Toxoplasma gondii Rhopty Discharge Correlates with Activation of the Early Growth Response 2 Host Cell Transcription Factor

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Toxoplasma gondii is an ubiquitous apicomplexan parasite that can cause severe disease in fetuses and immune-compromised patients. Rhoptries, micronemes, and dense granules, which are secretory organelles unique to Toxoplasma and other apicomplexan parasites, play critical roles in parasite growth and virulence. To understand how these organelles modulate infected host cells, we sought to identify host cell transcription factors triggered by their release. Early growth response 2 (EGR2) is a host cell transcription factor that is rapidly upregulated and activated in Toxoplasma-infected cells but not in cells infected with the closely related apicomplexan parasite Neospora caninum. EGR2 upregulation occurred only when live parasites were in direct contact with the host cell and not from exposure to cell extracts that contain dense granule or micronemal proteins. When microneme-mediated attachment was blocked by pretreating parasites with a calcium chelator, EGR2 expression was significantly reduced. In contrast, when host cells were infected with parasites in the presence of cytochalasin D, which allows rhoptry secretion but prevents parasite invasion, EGR2 was activated. Finally, we demonstrate that Toxoplasma activation of host p38 mitogen-activated protein kinase is necessary but not sufficient for EGR2 activation. Collectively, these data indicate that EGR2 is specifically upregulated by a parasite-derived secreted factor that is most likely a resident rhopty protein.
the involvement of a parasite-derived secreted factor, which is consistent with the observation that jun-B and c-jun transcript abundance correlated with expression of ROP16 gene virulence alleles (54).

In this report, we use a battery of cell biological and biochemical assays to demonstrate that EGR2 is activated by a Toxoplasma-derived secreted factor that is most likely localized to the rhoptries. This factor is distinct from known rhoptry factors that translocate into the host cytosol. EGR2 activation appears to be Toxoplasma specific since EGR2 was not upregulated in cells infected with Neospora caninum, which is a closely related apicomplexan parasite. Finally, we show that host cell p38 mitogen-activated protein kinase (MAPK) signaling is necessary but not sufficient for EGR2 activation.

**MATERIALS AND METHODS**

**Cell culture and reagents.** Mouse embryonic fibroblasts and human foreskin fibroblasts (HEF) were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, I-glutamine, and penicillin-streptomycin. Cytochalasin D-resistant epithelial cells (CB cells) (66) were obtained from David Sibley and were cultured in this same growth medium. Drug resistance was verified by routinely phenylalanine staining mock-treated or cytochalasin D-treated cells. In addition, cells were passaged for no more than 6 weeks. Actinomycin D, cycloheximide, cytochalasin D, and BAPTA-AM (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis[acetoxymethylester]) were from Sigma-Aldrich (St. Louis, MO). SB203580, U0126, SP600125, and LY294002 were from Calbiochem (San Diego, CA). Recombinant lethal factor and protective antigen purified (St. Louis, MO); SB203580, U0126, SP600125, and LY294002 were from Calbiochem and were provided by Jimmy Ballard, and lethal toxin was prepared by mixing equimolar amounts of lethal factor and protective antigen as previously described (55).

**Parasite preparations.** The RH (type I), GT1 (type I), Pru (type II), and CTG (type III) Toxoplasma strains and the NC-1 Neospora (from Dan Howe) strain were propagated in HEF as previously described (4). All parasites and host cell lines were tested once every 2 months for mycoplasma using the MycoAlert Assay Kit (Lonza, Basel, Switzerland) and found to be negative. Unless stated, experiments were performed at a multiplicity of infection (MOI) of 10:1 (parasites/host cells). Parasites were prepared by passing them through a 27-gauge needle twice to lyse host cells and then were extensively washed. Heat-killed parasites were prepared by incubating purified parasites at 50°C for 20 min. Excreted-secreted antigens (ESA) were prepared by incubating parasites for 30 min in 50 mM HEPES, pH 7.2, in the presence of 200 mM ethanol (12). Soluble tachyzoite antigen (STAg) was prepared in the presence of a protease inhibitor cocktail (Calbiochem) as previously described (27). However, to allow for membrane-associated factors to be present in our STAg, the sonicated lysates were centrifuged at 16,000 × g for 20 min at 4°C instead of 100,000 × g. Conditioned medium was collected from HFF that had been infected with Toxoplasma for 48 h and filtered through a 0.2-μm filter to remove parasites and host cells (4).

