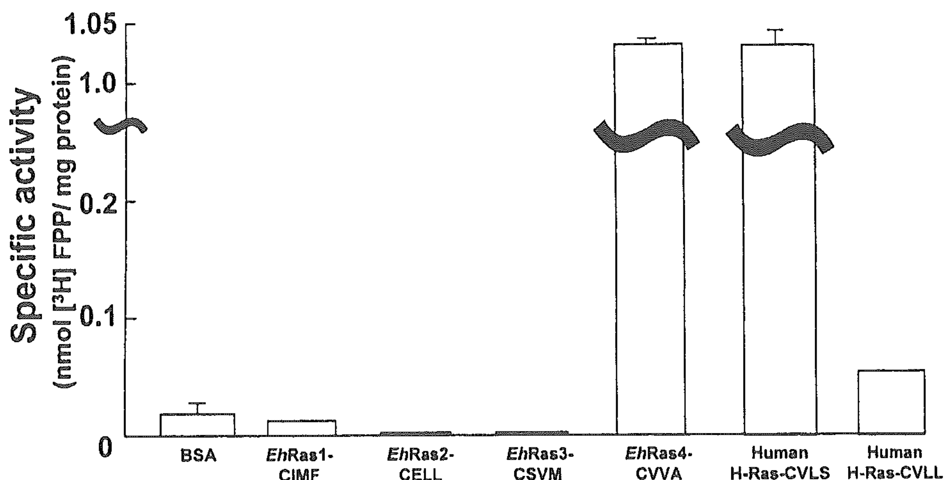
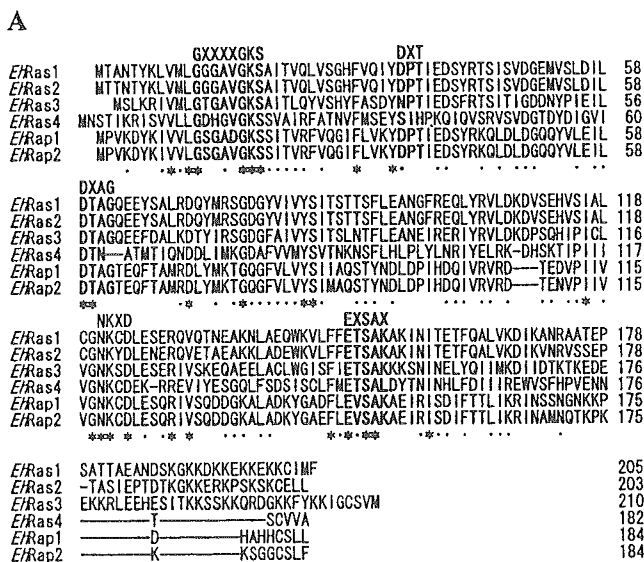


FIG. 4. **Substrate specificity of recombinant FT of *E. histolytica*.** The specific activity of the recombinant *Eh*FT was determined from the incorporation of [^{3}H]farnesyl pyrophosphate into the recombinant *Eh*Ras1, human wild type H-Ras-CVLS, and mutant type H-Ras-CVLL. Means ± S.E. of quadruplicates are shown.



B

	*Eh*Ras1	*Eh*Ras2	*Eh*Ras3	*Eh*Ras4	*Eh*Rap1	*Eh*Rap2
*Eh*Ras1		86	51	26	44	47
*Eh*Ras2			48	26	43	44
*Eh*Ras3				30	38	39
*Eh*Ras4					26	26
*Eh*Rap1						91
*Eh*Rap2						

FIG. 5.**Similarity among *Eh*Ras1-4 and *Eh*Rap1-2.** *A*, sequence alignment of *Eh*Ras1-4 and *Eh*Rap1-2 using the ClustalW program. Sequences over the alignment are GTP binding consensus sequences. *Asterisks* (*) and *dots* (.) indicate identical amino acids and conserved amino acid substitutions, respectively. GXXXXGKS and DXT depict GXXXXGK(S/T) and D(*X*)$_n$T consensus sequences, respectively. DDBJ/EMBL/GenBank™ accession numbers are given in Fig. 6. *B*, percentage identities among *Eh*Ras1-4 and *Eh*Rap1-2.

and 0.13 ± 0.03 μM, respectively (35).

Sensitivity of Recombinant EhFT to Human FT Inhibitors— We examined the sensitivity of *Eh*FT to known FT inhibitors. As shown in Table I, *Eh*FT was virtually insensitive at up to 30 μM except to FPT inhibitor-II and FPT 276 when recombinant *Eh*Ras4 was used as substrate. The lack of sensitivity of *Eh*FT

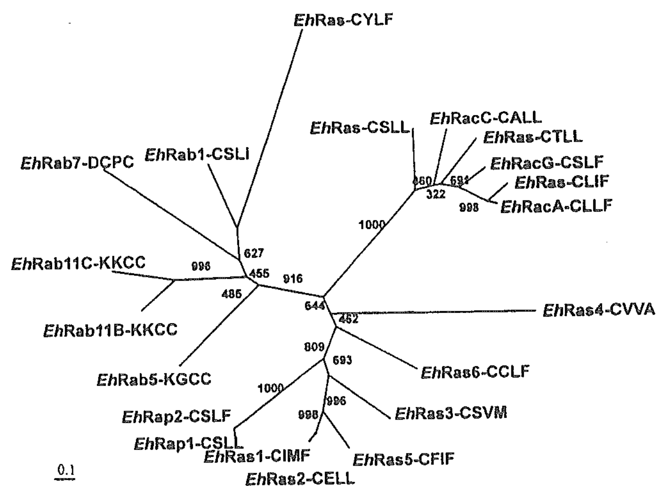


FIG. 6. **Phylogenetic tree of representative members of small GTPases of *E. histolytica* constructed by the Neighbor-joining method.** DDBJ/EMBL/GenBank™ accession numbers of these proteins are: *Eh*Ras1, AAA21446; *Eh*Ras2, AAA21447; *Eh*Ras3, AB112425; *Eh*Ras4, AB112426; *Eh*Rap1, AAA21444; *Eh*Rap2, AAA21445; *Eh*RacA, Q24814; *Eh*RacC, Q24816; *Eh*RacG, O76321; *Eh*Rab1, BAB40669; *Eh*Rab5, BAB40673; *Eh*Rab7, BAB40674; *Eh*Rab11b, BAB40678; *Eh*Rab11C, BAB40679. The other proteins without accession numbers shown in the tree are putative Ras superfamily small GTPases found in the *E. histolytica* genome data base. *Eh*Ras7-10 are labeled as *Eh*Ras-XXXX, because there is no sufficient evidence to support that these proteins are Ras homologues.

to FT inhibitors was not dependent on the substrate; *Eh*FT was also insensitive to these inhibitors when H-Ras-CVLS was used as a substrate.

DISCUSSION

In this study, we have demonstrated for the first time the molecular identity of FT in enteric parasitic protozoa. Although all major subgroups of small GTPases, *i.e.* Ras, Rap, Rho, Rac, Rab, Arf, and Ran, have been identified (12, 14–21) in *E. histolytica* and some of their functions studied (17, 18), this is the first demonstration and characterization of an isoprenylation enzyme essential for correct membrane topology and organelle targeting of these small GTPases. We identified common features of FT hitherto recognized in other eukaryotes: both FTα and FTβ contained well-conserved signature domains such as BET4 and CAL1 domains, and the repeats, *i.e.* protein prenyltransferase α-subunit repeats and prenyltransferase/ squalene oxidase repeats (Fig. 1). *Eh*FT forms a heterodimeric

TABLE I
Inhibition of recombinant EhFT by inhibitors of human FT

The enzymic activity of recombinant *Eh*FT using *Eh*Ras4-CVVA and human H-Ras-CVLS as protein substrates was determined by incorporation of [^{3}H]FPP in the absence and presence of FT inhibitors to calculate IC_{50} values.

Inhibitors	IC_{50}		
	E. histolytica FT		Human FT[a]
	EhRas4	Human H-Ras	Human H-Ras
	μM		
Prenyl analogues			
FPT inhibitor-I	>30	>30	0.075[b]
FPT inhibitor-II	2.0	2.7	0.075[b]
Gliotoxin	>30	>30	1[c]
α-Hydroxyfarnesyl-phosphonic acid	>30	>30	0.03[d]
Peptidomimetic			
FTI-276	2.4	0.9	0.0005[e]

[a] Reported IC_{50} values of human FT for the inhibitors using human H-Ras-CVLS as a protein substrate are shown for comparison.
[b] Ref. 47.
[c] Ref. 48.
[d] Ref. 49.
[e] Ref. 50.

complex with a ratio of 1:1 between α- and β-subunits, similar to the case in other organisms, as shown by co-purification (Fig. 3). Phylogenetic analyses indicate that both *Eh*FTα and *Eh*FTβ are equally distant from homologues from other organisms. This may partially explain some of the unique biochemical characteristics of the amebic FT not shared by the mammalian counterpart. It is also worth noting that trees of both α- and β-subunits are similar (Fig. 2), suggesting that the FT subunits co-evolved independently at a comparable rate in these organisms.

We identified *Eh*Ras4-CVVA as one of the intrinsic substrates of FT in *E. histolytica*. Although it was not possible to test all small GTPases as substrates for *Eh*FT, we showed that *Eh*FT exclusively utilized *Eh*Ras4 as a farnesyl acceptor. In contrast, recombinant *Eh*Ras1-3, Rap2, RacC, and Rab7 were not farnesylated by recombinant *Eh*FT (Fig. 5) or the whole cell extract. The fact that the amebic lysate contained the activity to transfer the farnesyl residue to *Eh*Ras4, but not other *Eh*Ras isotypes, reinforces the notion that the FT characterized in the present study is the sole FT in this organism and also specific for this Ras protein. We also tentatively concluded that FT-mediated farnesylation is not a major lipidation of Ras protein in this organism. It was unexpected that *Eh*FT did not utilize *Eh*Ras3, which terminates with CSVM, as a farnesyl acceptor, because mammalian and yeast small GTPases containing a C-terminal Ser, Met, Gln, Cys, or Ala were shown to prefer to be farnesylated (4). An unexpected substrate specificity was also previously reported for FT from another protozoan parasite *T. brucei*, which farnesylates Ras protein with CVIM, but not CVLS (43). The fact that *Eh*FT prefers smaller amino acids at the C terminus of *Eh*Ras (CVVA and CVLS) indicates that the amebic FT may possess a smaller binding cleft for the Ras C terminus.

Among newly found putative Ras-like proteins, *Eh*Ras3–6 were the only ones that contained a terminal *CaaX* and also showed a closer kinship to *Eh*Ras1 and *Eh*Ras2 than to other small GTPases (*i.e.* Rap, Rac/Rho, and Rab) (Fig. 6). This observation, together with the lack of farnesylation by *Eh*FT of *Eh*Ras1–3, Rap2, RacC, and Rab7, indicates that *Eh*Ras4 protein is the sole Ras protein farnesylated by *Eh*FT. It is also conceivable that *Eh*Ras1–3 proteins with the C-terminal Phe, Leu, or Met, respectively, are farnesylated by GGT-I, as shown for rat RhoB-CKVL (44). This is also the case for *Eh*Ras2-CELL, which has been shown to be farnesylated by the recombinant *E. histolytica* GGT-I in our preliminary experiment (data not shown). Although the C terminus of the previously identified amebic Ras/Rap (*i.e. Eh*Ras1-2 and *Eh*Rap1-2) (12) was presumed to be geranylgeranylated, a study using rabbit reticulocytes lysates (as a source of enzyme) and recombinant *Eh*Ras1 and *Eh*Rap2 showed that these proteins were not geranylgeranylated, but farnesylated (13). Considering that recombinant *Eh*FT neither farnesylates nor geranylgeranylates *Eh*Ras1 and *Eh*Rap2, we have to conclude that the results of the previous report (13) are likely a consequence of artifactual farnesylation by heterologous prenylase(s), as observed for *Eh*Ras2-CELL, which was farnesylated by the rat GGT-I (data not shown). Alternatively, it is conceivable that the farnesylation of these small GTPases by GGT-I may require an unidentified accessory factor, like Rab escort protein for GGT-II (3), in *E. histolytica*. Altogether, these results suggest that the substrate specificity of prenyltransferases varies widely among organisms. Further studies, including the cloning and enzymatic characterization of GGT-I of *E. histolytica* to determine if *Eh*Ras proteins are geranylgeranylated by the amebic GGT-I, are now underway.

