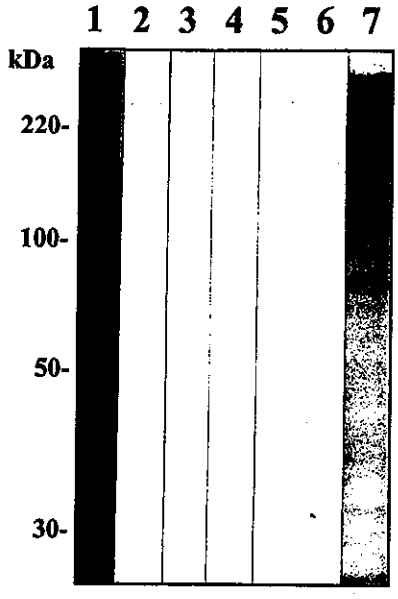

FIG. 3. Western immunoblot analysis of reactivities of human monoclonal antibody Fab fragments with trophozoites of *E. histolytica* HM-1:IMSS. Cell lysates were subjected to SDS-PAGE in a 7.5% polyacrylamide gel under nonreducing conditions and transferred to polyvinylidene difluoride membranes. The protein bands in lane 1 were stained with Coomassie brilliant blue. Lane 2, CP33; lane 3, CP33-H/L-CP17; lane 4, CP33-H/L-LA22; lane 5, CP33-H/L-CP26; lane 6, *E.coli* lysates (vector control); lane 7, plasma from a patient with an amebic liver abscess. The preparations in lanes 2 to 7 were treated with HRP-conjugated sheep antibody to human IgG $F(ab')_2$. The numbers on the left indicate the molecular masses of size markers.

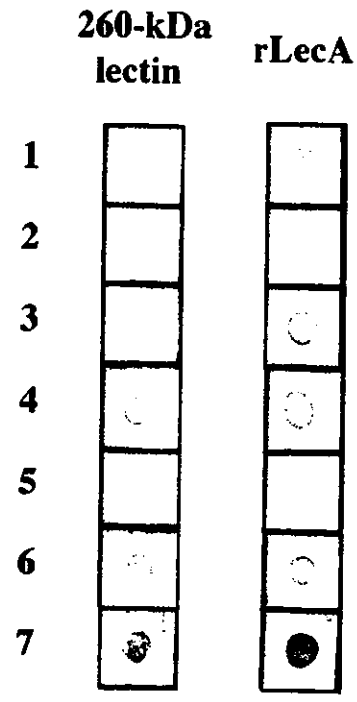

FIG. 4. Dot immunoblot analysis of reactivities of human monoclonal antibody Fab fragments with an affinity-purified 260-kDa lectin and with rLecA. One microgram of the 260-kDa lectin and 1 μg of rLecA were blotted onto a nitrocellulose membrane. The spots were treated as follows: line 1, CP33; line 2, CP33-H/L-CP17; line 3, CP33-H/L-LA22; line 4, CP33-H/L-CP26; line 5, control human Fab; line 6, IgG purified from plasma of a patient with an amebic liver abscess; line 7, anti-260-kDa lectin rabbit antibody. Then the preparations were treated with HRP-conjugated sheep antibody to human IgG $F(ab')_2$ (lines 1 to 6) or HRP-conjugated goat antibody to rabbit IgG (line 7).

with *E. dispar*. The epitope recognized by CP33 was located in a cysteine-rich domain of the heavy subunit of the Gal/GalNAc lectin. To date, seven different mouse monoclonal antibodies specific for nonoverlapping epitopes on the cysteine-rich domain of the heavy subunit of lectin have been identified (28, 35). Three of the seven murine antibodies inhibited amebic adherence to target cells, but two enhanced adherence by causing a marked increase in the galactose-binding activity of the lectin. Since the human antibodies prepared in this study had an inhibitory effect on amebic adherence to CHO cells, these antibodies must recognize an adherence-inhibiting epitope in the cysteine-rich domain (28). This conclusion is also supported by the fact that CP33 did not react with *E. dispar*, as previously it has been shown that the adherence-inhibiting epitopes are *E. histolytica* specific (28, 33). Demonstration of the ability of the Fabs to inhibit erythrophagocytosis indicates involvement of the Gal/GalNAc lectin in this process for the first time. Erythrophagocytosis is of interest as it is a characteristic property that distinguishes *E. histolytica* from the nonpathogenic parasite *E. dispar*.