**Real-time PCR.** Total RNA was isolated using the PureScript RNA purification system (Gentra Systems, Minneapolis, MN) and treated with RNA-free DNase (Ambion, Austin, TX). The RNA was then passed through an RNA affinity column (Ambion) to inactivate and remove the DNase. Total RNA was reverse transcribed into cDNA using random primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). CDNA was diluted 1:10 and mixed with Power Sybr green PCR master mix (Applied Biosystems, Foster City, CA), and PCRs were performed in an ABI 7500 Fast real-time PCR machine (Applied Biosystems). The efficiency of each primer set (Table 1) was determined to be between 80 and 120% of theoretical exponential amplification from cDNA dilutions. The absence of genomic DNA contamination was also verified by using DNase-treated RNA that was not reverse transcribed. Experiments were performed in triplicate, and each experiment was repeated at least three independent times. The threshold cycle (Ct) for β-actin and each target gene was determined by using the thermal cycler’s software, and changes in relative expression levels were determined with the following equation: ΔCt(target) = Ct(β-actin)target – Ct(β-actin)target, ΔCt(target) = Ct(β-actin)target – Ct(β-actin)reference. Relative expression levels were then expressed as changes using the equation 2−ΔΔCt. 

**Luciferase assays.** Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with plasmids in which the firefly luciferase gene is cloned downstream of the consensus EGR elements (52) (pEGR4x-Luc from John Svanen, University of Wisconsin) or AP-1 elements (pAP1-luc from Stratagene). Cells were cotransfected with a plasmid expressing Renilla luciferase under the control of the thymidine kinase promoter (pTK-Rel). Cells (1 × 105) were added to 24-well plates in a solution containing the transfection reagent and DNA. The cells were allowed to adhere for 1 h at 37°C, extensively washed, and then grown for 24 h in normal growth medium. For some experiments, parasites were added to a Transwell apparatus containing a 0.45-μm filter (Becton Dickinson, Franklin Lakes, NJ) and inserted above transfected cells to prevent parasite-host contact while allowing diffusion of soluble molecules. Lysates were harvested 16 h postinfection, and luciferase activity was measured with the Dual-Glo luciferase assay kit (Promega, Madison, WI) using a luminometer (TD-20/20; Turner BioSystems, Sunnyvale, CA).

**Western blots.** Mouse embryonic fibroblasts were washed in 1× phosphate-buffered saline (pH 7.2) before whole-cell lysates were collected using ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate) plus a protease inhibitor cocktail (Boehringer, Mannheim, Germany). The protein concentration of each sample was determined with the DC protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with antibodies against MIC2 (from Vernon Carruthers), GRA7 (from John Boothroyd), and p38 MAPK and phospho-p38 MAPK (Cell Signaling Technology, Danvers, MA).

**Evacuole assays.** Evacuole assays were performed essentially as described previously (46). Briefly, parasites were incubated at room temperature in 1 μM cytochalasin D in Endo buffer (44.7 mM K2SO4, 10 mM MgSO4, 106 mM sucrose, 5 mM glucose, 20 mM Tris, 0.35% [wt/vol] bovine serum albumin, pH 8.2) (22). After 10 min, the parasites were added to HFF and incubated for 20 min at 37°C to allow parasites to adhere to the host cell. The buffer was then removed and replaced with prewarmed invasion medium (Dulbecco's modified Eagle medium plus 3% fetal bovine serum) containing cytochalasin D. The cells were incubated at 37°C for 15 min and then fixed with ice-cold methanol. Evacuoles were detected by immunofluorescence using anti-Rop2,3,4, a monoclonal antibody (from Anthony Sinai) (40).

**RESULTS**

**Toxoplasma upregulates host EGR2 expression and activates EGR2 reporter activity.** We previously reported that some of the most rapidly upregulated transcripts in Toxoplasma-infected cells were canonical immediate early genes encoding transcription factor subunits (4). To assess the magnitude and timing of the changes in c-jun, jun-B, EGR1, and EGR2 expression in Toxoplasma-infected cells, total RNA was isolated 1, 2, 4, and 6 h after host cells were infected with tachyzoites of the RH strain. Real-time quantitative PCR was then performed to measure changes in expression of each gene relative to β-actin, whose abundance is not affected by parasite infection (4). The abundance of all four transcripts increased within 4 h postinfection. But the EGR2 gene was the only gene whose transcript abundance continued to increase 6 h postinfection.