Although we did not show a specific role for *Eh*Ras4, this protein shares all the conserved domains characteristic of Ras (42) except for incomplete DXAG and $D(X)_nT$ consensus sequences, and showed a close kinship to other *Eh*Ras proteins in the phylogenetic reconstruction (Fig. 6). We demonstrated that *Eh*Ras4 was capable of binding GTP (data not shown), verifying its identity as a small GTP-binding protein. *Eh*Ras1-4 lack a cysteine residue located 5–8 amino acids upstream of the C terminus to be palmitoylated in H- and N-Ras (45), which was shown to be essential for membrane association. In addition, *Eh*Ras4, in contrast to *Eh*Ras1-3, also lacks the so-called polybasic region (Fig. 5*A*), which was found in K-RasB and attributed to membrane association (46). The polybasic region was also implicated in interaction with a negatively charged patch on the surface of FTβ, which is located in close proximity to the region responsible for the binding to the Ras C terminus (38). Interestingly, *Eh*FTβ, which shows low affinity to *Eh*Ras1-3 with the polybasic region and high affinity toward *Eh*Ras4 without it, possesses a number of substitutions of negatively charged with either positively-charged or neutral amino acids particularly in helixes 3–5 (38) (*e.g.* D91S, E94M, E112R, D115G, E116Y, E131R, E166V, E167N, and D170Q, corresponding to rat FTβ). It is conceivable that these substitutions compensate for the repulsive force that interferes with proper binding, which would partially explain the observed Ras specificity of the amebic FT.

In addition to its unique (*i.e. Eh*Ras4-specific) acceptor specificity, the amebic FT revealed notable differences in sensitivity against compounds known to inhibit human FT by distinct mechanisms (Table I). Marked differences in sensitivity to FPP analogues were unexpected since all the aromatic amino acids (Trp^{102}, Tyr^{105}, Trp^{106}, Tyr^{154}, Tyr^{205}, Phe^{253}, Phe^{302}, Trp^{303}, Tyr^{361}, and Tyr^{365} of rat FTβ) that were shown to be located in the hydrophobic cleft at the center of the α-α barrel and implicated to be essential for the interaction with FPP within the FPP-binding pocket (41) were conserved. FPT inhibitors I, II, and α-hydroxyfarnesyl phosphonic acid share the common farnesyl (C15) portion (36), which interacts with these aromatic residues lined on this hydrophobic cleft (38). Therefore, the lack of sensitivity of *Eh*FT against these FPP analogues suggests that the binding specificity of these compounds does not depend on the structure of the FPP-binding pocket *per se*, but on the neighboring spacial and electrostatical environment. The fact that *Eh*FT is >10-fold more resistant to FPT inhibitor I and

α-hydroxyfarnesyl phosphonic acid than FPT inihibitor II whereas human FT is equally sensitive to these inhibitors agrees well with the notion that *Eh*FT has higher affinity to FPT inhibitor II. Considering the major structural differences between FPT inhibitors I and II: the presence of the *O*-ester linkage and the absence of the C-terminal residue in FPT inhibitor II, the observed differences in sensitivity may be partially explained by the substitutions of negative with neutral/positive amino acids found in the amebic FT described above. It is conceivable that *Eh*FT is not sensitive to the C*aa*M peptidomimetic FTI-276 (>1000-fold less than human FT) since *Eh*Ras3-CSVM was not a substrate of *Eh*FT. Finally, exploitation of critical differences in the affinity toward substrates and inhibitors between the mammalian and amebic FT should enable us to discover or design novel inhibitors selective for *Eh*FT, leading to the development of new chemotherapeutics against amebiasis.

Acknowledgments—We thank Naohiro Watanabe, Jikei, for valuable discussions and Yumiko Saito-Nakano and Fumie Tokumaru, NIID, for the *Eh*Rab5, *Eh*Rab7, and *Eh*Rap2 recombinant protein and helpful discussions. We also thank Vahab Ali, NIID, for the analysis of FT on gel filtration chromatography and Tomomi Okita, Jikei, for technical assistance. The data base search was conducted with a 7× *E. histolytica* genome data base available at the Institute for Genomic Research (TIGR) and Sanger Institute with financial support from NIAID, National Institutes of Health and The Wellcome Trust.

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Parasitol Res (2003) 91: 204–208
DOI 10.1007/s00436-003-0955-x

ORIGINAL PAPER

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Involvement of signaling through protein kinase C and phosphatidylinositol 3-kinase in the excystation and metacystic development of *Entamoeba invadens*

Received: 7 April 2003 / Accepted: 11 June 2003 / Published online: 16 August 2003
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Abstract Using an axenic excystation system in vitro, we examined the effect of protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K), which are signaling molecules responsible for numerous cellular responses, on the excystation and metacystic development of *Entamoeba invadens*. Excystation, which was assessed by counting the number of metacystic amoebae after the induction of excystation, was inhibited by the PKC inhibitors staurosporine, chelerythrine chloride and calphostin C in a concentration-dependent manner during incubation, compared with the controls. As cyst viability was not affected by these inhibitors, reduced excystation was not due to their direct toxic effects on cysts. Metacystic development, when determined by the number of nuclei in the amoebae, was delayed by these PKC inhibitors, because the percentage of 1-nucleate amoebae was lower than in controls at day 3 of incubation. Wortmannin, a potent inhibitor of PI3K, also inhibited excystation and metacystic development of *E. invadens* in a concentration-dependent manner, compared with the controls. These results indicate that signaling through PKC and PI3K contributes to the excystation and metacystic development of *E. invadens*.

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Introduction

Excystation and metacystic development of *Entamoeba* spp are necessary for infection. However, studies on these processes in the human parasite *E. histolytica* are difficult, because there is no axenic encystation medium available for this parasite (López-Romero and Villagómez-Castro 1993). In this regard, the axenic excystation in vitro of *E. invadens*, a reptilian parasite, is a useful model for the excystation of *E. histolytica* because excystation and metacystic development of *E. invadens* (Geiman and Ratcliffe 1936) are entirely in agreement with those of *E. histolytica* (Dobell 1928; Cleveland and Sanders 1930). Transfer of cysts of *E. invadens* from an encystation medium to a growth medium induces excystation (McConnachie 1955; Rengpien and Bailey 1975; Garcia-Zapien et al. 1995), so that the signal of a change in medium should be transduced from the membrane to the nucleus, to initiate excystation. However, no studies on signaling in the excystation of *E. invadens* have so far been reported.

Protein kinase C (PKC), a phospholipid-dependent serine/threonine kinase, has a crucial role in signal transduction for a variety of cellular responses, including cell proliferation and differentiation (Nishizuka 1986). The involvement of PKC has been inferred from the use of specific inhibitors of the enzymes. Use of these PKC inhibitors demonstrates that PKC plays an important role in the adhesion and killing of target cells by *E. histolytica* (Weikel et al. 1988; Santiago et al. 1994). In addition, we have demonstrated evidence for the participation of PKC in the growth and encystation of *E. invadens* (Makioka et al. 2000).

Phosphatidylinositol 3-kinase (PI3K) catalyzes the phosphorylation of inositol phospholipids as well as at position 3, to generate phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P_3], via PI 3-monophosphate and PI 3,4-bisphosphate. These lipid products bind specific protein molecules for the manifestation of various cellular functions, including cell adhesion, vesicular

trafficking, actin rearrangement, cell growth and cell survival (Toker and Cantley 1997). Wortmannin, a fungal metabolite, is a potent inhibitor of the PI3K family of enzymes and has proved a valuable reagent for studying PI3K-dependent responses (Toker and Cantley 1997). Wortmannin markedly inhibited phagocytosis by *E. histolytica* of bacteria, red blood cells and mucin-coated beads (Ghosh and Samuelson 1997), demonstrating an important role of PI3K in phagocytosis by this parasite. We previously demonstrated a possible role for PI3K in the signaling involved in the growth and encystation of *E. invadens* (Makioka et al. 2001a). Taken together, we considered it of interest to examine the effect of PKC and PI3K inhibitors on the excystation and metacystic development of *E. invadens*. Here, we report the participation of PKC and PI3K in these processes of *E. invadens*.

Materials and methods

Trophozoites of *Entamoeba invadens* strain IP-1 were cultured in axenic growth medium BI-S-33 (Diamond et al. 1978) at 26 °C. To obtain cysts, trophozoites (5×10^5 cells/ml) were transferred to an encystation medium called 47% LG (LG is BI without glucose; Sanchez et al. 1994). After 3 days of incubation, the cells were harvested and treated with 0.05% sarkosyl (Sigma Chemical Co., St. Louis, Mo.) to destroy the trophozoites (Sanchez et al. 1994). The remaining cysts were washed with phosphate-buffered saline (PBS), counted and suspended in a growth medium. Viability of the cysts was determined by trypan blue dye exclusion. For experiments on the effect of PKC inhibitors on excystation, duplicate cultures of 5×10^5 cysts/ml included various concentrations (from 1n*M* to 50 μ*M*) of the drugs and were incubated for 3 days. Metacystic amoebae were counted in a hemocytometer at 5, 24, 48 and 72 h and their viability determined by trypan blue dye exclusion. Viable metacystic amoebae and cysts were clearly distinguished as light yellow and light blue in color, respectively. The former was also identified by positive motility. Four PKC inhibitors: staurosporine (Tamaoki et al. 1986), chelerythrine chloride (Herbert et al. 1990; hereafter termed chelerythrine), calphostin C (Kobayashi et al. 1989) and d-*erythro*-sphingosine (Hannun et al. 1986; hereafter termed sphingosine) were used. Wortmannin was also used at concentrations of 0.1–1.0 μ*M*. All of these chemicals were purchased from Sigma and were initially dissolved in dimethyl sulfoxide (DMSO). The control cultures received the same volume of DMSO. For the estimation of metacystic development of *E. invadens*, duplicate cultures (5×10^5 cysts/ml) with and without PKC inhibitors or wortmannin were incubated for 3 days. Cells were harvested at 5, 24 and 72 h and stained with modified Kohn (Kumagai et al. 2001). The number of nuclei per amoeba was determined by counting at least 100 amoebae twice.

Results

Effect of PKC inhibitors on the number of metacystic amoebae

The effects of four PKC inhibitors on the number of metacystic amoebae are shown in Fig. 1. For staurosporine, the number of metacystic amoebae at 5 h of incubation with 1–10 n*M* was comparable with that of the controls. Amoebae cultured without the drug increased in number from 5 h to 48 h. In contrast, little increase in the number of metacystic amoebae occurred in cultures exposed to 1–10 n*M* staurosporine. The number of metacystic amoebae during incubation with 5–20 μ*M* chelerythrine was reduced in a concentration-dependent manner, compared with the controls. No increase in the number of amoebae occurred from 24 h to 72 h in cultures with 20 μ*M* chelerythrine. Calphostin C also reduced the number of metacystic amoebae in a concentration-dependent manner, with the decrease in the number of amoebae from 24 h to 72 h in cultures with ≥5μ*M* drug. In contrast, sphingosine showed little effect on the number of metacystic amoebae; and rather an increase in the number of amoebae occurred at 24 h and 48 h in cultures with 50 μ*M* sphingosine, compared with the controls.

Effect of PKC inhibitors on cyst viability in growth medium

As shown in Fig. 2, the number of viable cysts in cultures containing PKC inhibitors was comparable with that not exposed to the drugs during incubation.