In a previous study, the immunogobulin gene library derived from a patient with an amebic liver abscess was screened by the methods used in the present study. The positive rate of the first screening was 0.054% (27 of 5×10^4 of clones were positive), which is 5.7-fold higher than the rate (0.0095%) observed in this study (9). However, there was only one positive clone in the second screening by IFA with intact trophozoites. This suggests that the proportion of antibodies recognizing the trophozoite surface in the symptomatic patient with a liver abscess was smaller than the proportion in the asymptomatic cyst passer, even though the anti-*E. histolytica* antibody titer was higher in the symptomatic patient.

In the present study, when the heavy or light chain of CP33 was recombined with genes from the two libraries and rescreened, the positive rates were higher in the LA library. This may have been due to the symptomatic patient with the amebic liver abscess having a high antibody titer. However, the relative values were decreased to 2- or 4-fold from 5.7-fold. We concluded from these results that asymptomatic cyst passers have a high ratio of antibodies recognizing the adherence-inhibiting epitope of the heavy-subunit lectin compared with the ratio in

TABLE 3. Association and dissociation constants for the binding of recombinant human Fabs to the 260-kDa lectin and rLecA, as measured by surface plasmon resonance[a]

Fab	260-kDa lectin		rLecA	
	K_a (1/M)	K_d (M)	K_a (1/M)	K_d (M)
CP33	1.06×10^8	9.40×10^{-9}	7.19×10^7	1.39×10^{-8}
CP33-H/L-CP17	1.19×10^8	8.42×10^{-9}	6.43×10^7	1.56×10^{-8}
CP33-H/L-CP26	2.09×10^8	4.78×10^{-9}	1.19×10^8	8.40×10^{-9}
CP33-H/L-LA22	2.85×10^8	3.51×10^{-9}	1.29×10^8	7.73×10^{-9}

[a] K_a, association constant; K_d, dissociation constant.

TABLE 4. Effect of recombinant Fabs on erythrophagocytosis by *E. histolytica*[a]

Fab	% Of amebae with red blood cells (*P* vs control)	No. of red blood cells per ameba (*P* vs control)
Control	68.5 ± 5.9	2.70 ± 0.53
CP33	35.0 ± 6.1 (<0.005)	0.64 ± 0.17 (<0.005)
CP33-H/L-CP17	36.9 ± 3.5 (<0.002)	0.70 ± 0.07 (<0.005)
CP33-H/L-CP26	46.8 ± 6.2 (<0.02)	1.07 ± 0.25 (<0.01)
CP33-H/L-LA22	38.5 ± 7.8 (<0.01)	0.76 ± 0.15 (<0.005)

[a] Trophozoites (10^4 cells) were treated with 100 μg of Fab before incubation with human erythrocytes (10^6 cells). The results are means ± standard deviations for data from three experiments.

symptomatic patients. These adherence-inhibiting antibodies may help prevent the invasion of trophozoites into tissues in cyst passers, although no information was obtained in this study concerning antibodies to other adherence-inhibiting epitopes.

When the heavy chain of CP33 was combined with the light chains from the libraries, the positive rates for screening by colony blotting were 10- to 20-fold higher than the positive rates for screening of combinations of light chains of CP33 with heavy chains. This suggests that the heavy chain is more important for the binding of antibodies to the lectin. Indeed, the fact that the V-segment gene sequence of CP33-H contains many somatic mutations and the fact that no gene homologous with CP33-H has been reported are in accord with the observation that CP33 is reactive specifically with *E. histolytica*. Heavy-chain dominance in determining antigen binding has been demonstrated for antibodies to gp120 and to the reverse transcriptase of human immunodeficiency virus type 1 (HIV-1) (6, 26). In contrast, it has been reported that DNA binding activity is determined by the light chains in human anti-double-stranded DNA IgG Fab clones (41).