- **Table 1. Real-time PCR primers**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
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<tr>
<td>cJun FWD</td>
<td>TGAAGCTGTGCTCCTCGTCG</td>
</tr>
<tr>
<td>cJun REV</td>
<td>ATCAGCAGCATTCCACCTTC</td>
</tr>
<tr>
<td>junB FWD</td>
<td>GGTGGAAGTTCAAGAGCTACTTA</td>
</tr>
<tr>
<td>junB REV</td>
<td>CGGTTCTCTTGGCCTTGAGGC</td>
</tr>
<tr>
<td>EGR1 FWD</td>
<td>CAGCCCTCTGGAGCATGACCAAT</td>
</tr>
<tr>
<td>EGR1 REV</td>
<td>CGAGGAGAGAACGTAGGACAA</td>
</tr>
<tr>
<td>EGR2 FWD</td>
<td>GTTCCGGATCTGGATCGGAAACCTT</td>
</tr>
<tr>
<td>EGR2 REV</td>
<td>GCAAATCTGGCCCAACATAGTC</td>
</tr>
<tr>
<td>NAB1 FWD</td>
<td>CTCCTCTGATAGCTCTGATGGACA</td>
</tr>
<tr>
<td>NAB1 REV</td>
<td>GACTTGGCTCCTGAGTAGTAAG</td>
</tr>
<tr>
<td>β-actin FWD</td>
<td>GGAGCCAGGATCTACACTTIGT</td>
</tr>
<tr>
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<td>CCAAGGAGTTCTACGATCCAAAGA</td>
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fection, while the abundance of the other three plateaued (Fig. 1A).

To determine whether increases in the abundance of c-jun, jun-B, EGR1, and EGR2 correlated with increases in transcription factor activity, host cells were transfected with AP-1 or EGR luciferase reporter plasmids as well as a Renilla-luciferase reporter to normalize for transfection efficiency since this reporter is not modulated by Toxoplasma infection (60). The transfected cells were infected for 16 h, and then cell lysates were prepared and luciferase activity was measured. Consistent with the real-time PCR data, AP1- and EGR-dependent luciferase activity was significantly increased after host cells were infected with parasites (Fig. 1B). Since their genes were rapidly upregulated and because expression of jun-B and c-jun correlated with expression of active ROP16 alleles (54), we hypothesized that EGR1 and EGR2 were regulated by a parasite-derived secreted factor. We chose to primarily focus the remainder of this study on the EGR2 gene since, unlike the other genes, its expression continuously increased during infection. In addition, our previous microarray analysis (4) and subsequent real-time PCR assays (data not shown) indicated that several EGR2 target genes such as the NAB1 gene (61, 62) were upregulated in parasite-infected cells.

Next, we performed dose-response assays by infecting cells with increasing numbers of parasites. Six hours later, RNA was isolated and used to measure EGR2 transcript levels by real-time PCR. The data indicated that EGR2 was upregulated in a dose-dependent manner (Fig. 1C). To address the possibility that EGR2 activation was a general response of a host cell to infection, we tested if the closely related apicomplexan parasite Neospora caninum also upregulated host EGR2. Cells were infected with Toxoplasma or increasing numbers of Neospora cells for 18 h before RNA was harvested. Real-time PCR was then used to quantitate changes in EGR2 transcript abundance. In contrast to levels in Toxoplasma-infected cells, EGR2 mRNA levels remained unchanged in Neospora-infected cells regardless of the infection time or dose (Fig. 1D). EGR2 induction was not host cell type specific since EGR2 was also upregulated in macrophages and epithelial cells, which are cell types that Toxoplasma infects in vivo (not shown). Neospora also failed to upregulate the EGR luciferase reporter (Fig. 1E). Like EGR2 mRNA, c-jun and EGR1 mRNAs were also not increased in Neospora-infected cells, but jun-B mRNA was slightly upregulated (approximately twofold) in cells infected with Neospora at a MOI of 40:1 but not at lower MOIs (not shown).

Since endotoxin can also activate EGR-dependent transcription (15, 63), we needed to demonstrate that endotoxin was not responsible for upregulating EGR2 in our experiments. Thus, EGR reporter-transfected host cells were incubated with untreated parasites or with parasites heat killed under conditions that would not affect endotoxin. In contrast to live parasites, heat-killed parasites were unable to upregulate EGR-depen-
dent luciferase expression (Fig. 2A), indicating that endotoxin was not responsible for activating EGR2.