Effect of PKC inhibitors on metacystic development

As shown in Fig. 3, the percentages of metacystic amoebae with four nuclei at 5 h in cultures with PKC

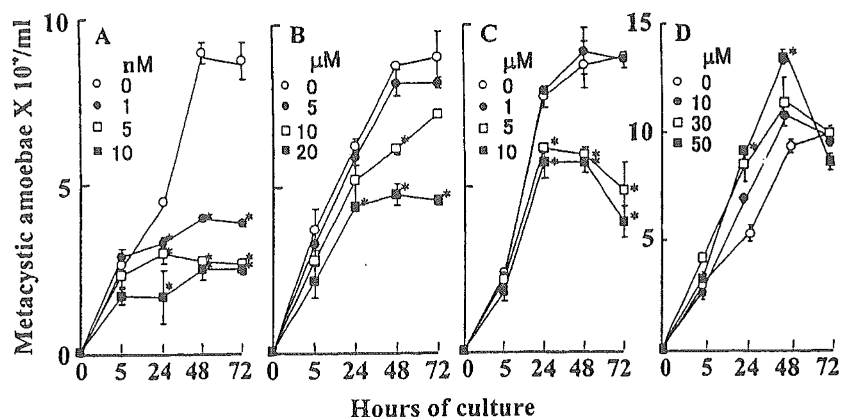

Fig. 1A–D Effect of protein kinase C (PKC) inhibitors on the number of metacystic amoebae of *Entamoeba invadens*. Cysts were transferred to a growth medium containing various concentrations of four PKC inhibitors: staurosporine (A), chelerythrine (B), calphostin C (C) and sphingosine (D). The means ± SE of metacystic amoebae for duplicate cultures are plotted (*each asterisk* indicates $P < 0.05$)

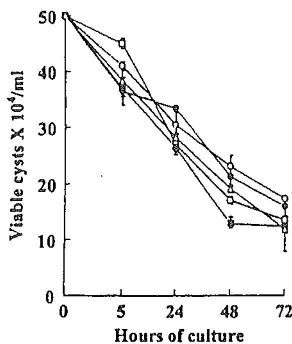

Fig. 2 Effect of PKC inhibitors on cyst viability of *E. invadens* in the growth medium. Experimental conditions were the same as those for Fig. 1. The means ± SE of viable cysts for duplicate cultures are plotted. *White circles* Control, *black circles* staurosporine (10 n*M*), *white squares* chelerythrine (20 μ*M*), *black squares* calphostin C (10 μ*M*), *white triangles* sphingosine (50 μ*M*)

inhibitors were similar to those of the controls. At 24 h, 11% were 4-nucleate and 88% were 1- to 3-nucleate in cultures minus PKC inhibitors, whereas 25% of amoebae were 4-nucleate and 75% were 1- to 3-nucleate in cultures containing staurosporine, suggesting a slower development of metacystic amoebae in the presence of the drug. In addition, the percentage of 1-nucleate amoebae in cultures with chelerythrine and calphostin C was lower than in cultures not exposed to these drugs, whereas that in cultures with sphingosine was not. In the controls at 72 h, only 3% of amoebae were 4-nucleate and the percentage of 1-nucleate amoebae reached 82%. However, in cultures with staurosporine and chelerythrine, respectively 16% and 11% of amoebae were still 4-nucleate and 49% and 63% were 1-nucleate. Calphostin C and sphingosine showed little effect.

Effect of wortmannin on the number of metacystic amoebae and cyst viability

The number of metacystic amoebae at 5 h of incubation with 0.1–1.0 μ*M* wortmannin was reduced in a concentration-dependent manner, compared with the control (Fig. 4A). At 24 h, the number of metacystic amoebae was significantly reduced in cultures with ≥0.5 μ*M* drug, compared with the controls. At 48 h and 72 h, amoebae cultured with ≥0.1 μ*M* wortmannin decreased in number, compared with the controls. As shown in Fig. 4B, wortmannin at 0.1–1 μ*M* had little or no effect on cyst viability during incubation.

Effect of wortmannin on metacystic development

As shown in Fig. 5, the percentage of 4-nucleate amoebae in cultures with wortmannin at 5 h was almost the same as that in the controls, whereas it was significantly higher than in the controls at 24 h and 72 h, suggesting the inhibition of metacystic development by wortmannin.

Discussion

The results indicate the participation of PKC in the excystation and metacystic development of *Entamoeba invadens*, although the four PKC inhibitors used differed in their potency. Staurosporine was most potent for the inhibition of excystation and metacystic development, whereas sphingosine showed no inhibitory effect. The reason for this difference is not clear. In this regard, preincubation of *E. histolytica* trophozoites with sphingosine is necessary to abolish phorbol myristate acetate (PMA) stimulation and the basal cytolytic activity of the parasite (Weikel et al. 1988). It has also been reported that prolonged incubation might be necessary for the incorporation of sphingosine into cells (Merrill et al. 1986). Therefore, it is probable that the difference in experimental conditions may affect the effect of sphingosine.

The process of excystation includes a loosening and separation of the amoeba from the cyst wall; and the amoeba begins to move about within the cyst. The amoeba then flows back and forth through a small pore in the cyst wall and escapes from the cyst. Thus,

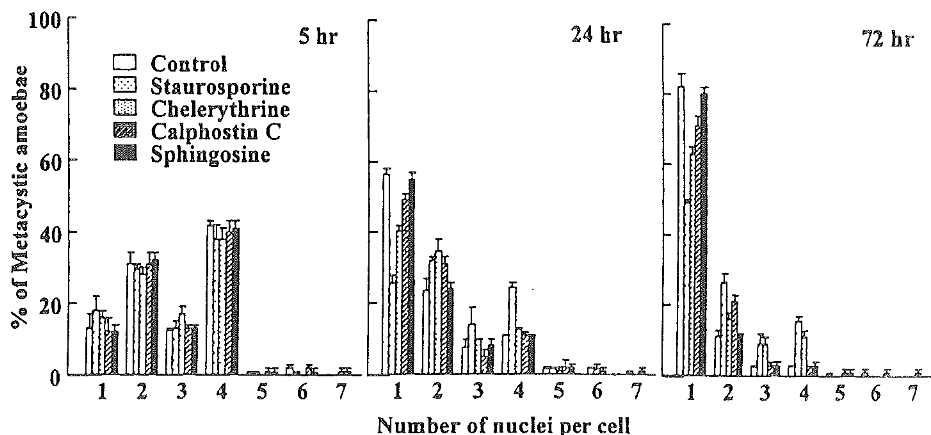

Fig. 3 Effect of PKC inhibitors on the metacystic development of *E. invadens*. Cysts were transferred to a growth medium with or without 10 n*M* staurosporine, 20 μ*M* chelerythrine, 10 μ*M* calphostin C or 50 μ*M* sphingosine. The numbers of nuclei per metacystic amoeba stained with modified Kohn at 5, 24 and 72 h of incubation were counted and the percentage of amoebae in each class (1- to 7-nucleate) was determined

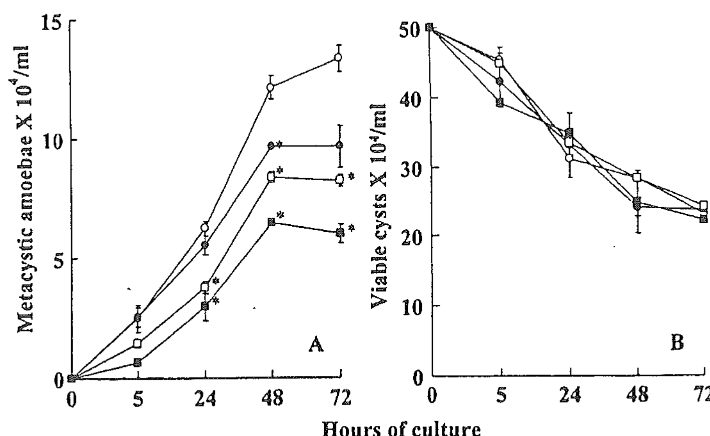

Fig. 4A, B Effect of wortmannin on the number of metacystic amoebae and cyst viability of *E. invadens*. Cysts were transferred to a growth medium containing various concentrations of wortmannin. The means ± SE of metacystic amoebae (A) and viable cysts (B) for duplicate cultures are plotted (*each asterisk* indicates $P < 0.05$). Concentrations are shown by *white circles* (0 μM), *black circles* (0.1 μM), *white squares* (0.5 μM) and *black squares* (1.0 μM)

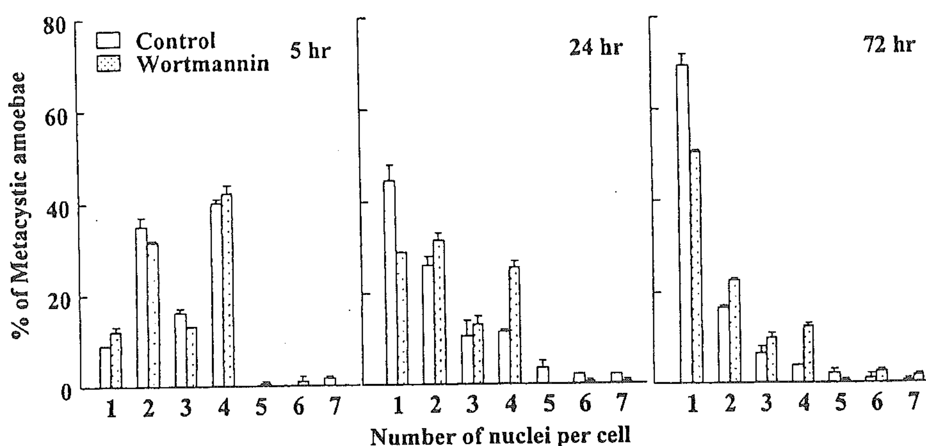

Fig. 5 Effect of wortmannin on the metacystic development of *E. invadens*. Cysts were transferred to a growth medium with or without 1 μM wortmannin. The numbers of nuclei per metacystic amoeba stained with modified Kohn at 5, 24 and 72 h of incubation were counted and the percentage of amoebae in each class (1- to 7-nucleate) was determined

reorganization of the actin cytoskeleton is necessary for these excystation events. We demonstrated that the actin-modifying drugs latrunculin A and jasplakinolide inhibited the excystation and metacystic development of *E. invadens* (Makioka et al. 2001b). Regarding the relation between the reorganization of the actin cytoskeleton and PKC, Santiago et al. (1994) demonstrated an interaction of *E. histolytica* trophozoites with the fibronectin-induced reorganization of the actin cytoskeleton and an increase in proteolytic activities through the activation of PKC pathways. Therefore, signaling through PKC would be related to the reorganization of the actin cytoskeleton necessary for excystation. We recently demonstrated that extracellular calcium ions, amoebic intracellular calcium flux, calcium channels and a calmodulin-dependent process contribute to the excystation and metacystic development of *E. invadens* (Makioka et al. 2002). This would relate to not only Ca^{2+} signaling but also signaling through PKC, because Ca^{2+} functions as a cofactor for PKC activation. Although PKC activity, the presence of a 68-kDa protein cross-reacting with anti-PKC antibodies and PKC homologous gene fragments were found in *E. histolytica* (De Meester et al. 1990; Que et al. 1993), it has not yet been determined which isoforms of PKC are present in this parasite.

The results indicate that wortmannin-sensitive signaling is also involved in the excystation and metacystic development of *E. invadens*. As cyst viability was not affected by wortmannin, reduced excystation is not due to its toxic effect on cysts. Cellular responses following signaling through $PI(3,4,5)P_3$, one of the products of PI3K, include actin rearrangement (Toker and Cantley 1997). Therefore, it is most probable that a signaling cascade through $PI(3,4,5)P_3$ is involved in the excystation of *E. invadens*.

In summary, the present study indicates that PKC and PI3K participate in the excystation and metacystic development of *E. invadens*, providing the first evidence of a signaling mechanism in *Entamoeba* spp.

Acknowledgements We would like to thank Prof. N. Watanabe for his support of this work, Dr. L. S. Diamond for supplying the *E. invadens* and T. Okita for technical assistance. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports and Technology of Japan, and by a Health Science Research Grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare of Japan.

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JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2004, p. 1069–1074 Vol. 42, No. 3
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.3.1069–1074.2004
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Evaluation of Recombinant Fragments of *Entamoeba histolytica* Gal/GalNAc Lectin Intermediate Subunit for Serodiagnosis of Amebiasis

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Received 18 September 2003/Returned for modification 10 November 2003/Accepted 24 November 2003

We have recently identified a 150-kDa surface antigen of *Entamoeba histolytica* as an intermediate subunit (Igl) of galactose- and *N*-acetyl-D-galactosamine-inhibitable lectin, which is a cysteine-rich protein consisting of 1,101 amino acids (aa) and containing multiple CXXC motifs in amino acid sequences. In the present study, full-length Igl except for the signal sequences (aa 14 to 1088) and three fragments of Igl—the N-terminal part (aa 14 to 382), the middle part (aa 294 to 753), and the C-terminal part (aa 603 to 1088)—were prepared in *Escherichia coli*, and the reactivity of these recombinant proteins with sera from patients with amebiasis was examined by means of enzyme-linked immunosorbent assay (ELISA). Sera from 57 symptomatic patients with amebic liver abscess or amebic colitis, sera from 15 asymptomatic cyst passers, sera from 40 individuals with other protozoan infections, and sera from 50 healthy controls were used. The sensitivity and specificity of the recombinant full-length Igl in the ELISA were 90 and 94%, respectively. When three fragments were used as antigens in the ELISA, the sensitivities were 56% in the N terminus, 92% in the middle part, and 97% in the C terminus. The specificities of the three antigens were 96% in the N terminus and 99% in both the middle and C-terminal fragments. These results demonstrate that Igl is well recognized in not only symptomatic but also asymptomatic patients with *E. histolytica* infection and that the carboxyl terminus of Igl is an especially useful antigen for the serodiagnosis of amebiasis.

