binding reactions for EMSAs were performed with a radiolabeled oligonucleotide probe containing the NF-κB consensus sequence and nuclear protein extracts from uninfected, infected or TNFα-treated wild-type (WT) MEFs. Antibodies to NF-κB members p50, p65, RelB, c-Rel and p52 were used for supershift assays. An increase in NF-κB binding activity was observed in infected MEFs and TNFα-treated cells compared with uninfected controls. Supershifts of NF-κB complexes confirmed the presence of p50/p65 heterodimers in infected and TNFα-treated cells. Supershifts were also observed with antibodies to RelB and p52 in infected cells. (B) Analysis of NF-κB translocation by EMSA in p65$^{-/-}$ MEFs infected with *T. gondii*. Nuclear extracts from uninfected, infected and TNFα-treated p65$^{-/-}$ cells revealed similar levels of NF-κB binding activity. Supershift assays of NF-κB complexes showed the presence of p50/p50 homodimers in the three experimental conditions, but p52 was only observed in *T.-gondii*-infected cells. As expected, no supershift was detected with anti-p65 antibodies. Abbreviation: n.s., non-specific complex.

T. gondii infection promotes phosphorylation of IκB

Activation of NF-κB is mediated by many stimuli, which converge at the level of phosphorylation and degradation of IκB (Karin, 1999). Consistent with the results obtained in the analysis of NF-κB activation by *T. gondii*, elevated levels of P-IκB were observed in infected MEFs (Fig. 6A, top). Interestingly, although treatment of uninfected cells with TNFα resulted in the degradation of IκB, levels of IκB in *T.-gondii*-infected cells were comparable to those in uninfected cells (Fig. 6A, middle). These observations might be related to a greater stability of P-IκB in infected cells than in the TNFα control. Alternatively, only a subset of the total intracellular pool of IκB might be phosphorylated in response to infection, leaving a proportion of molecules unphosphorylated and not destined for degradation. Treatment with the proteosome inhibitor MG132 caused accumulation of P-IκB and prevented degradation of the protein in TNFα-stimulated cells (Fig. 6B, top, middle). In contrast to this, no dramatic increase in P-IκB accumulation was observed in *T.-gondii*-infected cells compared with uninfected cells in the presence of MG132 (Fig. 6B, top). Taken together, these results suggest that, unlike TNFα, the complete pool of IκB is not being phosphorylated upon infection, therefore supporting the hypothesis of a subset of molecules undergoing phosphorylation induced by *T. gondii*. One possibility for this phenotype is that phosphorylation of IκB in infected cells is localized and does not involve total cellular IκB.

P-IκB localizes to the *T. gondii* PVM

The subcellular localization of P-IκB in *T.-gondii*-infected cells was examined by immunofluorescence. Using a monoclonal antibody to P-IκB, a distinctive pattern of P-IκB was observed around the *T. gondii* PVM (Fig. 7A,C, white arrows). The staining pattern was extensive but had discontinuities, suggesting either domains of activity or selective exclusion. Such exclusion could be mediated by PVM-associated organelles (Sinai et al., 1997). To some extent, P-IκB was also observed in the host cell nucleus, consistent with the activation of the NF-κB cascade. Uninfected cells (Fig. 7D, black arrows)

