Initiation and termination of NF-κB signaling by the intracellular protozoan parasite *Toxoplasma gondii*

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Accepted 7 April 2005
Journal of Cell Science 118, 3501-3508 Published by The Company of Biologists 2005
doi:10.1242/jcs.02428

Journal of Cell Science

Summary

Signaling via the NF-κB cascade is critical for innate recognition of microbial products and immunity to infection. As a consequence, this pathway represents a strong selective pressure on infectious agents and many parasitic, bacterial and viral pathogens have evolved ways to subvert NF-κB signaling to promote their survival. Although the mechanisms utilized by microorganisms to modulate NF-κB signaling are diverse, a common theme is targeting of the steps that lead to IκB degradation, a major regulatory checkpoint of this pathway. The data presented here demonstrate that infection of mammalian cells with *Toxoplasma gondii* results in the activation of IKK and degradation of IκB. However, despite initiation of these hallmarks of NF-κB signaling, neither nuclear accumulation of NF-κB nor NF-κB-driven gene expression is observed in infected cells. However, this defect was not due to a parasite-mediated block in nuclear import, as general nuclear import and constitutive nuclear-cytoplasmic shuttling of NF-κB remain intact in infected cells. Rather, in *T. gondii*-infected cells, the termination of NF-κB signaling is associated with reduced phosphorylation of p65/RelA, an event involved in the ability of NF-κB to translocate to the nucleus and bind DNA. Thus, these studies demonstrate for the first time that the phosphorylation of p65/RelA represents an event downstream of IκB degradation that may be targeted by pathogens to subvert NF-κB signaling.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/118/15/3501/DC1

Key words: *Toxoplasma gondii*, NF-κB, Immune regulation, Innate immunity, Host-pathogen interactions, Intracellular signaling

Introduction

The ability of an organism to control infection depends on its capacity to mount an appropriate immune response. The NF-κB family of transcription factors, a central component of innate and adaptive immunity, is responsible for the activation of many genes required in situations of infection, stress and injury. This family consists of a group of evolutionarily conserved proteins that pair to form combinations of hetero- and homo-dimers, which trigger distinct transcriptional programs in response to diverse stimuli. One of the major checkpoints of NF-κB activation is mediated by IκB proteins, which are the primary inhibitors of inducible NF-κB signaling. Thus, in response to various microbial and inflammatory stimuli, the IκB kinase (IKK) (Karin and Ben-Neriah, 2000), a complex composed of IKKα, IKKβ and IKKγ, phosphorylates IκB, which tags it for polyubiquitination and degradation by the proteosome. This event liberates NF-κB proteins, exposes their nuclear localization sequences, and allows them to translocate to the nucleus, where they can regulate gene transcription and initiate immune responses (Karin and Ben-Neriah, 2000; Rothwarf and Karin, 1999; Silverman and Maniatis, 2001).

Because of the important role played by NF-κB signaling in cell survival, innate recognition of microbial products and immunity to infection, this pathway exerts a strong selective pressure on infectious agents. Consequently, numerous parasites, bacteria and viruses have developed strategies to modulate this pathway to promote pathogen survival (Li, 2003; Santoro et al., 2003; Tato and Hunter, 2002). Although the mechanisms utilized by pathogens to exploit NF-κB signaling are diverse, a common theme is the manipulation of IκB degradation. For example, *Yersinia pestis* inhibits the activation of NF-κB and limits proinflamatory responses by interfering with activation of IKKβ and so prevents IκB degradation (Orth et al., 2000). By contrast, *Theileria sp.* induce constitutive activation of the IKK signalosome, resulting in NF-κB activity and enhanced proliferation and survival of infected cells (Heussler et al., 2002).

Toxoplasma gondii is an obligate intracellular parasite, and an important opportunistic pathogen of humans, particularly in patients with defects in T-cell function. This parasite is also a member of the ancient phylum Apicomplexa, which includes multiple pathogens responsible for considerable morbidity and mortality in humans and animals such as *Plasmodium* (the causative agent of malaria), *Theileria* and *Cryptosposidium*. Although previous studies have revealed an important role for NF-κB in the development of innate and adaptive immunity to *T. gondii* (Caamano and Hunter, 2002), there is evidence that

T. gondii can interfere with host cell signaling in the cells it infects and that this represents a parasite strategy to evade the innate immune response (Goebel et al., 2001; Luder et al., 1998; Luder et al., 2001; Shapira et al., 2004). For example, cells infected with *T. gondii* are refractory to LPS-induced expression of pro-inflammatory genes such as TNF-α, i-NOS and IL-12, which are required for resistance to this parasite (Butcher et al., 2001; Denkers, 2003; Robben et al., 2004; Shapira et al., 2004; Shapira et al., 2002). There are several reports that these genes are regulated by NF-κB (as well as by other pathways) and the failure of infected cells to produce these molecules is consistent with the lack of NF-κB activity in infected cells both in vitro and in vivo (Butcher et al., 2001; Denkers, 2003; Robben et al., 2004; Shapira et al., 2004; Shapira et al., 2002). However, despite these studies, the point at which *T. gondii* interferes with the NF-κB pathway remains unclear (Butcher et al., 2001; Denkers et al., 2003; Shapira et al., 2004; Shapira et al., 2002).