Amebiasis caused by infection with *Entamoeba histolytica* is an important parasitic disease in both developing and developed countries. It has been estimated that *E. histolytica* causes 50 million cases of colitis and liver abscess, resulting in 40,000 to 110,000 deaths annually (27). In cases of liver abscesses, diagnosis and treatment at an early stage are required to prevent fatal infection. Whereas the detection of trophozoites in liver pus is not easy, a serological test is practical as a sensitive and noninvasive means of diagnosis of amebic liver abscess (11, 21). On the other hand, in cases of asymptomatic cyst passers, *E. histolytica* and *E. dispar*, which is a nonpathogenic commensal ameba, must be distinguished by PCR analysis or by the detection of *E. histolytica*-specific antigens (2). However, since positive serology is found in most asymptomatic cases infected with *E. histolytica*, serological tests are also applicable (24). Thus, serodiagnosis is an important laboratory diagnostic tool for amebiasis, as well as microscopic detection of the pathogen.

Recently, several recombinant *E. histolytica* antigens were prepared, and their usefulness for serodiagnosis has been reported (12, 14, 18–20, 22, 28). One of the useful antigens is the 170-kDa heavy subunit (Hgl) of galactose- and *N*-acetyl-D-galactosamine-inhibitable lectin, which is the key factor in amebic adherence and subsequent pathogenesis (16). Hgl is a transmembrane protein that assumes a heterodimeric conformation that conforms with glycosylphosphatidylinositol (GPI)-anchored 31/35-kDa light subunit by disulfide bonds (15). We recently identified a GPI-anchored 150-kDa intermediate subunit (Igl) of lectin, which is noncovalently associated with Hgl (4, 8). A mouse monoclonal antibody specific for Igl significantly inhibits adherence and cytotoxicity of trophozoites to mammalian cells, erythrophagocytosis, and liver abscess formation in hamsters (5, 7, 23). Igl is a cysteine-rich protein that consists of 1,101 amino acids (aa) and contains multiple CXXC motifs in amino acid sequences (4). In the previous study, we examined the reactivity of sera from ameba-infected patients to affinity purified Igl by Western immnoblot analysis. The native Igl was recognized by all sera from not only symptomatic patients but also asymptomatic cyst passers (8). However, the possibility that copurified Hgl might affect the reactivity of Igl could not be excluded. Therefore, in the present study, recombinant Igl was prepared in *Esherichia coli*, and its reactivity with sera from patients with amebiasis was examined. We also report here on the different reactivity of partial Igl fragments with the sera.

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MATERIALS AND METHODS

Plasmid constructs for recombinant proteins. The DNA fragment coding for the full-length Igl, except for the N terminus and C terminus signal sequences

TABLE 1. List of oligonucleotide primers used in this study

Primer	Positions[a]	Sequence (5' to 3')[b]
EhIgl-S14	40–59	CCCTCGAGGATTATACTGCTGATAAGCT
EhIgl-S294	880–898	CCCTCGAGACAGAAGAAAATAAATGTA
EhIgl-AS382	1129–1146	CCCTCGAGTTAAAGTTTGCATGGTCCATC
EhIgl-S603	1807–1827	CCCTCGAGGAAGGACCAAATGCAGAAGAT
EhIgl-AS753	2244–2259	CCCTCGAGTTATAGCCTTTGTTCAGTG
EhIgl-AS1088	3247–3264	CCCTCGAGTTAAATGCCTTTAGCTCCATT

[a] Nucleic acid numbering is based on the *E. histolytica* Igl1 gene sequence (AF337950).

[b] Nucleotides added for cloning and translation termination are underlined.

(F-Igl, aa 14 to 1088 of *E. histolytica* Igl1), was obtained by PCR amplification of a plasmid containing the gene encoding Igl1 of *E. histolytica* HM-1:IMSS strain (4). DNA fragments, coding for three overlapping parts of Igl—the N-terminal part (N-Igl, aa 14 to 382), the middle part (M-Igl, aa 294 to 753), and the C-terminal part (C-Igl, aa 603 to 1088)—were also amplified by PCR. The oligonucleotide primers used are listed in Table 1. Twenty cycles of PCR were performed as follows: denaturation at 94°C for 15 s (135 s in cycle 1), annealing at 55°C for 30 s, and polymerization at 72°C for 60 s (360 s in cycle 20). Each of the amplified DNA fragments were digested with *Xho*I, purified, and then ligated with the pET19b vector (Novagen, Madison, Wis.). The plasmids were introduced into competent *Escherichia coli* JM109 cells and then clones containing the right direction of inserts were selected.

Expression, purification, and refolding of recombinant proteins. *E. coli* BL21 Star(DE3)pLysS cells (Invitrogen, Carlsbad, Calif.) were transformed with the cloned plasmids. Each bacterial clone was cultured in 400 ml of Luria-Bertani (LB) medium containing ampicillin until an optical density at 600 nm (OD_{600}) of 0.6 was achieved. Isopropyl-β-D-thiogalactopyranoside was added to the bacterial cultures to a final concentration of 1 mM, and the cultures were then incubated at 37°C for 3 h. Preparation of inclusion bodies and refolding of the proteins were performed by a using the Protein Refolding Kit (Novagen) essentially according to the manufacturer's recommendations. The bacteria were pelleted by centrifugation and suspended in 16 ml of wash buffer (20 mM Tris-HCl [pH 7.5], 10 mM EDTA, 1% Triton X-100). The suspension was sonicated and then centrifuged. This washing step was repeated five times to obtain the inclusion body. The pellet of the inclusion body was suspended in solubilization buffer (500 mM 3-(cyclohexyamino)-1-propanesulfonic acid (CAPS) [pH 11], 0.3% *N*-lauroylsarcosine) and incubated at room temperature for 15 min. After centrifugation, the supernatant was dialyzed in dialysis buffer (20 mM Tris-HCl [pH 8.5], 0.1 mM dithiothreitol) overnight at 4°C. Dialysis was continued in the buffer without dithiothreitol for 9 h, then in redox refolding buffer (0.2 mM oxidized glutathione, 1 mM reduced glutathione) overnight at 4°C, and finally for 3 h at room temperature.

SDS-PAGE and Western immunoblot analysis. Recombinant proteins were treated with an equal volume of the sample buffer (13) containing 2 mM phenylmethylsulfonyl fluoride, 2 mM TLCK (*N*α-*p*-tosyl-L-lysine chloromethyl ketone), 2 mM *p*-hydroxymercuriphenyl sulfonic acid, and 4 μM leupeptin for 5 min at 95°C and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For the Western immunoblot analysis, the protein bands were transferred to polyvinylidene difluoride membranes (26). After a blocking step with 3% skim milk in 10 mM phosphate-buffered saline (PBS; pH 7.4), each strip was incubated with serum samples diluted 1:100 in PBS containing skim milk for 1 h. After being washed with PBS containing 0.05% Tween 20 (PBS-Tween), the strips were treated with horseradish peroxidase (HRP)-conjugated goat antibody to human immunoglobulin G (IgG; whole molecule; ICN Pharmaceuticals, Aurora, Ohio). Development was by the Konica Immunostaining HRP-1000 kit (Konica Co., Tokyo, Japan).

Serum samples. A total of 162 serum samples were studied. Sera from 23 patients with an amebic liver abscess and sera from 34 patients with amebic colitis but without a liver abscess were used as symptomatic cases. The diagnosis of these patients was based on their clinical symptoms, positive serology in an indirect immunofluorescent antibody test, ultrasound examination (liver abscess), endoscopic or microscopic examination (colitis), and prompt response to treatment with metronidazole except for a liver abscess patient who died. Sera from 15 asymptomatic cyst passers, obtained from children in institutions for the mentally retarded, were also studied. In these asymptomatic cyst passers, cysts were identified as *E. histolytica* but not *E. dispar* by PCR (17, 25). In addition, sera from 40 individuals with infection of other protozoa, *Blastocystis hominis* infection (23 cases), malaria (7 cases), toxoplasmosis (7 cases), and giardiasis (3 cases), were used. Most of the serum samples except for asymptomatic cyst passers were obtained from hospitalized patients and outpatients in Tokai University Hospital, Tokyo Metropolitan Komagome Hospital, and St. Luke's International Hospital in Tokyo. As a negative control, sera obtained from 50 healthy individuals with no known history of amebiasis and no parasites in their stools were also used. All of the serum samples were stored at −30°C or at −80°C before use.

ELISA. Crude antigen was prepared from trophozoites of *E. histolytica* HM-1: IMSS axenically grown in BI-S-33 medium (9). After 3 washes with ice-cold PBS, trophozoites were sonicated and centrifuged at 12,000 × *g* for 30 min. The supernatant was used as crude antigen. Enzyme-linked immunosorbent assay (ELISA) was performed in duplicate on serum samples in 96-well flat-bottom microtiter plates (Coaster, Corning, N.Y.). The wells of the ELISA plates, containing 1 μg of crude antigen or 100 ng of recombinant Igl antigens diluted with 50 mM sodium bicarbonate buffer (pH 9.6), were incubated overnight at 4°C. The plates were washed with PBS-Tween and then treated with PBS containing 1% skim milk for 1 h. A total of 100 μl of the serum samples diluted 1:400 with PBS were added to the wells, followed by incubation for 1 h at room temperature. After being washed, the wells were incubated with 100 μl of HRP-conjugated goat antibody to human IgG (whole molecule; ICN Pharmaceuticals) for 1 h at room temperature. After being washed with PBS-Tween, the wells were incubated with 200 μl of substrate solution (0.4 mg per ml of *o*-phenylenediamine in citric acid-phosphate buffer [pH 5.0] containing 0.001% hydrogen peroxide). After 30 min of incubation, the reaction was stopped by the addition of 50 μl of 2.5 M H_2SO_4, and the OD_{490} was recorded by using a Bio-Rad (Hercules, Calif.) model 550 microplate reader. The cutoff point for a positive result was defined as an ELISA value with >3 standard deviations above the mean of the healthy negative controls. OD values were plotted and analyzed by using computer graphics software Prism version 4.0 (GraphPad, San Diego, Calif.).

RESULTS

Expression of recombinant fragments of *E. histolytica* Igl. Igl constructs prepared in the present study were full-length Igl and three partial fragments of Igl: F-Igl (aa 14 to 1088), N-Igl (aa 14 to 382), M-Igl (aa 294 to 753), and C-Igl (aa 603 to 1088). In the design of the constructs, N-Igl and M-Igl, as well as M-Igl and C-Igl, shared overlapping regions to reduce the possible loss of antigenic epitopes in the recombinant fragments. In addition, the region where CXXC motifs were not found in Igl, aa 399 to 524, were included in only M-Igl. All of the four recombinant proteins were expressed in bacteria as inclusion bodies. The purity of the refolded proteins was analyzed by SDS-PAGE (Fig. 1). The apparent molecular masses of these proteins in 7.5% gel were 150 kDa for F-Igl, 53 kDa for N-Igl, 67 kDa for M-Igl, and 85 kDa for C-Igl. Since minor protein bands still existed in F-Igl, Western immunoblot analysis was performed to confirm the antigenicity of the recombinant protein (Fig. 2). Serum samples from four individuals each from among cases of amebic liver abscess, amebic colitis, and asymptomatic cyst passers were used in the analysis. All of these sera were reactive with the 150-kDa F-Igl band. On the other hand, two sera from patients with giardiasis did not react with the band of F-Igl as well as a serum from the healthy control.