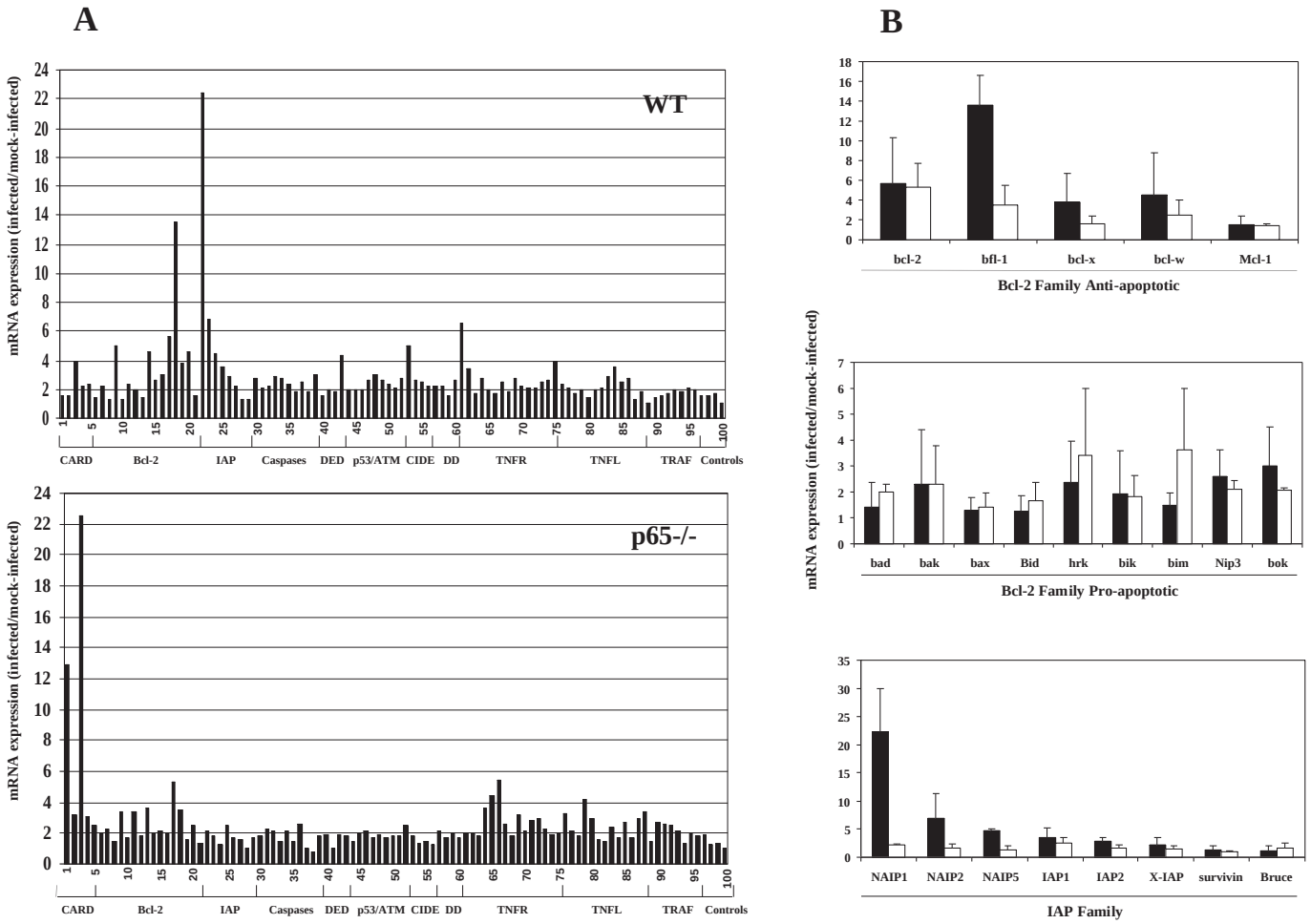

Fig. 4. Expression of host cell genes involved in the regulation of apoptosis in response to *T. gondii* infection. (A) Wild-type (WT) and p65$^{-/-}$ MEFs were infected with *T. gondii* for 20 hours and total RNA was isolated as described in the Materials and Methods section. Radiolabeled cDNA probes were hybridized to nylon membranes of the Mouse Apoptosis GEArray Q Series, which consist of a panel of 96 genes associated with apoptosis and four housekeeping genes (Superarray). Signals corresponding to the cDNAs hybridized to each gene were detected by phosphorimaging. Integrated densitometric values were calculated from each spot and normalized to actin values. On the *y* axis, ratios between infected and mock-infected values for each gene were calculated to depict changes in gene expression after infection. Bars represent the means of three experiments corresponding to the 100 genes in the array, which were grouped into families on the *x* axis. The identity of the complete set of genes and the levels of expression as a result of infection are shown in Table 1. A comparative analysis of wild-type and p65$^{-/-}$ MEFs revealed distinct patterns of gene expression in response to *T. gondii* infection. (B) Expression of apoptosis-related genes belonging to the Bcl-2 and IAP families. Differences between WT (black bars) and p65$^{-/-}$ (white bars) infected MEFs were most evident in the expression of the Bcl-2 anti-apoptotic gene *Bfl-1* (top) and the IAP members *NAIP1* and *IAP2* (bottom). Levels of expression of Bcl-2 proapoptotic genes were similar in both cell lines (middle). Bars denote ratios between infected and mock-infected gene expression levels and represent the means ± standard deviations of three separate experiments.

displayed little, if any, cytoplasmic P-IκB staining. To confirm the specificity of the P-IκB antibody, blocking experiments were performed with a peptide corresponding to a short amino acid sequence containing phosphorylated Ser32 of IκBα as described in Materials and Methods. As expected, incubation with the blocking peptide eliminated labeling by the anti-P-IκB antibody but had no effect on the parasite marker SAG1 (Fig. 7E-G).

Treatment with TNFα resulted in high levels of P-IκB in both infected and uninfected cells (Fig. 7I-K). Staining for P-IκB was observed in the host cell cytoplasm, nucleus and around the PVM (Fig. 7I). As described above, incubation with the blocking peptide resulted in the loss of P-IκB staining at both the PVM and the host cell, thereby confirming the specificity of the antibody. To rule out possible cross-reactivity to a parasite protein, immunoblot and immunoprecipitation analyses were performed with extracellular parasite extracts. No parasite-specific signals were detected by these methods (data not shown).

Localization of P-IκB to the PVM was confirmed by immunoelectron microscopy (Fig. 8). The reactivity of gold-labeled anti-P-IκB antibodies was detected mainly around the PVM, with no significant staining of the parasite being observed. The distinctive localization pattern of P-IκB suggests a highly specific mechanism is involved in the activation of NF-κB in *T.-gondii*-infected cells.