The experiments described here were aimed at understanding the events that underlie the ability of *T. gondii* to terminate the NF-κB pathway. These studies demonstrate that, despite IKK-dependent degradation of IκBα, infection of mammalian cells with *T. gondii* does not result in nuclear localization of NF-κB or the upregulation of NF-κB-dependent gene expression. However, this defect is not due to a parasite-mediated block in nuclear import, as general nuclear import and constitutive nuclear-cytoplasmic shuttling of NF-κB remain intact in infected cells. Rather, in *T. gondii*-infected cells, the termination of NF-κB signaling is associated with reduced phosphorylation of p65/RelA, an event involved in the ability of NF-κB to translocate to the nucleus and bind DNA. Although many pathogens regulate NF-κB by manipulating IκB degradation, the data presented here demonstrate for the first time that the phosphorylation of p65/RelA represents an event downstream of IκB degradation that can be exploited by pathogens to subvert NF-κB signaling.

Materials and Methods

Reagents

Mouse anti-p65 was from BD Transduction Laboratories (San Jose, CA) and rabbit anti-p50, c-Rel, p52, IKKα, IKKβ, and IKKγ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Protein G Sepharose was from Amersham Pharmacia (Piscataway, NJ). Human TNF-α was from Calbiochem (San Diego, CA).

Cell culture and parasites

Human foreskin fibroblasts (HFF) (American Type Culture Collection) were maintained in complete Dulbecco's modified Eagle medium (DMEM, Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (Hyclone Laboratories), 2 mM glutamine, 1000 U/mL penicillin, 10 g/mL streptomycin, 0.25 mg/mL fungizone, 1 mM sodium pyruvate, 1% (vol/vol) nonessential amino acids, and 50 ng/mL ciprofloxacin (Life Technologies). Tachyzoites of the virulent RH strain (expressing RFP where indicated) were maintained in vitro by infection of HFFs and biweekly passage. Tachyzoites from freshly lysed fibroblast cultures were washed once with PBS and resuspended in DMEM for in vitro assays. Parasites were added to cells (10:1 ratio) and at time points indicated (resulting in an 80-90% infection rate), supernatants were removed, cells washed with ice cold PBS, and whole cell extracts prepared.

NF-κB reporter cells

NF-κB reporter cells were generated as follows: NIH 3T3 cells were transfected using FuGENE transfection reagent (Roche, Indianapolis, IN), with pNF-κB-hrGFP (Stratagene, La Jolla, CA) plasmid containing a GFP reporter driven by a basic promoter element (TATA box) joined by 5 tandem repeats of NF-κB binding elements. Hygromycin selection was used to generate a population of cells that were then used to generate clones by limiting dilution. Clones used for experiments were selected based on high GFP expression following TNF-α treatment and low GFP expression under resting conditions.

Microscopy

For indirect immunofluorescence, coverslips were fixed for 10 minutes in 4% paraformaldehyde, permeabilized for 10 minutes in 0.25% Triton-X 100, blocked for 1 hour in PBS (pH 7.4) + 3% BSA fraction V (Fisher), incubated for 1 hour with primary antibody (in blocking solution), washed, and incubated for 1 hour in secondary antibody. Secondary antibodies used were Alexa goat anti-mouse 488 (Molecular Probes; 1:500) and Alexa goat anti-rabbit 594 (Molecular Probes; 1:500). Finally, parasite and host nuclear DNA were stained with 49,6-diamidino-2-phenylindole (DAPI) for 5 minutes (in PBS), samples were washed in PBS, and mounted on glass slides using Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) for examination using either a Zeiss Axiovert 35 equipped with a heated stage or a Leica DM IRBE equipped with a motorized filter wheel. Both inverted microscopes were equipped with motorized stage, 100W Hg-vapor lamp and Orca-ER digital camera (Hamamatsu, USA). Images were captured using Openlab 3.1 software (Improvision, Lexington, MA).

Analysis of the NF-κB pathway

All immunoprecipitation (IP) and immuno-blot (IB) analysis was performed as described previously (Zhong et al., 1997). Cells were lysed in buffer containing 200 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1 mM DTT, and protease inhibitors (Roche). For in vitro kinase assays, GST-IκB was generated and assays were performed as previously described (Zhong et al., 1997). Briefly, cells were stimulated with TNF-α (10 ng/ml), or infected for the times indicated, and whole cell lysates were prepared. Lysate proteins (approx. 500 μg) were immunoprecipitated with anti-IKK-γ antibody (Santa Cruz Biotechnology), and the immunoprecipitates were assayed for kinase activity using 3 μg recombinant GST-IκBα (1-54) as a substrate as described (Ruland et al., 2001). Electrophoretic mobility shift assays (EMSAs) were performed as described previously (Zhong et al., 1997). Briefly, samples contained 10 μg of whole cell extracts and were incubated (15 min; 26°C) with ^{32}P-labeled double-stranded oligonucleotides corresponding to the palindromic κB site (5′-GGGAATTCCC-3′), electrophoresed on a 5.5% polyacrylamide gel and then visualized by autoradiography. To asses phosphorylation of p65, HFF cells were labeled in vitro with ^{32}Pi as previously described (Zhong et al., 1997). Briefly, cells were labeled with 0.5 mCu ^{32}Pi for 2 hours before stimulation for 15 minutes. The cells were lysed, p65 was immunoprecipitated and separated on SDS-PAGE, and the dried gel was exposed for autoradiography over night or 3 hours for total phosphorylation (unprecipitated whole cell extracts).