The present study revealed that all the most similar V-segment germ lines of the cloned heavy chain belonged to the VH3 family. The VH3 gene family, with 22 functional genes, is the largest of the seven families (VH1 to VH7) and comprises about one-half of the expressed VH repertoire in adult peripheral B cells (13, 29). However, biases in gene family usage of the heavy-chain variable region have been reported in a number of diseases. For example, restricted VH3 germ line gene usage was observed in intravenous immunoglobulin-bound Fabs from a patient with thrombocytopenia that had progressed to systemic lupus erythematosus (31). It is known that VH3 antibodies are also important for defense against a variety of bacteria (1, 39, 40) and viruses (2, 17, 20). Although VH3 antibodies bind to HIV-1 gp120 in HIV-infected late-stage patients, VH3 gene family expression is reduced compared with the expression in healthy donors, but the other two main VH gene families, VH1 and VH4, show no significant variation in expression (14). When the gene usage of another neutralizing anti-*E. histolytica* lectin Fab, LA-01, which had previously been prepared from an LA library (9), was examined, it was found that the most similar germ lines of LA-01 were VH3-30, D1-26, and JH6c for the heavy chain and Vκ02/012 and Jκ5 for the light chain. This observation also supports the preferential usage of the VH3 gene family for the adherence-inhibiting epitope of the lectin. To our knowledge, this report is the first report demonstrating that VH3 antibodies are important in defense against parasitic infections.

In contrast to heavy-chain gene usage, the light-chain gene repertoire of human antibodies to HIV does not exhibit a family bias (15). In the present study, all 14 light chains from both libraries belonged to the Vκ1 family, in which the closest Vκ germ lines were 02/012 and L5, in spite of the selection of clones showing different patterns after restriction endonuclease digestion. This finding demonstrates that a limited repertoire of light-chain genes is required to create a functional binding site with the heavy chain of CP33. This is in accord with previous reports that some heavy chains prefer to pair with similar light-chain variable regions to form high-affinity binders (3, 21, 30).

In the present study, we found that there was only one amino acid residue that was different in CDR3 when CP33-L and L-CP17 were compared. One of the advantages of recombinant antibody technology is possible modification of the original antibody gene. By introduction of synthetic genetic variability in CDR3, which is an important region in antigen binding, it may be possible to increase the affinity and/or neutralizing activity of the antibody.

Whereas the advantage of a phage display system for the preparation of human antibodies has recently been demonstrated (5, 19, 36), the present study shows that screening by colony blotting and chain shuffling of cloned genes may be a useful way to find genes of immunoglobulins with high affinity to pathogens. Total analysis of antibody genes for the amebic lectin, including other adherence-inhibiting epitopes on the heavy and intermediate subunits of the lectin, should be helpful not only for understanding the mechanism of protective immunity but also for development of immunoprophylaxis against invasive amebiasis.

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Entamoeba invadens: inhibition of excystation and metacystic development by aphidicolin

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Abstract

The effect of aphidicolin, a specific inhibitor of the replicative DNA polymerases, on the excystation and metacystic development of *Entamoeba invadens* was examined. The protein profile of metacystic amoebae and their immunogenicity in the presence and absence of aphidicolin were also examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting. Excystation, which was assessed by counting the number of metacystic amoebae after the induction of excystation, was inhibited by aphidicolin in a concentration-dependent manner during incubation compared to the controls. Metacystic development, when determined by the number of nuclei in amoeba, was also inhibited by aphidicolin, because the percentage of 4-nucleate amoebae in cultures with aphidicolin during incubation was higher than that in cultures without the drug. The addition of aphidicolin to cultures at day 1 of incubation reduced the number of metacystic amoebae thereafter compared to cultures without the drug. The inhibitory effect of aphidicolin on excystation and metacystic development was reversed by removal of the drug. Pretreatment of cysts with aphidicolin before transfer to a growth medium containing the drug had no further effect on the excystation and metacystic development. Cellular proteins of metacystic amoebae with 4 nuclei, which were predominant even at day 3 in the cultures with aphidicolin, reacted strongly with rabbit anticyst serum absorbed with trophozoite proteins. In contrast, those of metacystic amoebae with 1 nucleus, which were predominant at day 3 in cultures without aphidicolin, no longer reacted with the absorbed anticyst serum, suggesting change in the expression of proteins during metacystic development.

Index Descriptors and Abbreviations: Entamoeba invadens; protozoa; aphidicolin; DNA polymerase; excystation; metacystic development; immunogenicity; DNA, deoxyribonucleic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide.