Table 1. Modulation of host cell genes involved in the regulation of apoptosis by *T. gondii*

Position	Gene*	WT†	p65$^{-/-}$†	Position	Gene*	WT†	p65$^{-/-}$†	Position	Gene*	WT†	p65$^{-/-}$†
	CARD family				Caspase family (cont.)				TNF receptor family (cont.)		
1	Apaf-1	1.5±0.8	12.9±4.1	35	Caspase-8	2.3±1.6	1.5±0.6	68	Cd30	1.8±1.3	1.9±0.6
2	Bcl-10	1.6±0.5	3.1±1.8	36	Caspase-9	1.9±1.5	2.9±0.5	69	4-1bb	2.8±2.4	3.1±2.3
3	Arc	4.0±3.6	22.6±14.0	37	**Caspase-11**	2.4±1.9	1.0±0.9	70	Dr6	2.2±2.6	2.1±1.0
4	Asc	2.3±2.6	3.1±0.6	38	Caspase-12	1.8±0.8	0.9±0.1	71	Trail-r	2.1±0.3	2.9±1.1
5	Nop30-like	2.3±2.0	2.5±0.3	39	Caspase-14	3.0±3.1	1.8±0.8	72	Tnfrsf11a	2.1±1.6	2.9±1.1
	Bcl2-family pro-apoptotic				Death effector domain family			73	Opg	2.4±1.9	2.3±0.3
6	Bad	1.4±1.0	2.0±0.3	40	Casper	1.6±0.9	1.9±1.4	74	Dr3/apo3	2.7±1.8	1.9±0.2
7	Bak	2.3±2.1	2.3±1.5	41	Flash	2.0±1.4	1.0±1.3	75	April	3.9±2.7	2.3±0.3
8	Bax	1.3±0.5	1.4±0.5	42	Fadd	1.9±1.0	1.9±0.4		TNF ligand family		
9	Bcl2l10	4.9±5.4	3.4±1.5	43	Bar like	4.3±4.1	1.8±0.1	76	**Tnfa**	2.4±1.3	3.3±0.8
10	Bid	1.3±0.6	1.7±0.7		p53 and ATM pathway			77	**Tnfb**	2.1±1.3	2.1±0.2
11	Hrk	2.4±1.6	3.4±2.6	44	p21Waf1	2.0±1.8	1.5±0.8	78	**LT-b**	1.7±1.9	1.8±0.7
12	Bik	1.9±1.7	1.8±0.8	45	Chk1	1.9±0.5	2.1±0.3	79	Ox40l	2.0±1.4	4.2±0.7
13	Bim	1.5±0.5	3.6±2.4	46	Mdm2	2.0±0.4	2.1±0.7	80	**Cd40l**	1.4±0.3	2.9±1.8
14	Blk	4.6±4.6	2.1±0.5	47	Chk2	2.6±1.6	1.6±0.1	81	**Fasl**	2.0±1.4	1.6±0.3
15	Nip3	2.6±1.0	2.1±0.4	48	Gadd45	3.1±1.1	1.9±0.4	82	Cd27l	2.1±1.6	1.4±0.5
16	Bok	3.0±1.5	2.1±0.1	49	Hus1	2.6±0.9	1.7±0.1	83	Cd30l	2.9±2.2	2.3±0.4
	Bcl-2 family anti-apoptotic			50	Rpa	2.3±0.8	1.8±0.3	84	4-1bbl	3.5±3.2	1.7±0.7
17	**Bcl-2**	5.7±4.6	5.3±2.4	51	**P53**	2.0±1.2	1.8±0.2	85	Trail	2.5±2.3	2.7±0.1
18	**Bfl-1**	13.6±3.0	3.5±2.0	52	Atm	2.7±1.3	2.5±0.4	86	Tnfsf11	2.8±2.8	1.7±0.3
19	**Bcl-x**	3.8±2.9	1.6±0.9		CIDE family			87	Apo3L	1.4±0.8	2.9±0.7
20	**Bcl-w**	4.5±4.3	2.5±1.5	53	Cide-a	4.9±3.7	1.8±0.1	88	Tnfsf14	1.8±1.4	3.4±0.9
21	Mcl-1	1.5±0.9	1.4±0.2	54	Cide-b	2.7±1.5	1.4±0.3		TNFR associated factor family		
	IAP family			55	Dffa	2.4±2.1	1.4±0.6	89	**Traf1**	1.1±0.8	1.5±0.4
22	Naip1	22.4±7.6	2.2±0.1	56	Dff40	2.2±1.3	1.2±1.3	90	**Traf2**	1.5±0.7	2.7±1.1
23	Naip2	6.9±4.5	1.8±0.6		Death domain family			91	Traf3	1.5±0.2	2.6±0.9
24	Naip5	4.8±0.3	1.3±0.8	57	Cradd	2.2±1.2	2.1±0.7	92	Traf4	1.6±0.6	2.5±0.1
25	**Iap1**	3.6±1.6	2.5±1.1	58	Dapk	2.2±1.7	1.7±0.5	93	Traf5	1.9±1.1	2.2±1.2
26	**Iap2**	2.9±0.7	1.7±0.5	59	Myd88	1.5±0.4	2.0±0.3	94	Traf6	1.8±0.8	1.3±0.8
27	**X-iap**	2.2±1.4	1.6±0.4	60	Rip	2.7±1.3	1.7±0.6	95	Tank	2.0±1.0	2.0±0.2
28	Survivin	1.3±0.8	1.0±0.2		TNF receptor family			96	Trip	2.0±0.9	1.8±0.2
29	Bruce	1.3±0.7	1.7±0.9	61	**Tnfr1**	6.5±4.2	2.1±0.3		Housekeeping genes		
	Caspase family			62	Tnfr2	3.4±1.0	2.1±0.9	97	Gapdh	1.6±0.8	1.9±0.2
30	Caspase-1	2.8±2.2	1.8±0.6	63	Ltbr	1.7±0.2	1.8±0.4	98	Ppia	1.6±0.4	1.3±0.2
31	Caspase-2	2.1±1.2	2.3±0.2	64	Ox40	2.7±1.8	3.6±0.7	99	Rpl13A	1.6±0.3	1.3±0.3
32	Caspase-3	2.2±0.5	2.2±0.1	65	**Cd40**	2.0±1.3	4.6±0.4	100	Beta-actin	1.0±0.0	1.0±0.0
33	Caspase-6	2.9±2.1	1.5±0.4	66	**Fas antigen**	1.7±0.5	5.4±1.8				
34	Caspase-7	2.8±1.5	2.2±1.8	67	Cd27	2.5±2.5	2.6±0.4				