ELISA reactivity of recombinant fragments with sera from patients with amebiasis. The reactivities of four recombinant proteins—F-Igl, N-Igl, M-Igl, and C-Igl—with patients' sera were examined by ELISA and compared to that of crude antigen (Fig. 3). When crude antigen was used in the ELISA, all of the sera from individuals with *E. histolytica* infection were scored as positive (sensitivity, 100%), and 5 of 90 negative control sera were judged as positive (specificity, 94%). By the use of F-Igl as the ELISA antigen, all of the sera from asymptomatic cyst passers were positive, but 2 of 23 sera from pa-

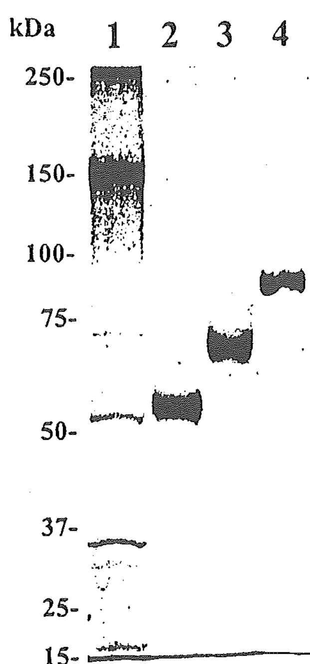

FIG. 1. SDS-PAGE analyses of recombinant Igl. A total of 4 μg of refolded proteins was electrophoresed in a 7.5% gel under reducing conditions. Protein bands were visualized with Coomassie brilliant blue. Lane 1, F-Igl; lane 2, N-Igl; lane 3, M-Igl; lane 4, C-Igl. The numbers to the left indicate molecular masses of size markers.

tients with liver abscess and 5 of 34 sera from patients with colitis were scored as negative (sensitivity, 90%). In the negative controls, five of 90 (four of *B. hominis* infections and one of the healthy controls) results were false positives (specificity, 94%). When three fragments were used as antigens, the mean OD values in the sera from patients with amebiasis were as follows: C-Igl > M-Igl > N-Igl. In the ELISA with N-Igl, sera from 9 cyst passers, sera from 6 patients with liver abscess, and sera from 17 patients with colitis were judged to be negative (sensitivity, 56%). Three sera from patients with malaria and a serum from *B. hominis*-infected individuals were positive (specificity, 96%). When M-Igl was used as an antigen, all of the cyst passers and patients with liver abscess were scored as seropositive, but six of the patients with colitis were seronegative (sensitivity, 92%). In the controls, only one of the *B. hominis*-infected individuals was seropositive (specificity, 99%). With the use of C-Igl, there were two false-negative cases, both of which were patients with colitis (sensitivity, 97%), whereas there was only one false-positive case, which was from a patient with malaria (specificity, 99%). OD values obtained with the ELISA using C-Igl were compared to those obtained with the crude antigen-based ELISA (Fig. 4). There was a significant correlation between the results of these two ELISA tests ($r = 0.8115$; $P < 0.0001$).

DISCUSSION

The results of the present study demonstrate that recombinant Igl is recognized well not only by sera from symptomatic patients with amebic liver abscess and amebic colitis but also by sera from asymptomatic patients in both Western immunoblotting and ELISA. This observation is coincident with the previous data in which affinity-purified native protein was recognized by sera from symptomatic and asymptomatic individuals in a Western immunoblot analysis (8).

In the ELISA system reported in the present study, only 100 ng of antigen was used for the coating of each well of the microplates. The OD values in this concentration were almost comparable with those in 1 μg of crude antigen. This fact demonstrates that the recombinant Igls possess high antigenicity. To date, several recombinant proteins of *E. histolytica* were prepared, and their efficacy in serodiagnosis was examined. When recombinant fragments derived from Hgl were used, sensitivities were 90 to 95% (18, 19, 28). With the use of other antigens, sensitivities were 100% for a 125-kDa surface antigen (14), 88% for 43.5-kDa alcohol dehydrogenase (12), and 82% for serine-rich 46- to 52-kDa antigens (SREHP) (22). In contrast, when a cysteine-rich 29-kDa surface antigen was used, sensitivity of the sera from patients with liver abscess was 76% but only 8% in samples from patients with colitis (20). In comparison with these previous reports, the sensitivity of C-Igl demonstrated in the present study (99%) was considerably higher. Although it is not clear at present how Hgl and Igl associate as a surface lectin complex, the present study demonstrated that Igl, as well as Hgl, is a valuable molecule for diagnostic purposes.

The most interesting observations in the present study were the different reactivities of three Igl fragments with sera from individuals with *E. histolytica* infections. Although high sensi-

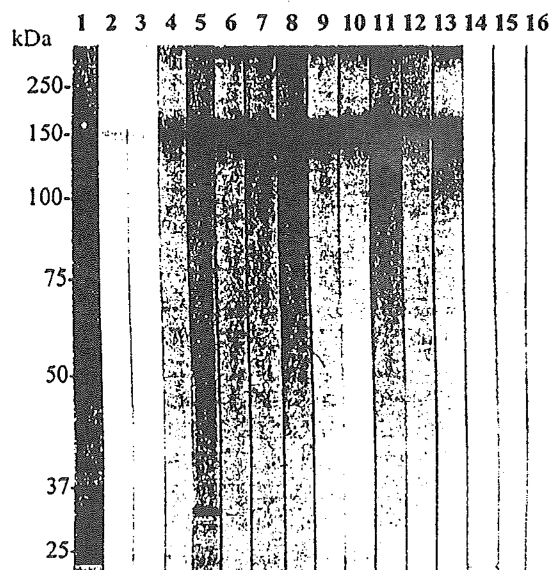

FIG. 2. Western immunoblot analysis of human sera reactivity with F-Igl. Purified F-Igl was subjected to SDS-PAGE in a 7.5% gel under reducing conditions and then transferred to polyvinylidene difluoride membranes. Protein bands of lane 1 were stained with Coomassie brilliant blue. Lanes 2 to 5, sera from individuals who were asymptomatic *E. histolytica* cyst passers; lanes 6 to 9, sera from patients with amebic colitis; lanes 10 to 13, sera from patients with amebic liver abscess; lanes 14 and 15, sera from patients with giardiasis; lane 16, serum from healthy controls. Sera were analyzed following the addition of HRP-conjugated goat antibody to human IgG (whole molecule) as the second antibody. The numbers to the left indicate molecular masses of size markers.

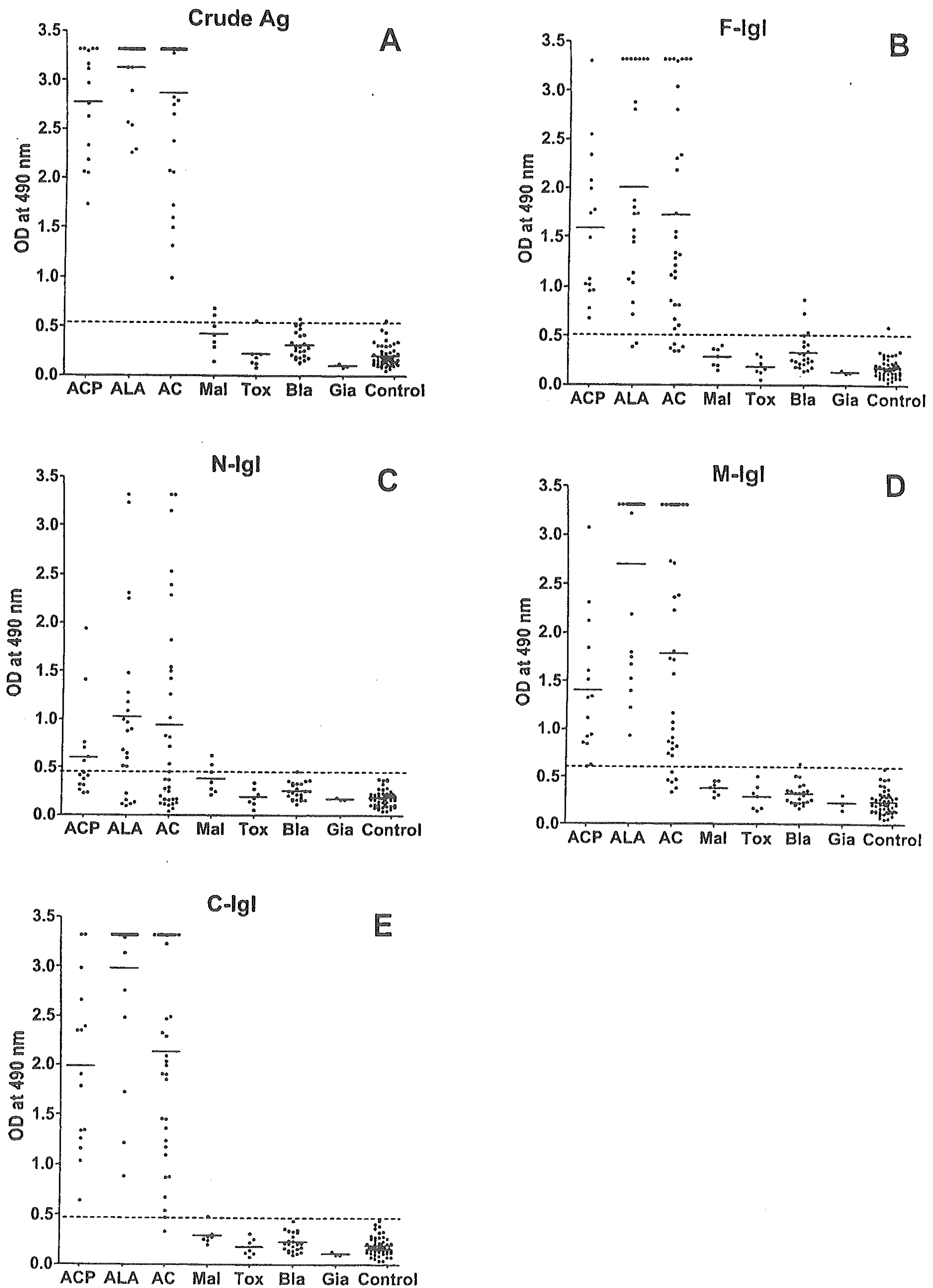

FIG. 3. ELISA reactivities of crude antigen and recombinant Igls from *E. histolytica* with sera from various patients. ELISA plates were coated with 1 μg per well of crude antigen (A) or 100 ng per well of F-Igl (B), N-Igl (C), M-Igl (D), or C-Igl (E). Serum samples used were as follows: ACP, asymptomatic *E. histolytica*-cyst passer (n = 15); ALA, amebic liver abscess (n = 23); AC, amebic colitis (n = 34); Mal, malaria (n = 7); Tox, toxoplasmosis (n = 7); Bla, *Blastocystis hominis* infection (n = 23); Gia, giardiasis (n = 3); Control, healthy controls (n = 50). HRP-conjugated goat antibody to human IgG (whole molecule) was used as the second antibody. The horizontal bars indicate the arithmetic means of the groups. The dashed lines indicate the cutoff values (mean value of healthy control sera plus three standard deviations).

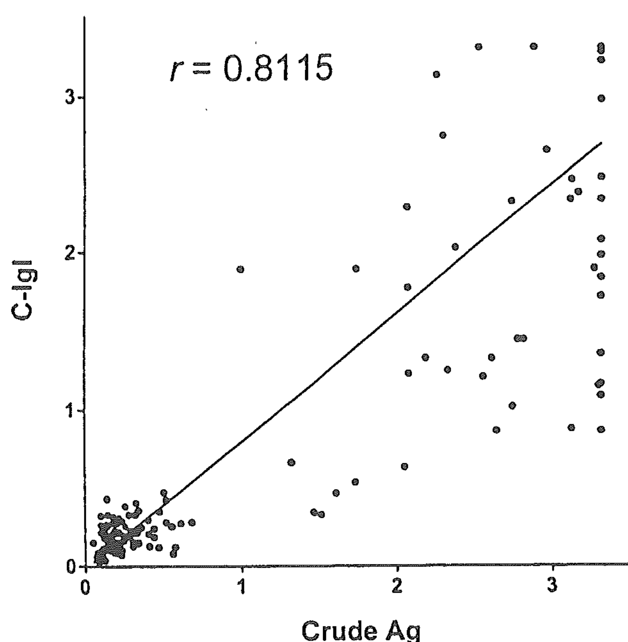

FIG. 4. Correlation between ELISA reactivities to C-Igl and crude antigen in sera from *E. histolytica*-infected individuals and controls. The assay was as described in Fig. 3.

tivity was observed in the use of M-Igl and C-Igl as antigen, N-Igl was recognized by only about a half of the patients with amebic infections. One of the possible explanations is that the antigenic epitope(s) located in N-Igl may not be exposed to the surface in native Igl. Another possibility is that antigenic differences among *E. histolytica* isolates may exist in the N terminus of Igl. In the previous study, monoclonal antibodies EH3015 and EH3023 were reactive with all of the 47 *E. histolytica* isolates but with none of the *E. dispar* isolates, indicating the existence of a common epitope in *E. histolytica* isolates (23). On the other hand, a difference in the reactivity of monoclonal antibodies EH3056 and EH3126 was observed among *E. histolytica* isolates, suggesting that qualitative and/or quantitative differences of Igl may exist. In Hgl genes, it has been demonstrated recently that only slight genetic diversity exists even in the isolates showing distinct diversity in the SREHP genes (3). Since the primary structure of Igl has been clarified in only the HM-1:IMSS strain at present, sequence analysis of Igl in various strains of *E. histolytica* will be required in further studies.