Microinjections

Alexa-488-BSA and Alexa-594-BSA were from Molecular Probes (Eugene, OR). NLS-containing peptides were generated by Bioworld (Dublin, Ohio), and were crosslinked to Alexa-BSA as previously described (Adam et al., 1990). A pressure injector system (World Precision Instruments, Sarasota, FL) was used to load Alexa-594-

conjugated peptide into cells bathed in an external solution containing (in mM) 145 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 glucose, pH 7.3. Cells to be injected were visualized using DIC optics in a chamber mounted on the stage of an inverted fluorescence microscope (Leica DMIRBE). Microinjection pipettes were fabricated with a tip diameter of 0.5 μM from boroscillicate capillary glass using a horizontal pipette puller (Sutter Instruments, Novato, CA). Fluorescence images of peptide localization were acquired using an intensified CCD camera (XR Mega 10, Stanford photonics) attached to the microscope side port and images acquired using QED (Pittsburgh, PA) imaging software.

Results

T. gondii does not induce nuclear accumulation of NF-κB or NF-κB-driven gene expression

Although this group and others have provided evidence that infection with *T. gondii* fails to activate NF-κB and renders cells refractory to NF-κB inducing stimuli (Butcher and Denkers, 2002; Butcher et al., 2001; Shapira et al., 2004; Shapira et al., 2002), others have reported that infection of cells with *T. gondii* results in activation of this pathway (Molestina et al., 2003). As part of previous studies, the effect of *T. gondii* on the expression of NF-κB-dependent cytokines such as IL-12 and TNF-α were assessed. However, since multiple other signaling pathways also contribute to the production of these cytokines, a reporter cell line that expresses GFP under the control of a promoter containing multiple NF-κB binding sites was generated and used to test whether infection with *T. gondii* led to NF-κB transcriptional activity. As expected, treatment of these cells with TNF-α or LPS induced expression of GFP (Fig. 1A, central panel, and data not shown). When these cells were infected with transgenic parasites that express RFP, the population of infected cells was clearly visualized by FACS, but neither the infected nor uninfected cells in these cultures expressed GFP as late as 18 hours post-infection (Fig. 1A, right-hand panel). Consistent with these results, immunofluoresence studies revealed that whereas stimulation of HFFs with TNF-α resulted in nuclear accumulation of p65/RelA (Fig. 1B, top panels), infection of these cells with *T. gondii* did not lead to the nuclear accumulation of p65/RelA at early or late time points (Fig. 1B; for lower magnification images containing greater number of cells see supplementary material Fig. S2b). Further densitometric analysis of immufloresence images, as well as immunoblot analysis of cytoplasmic and nuclear fractions for the presence of NF-κB family members, confirmed that infection with *T. gondii* does not result in the nuclear accumulation of p65/RelA (supplementary material Fig. S2a). Similar results were also observed for the NF-κB family members c-Rel and p50 (data not shown). Furthermore, the lack of NF-κB nuclear accumulation was also observed in *T. gondii*-infected NIH 3T3, MEF, HeLa and COS cell lines, as well as primary cultures of mouse and human macrophages (unpublished results), revealing that this is a characteristic of multiple cell types infected with this parasite. Interestingly, similarly to the reported ability of *T. gondii* to inhibit LPS signaling, treatment of infected cells with TNF-α does not lead to the upregulation of the NF-κB reporter gene or the nuclear accumulation of p65/RelA (supplementary material Fig. S1).

T. gondii activates hallmarks of NF-κB signaling

Although the studies in the previous section demonstrate that *T. gondii* fails to activate NF-κB-mediated transcriptional activity, it has been reported that this infection does lead to the degradation of IκB (Butcher et al., 2001). In addition, it has been suggested that a parasite kinase may be responsible for this activity (Molestina and Sinai, 2005; Sinai et al., 2004). Therefore, experiments were performed to determine whether infection with *T. gondii* stimulates the activation of the host IKK complex and whether this is responsible for the infection-induced degradation of IκB. The IKK-dependent degradation of IκB represents a classic hallmark of canonical NF-κB signaling (Li et al., 1999). Treatment of HFFs with TNF-α or infection with *T. gondii* resulted in robust IKK activity and degradation of IκBα (Fig. 2A). However, whereas NF-κB-dependent re-synthesis of IκBα was observed 30 minutes following TNF-α treatment, this was substantially delayed in *T. gondii*-infected cells. This result is consistent with previous observations (Butcher et al., 2001) and the failure of infection to activate NF-κB-dependent gene transcription (Fig. 1A). The eventual re-synthesis of IκB is probably a function of other signaling pathways activated by *T. gondii* that are involved in this process (Butcher et al., 2001). Nevertheless, infection of cells that lack IKKα/β with *T. gondii* did not result in degradation of IκB (Fig. 2B). Together, these data demonstrate that infection with *T. gondii* initiates activation of the NF-κB signaling pathway characterized by a robust host IKK activity that is indispensable for degradation of IκBα.