*Target genes of NF-kB are shown in bold font (reviewed at http://people.bu.edu/gilmore/nf-kb/).

†Densitometric values were normalized to actin and levels of gene expression are shown as an infected/mock-infected ratio. Data represent means±s.d. of three separate experiments.

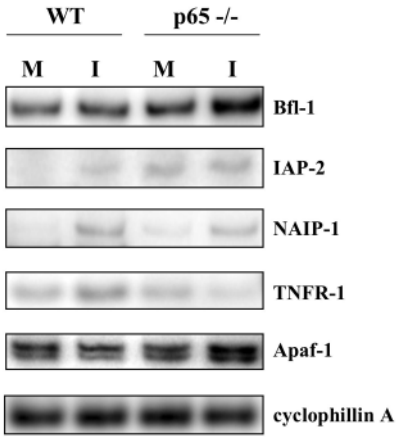

Fig. 5. RT-PCR analysis of gene expression in response to *T. gondii* infection. Total RNA (1 μg) from mock-infected and infected MEFs was used in a RT-PCR reaction in the presence of [α-^{32}P]dCTP as described in Materials and Methods. Signals corresponding to the different PCR products were detected by phosphorimaging. Results depict a representative experiment of three performed.

Discussion

NF-κB has been widely regarded as a key transcription factor playing a crucial role in modulating both innate and adaptive immune responses. In addition, activation of NF-κB is extensively considered to play an antiapoptotic function in cellular responses to diverse injurious stimuli (Karin and Lin, 2002). Microbial pathogens rely on different strategies to manipulate the NF-κB pathway in order to balance both detrimental proinflammatory and beneficial antiapoptotic consequences of NF-κB activation (Tato and Hunter, 2002).

The present study demonstrates that *T. gondii* induces rapid translocation of NF-κB to the nucleus of infected fibroblasts. This response was sustained at later stages of infection and was specific to parasite infection as indicated by confocal microscopy experiments. Single-cell analysis of NF-κB translocation within a mixed population resulted in a significant proportion of infected cells displaying p50 and p65 nuclear staining compared with uninfected cells. These results argue against a ‘bystander effect’ causing the translocation of NF-κB as a result of host cell activation owing to initial interactions between fibroblasts and *T. gondii*, or of parasite

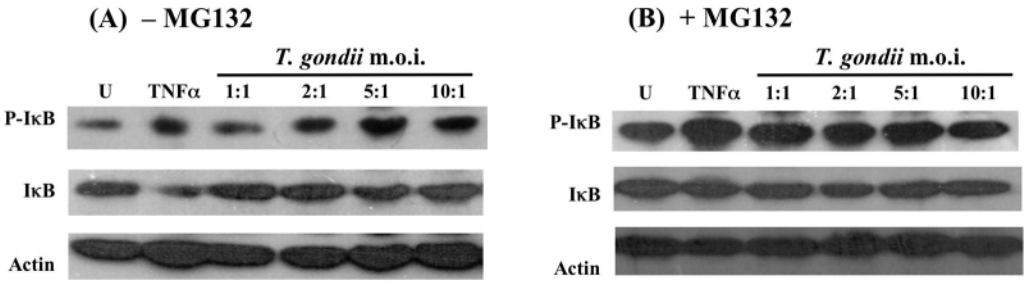

Fig. 6. Effects of *T. gondii* infection on the phosphorylation of IκB. MEFs were infected at increasing m.o.i. for 20 hours. Treatment of uninfected cells with TNFα (20 ng ml^{-1}, 20 minutes) was used as a positive control. Immunoblots were performed with mouse monoclonal anti-P-IκBα, polyclonal rabbit anti-IκBα, and polyclonal rabbit anti-actin as described in the Materials and Methods section. (A) Elevated levels of P-IκB were observed in infected MEFs but, unlike the TNFα control, levels of IκB were not decreased. (B) Treatment with 50 μM MG132 proteosome inhibitor for 2 hours prior to TNFα stimulation caused an accumulation of P-IκB and prevented degradation of the protein. In contrast to this, no apparent increase in P-IκB accumulation was observed in *T.-gondii*-infected cells treated with 50 μM MG132 for 2 hours prior to immunoblot analysis.