1. Introduction

Aphidicolin is a mycotoxin produced by fungi such as *Cephalosporium aphidicola* and *Nigrospora oryzae*. This tetracyclic diterpenoid is known as a specific inhibitor of nuclear replicative DNA polymerases in eukaryotic cells (reviewed by Spadari et al., 1982; Wang, 1991). Aphidicolin blocks eukaryotic cells in the S phase by inhibiting the replicative DNA polymerase and allows G2, M, and G1 cells to accumulate specifically at the G1/S border; it does not reduce cell viability and its action is reversible (Pedrali-Noy et al., 1980).

It was previously demonstrated that aphidicolin inhibits *Entamoeba histolytica* growth and DNA synthesis and induces synchronous growth of the parasite in the recovery phase after the removal of aphidicolin (Makioka et al., 1998). The inhibitory effects of aphidicolin were also demonstrated on the growth and encystation of *Entamoeba invadens* and the reversibility of its action (Kumagai et al., 1998). Thus aphidicolin is considered a useful tool for studies on cellular processes relating to DNA synthesis.

Excystation and metacystic development are necessary for *Entamoeba* infection and their processes were described for *E. histolytica* previously (Cleveland and Sanders, 1930; Dobell, 1928). However, knowledge of the excystation as well as the encystation is limited since no axenic encystation medium is available for this

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parasite (López-Romero and Villagómez-Castro, 1993). Studies have been conducted on the axenic in vitro encystation of *E. invadens*, a reptilian parasite, because of its close similarity with *E. histolytica* in morphology and life cycle (McConnachie, 1969). Since the excystation and metacystic development of *E. histolytica* (Cleveland and Sanders, 1930; Dobell, 1928) and those of *E. invadens* (Geiman and Ratcliffe, 1936) look similar, in vitro excystation of *E. invadens* may also become a useful model for excystation of the human parasite. Excystation is the process whereby the whole organism escapes from the cyst through a minute perforation in the cyst wall. Metacystic development is the process whereby a hatched metacystic amoeba with 4 nuclei divides to produce 8 amoebulae, which grow to become trophozoites (Cleveland and Sanders, 1930; Dobell, 1928; Geiman and Ratcliffe, 1936). The transfer of *E. invadens* cysts in an encystation medium to a growth medium could induce in vitro excystation (Garcia-Zapien et al., 1995; McConnachie, 1955; Rengpien and Bailey, 1975). No study has been conducted to follow the changes in the protein profile and their immunogenicity during excystation and metacystic development. Rabbit antisera were previously prepared against trophozoites and cysts, and the appearance of cyst-specific proteins was demonstrated in encysting *E. invadens* and their immunogenicity (Makioka et al., 2000). The present study examined the effect of aphidicolin on the excystation and metacystic development of *E. invadens* and also followed the changes occurring in the protein profile during these processes in the presence of aphidicolin by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting using these antisera. Here, it is reported that aphidicolin inhibits the excystation and metacystic development of *E. invadens* and also affects expression of proteins of metacystic amoebae that are recognized by cyst and trophozoite-specific antisera during development.

2. Materials and methods

Trophozoites of the IP-1 strain of *E. invadens* 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, 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 excystation and metacystic development of *E. invadens*, duplicate cultures of 5×10^5 cysts/ml were incubated with 0.1–10 μg/ml aphidicolin for 3 days. Metacystic amoebae were counted in a hemocytometer at 5 h (0 and 10 μg/ml only), days 1, 2, and 3 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. Aphidicolin, purchased from Sigma, was initially dissolved in dimethyl sulfoxide (DMSO). The control cultures received the same volume of DMSO.

Metacystic development was determined by the number of nuclei per amoeba. Cells were harvested at days 1 and 3 in cultures with or without 10 μg/ml aphidicolin and stained with modified Kohn (Kumagai et al., 2001). The number of nuclei per amoeba was determined by double-counting at least 100 amoebae.

For experiments on the effect of aphidicolin on excystation and metacystic development after the induction of excystation, duplicate cultures (5×10^5 cysts/ml) were incubated for 1 day, then aphidicolin was added to one culture at a concentration of 10 μg/ml. The cultures were incubated for an additional 2 days, and the metacystic amoebae were counted.