IκB family members are not responsible for the inhibition of nuclear accumulation of NF-κB in *T. gondii*-infected cells

The degradation of IκB is an event that is considered one of the last checkpoints before the nuclear localization and transcriptional activity of NF-κB. However, in *T. gondii*-infected cells, NF-κB fails to accumulate in the nucleus despite induction of IKK-dependent degradation of IκBα. However, IκB family members (IκBα, IκBβ, IκBε) can have redundant functions and can serve compensatory roles under appropriate conditions (Li and Verma, 2002). Indeed, IκBβ and IκBε are known to play a critical role in the kinetics of NF-κB activation (Hoffmann et al., 2002; Li and Verma, 2002). One possible explanation for the failure of NF-κB to accumulate in the nucleus of infected cells is that IκBβ and IκBε may function as molecular sieves in parasitized cells, binding to free NF-κB dimers in the cytoplasm. To address this possibility, cells doubly deficient (dKO) for IκB family members were utilized. Specifically, cells expressing only IκBα (IκBβ/ε dKO), IκBβ (IκBα/ε dKO) or IκBε (IκBα/β dKO) were infected with *T. gondii* and immunofluorescence was used to determine whether nuclear accumulation of NF-κB was now observed in infected cells. In these studies, despite the infection-induced degradation of IκBα (data not shown), nuclear localization of NF-κB was not observed in any of the cell lines tested (Fig. 3 and data not shown; for lower magnification images containing greater number of cells see supplementary material Fig. S3). These results suggest that it is unlikely that IκB proteins act as 'molecular sieves' to prevent the nuclear localization of NF-κB in *T. gondii*-infected cells.

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General nuclear import and constitutive nuclear-cytoplasmic shuttling of NF-κB are intact in *T. gondii*-infected cells

It has previously been suggested that *T. gondii* may inhibit NF-κB signaling by manipulation of the host cell nuclear import machinery (Butcher et al., 2001). To test this, *T. gondii*-infected cells were microinjected with SV40-NLS crosslinked to BSA and its ability to accumulate in nuclei was assessed using real-time imaging. SV40 contains a 'classical' NLS that translocates to nuclei in an importin-α- and importin-β-dependent manner and is used to assess nuclear pore function (Adam et al., 1990). Microinjection of uninfected cells with this construct resulted in rapid accumulation in the nucleus, reaching nucleo-cytoplasmic equilibrium within 10 minutes following microinjection (data not shown). Cells infected for 4 hours and then microinjected with BSA-NLS, exhibited similar nuclear accumulation of this complex (Fig. 4A). Moreover, the BSA-NLS complex accumulated in the nucleus of infected cells regardless of the number of parasites in the cell or the time following infection (data not shown). Therefore, these data indicate that general nuclear import functions in infected cells are intact and that inhibition of general nuclear import is an unlikely explanation for the lack of NF-κB activity in these cells. However, through the use of leptomicyne B (LMB), a drug that disrupts CRM1-mediated nuclear export of proteins, several groups have recently demonstrated that, in the absence of stimulation, constitutive nucleo-cytoplasmic shuttling of NF-κB occurs (Birbach et al., 2002; Huang et al., 2000). Therefore, additional studies were performed to assess whether *T. gondii* inhibits this process. As expected, when uninfected cells were treated with LMB for 4 hours, nuclear accumulation of NF-κB was observed (Fig. 4B, top panel). Likewise, when infected cells were treated with LMB, nuclear accumulation of NF-κB was observed (Fig. 4B, bottom panel). These results indicate that *T. gondii* does not interrupt the constitutive nucleo-cytoplasmic shuttling of NF-κB dimers that occurs in resting cells. Thus, although infection stimulates classic hallmarks of NF-κB activation, defects in constitutive nuclear import machinery or compensatory effects of different IκB family members do not explain the failure of NF-κB to accumulate in the nucleus and mediate transcription in infected cells.

Infection with *T. gondii* does not induce NF-κB dimers capable of binding DNA and abrogates phosphorylation of p65/RelA

A normal consequence of IκB degradation is the liberation of NF-κB dimers in the cytosol that can bind to NF-κB consensus cites on DNA. To determine whether *T. gondii*-induced degradation of IκB yields active NF-κB dimers capable of