In cells treated with various growth factors and cytokines, the EGR2 gene is defined as an immediate early gene since its transcriptional upregulation does not require de novo protein synthesis (31). To determine whether the EGR2 gene behaved as an immediate early gene in Toxoplasma-infected cells, host cells were mock treated or pretreated with either the transcription inhibitor actinomycin D or the protein synthesis inhibitor cycloheximide. The cells were then infected with parasites. After 6 h, RNA was isolated and EGR2 transcript levels were measured by real-time PCR.

Toxoplasma did not increase EGR2 mRNA abundance in actinomycin D-treated cells. Importantly, we noted a significant decrease in EGR2 mRNA levels in the mock-infected, actinomycin D-treated cells, indicating that the drug efficiently blocked transcription. When we blocked protein synthesis by treating cells with cycloheximide, which we confirmed blocked protein synthesis by >95% (not shown), EGR2 was upregulated as strongly as it was in cells not treated with cycloheximide (Fig. 2B). Together, these data demonstrate that Toxoplasma specifically upregulates EGR2 and that the EGR2 gene is an immediate early gene in Toxoplasma-infected cells.

EGR2 is not stimulated by factors expressed on the parasite surface or by soluble secreted factors. The rapid upregulation of EGR2 suggested that it was activated by a parasite-secreted factor. Although several host cell transcription factors have been identified and postulated to be regulated by parasite-secreted factors, direct evidence is lacking. Thus, we first asked whether direct contact between the parasite and host cell is required to stimulate EGR2. Host cells transfected with the EGR luciferase reporter were either infected with parasites or plated in the Transwell chamber (to prevent direct parasite-host cell contact), and purified extracellular parasites were added to the upper compartment. To allow for the dilution and the distance separating parasites from the host cells, fivefold-more parasites were added to the upper compartment of the Transwell chamber than were added directly to the cells. Lysates were prepared 16 h later, and luciferase activity was measured. In contrast to the case where parasites were directly added to host cells, EGR2 was not stimulated by parasites plated in the Transwell chamber (Fig. 3A, + Transwell).

Micronemes consist of soluble proteins or transmembrane proteins, most of which are proteolytically cleaved and shed from the parasite surface as parasites penetrate into the host cell. To test whether the EGR2-inducing factor was located

FIG. 2. EGR2 is activated by live parasites and behaves as an immediate early gene in Toxoplasma-infected cells. (A) Luciferase activity was measured in pEGR4x-Luc-transfected host cells infected with either live or heat-killed parasites. (B) Host cells were pretreated with either 1 µg/ml actinomycin D (Actino D) or 20 µg/ml cycloheximide (CHX) for 30 min and then infected with parasites. After 6 h, RNA was isolated and EGR2 transcript levels were measured by real-time PCR.

FIG. 3. Parasite-derived soluble secreted factors are unable to activate EGR2. (A) pEGR4x-Luc-transfected cells were either infected with Toxoplasma or were incubated with parasites placed in a Transwell chamber (+Transwell), ESA, STAg, or conditioned medium (CM). After 16 h, lysates were prepared and luciferase activity was measured. (B) Western blots of supernatants from parasites incubated in either the absence (unstimulated) or presence of ethanol (ESA) for 30 min or from sonicated parasite lysates (STAg). The arrows indicate either full-length (unreleased) or mature (secreted and processed) MIC2. α, anti-.
within micronemes, EGR-luciferase reporter-transfected host cells were treated with a preparation of ESA. ESA is prepared by incubating extracellular parasites with ethanol, which specifically stimulates microneme discharge, but not rhoptry or dense granule release, and protein processing (12) (Fig. 3B). Again, the addition of fivefold-more parasite equivalents of ESA failed to upregulate EGR-dependent luciferase expression (Fig. 3A, ESA). To account for proteins not released into ESA, host cells were incubated with STAg, which was prepared by repeatedly sonicating parasites. Similar to ESA, STAg was unable to stimulate the EGR2 reporter (Fig. 3A, STAg). These data indicate that the EGR2-inducing factor is not released into ESA or that, if it is released, as it would be in STAg, it is unable to interact with its host cell receptor because either the factor needs to be processed or the host cell receptor is not expressed on the surface of the host cell.