For experiments on the reversibility of the effect of aphidicolin, duplicate cultures containing 10 μg/ml aphidicolin were incubated for 1 day. Cells were then centrifuged at 400*g* for 5 min after chilling on ice and the spent medium removed. Cells were washed twice with a growth medium and then suspended in a fresh growth medium. In control cultures, cells were similarly treated without replacement of the medium. The cultures were incubated for an additional 2 days, and the metacystic amoebae were counted.

To examine the effect of pretreatment with aphidicolin on excystation and metacystic development, cysts were first incubated for 30 min in an encystation medium with or without 10 μg/ml aphidicolin, then transferred to a growth medium with or without the same concentration of drug, and the metacystic amoebae were counted daily.

To examine the change in the protein profile and immunogenicity of metacystic amoebae during development, cellular proteins of metacystic amoebae were prepared as follows: cells were harvested at days 1 and 3 of incubation in cultures with and without 10 μg/ml aphidicolin, washed by centrifugation in PBS, and treated at room temperature for 10 min with 0.05% Sarkosyl containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino) butane (E-64), 1 μg/ml *N*-tosyl-L-lysyl chloromethyl ketone (TLCK), 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 mM benzamidine hydrochloride to disrupt the metacystic amoebae. All these proteinase inhibitors were purchased from Sigma. After centrifugation, the lysates of metacystic amoebae were obtained as supernatants. The lysates of trophozoites were similarly prepared.

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the lysates of metacystic amoebae and trophozoites were mixed with the sample buffer (Laemmli, 1970), cysts were dissolved in the sample buffer and subjected to electrophoresis: 10^5 equivalent of each per lane. Electrophoresis was performed using a Tris–glycine buffer (pH 8.3) containing 0.1% SDS (Laemmli, 1970) on 12.5% polyacrylamide slab gels. The gels were stained with Coomassie blue R-250.

For immunoblotting, the nitrocellulose filters were exposed for 1 h to a 1:100 dilution of rabbit antitrophozoite serum, anticyst serum or the anticyst serum absorbed with trophozoite proteins, in PBS containing 0.05% Tween 20 (PBS/Tween). The preparation of these antisera was described previously (Makioka et al., 2000). The filters were washed with PBS/Tween, incubated for 1 h with peroxidase-conjugated goat anti-rabbit immunoglobulins (ICN Pharmaceuticals) diluted in 1:1000 in PBS/Tween, washed again, and developed with PBS containing 4-chloro-1-naphthol and hydrogen peroxide.

All the experiments of the present study were performed at least three times and similar results were obtained. Therefore, the data presented in the results are representative.

3. Results

The effect of aphidicolin on the number of metacystic amoebae of *E. invadens* after the transfer of cysts to a growth medium was examined. At 5 h of incubation, the number of metacystic amoebae in cultures without aphidicolin was 1.84 ± 0.19 ($\times 10^4$/ml), whereas it was 0.96 ± 0.06 ($\times 10^4$/ml) ($P < 0.05$) in cultures containing 10 μg/ml of the drug. At day 1, little decrease in the number of metacystic amoebae occurred in cultures with 0.1 μg/ml aphidicolin, whereas it significantly decreased in cultures with more than 0.5 μg/ml drug compared to the controls (Fig. 1). Amoebae further increased in number from day 1 to day 3 of incubation in cultures without the drug. In contrast, metacystic amoebae remained unchanged in number from day 1 to day 3 in cultures containing 5 and 10 μg/ml.

The effect of aphidicolin on cyst viability is shown in Fig. 2. The number of viable cysts in cultures containing aphidicolin during incubation was comparable to or greater than that of controls.

The effect of aphidicolin on metacystic development was examined by counting the number of nuclei per cell. As shown in Fig. 3, 53% of metacystic amoebae were 4-nucleate at 5 h of incubation in both cultures with and without aphidicolin. The percentage of 4-nucleate amoebae in the control cultures then decreased to 17 and 2% at days 1 and 3, respectively, following increased percentages of 1- to 3-nucleate amoebae. In contrast, the