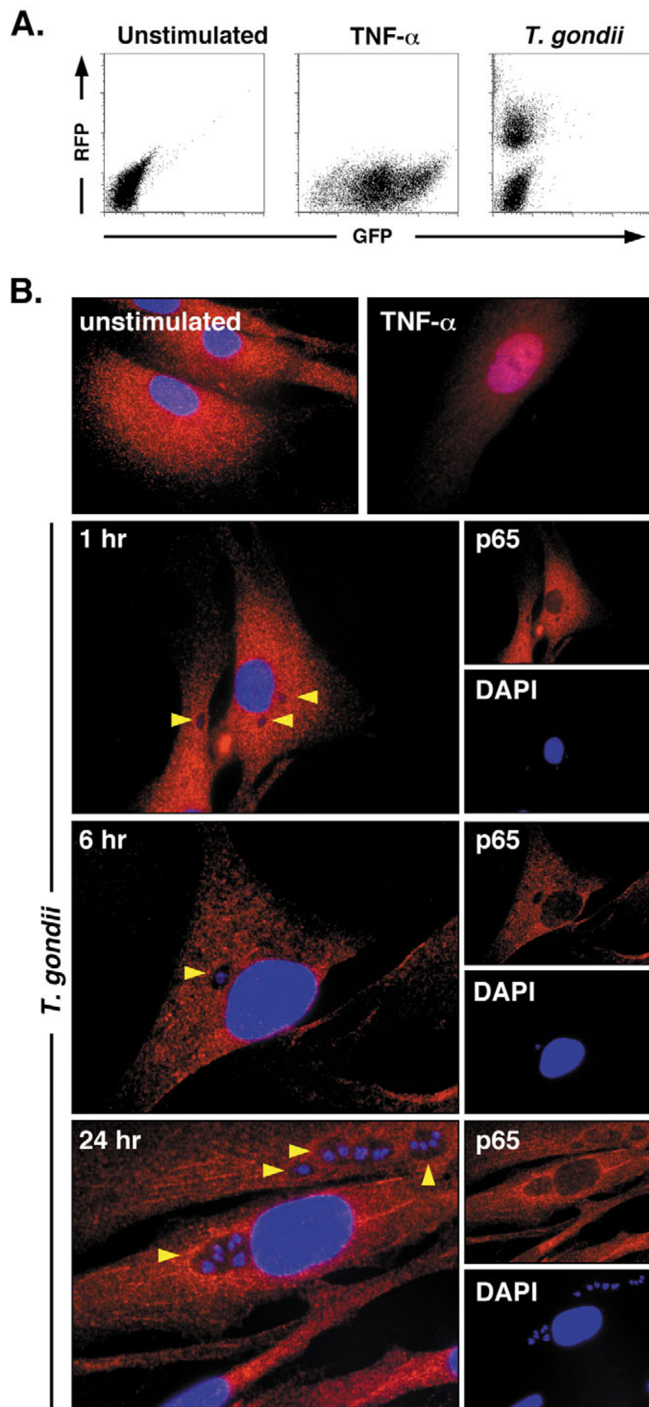

Fig. 1. Infection with *T. gondii* fails to induce NF-κB-dependent gene expression and does not result in nuclear accumulation of NF-κB. (A) Clonal 3T3 cells expressing GFP driven by a promoter containing NF-κB binding sites were used to assess NF-κB responsiveness during *T. gondii* infection. Cells were either left untreated (left panel), stimulated with TNF-α (10 ng/ml) for 18 hours (middle panel), or infected with p30-RFP-expressing Rh parasites for 18 hours (right panel); parasites were generated as previously described (Striepen et al., 2001). Expression of GFP and RFP was assessed by flow cytometry using BD FACS Calibur and analyzed using Cell Quest (Becton Dickinson, Mountain View, CA).
(B) Indirect immunofluorescence analysis of *T. gondii*-infected HFF cells demonstrates lack of nuclear accumulation of NF-κB. Anti-p65 (red) staining reveals cytoplasmic localization in uninfected cells and nuclear accumulation in cells stimulated with TNF-α for 1 hour. 'DAPI' indicates staining of host cell and parasite nuclei (blue). Yellow triangles indicate parasite-containing vacuoles, and infection times are indicated in the top left corner of each panel. The absence of nuclear accumulation of NF-κB in infected cells correlates with the lack of GFP expression in panel A.

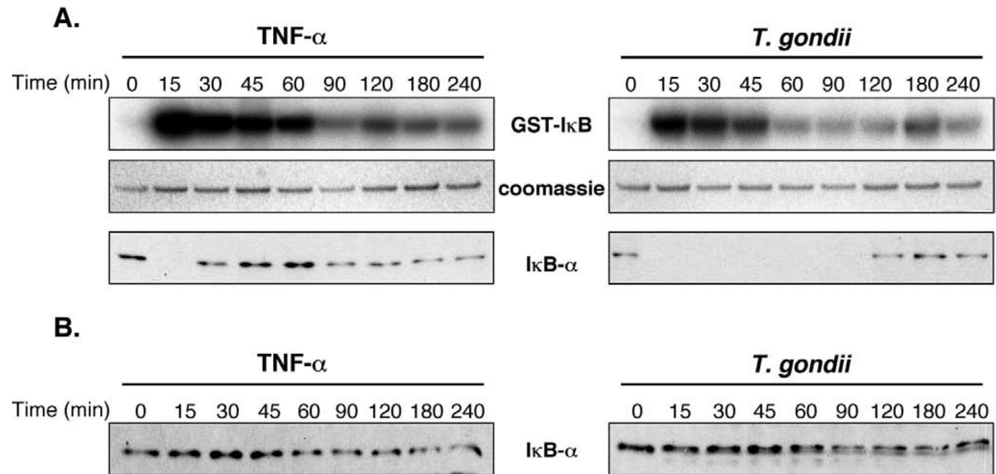

Fig. 2. Infection with *T. gondii* induces IKK-dependent degradation of IκB. (A,B) Whole cell extracts from HFF cells (A) or IKKα/β dKO 3T3 cells (B) treated with TNF-α (left panels), or infected with *T. gondii* (right panels) were prepared at the times indicated. To assess IKK activity, in vitro kinase assays were performed as previously described (Zhong et al., 1997) (A, top panels) and gels were Coomassie Blue stained to confirm equal loading (A, middle panels). Immunoblots with anti-IκBα antibody (A, bottom panels) were performed to assess total levels in treated cells.

binding DNA, electrophoretic mobility shift assays (EMSA) were performed on whole cell extracts from TNF-α treated or infected HFF cells. Whereas TNF-α stimulation led to increased levels of NF-κB binding activity (Fig. 5A, left), infection with *T. gondii* did not result in the expected appearance of NF-κB complexes capable of binding DNA (Fig. 5A, right; to view the entire gel, including unbound probe, see supplementary material Fig. S4). The lack of NF-κB activity in infected cells was not due to a reduction in cellular p65/RelA, as total levels of p65/RelA remained the same through the course of infection (Fig. 5B).