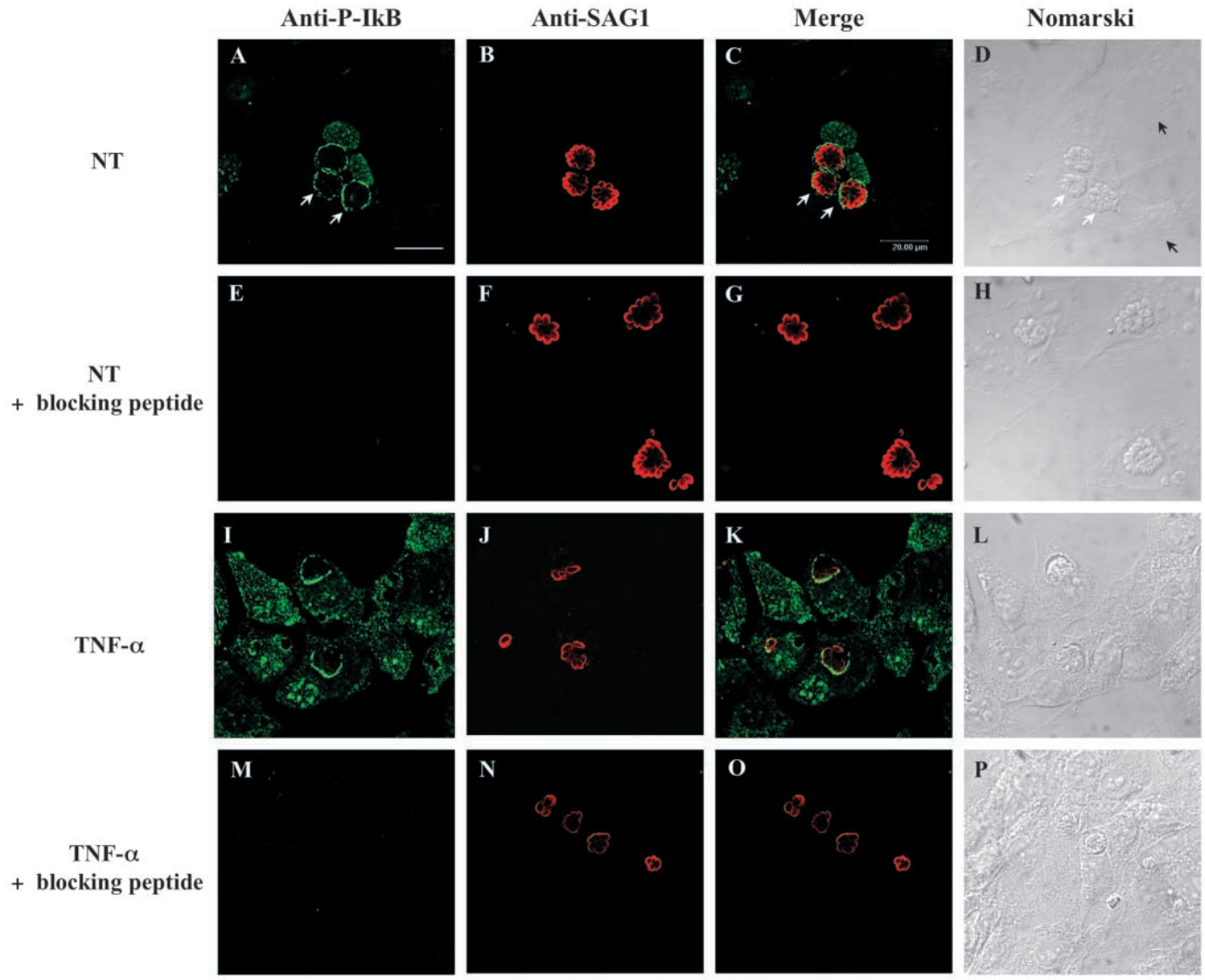

Fig. 7. Localization of P-IκB to the *T. gondii* PVM. MEFs grown on glass coverslips were infected at a m.o.i. of 5:1 for 20 hours. Double immunofluorescence was performed with mouse monoclonal anti-P-IκBα (green) and mouse monoclonal anti-*T.-gondii* SAG1 (red). A distinctive pattern of P-IκB was observed around the *T. gondii* PVM (A,C, white arrows). Uninfected cells (D, black arrows) displayed little, if any, cytoplasmic P-IκB staining. Blocking experiments with a peptide corresponding to a short amino acid sequence containing phosphorylated Ser 32 of IκBα eliminated labeling by the anti-P-IκB antibody but had no effect on the parasite marker SAG1 (E-G). Treatment with TNFα resulted in high levels of P-IκB in both infected and uninfected cells (I,K). Staining for P-IκB was observed in the host cell cytoplasm, nucleus and around the PVM. Incubation with the blocking peptide resulted in the loss of P-IκB staining both at the PVM and host cell (M-O). Scale bar, 20 μm.