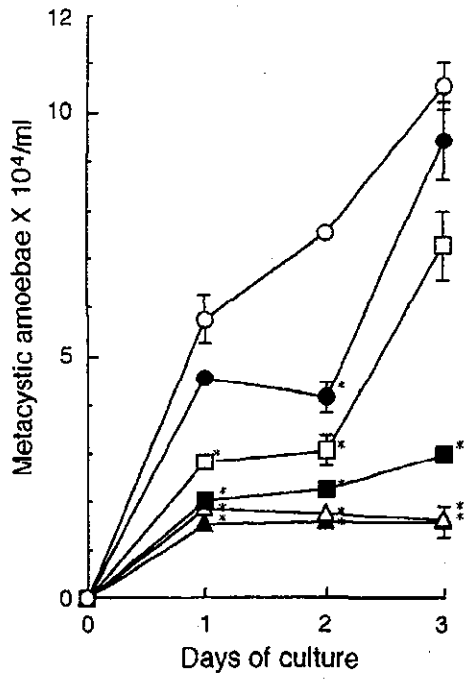

Fig. 1. Effect of aphidicolin on the number of metacystic amoebae of *E. invadens*. Cysts were transferred to a growth medium containing various concentrations of aphidicolin. The mean number ± SE of metacystic amoebae for duplicate cultures is plotted (*$P < 0.05$). Concentrations of 0, 0.1, 0.5, 1, 5, and 10 μg/ml are shown by open circles, solid circles, open squares, solid squares, open triangles, and solid triangles, respectively.

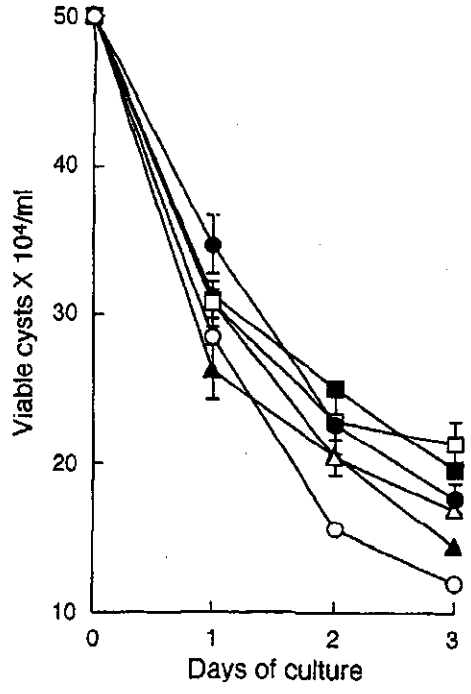

Fig. 2. Effect of aphidicolin on cyst viability in the growth medium. Experimental conditions were the same as those for Fig. 1. The mean number ± SE of viable cysts for duplicate cultures is plotted. Concentrations of 0, 0.1, 0.5, 1, 5, and 10 μg/ml are shown by open circles, solid circles, open squares, solid squares, open triangles, and solid triangles, respectively.

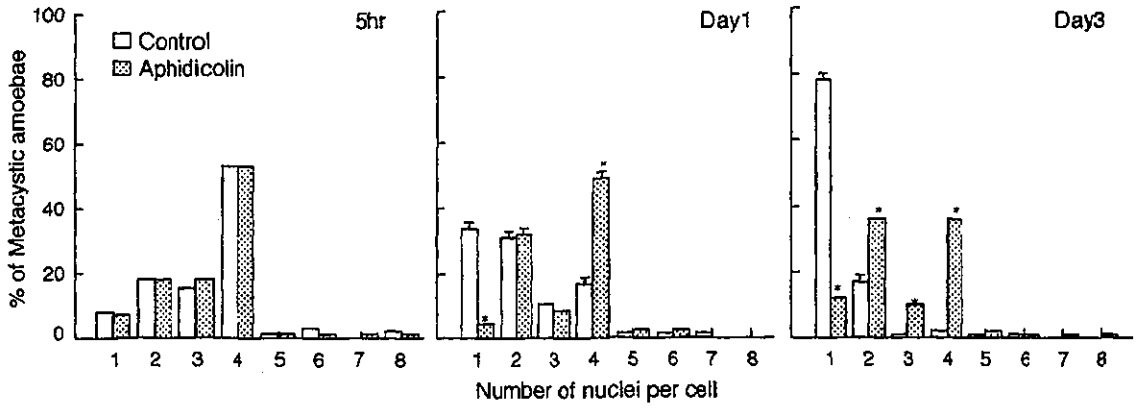

Fig. 3. Effect of aphidicolin on the metacystic development of *E. invadens*. Cysts were transferred to a growth medium with or without 10 μg/ml aphidicolin. The numbers of nuclei per metacystic amoeba stained with modified Kohn at 5 h and days 1 and 3 of incubation were counted and the percentage of amoebae was determined (*$P < 0.05$).

percentage of 4-nucleate amoebae in cultures with aphidicolin was still 49 and 36% at days 1 and 3, respectively, suggesting the inhibition of metacystic development by aphidicolin.