Together with the observation that general nuclear import is intact in infected cells, the results described above indicate that *T. gondii* targets the ability of NF-κB to accumulate in the nucleus and bind DNA. Although our understanding of the events that regulate the strength and duration of NF-κB transcriptional activity is in its infancy, recent evidence indicates that, in response to various stimuli, the phosphorylation of p65/RelA is critical in regulating DNA binding and subsequent transactivation (Chen and Greene, 2004; Zhong et al., 2002; Zhong et al., 1997). Consistent with these observations, stimulation of HFF cells with TNF-α resulted in an increase in total levels of phosphorylated proteins and specific phosphorylation of p65/RelA (Fig. 5C). However, although infection with *T. gondii* also stimulated an increase in the total levels of phosphorylation in these cells, specific phosphorylation of p65/RelA was not detected (Fig. 5C). These findings represent the first identification of a specific step in the NF-κB signaling pathway that is deficient in cells infected with *T. gondii*.

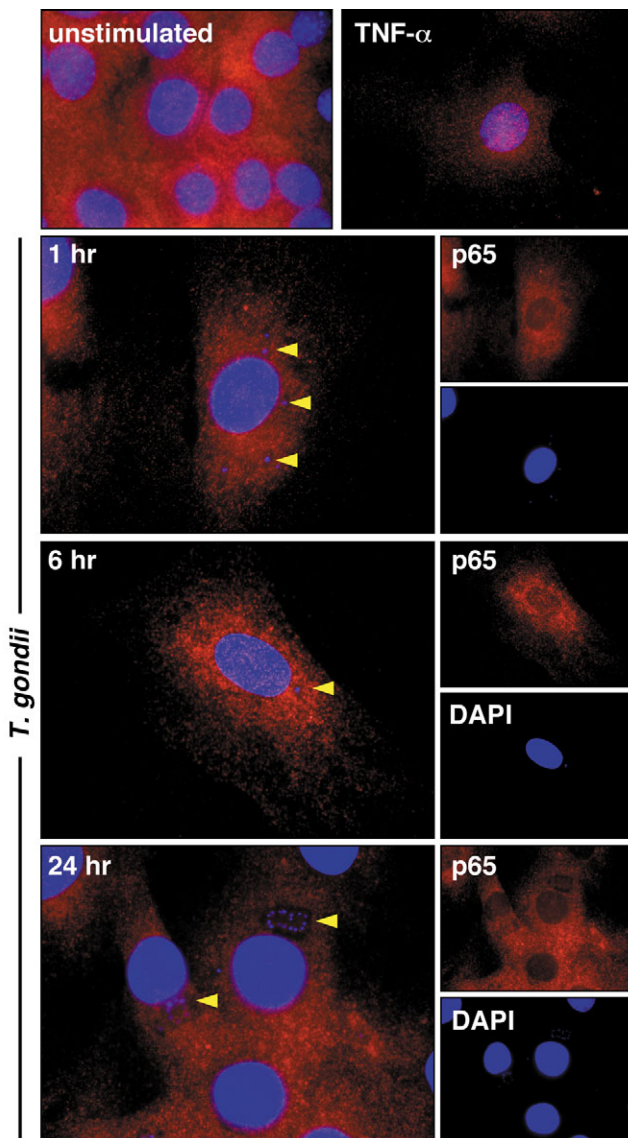

Fig. 3. The ability of *T. gondii* to prevent the nuclear accumulation of NF-κB does not depend on IκB family members. Indirect immunofluorescence analysis of *T. gondii*-infected 3T3 cells doubly deficient in IκBβ and IκBε demonstrates lack of nuclear accumulation of NF-κB. Anti-p65 (red) staining reveals cytoplasmic localization in uninfected cells and nuclear accumulation in cells stimulated with TNF-α for 1 hour. 'DAPI' indicates staining of host cell and parasite nuclei (blue). Yellow triangles indicate parasite-containing vacuoles, and infection times are indicated in the top left corner of each panel. Similarly, nuclear accumulation was not observed following infection of IκBα/IκBβ or IκBα/IκBε dKO cells (data not shown). Yellow triangles indicate parasite-containing vacuoles, and infection times are indicated in the top left corner of each panel.