CXXC motifs existing in Igl have also been observed in the variant-specific surface antigen of *Giardia intestinalis* (1, 10). Therefore, it is possible that common epitopes between *E. histolytica* Igl and *G. intestinalis* variant-specific surface antigen may exist. In the present study, however, sera from patients with giardiasis did not react with recombinant Igls in both the Western immunoblotting and ELISA, although the number of sera examined was limited. Whereas several commercial kits using crude antigens are now available for serodiagnosis of amebiasis, an expected merit of recombinant proteins for diagnostic purposes must be their higher specificity. Indeed, specificity of the ELISA with C-Igl (99%) was higher than that of crude antigen-based ELISA (94%) in the present study when the cutoff point was defined as an OD value with three standard deviations above the mean of the healthy negative controls. However, a comparison of Fig. 3A and E suggests that discrimination between amebic cases and controls is more evident with the use of the crude antigen. Therefore, we cannot exclude the interpretation that, if the cutoff point is set above the mean plus three standard deviations of the controls, the specificity of the ELISA with crude antigen increases. Another advantage of the recombinant protein is that the use of defined proteins in serodiagnosis will facilitate standardization of the assays. In addition, production of a recombinant protein in large quantities may be an economically effective method compared to the cultivation of trophozoites (14, 18, 22).

When F-Igl was used as the antigen, whereas two sera from patients with amebic liver abscess and five sera from patients with amebic colitis were negative, none of the sera from asymptomatic cyst passers was negative. In the cases of ELISA with M-Igl and C-Igl as antigens, false-negative results were detected in only the colitis cases. These observations suggest that the antibodies which recognized the epitopes located in M-Igl and C-Igl may function to prevent the invasion of trophozoites into host tissues. Indeed, when hamsters have been immunized with native Igl, liver abscess formation has been significantly inhibited (6). Therefore, a partial fragment of Igl, such as M-Igl and C-Igl, may also be one of the candidate vaccines for amebiasis.

In conclusion, recombinant Igl was well recognized by sera from patients with amebiasis but not by sera from patients with other protozoan infections. In particular, the C terminus fragment (aa 603 to 1088) of Igl was valuable for the serodiagnosis of amebiasis.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science and by grants from the Ministry of Health, Labour, and Welfare of Japan. X.-J.C. is a recipient of the Japanese Society for the Promotion of Science Postdoctoral Fellowship for Foreign Researchers.

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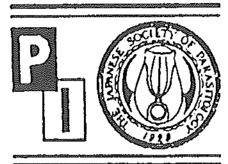

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Isolation and functional analysis of a *chk2* homologue from *Entamoeba histolytica* ☆

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Received 22 March 2004; accepted 12 August 2004
Available online 1 October 2004

Abstract

Mammalian Chk2 is a Ser/Thr kinase required for cell-division arrest induced by DNA damage. We found six new kinase genes of *Entamoeba histolytica* by analysis in silico. One of the kinase genes was a homologue of human *chk2* gene. The *chk2* homologue gene (*Eh chk2*) was expected to encode 398 amino acids and showed nearly 50% homology to human Chk2 in amino acid sequence. *Eh chk2* had a catalytic domain of Ser/Thr kinase and a fork head-associated (FHA) domain that is highly conserved among Chk2 homologues in vertebrates. To examine the biological functions of *Eh chk2*, we synthesized *Eh chk2* mRNA in vitro and injected it into immature frog eggs (*Xenopus laevis* oocytes) as a model system of cell division. *Eh chk2* markedly delayed the cell division of frog eggs by disrupting transition of G2 phase to M phase. *Eh chk2* also inhibited the activation of p42 MAPK and Cdc2 kinase which are representative events induced by cell division. These results suggest that *Eh chk2* gene should be a cell-division regulator in *E. histolytica*.
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Keywords: *Entamoeba histolytica*; Chk2; Kinase; Cell division

1. Introduction

Entamoeba histolytica, an enteric protozoan parasite, is the cause of human amebiasis [1–3]. Amoebic trophozoites of *E. histolytica* proliferate in the human colon or liver and often cause tissue destruction. Although proliferation of trophozoites is an important factor in amoebic diseases, regulation of cell division is not fully understood at the molecular levels.

The regulation of cell division is studied well in eukaryotic cells. In eukaryotes, a cell cycle is separated into two major phases, the resting interphase and mitotic M phase. Once interphase cells enter in M phase, they start dividing irreversibly. The key regulator of cell division is a complex of two proteins, CyclinB and Cdc2. Cdc2 is a member of cyclin-dependent protein kinases (Cdks). The activity of Cdc2/CyclinB complex is inhibited in interphase. Once Cdc2/CyclinB is activated, the cells enter M phase and start dividing [4–8]. The activity of Cdc2/CyclinB is directly regulated by Cdc25 phosphatase. Cdc25 phosphatase dephosphorylates Tyr-15 of Cdc2 and activates it for progression into M-phase [9]. When DNA is damaged or replicated defectively, the cell cycle is arrested at a checkpoint in the interphase by inhibiting the activity of Cdc25 phosphatase. In arrested cells, Cdc25 is phosphorylated at Ser-216 by Chk1 or Chk2 kinase (a homologue of yeast Cds1) to lose its activity [10]. Chk2 is an important kinase which inhibits cell cycle at checkpoints, especially in DNA damage at S phase or G2 phase. The activity of mammalian homologue of Chk2 appears to require a checkpoint protein kinase, Ataxia telangiectasia mutated (ATM), which phosphorylates and activates Chk2 [11–13].

In *E. histolytica*, some cell-cycle regulating genes, *Eh cdc2*, *ras*, *rap*, *mfk1* and *rho* homologue, have been isolated [14–18], however, with the exception of *Eh cdc2*, these

☆ Nucleotide sequences data reported in this paper are available in the GenBank data base under the accession numbers: AB118100 (Eh1119), AB118101 (Eh1751), AB118102 (Eh1826), AB118103 (Eh2109), AB118104 (Eh6121), AB118573 (*Eh S6*) and AB118105 (*Eh chk2*).

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doi:10.1016/j.parint.2004.08.002

genes are no central regulator genes that directly control the activity of Cyclin–Cdk complex. We isolated a homologue of S6 kinase (*Eh S6*) from *E. histolytica* cDNA library (accession No: AB118573). In this study, using the information of amino acid sequences of *Eh S6*, we found other six new kinase genes of *E. histolytica* including a homologue of human *chk2* (*Eh chk2*) by analysis in silico. The isolated *Eh chk2* gene had a fork head-associated (FHA) region which is characteristic of human *Chk2*. *Eh chk2* mRNA transcribed in vitro could induce a delay of cell division in immature frog eggs. *Eh chk2* gene should be a cell-division regulator in *E. histolytica*.

2. Materials and methods

2.1. Preparation of genomic DNA and Polymerase chain reaction (PCR)

E. histolytica strain HM1:IMSS was cultured in TYI-S-33 medium axenicly at 35.5 °C in vitro [19]. Genomic DNA was prepared from *E. histolytica* trophozoites (10^8) with a standard method [18]. We searched new kinase genes with *Eh S6* sequence in the *E. histolytica* genome database of the institute for genomic research (TIGR; http://www.tigr.org/tdb/e2k1/eha1/). TIGR database informs that preliminary sequence data for *E. histolytica* is deposited regularly into the GSS division of GenBank. We performed PCR to amplify cDNA fragments of kinases that were found by the DNA homology search in TIGR database. The PCR was 30 cycles of 90 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min with 0.1 μg of the genomic DNA and primers described below. The PCR products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. PCR primers used are as follows:

Eh1119 forward primer: 5′-GAACTCGAGACCATGAGAAGAACTTTTCAGTT-3′
Eh1119 reverse primer: 5′-CCTCTAGATAAACACTTCTTCGGATAGTTCA-3′
Eh1751 forward primer: 5′-TTTCTCGAGACCATGAGTGATGGAAATGAAAT-3′
Eh1751 reverse primer: 5′-TTTTCTAGACAAACTCAGTTCATATTTAAAAT-3′
Eh1826 forward primer: 5′-AAACTCGAGACCATGTCAAGTGAAACTCCAAC-3′
Eh1826 reverse primer: 5′-AAGTCTAGACTTTAACCCATTGTCTTTCCTTG-3′
Eh2109 forward primer: 5′-AGTCTCGAGACCATGTCAAAGGAAGTTGGAGA-3′
Eh2109 reverse primer: 5′-AAGTCTAGAAAATATCAAAAATAAAAACAAAC-3′
Eh6121 forward primer: 5′-CAACTCGAGACCATGAAAATATTAAATAAAAC-3′
Eh6121 reverse primer: 5′-AAATCTAGAGGTTGTTATTTACCCACATATGT-3′
Eh chk2 forward primer: 5′-TAACTCGAGACCATGTGGGGAAAATTTGTTTG-3′
Eh chk2 reverse primer: 5′-GACTCTAGAAATTAGACTTAAATAATATCTTT-3′
β-actin forward primer: 5′-CGCCTCGAGACCATGGATGATGATATCGCCGC-3′
β-actin reverse primer: 5′-GCCATCCTGCGTCTGGACCT-3′

All primer sets contain a kozak sequence [20] and *Xho*I site in their forward primers and *Xba*I site in their reverse primers.

2.2. DNA sequence analysis

DNA sequences were determined with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Tokyo, Japan) using an ABI 310 DNA sequencer (Perkin Elmer, CT, USA). Sample mixtures (10 μl), which contained 10 ng of cDNA, 1.6 pmol of forward and reverse PCR primers and 4 μl of the kit reagent, were reacted for sequencing PCR. The PCR was 25 cycles of 96 °C for 30 s, 50 °C for 15 s and 60 °C for 4 min.

2.3. Construction of recombinant plasmids and in vitro mRNA transcription

Six new kinase genes, which have an entire open reading frame, were amplified by PCR with primers designed for cutting out. After appropriate PCR, PCR products of the kinase genes were digested with restriction enzymes, *Xba*I and *Xho*I, and subcloned into pT7G vector. The amplified pT7G recombinant plasmids were digested with only *Xho*I and then transcribed in vitro with T7 RNA polymerase using a MEGAscript T7 kit (Ambion, Austin, USA) to obtain 5′-capped mRNAs.

2.4. Detection of functions of kinase mRNA in Xenopus eggs

Eggs were collected from an ovary of *Xenopus laevis*. Stage VI immature eggs were defolliculated by treatment with 1.5 mg/ml of collagenase (Wako, Osaka, Japan). One shot of 60 nl of 0.2 mg/ml of mRNA, which was transcribed in vitro and solubilized in RNase-free water, was injected into about 20 eggs. The mRNA-injected eggs were exposed to 1 ng/ml of progesterone hormone (Wako) after 12 h of injection and cultured in modified Barth's solution at 23 °C [21,22]. Entry into M-phase was estimated by the germinal vesicle breakdown (GVBD), which accompanied a small white spot on the animal pole of eggs. Percentage of white spotted eggs was obtained by several intervals.

