Potential Involvement of Extracellular Signal-regulated Kinase 1 and 2 in Encystation of a Primitive Eukaryote, *Giardia lamblia*

STAGE-SPECIFIC ACTIVATION AND INTRACELLULAR LOCALIZATION

Received for publication, September 10, 2002, and in revised form, October 22, 2002
Published, JBC Papers in Press, October 22, 2002, DOI 10.1074/jbc.M209274200

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*This work was supported by the Office of Research and College of Health and Public Affairs, University of Central Florida. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Mitogen-activated protein kinase (MAPK) pathways are major signaling systems by which eukaryotic cells convert environmental cues to intracellular events such as proliferation and differentiation. We have identified *Giardia lamblia* homologues of two members of the MAPK family, ERK1 and ERK2. Functional characterization of giardial ERK1 and ERK2 revealed that both kinases were expressed in trophozoites and encysting cells as 44- and 41-kDa polypeptides, respectively, and were catalytically active. Analysis of the kinetic parameters of the recombinant proteins showed that ERK2 is ~5 times more efficient than ERK1 in phosphorylating myelin basic protein as a substrate, although the phosphorylating efficiency of the native ERK1 and ERK2 appeared to be the same. Immunofluorescence analysis of the subcellular localization of ERK1 and ERK2 in trophozoites showed ERK1 staining mostly in the median body and in the outer edges of the adhesive disc and ERK2 staining in the nuclei and in the caudal flagellum. Our study also showed a noticeable change in the subcellular distribution of ERK2 during encystation, which became more punctate and mostly cytoplasmic, but no significant change in the ERK1 localization at any time during encystation. Interestingly, both ERK1 and ERK2 enzymes exhibited a significantly reduced kinase activity during encystation reaching a minimum at 24 h, except for an initial ~2.5-fold increase in the ERK1 activity at 2 h, which resumed back to the normal levels at 48 h despite no apparent change in the expression level of either one of these kinases in encysting cells. A reduced concentration of the phosphorylated ERK1 and ERK2 was also evident in these cells at 24 h. Our study suggests a functional distinction between ERK1 and ERK2 and that these kinases may play a critical role in trophozoite differentiation into cysts.

*Giardia lamblia*, an evolutionary primitive eukaryotic protozoan parasite and an intestinal pathogen of humans and animals, is one of the major causes of water-borne diseases worldwide (1). This flagellated protozoan undergoes complex life cycle stages while inside the host. Exposure to the highly acidic condition in stomach and proteases in the upper small intestine triggers excystation of trophozoites from the ingested cysts. Newly emerged trophozoites swim freely in the intestinal fluid and colonize the upper small intestine to replicate (2, 3). As enterocytes migrate to the tip of the villus and get sloughed off into the intestinal lumen, the attached trophozoites either reattach to new enterocytes to remain in the intestine or differentiate into infective cysts. Although the life cycle of this primitive eukaryote and physiological signals that regulate induction of excystation and encystation have been studied extensively, the molecular mechanisms by which trophozoites sense and respond quickly to the environmental signals in the intestine to initiate encystation remain largely unknown.

Encystation is an adaptive process to cope with the depletion of nutrients, specifically cholesterol, in the presence of high bile concentration in the lower small intestine (4, 5). It is accomplished in three steps as follows: (i) induction of the encystation-specific gene expression, specifically those that are necessary for the synthesis and processing of cyst wall proteins (CWP) (6–9); (ii) synthesis and intracellular transport of cyst wall proteins through newly developed secretory organelles such as Golgi apparatus (10) and encystation-specific vesicles (11); and (iii) assembly of the extracellular cyst wall (12, 13). Encysting trophozoites undergo distinct morphological changes such as disappearance of the median bodies and disorganization of the adhesive disc and flagella. The attached trophozoites gradually round up and detach. They lose mobility and become refractile as the cyst wall is assembled. The vacuoles that lie beneath the trophozoite membrane become the periplasmic space separating the parasite from the cyst wall (14). Unlike differentiating mammalian cells, giardial differentiation into cysts initiates in the cells arrested at G2 (15). During encystation two rounds of DNA replication without an intervening cell division occur, and nuclear division precedes chromosomal duplication (15). The dramatic changes in the cytoskeleton reorganization and gene expression during encystation suggest the existence of an orchestrated signaling mechanism. We speculate that the activation or inactivation of microtubule-associated proteins and transcription factors by kinases might play an important role in differentiation of trophozoites into cysts in response to cholesterol starvation.