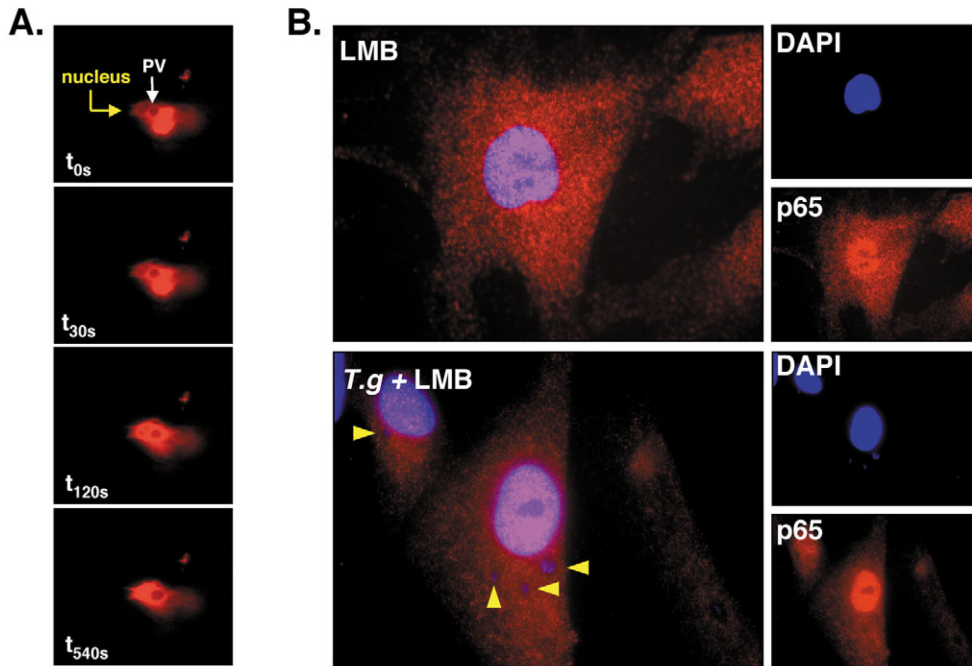

Fig. 4. *T. gondii* does not inhibit general nuclear import mechanisms or interrupt the constitutive nucleo-cytoplasmic shuttling of NF-κB. To address whether *T. gondii* regulates general nuclear import, infected HFFs were microinjected with SV40-NLS crosslinked to BSA (A) and assessed using real-time imaging. Microinjection of uninfected cells with this construct resulted in rapid accumulation in the nucleus, reaching nucleo-cytoplasmic equilibrium within 10 minutes following microinjection (data not shown) (A). Microinjection of *T. gondii*-infected cells with BSA-NLS complex. PV, parasitophorous vacuole; host cell nucleus indicated by arrow. (B) Treatment of uninfected cells (B, top panels) or infected cells (B, bottom panels; parasite containing vacuoles indicated by yellow triangles) with LMB for 4 hours resulted in the nuclear accumulation of p65/RelA.

Discussion

Numerous parasites, bacteria and viruses have been shown to inhibit NF-κB signaling in order to prevent or delay innate or adaptive immune responses, whereas others activate NF-κB to promote the survival of the cells they infect (Tato and Hunter, 2002). Although the mechanism of action for many of these pathogens remains poorly understood, a common theme is the targeting of steps that lead to IκB degradation (Tato and Hunter, 2002). By contrast, the studies presented here demonstrate that the ability of *T. gondii* to limit NF-κB signaling is downstream of IκB degradation and identify a defect in p65/RelA phosphorylation. It remains unclear, however, whether this is a parasite-mediated effect, or simply represents a failure of host cells to respond appropriately to *T. gondii*. For example, there may be a parasite product that interferes with the phosphorylation of NF-κB. Alternatively, there are multiple host kinases upstream of the phosphorylation of p65/RelA (Mattioli et al., 2004; Sakurai et al., 2003; Vermeulen et al., 2002; Yang et al., 2003; Zhong et al., 1997) and it is not known whether these are activated in infected cells. Furthermore, the acetylation status of p65/RelA, an event that regulates its transcriptional activity, and DNA-binding affinity, was not examined in *T. gondii*-infected cells and could play a role in the ability of the parasite to inhibit NF-κB responses (Quivy and Van Lint, 2004). Interestingly, treatment of infected cells with TNF-α revealed that infected cells appear not to be responsive to TNF-α stimulation with respect to NF-κB reporter gene expression and nuclear accumulation of p65/RelA – events that are probably regulated at the level of p65/RelA phosphorylation. Furthermore, in preliminary