Finally, we examined whether infection stimulated host cells to release autocrine-acting factors that could stimulate the EGR luciferase reporter. Thus, conditioned culture medium was collected from host cells that had been infected for 48 h. The conditioned medium was filtered through a 0.2-μm filter to remove any parasites or host cells and then added to EGR luciferase reporter-transfected host cells. We found that like ESA or STAg the conditioned medium could not stimulate EGR-dependent luciferase expression (Fig. 3A, CM). Collectively, these data indicate that the EGR2-inducing factor is not a parasite cell surface protein or a parasite- or host-derived soluble, secreted factor.

EGR2 activation correlates with rhoptry secretion. The above data indicate that EGR2 was activated by microneme-dependent adhesion or rhoptry-dependent secretion or after parasites had successfully invaded the host cell. *Toxoplasma* attachment to host cells is mediated by calcium-dependent secretion of micronemal adhesive proteins (11). Thus, parasites were pretreated with the calcium chelator BAPTA-AM to prevent microneme secretion and subsequent host cell attachment. The parasites were then added to host cells, and 6 h later EGR2 mRNA levels were measured by real-time quantitative PCR. We found that EGR2 expression was significantly lower in host cells infected with BAPTA-AM-treated parasites (Fig. 4A). These data indicate that microneme-dependent secretion and attachment are required for EGR2 activation. In addition, they provide further evidence that contact between parasite surface proteins and the host cell is not sufficient to upregulate EGR2.

Following microneme secretion and host cell attachment, *Toxoplasma* secretes its rhoptries and penetrates into its host cell via a process powered by the parasite’s actomyosin cytoskeleton (10). Penetration and rhoptry secretion can be uncoupled by treating parasites with the actin polymerization inhibitor cytochalasin D, which blocks host penetration but not rhoptry secretion (28). Rhoptry secretion by cytochalasin D-treated parasites into host cells results in the formation of tubular membranous structures termed evacuoles. To determine the percentage of evacuoles that formed under the conditions in which we performed our experiments, we stained host cells infected with cytochalasin D-treated parasites with anti-ROP2,3,4 antibody and found that ~60% of parasites attached to host cells formed evacuoles (Fig. 4B). Given that evacuoles are short-lived structures (28) and that we examined only a single time point, these data are consistent with other reports that evacuoles form in the majority of host cells in contact with cytochalasin D-treated parasites (28).

Next, mock-treated or cytochalasin D-treated parasites were added to EGR luciferase reporter-transfected cytochalasin D-resistant host cells. As expected, we found that parasite invasion was reduced >95% when parasites were treated with cytochalasin D (not shown). However, EGR-dependent luciferase expression was increased to similar levels when host cells were infected with either mock-treated or cytochalasin D-treated cultures (Fig. 4C). These data strongly suggest that EGR2 activation is correlated with rhoptry secretion and does not require host cell invasion.

**Toxoplasma activation of host p38 MAPK is necessary but not sufficient to activate EGR2.** Increases in intracellular Ca2+ levels as well as activation of MAPKs (p38, extracellular signal-regulated kinase [ERK], and c-jun N-terminal protein kinase) and phosphoinositide-3 kinase (PtdIns-3K) are signaling events triggered in *Toxoplasma*-infected cells (33, 34, 43). Because some of these pathways are also implicated in stimulating EGR-dependent transcription (31, 71), we sought to determine what role, if any, they played in *Toxoplasma* induction of EGR2. To specifically inhibit host cell Ca2+ signaling during *Toxoplasma* infection, we pretreated host cells with BAPTA-AM and then extensively washed the cells before infecting them with parasites in BAPTA-AM-free medium (43). EGR2 mRNA levels measured 6 h postinfection indicated that chelating host cell intracellular calcium increased *Toxoplasma*-stimulated EGR2 expression with marginal statistical significance (Student’s *t* test; *P* = 0.058) (Fig. 5A).