A variety of critical cellular functions in eukaryotic cells are mediated through the MAP kinase pathway in response to extracellular stimuli (reviewed in Ref. 16). The mammalian MAP kinases p44 and p42, also known as ERK1 and ERK2, are serine/threonine kinases and are the two best-studied members of the MAP kinase family. Full activation of ERK1 and ERK2

1 The abbreviations used are: CWP, cyst wall proteins; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MBF, myelin basic protein; ORF, open reading frame; DAPI, 4,6-diamidino-2-phenylindole; PBS, phosphate-buffered saline; SRE, sterol-response element; SREBP, SRE-binding protein; DIC, differential interference contrast.

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requires dual phosphorylation at the serine and tyrosine residues at the TXY motif by MEK1/2 (17, 18). Upon activation, both ERK1 and ERK2 translocate to the nucleus and phosphorylate a variety of proteins including transcription factors, such as AP-1 (19), Elk1 (20), Ets1 (21), and SREBP (22), that directly or indirectly stimulate cell proliferation or differentiation.

A differential expression and/or activity of various MAP kinase homologues has been observed in parasitic protozoa. The fluctuation of expression or activity of MAP kinases has also been implicated for proliferation, differentiation, or development (23). Some studies have shown that the activity of KFR1, a MAP kinase homologue of Trypanosoma brucei, is higher in the differentiating bloodstream form than the procyclic form (23). LMAPK, a MAPK homologue of Leishmania major, is expressed in promastigotes and amastigotes, but the activity is detected only in amastigotes. Similarly, expression of PMP2, a MAPK homologue identified in Plasmodium falciparum, has been detected only in gametocytes of malaria parasites (24). Here we report functional characterization of giardial homologues of ERK1 and ERK2. Our study provides evidence of differential subcellular localization and activation of giardial ERK1 and ERK2 in trophozoites and in encysting cells. Our results indicate, for the first time, that giardial ERK1 and ERK2 possess functional differences, when they are thought to have overlapping functions, and that ERK1 and ERK2 may be regulated differentially.

EXPERIMENTAL PROCEDURES

Materials—Unless specified, all materials were obtained from Sigma, Fisher, and Invitrogen.

Giardia Culture and Differentiation—G. lamblia (WB clone C6, ATCC) were cultured in TYI-S-33 medium with 10% bovine serum and bile (25). Encystation was induced essentially as described by Kane et al. (4). Briefly, encystation was induced by growing trophozoites for one culture cycle in TYI-S-33. Media were poured off along with unattached trophozoites and replaced with encystation medium containing 10 mg/ml bovine bile at pH 7.8 and incubated at 37 °C for 3 days. Encystation medium was replaced with TYI-S-33 medium at 24 h, and incubation was continued for 48 h. Encysting cells were harvested at different time points by chilling and centrifugation and subsequently used for protein extraction.

PCR Amplification—A 2.0-kb DNA fragment of ERK1 containing the entire open reading frame and a 530-bp fragment of ERK2 were obtained by PCR amplification of Giardia genomic DNA using primers (forward, 5′-GCTGATTGTGTTAG-3′, and reverse, 5′-GCCCGTAGCCCCAGTTC-3′). The amplification products were cloned into pET41. The ORF of gERK2 (1.086 kb) was cloned into NcoI and XhoI sites and expressed as glutathione S-transferase (GST)-tagged fusion proteins, respectively. Expressed proteins were purified through cobalt affinity column (TALON superflow resin, Clontech Laboratories) and used for in vitro kinase assays and generation of rabbit polyclonal antibodies through a commercial vendor (Cocalico, Reamstown, PA). The His tag (pET30 ERK2) and glutathione S-transferase tag (pET41 ERK1) sequences added 6 and 31 kDa of molecular masses to the fusion proteins, respectively.

Western Blot and Immunoprecipitation—Trophozoites or encysting cells at different time points were harvested, and crude cell extracts (50 μg) in Tris- HCl (100 mM, pH 7.0) were used for immunodetection of endogenous phosphorylated ERK1 and ERK2 using anti-ERK1 and anti-ERK2 polyclonal antibodies. Phosphorylated ERK1 and ERK2 were detected in the crude extracts with or without treatment with λ phosphatase (2000 units for 30 min at 30 °C) were detected by an anti-phospho-ERK polyclonal antibody that recognized both ERK1 and ERK2 (Cell Signaling Technologies). Expression of CWPI in encysting cells was detected by immunoblot analysis using anti-CWPI monoclonal antibody (kindly provided by Dr. Henry Stibbs, Waterborne Inc.). Positive signals were detected using a chemiluminescence kit (Pierce) and an anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody. For immunoprecipitation (28), trophozoites or encysting cells were lysed in TAN buffer (10 mM Tris acetate, pH 5.0, 1% Nonidet P-40, 100 mM NaCl, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium leupeptin, and 1 mM sodium aprotinin). Crude extracts were treated with rabbit serum followed by protein A-agarose to eliminate nonspecific binding of proteins to antibody. The protein A-agarose beads were collected by centrifugation, and the supernatants were incubated with the appropriate antibody. Protein-antibody complexes were captured by protein A-agarose beads, washed, and collected by centrifugation. Protein A-agarose beads bound to the immune complex were then resuspended in 100 mM Tris, pH 7.0, and used for subsequent experiments.