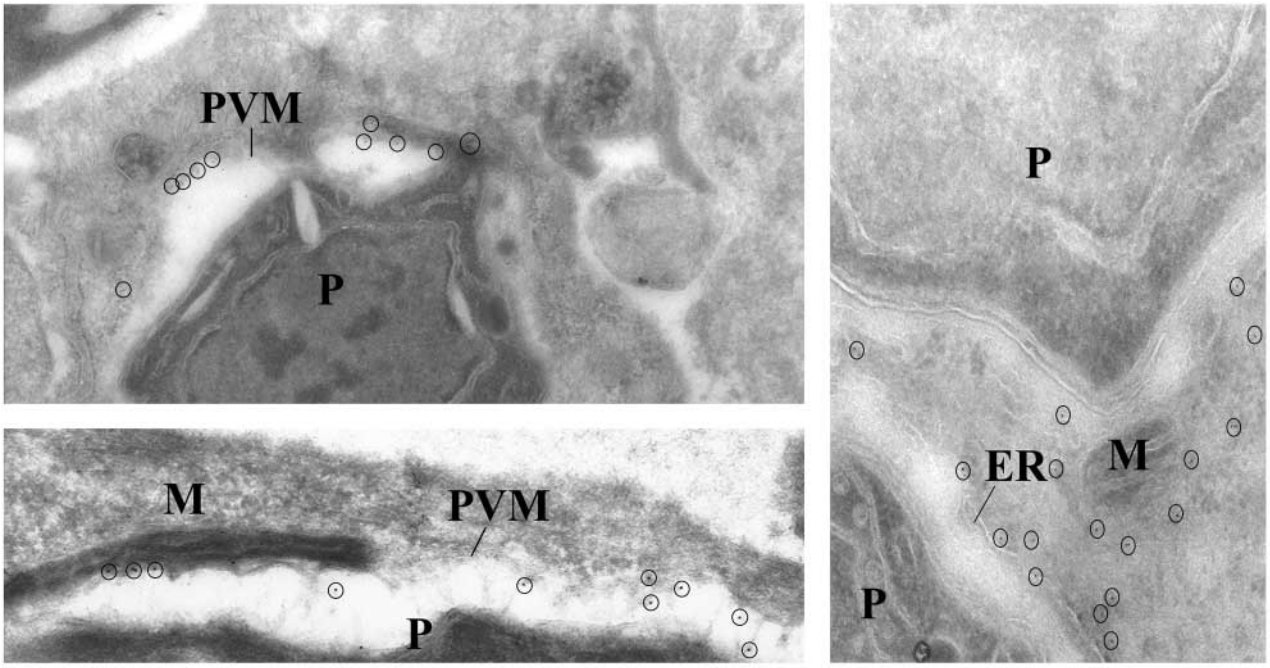

Fig. 8. Confirmation of P-IκB localization to the *T. gondii* PVM by immunoelectron microscopy. Infected fibroblasts were labeled with rabbit anti-P-IκB antibodies and 5 nm protein-A/gold particles. The reactivity of gold-labeled anti-P-IκB antibodies was detected mainly around the PVM (circles), with no significant staining of the parasite being observed. Abbreviations: ER, host endoplasmic reticulum; M, host mitochondria; P, parasite; PVM, parasitophorous vacuolar membrane. The localization pattern of P-IκB in the PVM of infected cells suggests a highly specific mechanism is involved in the activation of NF-κB by *T. gondii*. Scale bars, 0.5 μm.

products secreted into the medium. Although no extensive nuclear translocation of NF-κB subunits was detected, the low level of nuclear translocation in uninfected cells could be due to a paracrine pathway via TNFα or IL-1β production from neighboring cells infected with the parasite. In support of this hypothesis, our DNA array data showed an increase in TNFα gene expression as a result of *T. gondii* infection (Table 1), whereas Blader et al. reported an upregulation of IL-1β in microarrays of infected fibroblasts (Blader et al., 2001).

Previous studies have reported conflicting results on the ability of *T. gondii* to activate NF-κB. Shapira et al. reported activation of NF-κB (p50/p65) in peritoneal cells from infected C57BL/6 mice, but in vitro experiments with bone-marrow derived macrophages failed to show nuclear localization of p65 or c-Rel in response to a 2 hour *T. gondii* infection (Shapira et al., 2002). In a separate study, Butcher et al. reported a lack of NF-κB activation by *T. gondii* in in vitro peritoneal macrophages examined at 2 hours p.i., which correlated with a failure to stimulate TNFα and IL-12 (Butcher et al., 2001). Moreover, both studies reported an inhibition of LPS-induced activation of NF-κB and transcription of NF-κB-dependent genes such as *IL-6*, *IL-8*, *IL-12*, *TNFα* and *iNOS*. Discrepancies between our results and the studies mentioned above might be related to differences in the time points and cell types examined. In our study, *T.-gondii*-induced NF-κB translocation was apparent as early as 1 hour p.i. and the response remained active after 20 hour p.i., as determined by three different experimental techniques (Figs 1-3).