Next, EGR luciferase reporter-transfected host cells were pretreated with PtdIns-3K (LY294002), p38 MAPK (SB203580), ERK (U0126), and c-jun N-terminal protein kinase (SP600125) inhibitors and then infected with parasites. Sixteen hours later, luciferase activity was measured, and we found that, like chelating host cell calcium, the PtdIns-3K inhibitor potentiated EGR-dependent luciferase expression. In contrast, the p38 MAPK inhibitor significantly inhibited *Toxoplasma*-induced EGR-dependent luciferase expression (Fig. 5B), while the other MAPK inhibitors had no statistically significant effect. Because p38 MAPK inhibitors have also been shown to affect *Toxoplasma* (70), we wanted to specifically target the host cell enzyme. To do this, we took advantage of the fact that *Bacillus anthracis* lethal toxin is a metalloprotease that cleaves and inactivates the N terminal of MAPK kinases (MKKs) (2, 50, 69). Thus, EGR luciferase reporter-transfected host cells were pretreated with lethal toxin for 6 h, which we determined was the minimal time required to achieve maximal cleavage of MKKs (not shown). The host cells were washed to remove any unbound toxin, and parasites were then added for 16 h. In the absence of host MKKs, *Toxoplasma* stimulation of EGR-dependent luciferase expression was significantly reduced (Fig. 5C). Together, these data indicate that p38 MAPK is necessary for parasite induction of EGR2.

Next, we determined whether p38 MAPK activation was sufficient for EGR2 activation. Thus, host cells were treated with anisomycin, which is a well-characterized p38 MAPK activator (9). Though anisomycin robustly stimulated p38 MAPK phosphorylation (Fig. 6A), neither EGR2 mRNA levels nor EGR-dependent luciferase expression increased in anisomycin-treated cells (Fig. 6B and C). In summary, these data in-
Toxoplasma activation of p38 MAPK is necessary but not sufficient for activating EGR2.

**DISCUSSION**

Several host cell transcription factors including HIF1, STAT3, STAT6, and, in some cases, NF-κB are activated in Toxoplasma-infected host cells (17, 43, 44, 48, 54). Activation of at least some of these transcription factors has been postulated to be regulated by parasite factors localized to a variety of parasite organelles including the plasma membrane, rhoptries, micronemes, and dense granules. But data directly demonstrating this have been lacking. Here, we used a battery of biochemical and cell biological assays to characterize the parasite factor that activates EGR2, which is rapidly and specifically upregulated by Toxoplasma. The inability of EGR2 to be upregulated by heat-killed, BAPTA-AM-treated, or formaldehyde-fixed (not shown) parasites indicated that Toxoplasma surface proteins do not activate EGR2. In addition, the failure of ESA or STAg to stimulate EGR2 indicated that the EGR2-inducing factor is likely not a dense granule or micronemal protein. On the other hand, data from experiments using cytochalasin D-treated parasites strongly suggested that EGR2 activation correlates with rhoptry secretion.

Rhoptry proteins have two distinct functions. First, proteins like RON2 and RON4 function in concert with some micronemal proteins to form the moving junction, which is where parasite penetration into the host cell takes place (1). The host cell proteins that are organized into the moving junction are unknown, and thus it is not clear whether EGR2 activation occurs after engagement of these proteins. Second, rhoptry proteins interact with the host cell by being released in a...
manner analogous to bacterial type III secretion system release into the cytosol of infected host cells. These include (i) the ROP16 and ROP18 polymorphic virulence factors (53, 54, 64), (ii) a phosphatase 2C (PP2c) homolog that traffics to the host nucleus (26), and (iii) members of the ROP2 kinase-like protein family that interact with host mitochondria and the endoplasmic reticulum (59). ROP16 is likely not the EGR2-inducing factor since the EGR2 gene was not among the host genes differentially expressed by various polymorphic ROP16 gene alleles (54). In addition, EGR2 was upregulated by all three Toxoplasma clonal types, suggesting that, in contrast to ROP16 or ROP18, the EGR2-inducing factor is not polymorphic (not shown). We did, however, note that multiple isolates of the RH strain upregulated EGR2 significantly higher than any other parasite strain we tested, including a second type I strain, GT1 (E. D. Phelps and I. J. Blader, unpublished data). But because of potential differences (in, e.g., growth rate, invasion, and rhoptry secretion) between these strains that may not be associated with polymorphisms in the gene encoding the EGR2-inducing factor, knowing whether the factor is or is not truly polymorphic awaits its cloning. The rhoptry-localized PP2c homolog can also be excluded since there was no apparent difference in EGR2 expression between host cells infected with wild-type parasites and those infected with PP2c knockout parasites (26). Our data, however, cannot exclude ROP2 family members, and further experiments are needed to test this possibility.