Kinetin Assay—Purified recombinant gERK1 and gERK2 (5 μg) or semi-com pact protein A-agarose beads pellets of immunoprecipitated gERK1 and gERK2 (25 μg) were used in a 50-μl reaction containing 100 μM Tris-HCl, pH 7.5, 600 μM ATP, 1 mM MgCl2 (ERK1) or 10 mM MgCl2 (ERK2), 6 mM γ-32P-ATP, and 100 μM of myelin basic protein (MBP). The reaction mixture was incubated at 30 °C for 10 (ERK1) or 30 min (ERK1), and 30 μl (recombinant protein only) were spotted on phosphocellulose filter discs after addition of 5 μl of EDTA (100 mM) to stop the reaction. Filter discs were washed with 1% H3PO4, and bound radioactivity was detected in a liquid scintillation counter (Beckman Instruments). The remaining 20 μl of the reaction mix (recombinant protein) or the 50 μl of the reaction mix (immunoprecipitated protein) was combined with 4× Laemmli sample buffer and resolved in SDS-PAGE (12–14%). Phosphorylation of MBP was detected by autoradiography and PhosphorImager (Amersham Biosciences) analysis. The phosphatase-based luciferase assay (33) was performed with 35,000 to 70,000 cells/ml cultured on 12-mm glass coverslips placed in 24-well tissue culture dishes and allowed to grow for 65–70 h in AnaeroPack jar (Mitsubishi Gas Chemical Co.) at 37 °C. In some experiments, trophozoites were exposed to encystation medium, and cells were fixed after the specified time. Trophozoites or encysting cells attached on coverslips were harvested in 100% chilled methanol, washed 2% sodium D-glucuronate, and further permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. Permeabilized cells were blocked in blocking buffer (5% goat serum and 1% glycercer in PBS) and incubated with appropriate rabbit polyclonal antibody (ERK1 1:3000 and ERK2 1:6000 in blocking buffer). At the end of incubation, cells were washed with PBS and visualized with an Alexa 488-conjugated anti-rabbit IgG (Molecular Probes, Eugene, CA) secondary antibody diluted in blocking buffer (1:100). Cells were washed in PBS and counterstained with DAPI (0.1 μM). Cells were washed in PBS and post-fixed with 4% paraformaldehyde (Electron Microscopy Sciences), rinsed with PBS and mounted on Gelmount.
Localization of the target proteins was detected in a Delta Vision Image Restoration Microscope using SoftWoRx image analysis software. DIC images of the localization were captured in a Zeiss LSM 510 laser scanning confocal microscope.