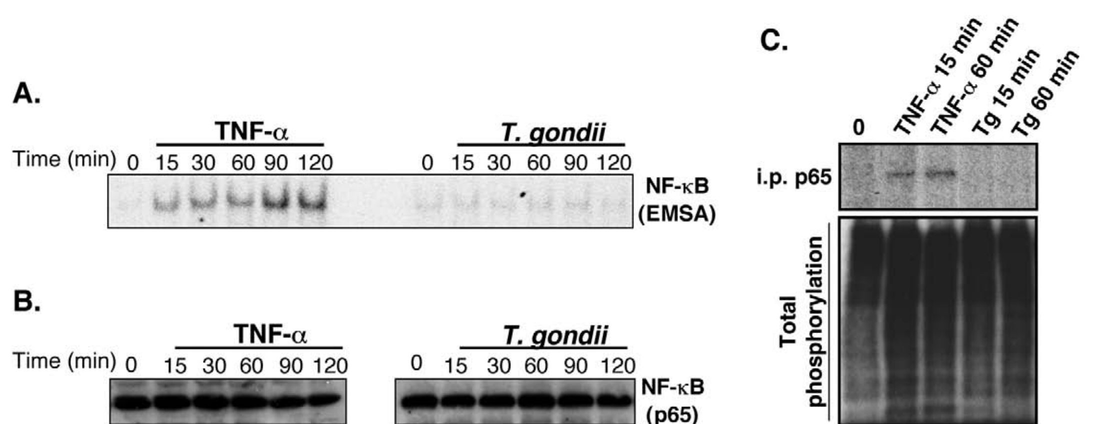

Fig. 5. Infection with *T. gondii* blocks the phosphorylation of p65/RelA and DNA binding. (A,B and C) Whole cell extracts from HFF cells treated with TNF-α (left panels), or infected with *T. gondii* (right panels) were prepared at times indicated. EMSA was performed to determine whether the degradation of IκB yields active NF-κB dimers (A). (B) Immunoblots with anti-IκBα antibody (A, bottom panels) were performed to assess total levels in treated cells. (C) HFFs were labeled with ^{32}Pi orthophosphate as previously described (Zhong et al., 1997) and were treated with TNF-α or infected with *T. gondii* for 15 minutes. Whole cell extracts were either immunoprecipitated overnight with anti-p65 antibody to determine p65/RelA-specific phosphorylation (upper panel) or separated on SDS-PAGE to determine total cellular phosphorylation (lower panel).

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experiments, the phosphorylation status of p65/RelA in infected cells treated with TNF-α was examined and appears to be deficient (data not shown). These observations are part of an ongoing effort to characterize NF-κB signaling in *T. gondii*-infected cells and future studies are aimed at identifying host and/or parasite factors that regulate NF-κB signaling.

Although much is known about the immune mechanisms that regulate resistance to *T. gondii*, it remains unclear how the initial interaction between the parasite and the host cell affects the development of protective immune responses. It is clear, however, that the development of these responses is dependent on various transcription factors including NF-κB. Thus, the utilization of various NF-κB-knockout mice has identified critical roles for these transcription factors in resistance to *T. gondii* (Caamano et al., 1999; Caamano et al., 2000; Franzosa et al., 1998). However, the observation that *T. gondii* fails to activate NF-κB has been controversial because of reports that infection of cells with *T. gondii* results in activation of this pathway at late time points, that this is required to prevent apoptosis of infected cells (Molestina et al., 2003) and that less virulent strains of *T. gondii* can induce low levels of NF-κB nuclear translocation (Robben et al., 2004). Although these results may represent differences between virulent (type I) and less virulent (type II) strains of *T. gondii*, we have not found any evidence for these observations (data herein and not shown). Furthermore, while Sibley and colleagues observed that the RH (type I) strain of *T. gondii* did not activate NF-κB (similar to the results reported here), they did observe that the less virulent (type II) Pruigniaud strain induced low levels of NF-κB that correlated with increased production of IL-12 (Robben et al., 2004). However, it is important to note that, in the assays used in this report, infection with tachyzoites of Pruigniaud did not lead to nuclear accumulation or phosphorylation of p65 (data not shown). Nevertheless, although the reasons for these differences remain unclear, the data presented here are consistent with previous studies showing that infection with this parasite does not lead to the activation of NF-κB (Butcher and Denkers, 2002; Butcher et al., 2001; Luder et al., 2001; Robben et al., 2004; Shapira et al., 2002). Also, one interpretation of these results is that the inhibition of NF-κB signaling by *T. gondii* may in fact protect from the pro-apoptotic signals mediated by this transcription factor. Moreover, since infected cells, in vitro or in vivo, have a reduced capacity to produce pro-inflammatory cytokines such as IL-12, it seems likely that the failure to activate NF-κB represents a strategy that promotes the early expansion and growth of this parasite.

While these findings identify p65/RelA phosphorylation as a novel target that can be manipulated by pathogens to limit NF-κB-dependent signaling, they also highlight the role of this post-translational event in the regulation of DNA binding and suggest that this process may influence nuclear import and retention of NF-κB. Several phosphorylation sites as well as multiple kinases are required for p65/RelA activation, association with CBP/p300, and activation of gene transcription. For example, phosphorylation of p65/RelA at S536 is mediated by IKKβ and/or IKKα and occurs in the cytoplasm, whereas phosphorylation of p65/Rel at S276 is regulated by PKA and cAMP (Mattioli et al., 2004; Yang et al., 2003; Zhong et al., 1997). Furthermore, the phosphorylation status of p65/RelA is determined by both kinases and phosphotases. For example, recent evidence suggests that protein phosphatase 2A (PP2A) is physically associated with the p65/RelA-IκB complex and can dephosphorylate p65/RelA under appropriate conditions (Yang et al., 2001). Signal-induced cytosolic p65/RelA phosphorylation provides a mechanism that ensures that only activated NF-κB can induce transcription, thereby maintaining NF-κB as an inducible transcription factor. Given the role of NF-κB signaling in the development of inflammatory and autoimmune disease, as well as cancer, much effort has been directed at the identification of drugs that can modulate this pathway. Elucidating the mechanism by which *T. gondii* interferes with the phosphorylation of p65/RelA will extend our understanding of the host-pathogen relationship, and should also yield novel insights into the molecular mechanisms of NF-κB signaling in mammalian cells and provide potential targets for drug design.

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