2.5. Western blot analysis

The treated eggs were dissolved in Laemmli's sample buffer [23] and heated at 98 °C for 3 min. And then, those

samples were applied to 12% SDS-polyacrylamide gel. Proteins on the gel were transferred to a nitrocellulose membrane (Hybond ECL: Amersham Pharmacia Biotech, USA) using a transfer apparatus (Cima Biotech, MN, USA). After transfer, the membrane was incubated with a blocking buffer of 20 mM Tris–HCl, 150 mM NaCl and 0.1% Tween-20, pH 7.5 (TBS-T) containing 4% skimmed milk (Gibco Oriental, Tokyo, Japan) at room temperature for 1 h. The membrane was incubated with anti-Cdc2 monoclonal antibody (1:5000; PSTAIR, Santa Cruz Biotechnology, USA), anti-phospho-p44/p42 MAP kinase (Thr-202/Tyr-204) E10 monoclonal antibody (1:5000; Cell Signaling Technology, MA, USA), or anti-ERK 1/2 polyclonal antibody (1:5000; Promega, WI, USA) in the blocking buffer at room temperature for 1 h. After washing with TBS-T, the membrane was incubated for 1 h with goat anti-rabbit IgG (H+L) antibody conjugated with horseradish peroxidase (1:5000; Promega) for Cdc2 detection, or with goat anti-mouse IgG antibody conjugated with horseradish peroxidase (1:5000; Promega) for p42 MAPK detection. Enzyme reaction was detected with enhanced chemiluminescence by an ECL kit (Amersham Pharmacia Biotech, Buckingham-

```
Ehchk2      1   ---------------------------------------------MWGKFVCEQFHKEIP
human       1   ------------------------------------------------------RD*SCE
drosophila  1   --------------MARDTQGTQGTQSQASNIWTQVESQPMEKIVWGRLYGKNIKI*SLD
elegans     1   MVRGTKRRRSSAEKPIVVVPVTRDDTMPVDEDLVVGESQCAASKPFAKLVGVRRGISS*D
rad53       1   -----------MENITQPTQQSTQATQRFLIEKFSQEQIGENIVCRVICTTGQIPIRDLS

Ehchk2      16  FNKGEMIIGR----------KAYEFLSSIVKVSVIHCIIKRSELPNLTVTTITDKSTNGT
human       7   YCFD*PLLK*-------------TDKYRTYSKKHFRIFREVG-PK*SYIAY*E*H*G***
drosophila  47  L*ND*FTA**GEANDLILTLNDLPEKILTRISK*HFI*KRAN-CELTNPVY*Q*L*R***
elegans     61  LADDHFVC**GSDDAPTNFNFSQVAKDVGLYRFISKIQFSIDRDTETRRIYLH*H*R***
rad53       50  ADISQVLKEKRSIKKVWTFGRNPACDYHLGNI*RLSNKHFQIL*GEDGNLLLN*I*****

Ehchk2      66  YLNGERLEKNLETYLSCFDEITFLNKITQPDYITFDYYDSTIIDLINKQCS---------
human       53  FV*T*LVG*GKRRP*NNNS**ALSLSRNKVFVFFDLTV*DQSVYPKALR-----------
drosophila  106 FV*N*KIGT*RMRI*KND*V*SLSHPTYKAFVFKDLSPNES*GLPEEIN-----------
elegans     121 LV*Q*MIG*G*SRE*MNG*L*SIGIPALIIFVYESADA*HHP--EELTK-----------
rad53       110 W***QKV***SNQL**QG****VGVGVESDILSLVIFINDKFKQCLEQNKVDRIRSNLKN

Ehchk2      116 ------------------------LFKKYQLGKYIGKGSFGVVREIMELATNTKFAIKII
human       101 --------------------------DE*IMS*TL*S*AC*E*KLAF*RK*CK*V*****
drosophila  154 --------------------------*T*YVNRKL*S*AY*L**LVYDTR*CQQ**M**V
elegans     167 --------------------------*YHVTSHSL***G**K*LLGYKKSDRSVV***QL
rad53       170 TSKIASPGLTSSTASSMVANKTGIFKDFSIIDEVV*Q*A*AT*KKAI*RT*GKT**V***

Ehchk2      153 NKE---------KAKNSLNQIHRECNTMKKINHPNSVKFKELFETNEMIFVIMELINGTT
human       136 S*RKFAIG--SAREADPALNVET*IEIL**L***CII*I*NF*DAED-YYIVL**ME*GE
drosophila  189 K*NMLSGARPSTNFSDPDR-VLN*AKI**NLS**CV*RMHDIVDKPDSVYMVL*FMR*GD
elegans     202 *---TQFSTRCSR*IAKTRD*RN*VEV***LS***I*AIYDWITVAKYSYMVI*YVG*GE
rad53       230 S*R---------*VIGNMDGVT**LEVLQ*L***RI*RL*GFY*DT*SYYMV**FVS*GD

Ehchk2      204 LEKVLKENSLS-----HQEKNDIIIELLQLLKYLHSIDIVHRDIKPENLMITRIKDEIHI
human       193 *FDKVVG*KR----LKEATCKLYFYQM*LAVQ***ENG*I***L****VLLSSQEEDCL*
drosophila  248 *LNRIIS*K*----LSEDISKLYFYQMCHAV****DRG*T***L**D*VLLETNDE*TLL
elegans     259 FFSKVVDSKYNRMGLGESLGKYFAFQ*IDAIL****VG*C********ILCSDKAERCIL
rad53       281 *MDFVAAHGAVG----EDAGRE*SRQI*TAI**I**MG*S***L**D*IL*E-QD*PVLV

Ehchk2      259 KLIDFGFGKELTGTIHAATLCGTPLYAAPELFEDKKTG----------YDARKIDIWSAG
human       249 *IT***HS*I*GE*SLMR******T*L***VLVSVG*----------AGYN*AV*C**L*
drosophila  304 *VS***LS*FVQKDSIMR********V***VLITGGR----------EAYTK*V****L*
elegans     319 **T***MA*NSVN--RMK*R****S*N***IVANEG-----------VEYTP*V****L*
rad53       336 *IT***LA*VQGNGSFMK*F***LA*V***VIRG*D*SVSPDEYEERNEYSSLV*M**M*

Ehchk2      309 VIIYIILTGRHPFCSNDYKIKELLDNIQHNSYSSFPCFNLLTDSQQLLLKGMFDSDVSKR
human       299 **LF*C*S*YP**SEHRTQVSLKDQITSGKYNFIPEVWAEVSEKALD*V*KLLVV*PKA*
drosophila  354 *VLFTC*S*TL**SDEYGTP-AAQQIKKGRFAYGH*SWKSVSQRAK**INQ*LIV*PER*
elegans     366 CVLF*TFS*YP**SEEYTDMTMDEQVLTG-RLIFHAQWRRI*VET*NMI*W*LTVEP*N*
rad53       396 CLV*V****HL**SGS-TQDQLYKQIGRGSYHEGPLKDFRISEEARDFIDSLLQV*PNN*

Ehchk2      369 FSASECLECLQRKRQKEISDVFCSSKKDII------------------------------
human       359 *TTE*A*RHPWLQDEDMKRKFQDLLSEENESTAL--------------------------
drosophila  413 P*IDDV*QSSWLGDAPMLQKAKRLM*L*GMEIEE--------------------------
elegans     425 P**V*LMSTQWM*CADCRTAKQDIL*SIKPISAA--------------------------
rad53       455 ST*AKA*NHPWI*MSPLG*QSYGDFSQISLSQSLSQQKLLENMDDAQYEFVKAQRKLQME
```

Fig. 1. Comparison of chk2 ORF between *Eh chk2*, human, Drosophila, *C. elegans* and *S. cerevisiae*. The names of databases and accession numbers of the homologues are as follows; human chk2 (GenBank, AF086904), Drosophila chk2 (GenBank, U87984), *C. elegans* chk2 (SWISS-PROT, CHK2_CAEEL), *S. cerevisiae* CHK2/RAD53 (PIR, A39616) Asterisk marks the identical amino acid residues. A FHA homology region is underlined. Highly conserved amino acid residues of FHA domain are bolded.

shire, UK) and an image analyzer LAS-1000 plus (Fujifilm, Tokyo, Japan).

3. Results

3.1. Cloning of a chk2 homologue of E. histolytica

Cell cycle is regulated by the interplay of many molecules, such as Cdks, Cyclins, and cell-cycle checkpoint kinases. Among those molecules, Ser/Thr kinases are the most important regulators. We isolated a S6 kinase of *E. histolytica* (*Eh S6*: AB118573). In order to look for new cell-division regulator genes of *E. histolytica*, we used TIGR *E. histolytica* genome database, which is an open database containing *E. histolytica* genome sequences. We performed a homology search in the *E. histolytica* genome database using a DNA sequence of conserved Ser/Thr kinase catalytic domain of the *Eh S6* gene. As a result, we found six new *E. histolytica* kinase genes that contain an entire open reading frame and a catalytic domain sequence of Ser/Thr kinase (Figs. 1 and 2) (Table 1). One of the newly assigned genes had 52% homology in overlapping region to the human *chk2* gene in amino acid sequence and designated *Eh chk2* (Fig. 1). The DNA sequence of *Eh chk2* contained about 1.2 kbp of no intron region within it.

```
A)
Eh1119      61  DKASICSRPEKKRKVERELAILRIIHHPNIIDYYASYETTKLLFVVQELLSGGELYTYVE
humansnf    59  **TRLD*--SNLE*IY**VQLMKLLN**H**KL*QVM**KDM*YI*T*FAKN**MFD*LT
Gthetasnf   47  K*DLFYDK*SLRL*IQ**ISVMKLMF**HV*KI*DVL*DS*Y**LII*YA*K***FN*LV

Eh1119     `121 KKKKLSLEESVKFLLQILSALKYIHKWQICHRDVKLENILLSHDCSTAKLCDFGMATYTG
humansnf   117  SNGH**EN*AR*KFW*****VE*C*DHH*V***L*T**L**DGNMDIKLADFGFGNF*KS
Gthetasnf  107  E*R**ENR*AL**FHE*I*G*E*C**HR*****L*******DMKLQIKIADFGMASLSIP

Eh1119     181  GMPLRDSCGSPFYAAPELFTQPTYDGCIADIWSLGVVFYVMIFGLMPFP--GETDEEFVE
humansnf   177  *E**STW****P*****V*EGKE*E*PQL********L**LVC*SL**D--*PNLPTLRQ
Gthetasnf  167  NIM*KTF****H**S**VVSNEP*N*IK*****C*IIL*ALVV*KL*YDEENDNMRKLFN
B)
Eh1751     276  VVVIQMEYCSGSNLSRIIESQELHYSKKNANEKINFYFKQIITGIQYIHSKNIIHGDLKP
humanTIK   361  CLF****F*DKGT*EQW**KR---RGE*LDKVLALEL*E**TK*VD*****KL**R****
mouseTIK   323  CLF****F*DKGT*EQWMRNR---NQS*VDKAL*LDLYE**V**VE*****GL**R****

Eh1751     336  ANIFR-DGDILKIGDFGYATMAKNRNK--CKFVGTPGYTAP-EVSSGDYDTSIDIYSLGI
humanTIK   418  S***LV*TKQV******LV*SL**DG*-RTRSK**LR*MS*EQI**Q**GKEV*L*A**L
mouseTIK   380  G***LV*ERHI******L**ALE*DG*SRTRRT**LQ*MS*EQLFLKH*GKEV**FA**L

Eh1751     392  ILLEMCMSCVTRSEFILGIELIKKR----------------QINETVSKYFPQLSQLILN
humanTIK   477  **A*LLHV*D*AF*TSKFFTDLRDG-IISDIFDKKEKTLLQKLLSKKPEDR*NT*EILRT
mouseTIK   440  **A*LLHT*F*E**K*KFF*SLR*GDFSNDIFDNKEKSLLKKLLSEKP*DR*ET*EILKT
C)
Eh1826     203  PEDQLEMIKREIQLMRQLHHKNIVKLFDVYENNE-ILYLILEYVEGGELYDRLVQGALN-
Eh2109     45   DP---QKLQG**KILKMVD*PY*I**Y**FDG*DGK**I*TDL*K****F**ISDKTFYP
humanMLK   1501 K*K--*N*RQ**SI*NC***PKL*QCV*AF*EKA-NIVMV**I*S****FE*IIDEDFEL

Eh1826     260  -ERQAACVLYQLVSAITYLHKNNIAHRDLKPENILCVYKN-KLYIKIADFGLSKDFST--
Eh2109     101  -*DK*KI*VKR*I***G***SM**V**********LKSPDDDTDVR*****F**MITEDA
humanMLK   1558 T**ECIKYMR*ISEGVE*I**QG*V*L*******M**N*T-GTR**LI****ARRLEN-A

Eh1826     317  SLLQTCCGTPSYVAPEIIKGDCYTCQCDIWSIGVITYLVLSGNLPFYDENEEVIFDKILD
Eh2109     161  QI*L*A****V*****VLNAKG*GMEV*M********VL*C*YP**FGDTLGE*LSAVCA
humanMLK   1616 GS*KVLF***EF****V*NYEPIGYAT*M******C*ILV**LS**MGD*DNETLANVTS
D)
Eh6121     66   HRFSIERTRFYAAELLIGLKYLHDAGIVYRDLKPENILLTDEGHVCITDFGLCKEG-LTE
DdisRac    213  KK*TED*V*Y*G**IVLA*EH**LS*VI********L***N***I*M*********L**P
humanRac   241  RV**ED*****G**IVSA*D***SGK*******L**LM*DKD**IK**********-I*D

Eh6121     125  KDQTNTFCGTPEYLAPEILLGNGYGFAVDWWSYGTLIYEMLLGLPPFFDNDVQTMYQKIV
DdisRac    273  T*K*G************V*Q*****KQ*****F*S*L****T*****YNQ***E**R**M
humanRac   300  AATMK************V*ED*D**R*****GL*VVM***MC*RL**YNQ*HEKLFEL*L

Eh6121     185  SDDVRFPKNTPPAIREFISALLQKDPEDRLT----NPDIMXKHPFFKNMDFEMVKAKKIK
DdisRac    333  MEKLS**HFIS*DA*SLLEQ**ER***K**A----D*NLIKR****RSI*W*QLFQ*N*P
humanRac   360  ME*IK**RTLSSDAKSLL*G**I***NK**GGGPDDAKEIMR*S**SGVNWQD*YD**LV
```