**RESULTS**

**Sequence Characterization and Expression Analysis of gERK1 and gERK2**

We have cloned giardial homologues of ERK1 (ORF 1.15 kb, 386 amino acids) by direct PCR amplification of the genomic DNA and ERK2 (ORF 1.086 kb, 362 amino acids) by screening a /H9261 ZAPII Giardia cDNA library. Upon screening the library using a 530-bp fragment as a probe, a 1.15-kb cDNA clone of ERK2 was obtained (GenBank™ accession number AY149274 for ERK1 and accession number AY149275 for ERK2). Multiple sequence analysis of the translated amino acid sequence of the ORF showed that *Giardia* ERK1 had 51% identity with *Dictyostelium discoideum* ERK1, 49% identity with *Chlamydomonas reinhardtii* MAPK, 42% identity with *Arabidopsis thaliana* MAPK, 41% identity with human ERK1, and 50% identity with human ERK8 with a probability score between e-100 and 1e-76. The deduced amino acid sequence of *Giardia* ERK2 ORF exhibited 66% identity with *D. discoideum* ERK2, 59% identity with *L. mexicana* MAPK2, 57% identity with *P. falciparum* MAPK1, and 50% identity with human ERK8 with a probability score between e-131 and 2e-94. The gERK2 cDNA clone contains a short 9-bp 5' and a 56-bp 3' untranslated sequence that are typical for *Giardia* transcripts. Multiple sequence alignment of the amino acid sequence of the ERK1/2 catalytic domain revealed that all 11 subdomains, characteristic of serine/threonine kinases, are present in both gERK1 and gERK2 (Figs. 1A and 2A). This includes the consensus motif GXXGXXV (Gly26–Val33 gERK1 and Gly25–Val32 gERK2) in subdomain I for anchoring non-transferable phosphates of ATP, the invariant Lys (Lys-48 ERK1 and Lys-47 gERK2) and the surrounding region (Val45–Ser51, ERK1, Val44–Ser50, ERK2) in subdomain II for binding and orienting ATP for maximum enzymatic activity, and the glutamic acid (Glu 66 ERK1, Glu65, ERK2) in the subdomain III involved in formation of a salt bridge with Lys48 and Lys47. The catalytic loops in both ERK1 and ERK2 are nearly 100% homologous with the HRDLKXXN motif (His143–Asn150 ERK1 and His138–Asn145 ERK2). The conserved TXY motif for dual phosphorylation (Thr 191–Tyr193 ERK1 and Thr180–Tyr182 ERK2) for the activation of the enzyme is also present. Five other amino acids, Phe54 (Phe53 gERK2), Arg62 (Arg61 gERK2), His121 (His116 gERK2), Lys134 (Lys129 gERK2), and Cys157 (Cys152 gERK2), considered to be
the signature residues for MAPK as they are not shared by any other kinases including the CDKs (32), are also present in gERK1 and gERK2. Molecular three-dimensional modeling of gERK1 and gERK2 revealed the presence of additional α-helices (Fig. 1, B and C, and Fig. 2, B and C) in giardial ERK1/2 when compared with rat extracellular signal-regulated kinase (pdb1erk) and phospho-ERK2 (pdb2erk) indicating significant structural differences between mammalian ERK1/2 and giardial ERK1/2. Functional implication of these additional α-helices is unclear at this moment.

Analysis of genomic organization by Southern blot and search of *Giardia* genome sequence database suggest that both gERK1 and gERK2 are single copy genes (data not shown). Northern blot analysis of gERK1 and gERK2 revealed that both are expressed as a single transcript of 1.3 kb for ERK1 and 1.2 kb for ERK2, respectively, in trophozoites (data not shown). Analysis of the protein products by Western blotting of cell extracts indicated that the expression of the 44-kDa gERK1 or the 41-kDa gERK2 did not fluctuate during growth and encystation (Figs. 8 and 9). The size of the native gERK1 and gERK2 was in agreement with the deduced amino acid sequences from the ORFs.

**Giardial ERK1 and ERK2 Were Catalytically Active**—For functional characterization of gERK1 and gERK2, the catalytic activity of the recombinant ERK1 and ERK2 was determined next using MBP as the substrate. *In vitro* kinase assays revealed that both recombinant ERK1 and ERK2 were catalytically active (Fig. 3) and phosphorylated MBP with different efficiency (specific activities: 3.7 ± 0.3 nmol of phosphate/mol of ERK1/s and 20.1 ± 0.06 nmol of phosphate/mol of ERK2/s). Recombinant ERK2 was 5 times more efficient than ERK1 in incorporating phosphates into MBP and was capable of autophosphorylation (Fig. 3, E and C). No autophosphorylation was noted for gERK1, which suggests that gERK1 possibly can maintain a basal level of activity without being activated by phosphorylation (Fig. 3 A). This is apparently reflected into the lower efficiency of phosphorylation of MBP by the recombinant gERK1, and therefore, the activity of the recombinant gERK1 may not represent the full phosphorylating potential of the endogenous gERK1. At the standard reaction conditions using 5 µg of the enzyme and varying concentrations of MBP, the apparent $K_m$ values for the MBP were in the micromolar range (31.8 ± 8.5 μM ERK1 and 33.1 ± 7.3 μM ERK2) (Fig. 3, B and E), which are close to that reported for the mouse ERK2 (33). Both ERK1 and ERK2 preferred ATP to GTP as the phosphate donor (ERK1, $K_m$ ATP 865.5 ± 36.0 μM; ERK2, $K_m$ ATP 32.1 ± 2.5 μM); however, ERK2 was 8 times more efficient in utilizing ATP than ERK1. Giardial ERK1 and...
ERK2 also favored Mg$^{2+}$ over Mn$^{2+}$ as the divalent cation (ERK1, $K_m$, 1.53 ± 0.4 mM; ERK2, $K_m$, 0.265 ± 0.001 μM) (Fig. 3).