Our data also suggest that the EGR2-inducing factor is not among other parasite factors implicated in modulating host gene expression. Besides EGR2 and AP-1, STAT3, STAT6, and hypoxia-inducible factor 1 (HIF1) are other host transcription factors activated in parasite-infected cells (8, 54, 60). As discussed above, ROP16 is unlikely to be the EGR2-inducing factor. Likewise, EGR2 and HIF1 are probably regulated by different factors since HIF1 can be activated by extracellular parasites (60). Toxoplasma can also positively or negatively modulate NF-κB (7, 16, 32, 47, 51, 57, 58). Whether Toxoplasma gondii regulation of NF-κB and activation of EGR2 are related is not clear and awaits further investigation.

It is also possible that EGR2 activation is triggered by parasite factors interacting with the host cell surface immediately before rhoptry secretion. This would most likely be achieved by a micronemal or parasite surface protein since they are the most likely to interact with host surface proteins. Our data allow us to exclude parasite surface proteins because heat-killed and BAPTA-AM-treated parasites could not activate EGR2. Eliminating micronemal proteins that act immediately before invasion is, however, more complicated since currently available technologies do not allow us to discriminate between intimate attachment, formation of the moving junction, and discharge of rhoptry proteins. We favor the EGR2-inducing factor being a resident rhoptry protein because of the precedent for these proteins to regulate host cell signaling and

FIG. 5. The host p38 MAPK pathway is required for EGR2 activation. (A) Cells were treated with 15 μM BAPTA-AM for 30 min, extensively washed, and then mock or parasite infected. After 6 h, RNA was isolated and EGR2 expression levels were measured by real-time PCR. (B) pEGR4x-Luc-transfected cells were mock treated or treated with LY294002 (LY; 25 μM), SB203580 (SB; 15 μM), U0126 (U; 10 μM), or SP600125 (SP; 25 μM) for 30 minutes. The cells were then infected, and 16 h later luciferase activity was measured. (C) pEGR4x-Luc-transfected cells were incubated in the absence or presence of B. anthracis lethal toxin for 6 h. The cells were extensively washed to remove extracellular toxin and then mock or parasite infected. After 16 h, lysates were prepared and luciferase was measured.
because high concentrations of ESA, which contains all known micronemal proteins (72), did not activate EGR2.

It is becoming increasingly clear that some host signaling and transcriptional pathways are rapidly activated in response to many different infectious agents, suggesting that they function as innate sentinels (5). Although some viruses and bacteria activate EGR2 (23, 36), EGR2 activation by *Toxoplasma* was specific since EGR2 was not activated by *Neospora* caninum. Although *Neospora* and *Toxoplasma* are closely related pathogens (21, 30), these data are consistent with other reports that these two genetically and morphologically similar parasites interact differentially with their host cells. For example, *Toxoplasma* interferes with gamma interferon-dependent gene expression while *Neospora* does not. In addition, *Neospora* is unable to modulate NF-κB (29, 35).

Several signaling pathways, including the PtdIns-3K and ERK pathways, can regulate EGR2 (31). To our knowledge, our data are the first to demonstrate a role for p38 MAPK in EGR2 activation. However, p38 MAPK activation alone was not sufficient to upregulate EGR2. This suggests that the parasite triggers at least two distinct signaling pathways that converge on the EGR2 gene promoter. We do not yet know whether both pathways are activated by a single factor or whether two distinct parasite-derived factors are involved. Alternatively, p38 MAPK signaling may be required to enable the EGR2-inducing factor to interact with its cellular target.

In this report, EGR2 was utilized as a reporter for parasite secretion-dependent activation of host cell transcription. A related and important question is what role EGR2, as well as EGR1, c-jun, and jun-B, plays during infection. EGR2 is best characterized as a critical regulator of peripheral nerve myelination and of hindbrain development (49, 56, 65). Attempts to elucidate the molecular basis for these phenotypes have identified several EGR2-regulated genes (38, 49, 67). These genes include genes that could help satisfy parasite nutritional needs including those that function in cholesterol and iron metabolism as well as those encoding growth factor receptors and other signaling proteins. In addition, EGR2 can regulate cell
survival by upregulating expression of the prosurvival BCL2 family member Mc11 and by promoting the pro tease-mediated degradation of the proapoptotic Bim gene (6). It is also noteworthy that, in some situations, EGR2 regulates pros apo totic genes such as the p53, BAK, BNP3, FasL, and PTEN genes (19, 67, 68). Therefore, EGR2 may function as a fac tor that either promotes parasite growth or protects host cells from infection. Our future work will focus on discriminating between these possibilities.

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