Fig. 2. Comparison of overlapping region of newly cloned *Eh* kinases and homologues. (A) Eh1119, human snf1 (GenBank, BC038504) and *Guillardia theta* snf1 (PIR, B90120). (B) Eh1751, human TIK (GenBank, U50648) and mouse TIK (PRF, 1715268A). (C) Eh1826, Eh2109 and human MLK (GenBank, AY339601). (D) Eh6121, *Dictyostelium discoideum* Rac (SWISS-PROT, KRAC_DICDI) and human Rac (SWISS-PROT, AKT3_HUMAN). Asterisk marks the identical amino acid residues.

Table 1
Newly cloned *Eh* kinases

Clone name	Length (bp)	Accession number	Segment number of TIGR	Homologue
Eh1119	1797	AB118100	316759	SNF1
Eh1751	1311	AB118101	317284	PKR(TIK)
Eh1826	1371	AB118102	318129	myosin L chain kinase
Eh2109	810	AB118103	316894	myosin L chain kinase
Eh6121	1398	AB118104	318097	Rac1
Eh chk2	1194	AB118105	317604	Chk2

The nucleotide sequence data reported in this paper appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession numbers as indicated. Reference segment numbers of TIGR database are shown. Homologues were deduced by the expected amino acid sequences.

Expected amino acid sequence of *Eh chk2* contained a FHA domain in its N-terminal region (Fig. 1) which is conserved region in eukaryotic Chk2 [24,25]. Amino acid sequences of FHA domain have diversity with 55–75 residues, but seven amino acid residues are highly conserved among eukaryotic FHA domains [26]. All of those conserved seven amino acid residues were found in the putative FHA domain of *Eh chk2* (Fig. 1).

3.2. Functions of Eh chk2 in vivo

Human Chk2 kinase is a critical inhibitor of cell division. We tried to ensure the in vivo function of *Eh Chk2* in cell-division regulation. For functional analysis, we used immature eggs of the amphibian, *Xenopus laevis*, which is a common system for cell-cycle analysis. The immature *Xenopus* egg is naturally arrested at G2 phase with low activities of p42 MAP kinase and Cdc2/CyclinB complex. Progesterone hormone stimulates the activities of MAP kinase and Cdc2/CyclinB complex, and makes egg enters cell division. This event accompanies chromosome condensation and nuclear envelope disruption (GVBD). The GVBD is easily observed by the appearance of the white spot on the top of a *Xenopus* egg. We transcribed mRNAs from newly found *E. histolytica* kinase genes (*Eh chk2*, Eh1751, Eh1826 and Eh6121) and *Xenopus* β-actin gene as a negative control. Each mRNA was injected into interphase eggs, which were incubated for 12 h and stimulated with progesterone hormone to induce an entry into M phase of cell division. We counted the appearance of white spots and measured intervals where GVBD was observed in 50% eggs. The time of 50%-GVBD was 190–200 min in eggs uninjected and eggs injected with β-actin, Eh1826 or Eh6121 mRNAs, whereas, the time of 50%-GVBD was prolonged to over 420 min in eggs injected with *Eh chk2* mRNA (Fig. 3). To our surprise, the time of 50%-GVBD was prolonged also in eggs injected with Eh1751 mRNA, a homologue of RNA-dependent protein serine/threonine kinase (PKR) [27–29] (Fig. 3).

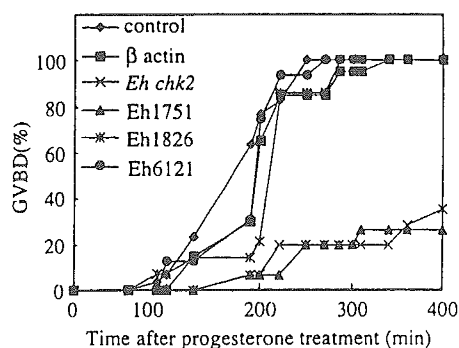

Fig. 3. *Eh chk2* mRNA induces a delay of cell division in *Xenopus* immature eggs. The mRNAs (6 ng each) encoding β-actin, *Eh chk2*, Eh1751, Eh1826 or Eh6121 were injected into 20 *Xenopus* eggs (control eggs were not injected). After progesterone treatment, the appearance of white spot in recipient eggs was counted at the indicated times. The percentage of the eggs, which showed white spot at the animal pole, was indicated as %GVBD.

Most vertebrate cells express both p42 MAPK (ERK2) and p44 MAPK (ERK1). In *Xenopus* eggs, however, only p42 MAPK is expressed. We then examined effects of *E. histolytica* kinases on activation of p42 MAPK and Cdc2, because both are phosphorylated and activated specifically in M phase of cell division [30]. Cellular proteins of eggs injected with those mRNAs were extracted and analyzed by western blotting. The protein bands of p42 MAPK were shifted up due to their phosphorylation and activation with the injections of β-actin, Eh1826 and Eh6121 mRNAs (Fig. 4, upper panel). Furthermore, the active forms of p42 MAPK were not detected in eggs injected with *Eh chk2*

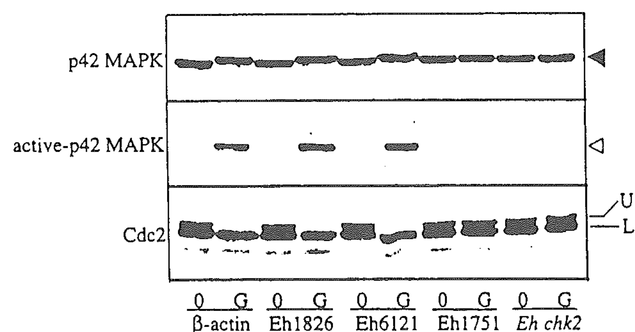

Fig. 4. Injection of *Eh chk2* mRNA delays the activations of p42 MAPK and Cdc2 kinase in *Xenopus* eggs. The eggs were injected with 6 ng of mRNAs of β-actin, Eh1826, Eh6121, Eh1751 or *Eh chk2* and treated with progesterone. The cellular proteins were collected just before progesterone treatment (represented as 0) and at 200 min after the treatment (G). GVBD was observed in about 50% of control eggs at 200 min. Total p42 MAPK was detected with anti-ERK 1/2 polyclonal antibody. The active forms of p42 MAPK and cdc2 kinase were detected with anti-phospho-p44/p42 MAPK (Thr-202/Tyr-204) E10 monoclonal antibody and anti-Cdc2 monoclonal antibody, respectively. The black arrow indicates both forms of activated and inactivated p42 MAPK. The white arrow indicates activated form of p42 MAPK. The upper band (U) of Cdc2 protein corresponds to the Tyr-15-phosphorylated form (inactive form) and the lower band (L) to the unphosphorylated form including active form of Cdc2. The weak band below the L band corresponds to Cdk2, a member of Cdk family.

mRNA and Eh1751 mRNA before GVBD (Fig. 4, middle panel). As for Cdc2 activation, the upper bands of Cdc2 inactive form, which is phosphorylated both Tyr-15 and Thr-14, disappeared with the injections of β-actin, Eh1826 and Eh6121 mRNAs, but not disappeared with the injections with *Eh chk2* mRNA and Eh1751 mRNA (Fig. 4, lower pannel). Therefore, *Eh chk2* mRNA and Eh1751 mRNA delayed the activation of both Cdc2 and p42 MAPK, whereas other *E. histolytica* kinase mRNAs did not show the effects. These results indicate that the *Eh chk2* gene has the function to prolong cell division in vivo and that the *Eh chk2* gene probably works in *E. histolytica* as a regulator of cell division as well as the human *chk2* gene.

4. Discussion

The life cycle of protozoan parasites, *E. histolytica*, has two stages; dormant cyst and amoebic trophozoite. The trophozoite proliferates by mitotic cell division and plays an essential role in amoebic diseases, although its growth regulation is not fully understood at the molecular levels. Identification of cell cycle regulator of *E. histolytica* will be helpful to control the amebiasis. We found six new genes of *E. histolytica* kinases including *Eh chk2*, a human *chk2* homologue in silico analysis. The human Chk2 plays a central role in G2 phase arrest of cell cycle by inhibiting Cdc25 phosphatase. Overexpression of human *chk2* (Cds1) gene was reported to delay the cell division in *Xenopus* eggs [31]. We showed that *Eh chk2* mRNA delayed the cell division in *Xenopus* eggs, whereas mRNAs of *Xenopus* β-actin or two other *E. histolytica* kinases did not (Fig. 3). In addition, *Eh chk2* mRNA inhibited p42 MAPK activation and Cdc2 activation, both of which are accompanied with cell division (Fig. 4). Our results indicate that the *Eh chk2* gene has similar functions to the human *chk2* gene.

The predicted amino acid sequence of *Eh chk2* contains a FHA domain in its N-terminal region. FHA domain is a protein–protein interaction motif which is widely found in checkpoint kinases of prokaryotes and eukaryotes. Rad53, a homologue of Chk2 kinase in a yeast *S. cerevisiae*, has two FHA domains and works in response to DNA damage [32–34]. The FHA domain in human Chk2 (Cds1) is indispensable region for its function [35]. The fact that predicted *Eh chk2* protein contains a FHA domain with highly conserved amino acid residues supports its functions are similar to human Chk2.

Interestingly, the injection of Eh1751 mRNA induced delay of cell division, and inhibited activation of p42 MAPK and Cdc2 in *Xenopus* eggs as well as injection of *Eh chk2* mRNA. DNA sequence of Eh1751 gene has homology to PKR kinase (interferon-induced double-stranded RNA-dependent kinase) (TIK). The expression of PKR is controlled in cell division. PKR is a potent inhibitor of cell growth in mammalian cells [36]. Our results suggest that Eh1751 is a PKR gene in *E. histolytica*.

We assessed the activity of *Eh chk2* gene in frog eggs in this report. The frog egg is a common system for examining functions of cell-cycle regulator genes in eukaryotes. However, more conclusive functions of *Eh chk2* gene should be confirmed in *E. histolytica* trophozoites. Overexpression or knockout of *Eh chk2* gene in the trophozoites will further clarify its biological functions [37–41]. It is known that the human Chk2 protein directly interacts with Cdc25 and inhibits its function, and the Chk2 is phosphorylated and activated by a check point kinase ATM [11–13]. Interaction between amoebic Cdc25 and Eh chk2 protein and requirement of ATM kinase for the activity of Eh chk2 will be interesting to be studied on. The identification and functional analysis of cell-cycle regulating genes of *E. histolytica* may give us a new resolution of amebiasis in the future.

Acknowledgements

We thank the Institute for Genomic Research (TIGR) for opening the *Entamoeba histolytica* Genome Database (http://www.tigr.org/tdb/e2k1/eha1/). TIGR informs that preliminary sequence data for *E. histolytica* is deposited regularly into the GSS division of GenBank and that the sequencing effort is part of the International *Entamoeba* Genome Sequencing Project and is supported by award from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Our work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (No. 12780542).

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