Although the kinetic parameters for the recombinant gERK2 were comparable with that for the mammalian ERK2, the catalytic efficiency of the recombinant gERK1 was relatively poor. To determine the catalytic activities of the endogenous ERK1 and ERK2, we immunoprecipitated ERK1 and ERK2 from the trophozoite extracts using anti-gERK1 and anti-gERK2 rabbit polyclonal antibodies. The specificity of the anti-ERK1 and -ERK2 antibodies was determined previously by Western blot analysis of the purified recombinant enzymes and crude trophozoite extracts (Fig. 4 and 5), which indicated a single peptide band of 75 kDa for the recombinant ERK1 and a 44-kDa polypeptide band for the endogenous ERK1 recognized by the antibody at a dilution of 1:4000 (Fig. 4A and B). Similarly, anti-ERK2 antibody recognized a single major polypeptide band of 47 kDa for the recombinant ERK2 and a 41-kDa band for the endogenous ERK2 recognized by the antibody at a dilution of 1:4000 (Fig. 4A and B). Neither ERK1 nor ERK2 antibodies cross-reacted with the recombinant gERK2 or gERK1, respectively (data not shown).

Immunoprecipitated ERK1 and ERK2 were subjected to in vitro kinase assays using MBP as the substrate. Results presented in Figs. 4 (C and D) and 5 (C and D) indicated that both endogenous ERK1 and ERK2 were catalytically active and capable of phosphorylating MBP.

**Giardial ERK1 and ERK2 Showed Differential Subcellular Localization**—To assess the functional role of giardial ERK1 and ERK2 and whether they have overlapping functions, we monitored intracellular distribution of ERK1 and ERK2 by immunofluorescence analysis using primary antisera specific for gERK1 and gERK2 and deconvolution restoration microscopy. Signals obtained for the ERK1 antibody indicated localization of ERK1 mostly in the flagellar basal bodies and in the median body. Localization of ERK1 was also seen in the adhesive disc, the ventral groove, and along the caudal flagella (Fig. 6A). On the other hand, signals for the ERK2 antibody were mostly at the cell membrane, at the anterior and caudal flagella, and in the nuclei (Fig. 7A). In support of the studies indicating that mammalian ERK1 and ERK2 are two microtubule-associated proteins (34), the overall pattern of gERK1 and gERK2 localization to the specific intracellular structures is suggestive of their possible association with cytoskeletal structures.

**Altered Localization, Phosphorylation, and Catalytic Activity of ERK1 and ERK2 during Encystation**—Giardia trophozoites undergo remarkable structural changes during encystation that are associated with altered intracellular events, such as disappearance of the median body, cell cycle arrest, appearance of encystation-specific vesicles, and increased synthesis and transport of cyst wall proteins. Because ERK1 and ERK2 from higher eukaryotes are known to be involved in a variety of cellular functions associated with cell proliferation and cellular differentiation (35, 36), we set out to analyze intracellular localization and catalytic activity of ERK1 and ERK2 at different stages of encystation by immunofluorescence analysis and in vitro kinase assays of the immunoprecipitated samples. Trophozoites were treated with the encystment media and har-
vested at 2, 6, 10, 24, and 48 h. A distinct change in the localization of ERK2 in the encysting cells was seen as early as 2 h of encystation, in which the antibody showed a punctate but even staining throughout the cytoplasm. No staining in the nuclei or in the flagella by the antibody was noted at 2, 24, and 48 h following exposure to the encystment media (Fig. 7, B–D). At 24 h, localization of ERK2 was seen mostly toward the posterior end of the cell and surrounding the large vesicle-like structures (Fig. 7C), which started to appear around 10 h during encystation (data not shown). At 48 h, more cells with vesicles were present, and similar staining of ERK2 was seen in the cytoplasm and at the perivesicular region. On the other hand, a more subtle change in the localization of ERK1 was noted during encystation, which includes lack of staining in the caudal flagella at 2 h onward and in the median body presumably because of the disappearance of the flagella and the median body at the early stage of encystation (Fig. 6, B–D). Also, accumulation of ERK1 in the ventral groove was evident in encysting cells at 24 h. No alteration in the staining of ERK1 in the adhesive disc and in the flagellar basal bodies was noted in any stages of encystation. The observation that ERK1 and ERK2 are targeted to different locations in trophozoites and that localization of both proteins changes during encystation suggests a possible functional difference between gERK1 and gERK2 and important roles of ERK1 and ERK2 during giardial differentiation.