The different results obtained in vitro among fibroblasts and macrophages might actually have implications in the context of in vivo infection. In the latter, an apparent blockade of NF-κB activation and proinflammatory cytokine production by *T. gondii* in infected macrophages might inhibit phagocytic functions that could be detrimental to the parasite (Butcher et al., 2001). On the other hand, NF-κB activation by *T. gondii* in fibroblasts, as reported herein, might be crucial for eliciting a survival response in host cells by stimulating an antiapoptotic phenotype as discussed below. Of note, translocation of NF-κB and production of IL-8 have been reported in epithelial cells infected with *T. gondii* (Kim et al., 2001). Such a response could exacerbate the inflammatory response but also increase leukocyte flow to the site of infection and favor dissemination of the pathogen in the host. Clearly, differences between immune and non-immune cells in response to *T. gondii* infection is a fascinating area of investigation with profound implications in both the acute and chronic stages of disease.

The NF-κB/ Rel family is composed of multiple members, several of which play specific functions in the cell-type-specific regulation of gene expression (Ghosh and Karin, 2002). In addition to p50 and p65, gel shift assays revealed the presence of p52 (NF-κB2) and RelB in nuclear extracts of infected cells. RelB associates with the NF-κB2 precursor p100 in the cytoplasm of resting cells. Upon cell stimulation, the IκB-like C-terminus of p100 becomes degraded, releasing p52/RelB dimers that translocate to the nucleus (Solan et al., 2002). Although translocation of p50/p65 dimers is mostly dependent on phosphorylation of IκB by the IKKβ subunit of the IKK complex, activation of p52/RelB relies on p100 phosphorylation by the IKKα subunit (Ghosh and Karin, 2002). It is noteworthy that, in addition to inducing p50/p65 translocation, *T. gondii* has the capability to activate p52/RelB, which is an important transcription factor involved in B-cell

proliferation and adaptive immunity (Caamano et al., 1998; Snapper et al., 1996). It should be mentioned that mice deficient in p52 or RelB are highly susceptible to toxoplasmosis (Caamano et al., 1999; Franzoso et al., 1998), which, in $p52^{-/-}$ mice, is characterized by an increase in lymphocyte apoptosis during chronic infection (Caamano et al., 2000).

The fact that our previous data showed a loss of apoptosis inhibition by *T. gondii* in $p65^{-/-}$ fibroblasts (Payne et al., 2003) prompted us to compare gene expression profiles between wild-type and $p65^{-/-}$ cells in response to infection, with a focus on genes involved in cell death or survival. In correlation with the activation of NF-κB, an increased expression of genes that are targets for this transcription factor was observed in infected MEFs by DNA array analysis. Interestingly, differences in the extent of gene expression were observed between wild-type and $p65^{-/-}$ cells, particularly among members of the Bcl-2, IAP, TNFR and CARD families. To a certain extent, these differences were also observed by RT-PCR analysis, but the divergence of gene expression between the cell lines was not as profound as observed with the DNA arrays, which could reflect differences in sensitivity between the two methods.

Using DNA arrays, expression of Bfl-1, IAP2, and TNFR1 was elevated over three fold in wild type cells compared to $p65^{-/-}$. The results from IAP2 expression confirm earlier findings of Blader et al., where upregulation of this gene was also observed in *T.-gondii*-infected fibroblasts (Blader et al., 2001). The parasite-mediated induction of Bfl-1 might prevent host cell death primarily through regulation of mitochondrial events such as membrane depolarization, cytochrome-*c* release, and caspase-9 activation (Chen et al., 2000; Lee et al., 1999). Indeed, we (L. Hardi, J. Carmen and A. P. Sinai, unpublished) and others (Goebel et al., 2001) find an inhibition of cytochrome-*c* release in infected cells. In addition, we find an inhibition of caspase-9 activity as well as a blockade of the initiator caspase 8 and executioner caspase 3 activities enforced in *T.-gondii*-infected cells (Payne et al., 2003).

Stimulation of the expression of IAP family members is predicted to lead directly to the inhibition of both initiator and executioner caspases (Devaraux and Reed, 1999). We find a specific induction in the expression of several members of the IAP family, a result that mirrors the findings of Blader et al. for IAP1 and IAP2 (Blader et al., 2001). A dramatic example of this is the induction of *NAIP1* expression in infected MEFs. *NAIP1* was initially identified as a candidate gene for the inherited neurodegenerative disorder spinal muscular atrophy and has been shown to inhibit both caspase 3 and caspase 7 in neurons and HeLa cells (Liston et al., 1996; Maier et al., 2002). The mechanisms involved in the activation of *NAIP1* expression by *T. gondii* warrants further investigations, particularly because, to our knowledge, there is no literature concerning a direct role for NF-κB in regulation of this gene. Our data suggest a role for the regulation of *NAIP1* by NF-κB, at the very least in the context of parasite infection.

A recent study reported a capability of *T. gondii* to regulate expression of TNFRs in vitro (Derouich-Guergour et al., 2002), as shown by an enhanced release of soluble TNFR1 from infected cells. The resistance of *T.-gondii*-infected fibroblasts to apoptosis induced by TNFα raises the possibility of a downregulation of TNFR1 or TNFR2 during infection. Such a scenario does not appear likely at the mRNA level because results from our array experiments revealed increased expression of *TNFR1* and *TNFR2* in response to *T. gondii*, an event that also correlates with the activation of NF-κB. Further studies are required to examine the effects of infection on membrane and soluble protein levels of TNFR1 and TNFR2, to determine whether a deficiency in TNFα signaling at the receptor level plays a role in the resistance to apoptosis.