To understand the roles played by gERK1 and gERK2 during encystation, we next monitored the catalytic activity of ERK1 and ERK2 in encysting cells, as it is well known that signals for both cell proliferation and differentiation are mediated through activation or inactivation of ERK1 and ERK2 (36, 37). Although immunoblot analysis indicated no significant change in the expression of either ERK1 or ERK2 in encysting cells at different stages of encystation (Figs. 8A, 9A, and 10C, panel a), altered activities of both kinases were apparent when phosphorylation of MBP by the immunoprecipitated enzymes was used to monitor the catalytic activity. At 2 h of encystation, a~2.5-fold increase in the activity of ERK1 was noted which gradually declined during 6 and 10 h reaching the lowest point at 24 h. At 48 h, the activity of ERK1 resumed back to the level of uninduced cells (Fig. 8, B–D). Interestingly, the activity of ERK2 dropped significantly at 2 h and remained at that level until 10 h. At 24 h, the activity of ERK2 in encysting cells reached the minimum level but recovered back to the uninduced level at 48 h, thus exhibiting a 5–8-fold increase in the activity within 24 h (Fig. 9, B–D). Also, it is noteworthy that the catalytic activity of both ERK1 and ERK2 in encysting cells was minimum at 24 h when the maximum number of cysts appeared in the culture medium.

Because the activity of ERK1 and ERK2 depends on the activating phosphorylation of these two proteins, we set out to assess the phosphorylation status of ERK1 and ERK2 at different stages of encystation using a phospho-ERK antibody generated against a 20-amino acids peptide antigen around the TXY motif of human p44 ERK1, which showed 65 and 52% identity with the same region of the giardial ERK1 and 2, respectively. Immunoblot analysis revealed that the concentration of phospho-ERK1 in encysting cells remained the same as that of the untreated trophozoites until 10 h except for a marginal increase at 2 h (Fig. 10C, panel b). A significant decrease in the phospho-ERK1 concentration was noted in the encysting cells at 24 h, which was possibly reflected into the decreased kinase activity at that stage. The concentration of phospho-ERK2 in encysting cells showed a slight decrease in 6 and 10 h but a dramatic drop at 24 h confirming the minimum kinase
activity as observed at that stage of encystation. Although at a much lower intensity, the band corresponding to phospho-ERK2 was detectable at 48 h representing an increased phosphorylation of ERK2, while concentration of the phosphorylated ERK1 remained low at 48 h. The expression of cyst wall protein 1 (CWP1) was used as a marker for the encystation process, which showed an increased expression of CWP1 at 10 and 24 h, as expected (Fig. 10C, panel c). Recognition of the phospho-gERK1 and -2 by the anti-phospho-ERK antibody was validated by the treatment of the crude trophozoite extracts with \( /H9261/-phosphatase, which resulted in disappearance of the polypeptide bands corresponding to the phospho-gERK1 and -2 (Fig. 10A). Furthermore, phospho-ERK antibody did not recognize the recombinant gERK1 and -2 (Fig. 10B).

DISCUSSION

ERK1 and ERK2 are two highly conserved ubiquitously expressed enzymes in a variety of organisms and play a pivotal role in regulating complex cellular processes in response to environmental cues. To understand the functional role of MAPK in growth and differentiation of Giardia trophozoites, we characterized giardial homologues of ERK1 and ERK2. Sequence analysis indicated that over 50% identity exists between gERK1/gERK2, and the orthologues from other model organisms and the Giardia homologues contain all subdomains that are conserved in other eukaryotes. However, a 10-amino acid insertion of unknown significance is present in ERK1 at the activation domain before the TXY motif, which is not shared by other ERK1 or ERK2 from different species (Figs. 1A and 2A). Computer-based three-dimensional modeling indicated distinct differences in the structures of giardial ERK1/2 when compared with the mammalian ERK1/2, although the overall structures were the same (Fig. 1, B and C, and Fig. 2, B and C). Both recombinant and native ERK1 and ERK2 were catalytically active and phosphorylated myelin basic protein, a widely used substrate for ERK1 and ERK2 in assay systems. Thus, the structural similarity and substrate specificity con-
immunoprecipitated ERK1 at different times of encystation. Data represented ERK1 in the crude extract prior to immunoprecipitation showing activating phosphorylation at the T

It is possible that the full activation of gERK1 requires the phosphorylation of MBP with apparently similar efficiency. 

could be partially achieved by the recombinant ERK2 through concentration of MgCl2 completely inhibited phosphorylation as the divalent cation. MnCl2 and a higher concentration of MgCl2 completely inhibited phosphorylation by both kinases (data not shown).