Although *T. gondii* infection resulted in an apparent protective phenotype in wild-type MEFs, expression of some proapoptotic genes of the Bcl-2 family was increased at least twofold in both wild-type and $p65^{-/-}$ cells. Of interest was the significant upregulation of *Apaf-1* in $p65^{-/-}$ cells only. These data suggest that a complex regulation pattern of both pro- and antiapoptotic genes can be induced by *Toxoplasma* in infected fibroblasts. It is likely that the increase in proapoptotic gene expression by infection alone is not sufficient enough to trigger cell death but, upon an apoptotic stimulus, a lack of NF-κB activity could have important defects in enhancing the expression level of survival genes.

Phosphorylation of IκB by the IKK complex is an essential step in the NF-κB signaling cascade (Karin, 1999). *T. gondii* infection promoted phosphorylation of IκB but, in contrast to the TNFα control, subsequent degradation of the protein was apparently lacking in infected cells. Because treatment with TNFα was, for a short period of time, comparable to *T. gondii* infection, it is plausible that IκB levels observed in infected cells were related to a reappearance of the protein after 20 hours p.i. Alternatively, phosphorylation of IκB induced by *T. gondii* infection might occur without proteolysis of IκB. During the conventional NF-κB pathway, IKK phosphorylates IκB at serines 32 and 36, and predisposes the protein for ubiquitination and degradation by the proteasome (Ghosh and Karin, 2002). However, NF-κB activation via tyrosine phosphorylation of IκB by an unspecified kinase without proteolytic degradation of IκB has also been shown to occur (Imbert et al., 1996; Livolsi et al., 2001). Our studies indicate that phosphorylation of IκB in *T.-gondii*-infected fibroblasts occurs at serines 32 and 36, because these were the targets of the monoclonal antibody used, but possible phosphorylation at tyrosine residues was not demonstrated.

A third possibility for the observable constant levels of IκB during infection could be that only a small proportion of the intracellular pool of the protein becomes phosphorylated and therefore susceptible for degradation. Treatment with the proteosome inhibitor MG132 did not cause a dramatic accumulation of P-IκB in infected cells, as opposed to cells stimulated with TNFα, supporting the idea of a subset of molecules undergoing phosphorylation. Surprisingly, a closer examination of the subcellular distribution of P-IκB in infected cells by confocal microscopy revealed localization of the phosphorylated protein primarily to the *T. gondii* PVM. Following stimulation with TNFα, P-IκB was observed throughout the cytoplasm and nuclei of infected cells in addition to the PVM. Taken together, our results indicate that *T. gondii* promotes a localized phosphorylation of IκB at the PVM rather than inducing a massive intracellular response, which might reflect a capability of the parasite to regulate the NF-κB cascade.

At present, the mechanisms involved in sequestration of IκB to the PVM are not clear and the parasite and host factors

involved in this interaction remain to be identified. Coincidentally, *Theileria parva*, an apicomplexan parasite that causes lymphoproliferative disease in cattle and is closely related to *T. gondii*, recruits the IKK complex of infected host cells to the surface of schizont forms of the parasite (Heussler et al., 2002). It has been proposed that this unusual mechanism results in continuous phosphorylation of IκB by IKK and constitutive activation of NF-κB, which promotes proliferation of infected lymphocytes and resistance to apoptosis (Heussler et al., 2001b). Whether or not IκB is recruited to the *T. gondii* PVM as a complex with IKK remains to be established. Still, the focal pattern of P-IκB association with the parasite, together with an absence of IκB degradation, suggests a highly specific mechanism is involved in the activation of NF-κB by *T. gondii* in infected cells.

T. gondii is widely regarded as one of the most successful parasites on earth because of its broad host range and ability to establish a lifelong chronic infection (Tenter et al., 2000). The induction of a survival response by *T. gondii* through NF-κB-dependent upregulation of antiapoptotic genes is indicative of a high level of adaptation between the parasite and the host. Direct consequences of the *T.-gondii*-mediated prevention of apoptosis might include modulation of the immune response and continued existence of the parasite, thereby setting the stage for the transition between the acute (tachyzoite) and chronic (bradyzoite) phases of infection. Finally, localization of phosphorylated IκB to the *T. gondii* PVM by a potential parasite-derived factor suggests a unique capability of this pathogen to manipulate the NF-κB pathway.

We thank D. Baltimore, M. Karin, N. Rice and L. Kasper for their gifts of cell lines and antibodies. We thank V. Rangnekar for advice on gel-shift assays. The technical assistance by the staff of the University of Kentucky College of Medicine Electron Microscopy and Imaging Suite is gratefully acknowledged. This work was supported by new faculty startup funds from the University of Kentucky Research Challenge Trust Fund (RCTF), American Cancer Society Institutional Research Grant (850001-13-IRG) and a grant from NIH/NIAID RO-1 AI49367 awarded to A.P.S. I.C. is supported by a Scientist Development Grant (0230079N) from the American Heart Association.

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