Because ERK1 and ERK2 phosphorylate a variety of proteins, intracellular localization of these kinases defines their functional specificity. Accordingly, the precise distribution of ERK1 in the basal bodies, the median body, and in the adhesive discs may signify a possible interaction of ERK1 with proteins, intracellular localization of these kinases defines their functional specificity. Accordingly, the precise distribution of ERK1 in the basal bodies, the median body, and in the adhesive discs may signify a possible interaction of ERK1 with proteins associated with these structures. Basal bodies of the flagellated cells are rod-like structures that correspond to the centrosomes of the higher eukaryotic cells and are associated with origins of the flagellar axonemes. In flagellated cells, basal bodies are involved in flagellar motility; therefore, localization of ERK1 into basal bodies presumably reflect its potential association with microtubule-associated proteins. This assumption is based on the findings that the association of mammalian ERK1 and ERK2 with the microtubule-binding protein tubulin played an important role in the spindle assembly checkpoint (38), the mammalian ERK interacts with the spindle microtubule motor CENP-E during mitosis (39), and that the active ERK associates with microtubules and regulates microtubule stability in fibroblasts and breast epithelial cells (40). It is possible that gERK1 may associate with tubulins in the median body and adhesive discs for the maintenance of microtubule stability. Staining with the gERK2 antibody showed a distinctly different pattern of gERK2 localization, in particular to the cell membrane, anterior and caudal flagella, and presumably to the nuclei, which suggests a possible interaction of gERK2 with microtubule-associated proteins in cytoskeletal structures different from what was observed with gERK1. It is well documented that mammalian ERK1 and -2 translocate to the nucleus upon activation where they phosphorylate specific transcription factors (16, 41). So far no information is available on the specific transcription factors in Giardia that are phosphorylated by ERK. Detailed studies are required to understand the function of the subset of ERK1 and ERK2 targeted to various cytoskeletal structures and to the nuclei in Giardia.

Analysis of the distribution of ERK1 and ERK2 in encysting cells revealed an evident relocation of ERK2 in the cytoplasm. Redistribution of ERK2 was noticeable as early as 2 h of encystation, which did not change significantly in the latter part of encystation, except for its localization around the perivesicular region of the large vesicle-like structures that were present in the encysting cells at 24 and 48 h. On the other hand, localization of ERK1 did not alter significantly except for the high intensity staining by the antibody in the ventral groove area at 24 h. The ventral groove is a shallow region, which extends from the adhesive disc to the caudal region of the body and is the site of origin of the ventral flagellar pair. It is believed that the ventral groove generates the force to develop a negative pressure inside the adhesive disc for the attachment of the parasite (14, 42). The rationale for the accumulation of a subset of ERK1 in this area is unclear. The disappearance of ERK1 staining in the median body at 24 and 48 h was due to disorganization of the median body during encystation. Interestingly, a parallel fluctuation in the kinase activity of the endogenous ERK2 and ERK1 was noted during encystation. A significantly reduced activity of ERK2 was maintained up to 24 h as long as the cells were exposed to the encystation medium. Interestingly, upon replacement of the encystation medium with the regular growth medium, a 5–8-fold increase in the activity was noted within 24 h. The activity of ERK1 instead was biphasic with an initial ~2.5-fold increase followed by a gradual decline until 24 h. Upon replacement of the regular medium at 24 h, the activity was restored to the level of uninduced cells. It is possible that the initial increase in the ERK1 activity may be required for the initiation but not for the maintenance of encystation. Also a parallel decrease in the concentration of phospho-ERK1 and -ERK2 at 24 h suggests that the activating phosphorylation of ERK1 and ERK2 might be necessary for the complete activation of giardial ERK1 and ERK2 as observed in mammalian ERK1/2.

The results obtained from this study also suggest that the high bile concentration in the encystation medium, which may induce cholesterol starvation, may be a factor that indirectly prevents ERK1 and ERK2 activation. However, it is interesting that the exposure to a high bile concentration and thereby low
cholesterol concentration generated signals that may have different effects on the activation of ERK1 and ERK2. This finding also suggests the possibility that the function of giardial ERK1 and ERK2 may be regulated through different mechanisms. Differential phosphorylation of ERK1 and ERK2 by the PCPH oncogene has been reported in 293T cells (43). It was proposed that in these cells, ERK1 activation might have been mediated through a Ras/MEK-independent pathway (43). Although Giardia homologues of MEK and MEKK have been identified, it is not known which signaling pathway(s) activates ERK1 and ERK2 in trophozoites and in encysting cells. In depth studies are needed to elucidate the regulation of activation of giardial ERK1/ERK2 during encystation.

The first step of encystation is the induction of expression of CWP proteins, which is triggered by the depletion of cholesterol. It has been shown that in higher eukaryotes, cholesterol homeostasis is maintained by the transcription activation of various genes through activation of transcription factors, SREBP1a and SREBP2, that bind to the sterol-response element (SRE) (44). The promoter of CWP1 and CWP2 contains degenerated SRE, and it has been hypothesized that CWP expression might be induced by interaction with the SRE-binding proteins (SREBP) (44). Importantly, recent studies (45) have shown that mammalian SREBP1a and SREBP2 are activated by ERK1 and ERK2, and their activation may be linked to the MAPK cascade. It is possible that the activation of CWP gene transcription during encystation is mediated through SREBP, which is in turn activated by ERK1. Recently, the observation that Giardia trophozoites express a cell-surface cholesterol-sensing receptor, receptor-Ck (26), suggests that a sterol-regulated signaling pathway might be present in Giardia. So far, no information on the function of giardial homologues of SREBP is available. Further study in this area will shed light on the involvement of ERK1 in transcription of CWP genes.

Based on our observations, we speculate that inactivation of ERK2 was necessary for the formation of cysts, whereas an increased activity of ERK1 was a prerequisite for the encystation process to begin. Phosphorylation status of ERK1 and ERK2 correlated best with the activity profile at 24 h supporting the conjecture of inactivation of ERK1 and ERK2 during encystation. However, despite the fact that the activity of ERK1 and ERK2 was back to the original level in cells at 48 h upon maintaining them in TYI-S-33 media for the last 24 h, no significant change in the localization of ERK1 or ERK2 was noted in encysting cells except for the lack of accumulation of ERK1 in the ventral groove. Functional significance of the

**Fig. 9. In vitro kinase assay with immunoprecipitated gERK2.** Trophozoites were allowed to encyst for the times indicated, and gERK2 was immunoprecipitated from the crude cell extracts. Immunoprecipitated ERK2 was used for in vitro kinase assays. A, immunoblot analysis of the ERK2 in the crude extract prior to immunoprecipitation showing the level of ERK2 expression. B, autoradiogram showing phosphorylation of MBP by the immunoprecipitated extracts. C, Coomassie Blue-stained SDS-PAGE of the kinase assay showing equal amounts of added MBP. D, densitometric analysis of the phosphorylated MBP by the immunoprecipitated ERK2 at different times of encystation. Data represent mean ± S.D. of three separate experiments.

**Fig. 10. Assessment of the concentration of phospho-ERK1/ERK2 during encystation.** A, detection of the phospho-ERK1 and -2 and dephosphorylated ERK1 and -2 in crude trophozoite extracts (30 µg) with and without treatment with λ phosphatase to determine the specificity of the phospho-ERK antibody. B, Western blot analysis of the recombinant ERK1 and -2 using anti-phospho-ERK and anti-gERK1 and -2 antibodies to confirm that phospho-ERK antibody does not recognize dephosphorylated ERK1 and -2. C, immunoblot analysis of the dephosphorylated ERK1 and ERK2 using anti-ERK1 (1:4,000) and ERK2 (1:30,000) antibodies together (panel a) and phosphorylated ERK1 and ERK2 using an anti-phospho ERK antibody (1:100) (panel b) in the crude extracts (50 µg) from encysting cells at different stages of encystation. Expression of CWP1 protein (panel c) using anti-CWP1 antibody (1:1 × 10^6) was used as a marker for encystation.
differential distribution and activity of ERK1 and ERK2 during encystation remains to be determined.

REFERENCES

1. Marshall, M. M., Naumovitz, D., Ortega, Y., and Sterling, C. R. (1997) Clin. Microbiol. Rev. 10, 67–85
2. Thompson, R. C., Reynoldson, J. A., and Mendis, A. H. (1993) Adv. Parasitol. 32, 71–160
3. Gillin, F. D., Reiner, D. S., and McCaffery, J. M. (1996) Annu. Rev. Microbiol. 50, 679–705
4. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
5. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
6. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
7. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
8. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
9. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
10. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
11. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
12. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
13. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
14. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
15. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
16. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
17. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
18. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
19. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
20. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
21. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
22. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
23. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
24. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
25. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
26. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
27. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
28. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
29. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
30. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
31. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
32. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
33. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
34. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
35. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
36. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
37. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
38. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869
39. Kane, A. V., Ward, H. D., Keusch, G. T., and Pereira, M. E. A. (1991) J. Infect. 142, 859–869