To examine the effect of aphidicolin on excystation and metacystic development after induction, aphidicolin (10 μg/ml) was added to cultures at day 1 of incubation and metacystic amoebae counted 1, 2, and 3 days later. As shown in Fig. 4, metacystic amoebae markedly decreased in number 1 and 2 days after the addition of aphidicolin compared with those in cultures without the drug. The reason for this decrease remains unclear. The percentages of 4- and 1- to 3-nucleate amoebae in cultures 1 day after the addition of aphidicolin were 8 and 88%, respectively, which were almost the same as those of 9 and 89%, respectively, in the control cultures.

To determine whether the inhibitory effect of aphidicolin on excystation and metacystic development was reversible, spent medium containing 10 μg/ml aphidicolin for 1 day of incubation was replaced with a drug-free growth medium. After removal of the drug, the number of metacystic amoebae increased to 91% of the control (Fig. 5). The recovery of the number of amoebae was associated with the increase in percentage of 1- to 3-nucleate amoebae (data not shown).

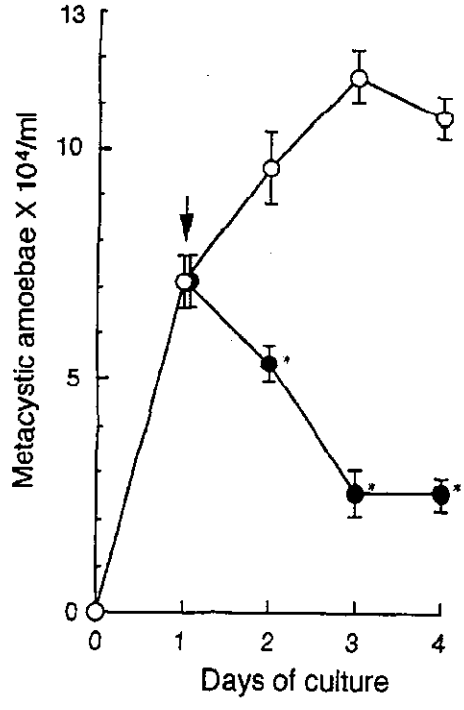

Fig. 4. Effect of aphidicolin on the excystation and metacystic development of *E. invadens* after the induction of excystation. At day 1 of incubation, aphidicolin (10 μg/ml) was added to cultures and metacystic amoebae counted for another 3 days (*$P < 0.05$).

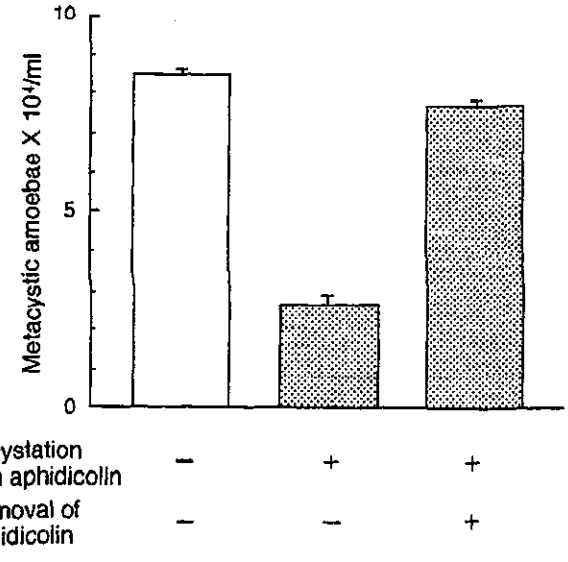

Fig. 5. Effect of the removal of aphidicolin on the excystation and metacystic development of *E. invadens*. After exposure of cysts to 10 μg/ml aphidicolin in a growth medium for 1 day, the drug was removed by replacement of the medium with a drug-free growth medium and the cultures were further incubated for 3 days. The mean number + SE of metacystic amoebae for duplicate cultures is plotted.

To examine the effect of pretreatment with aphidicolin on excystation and metacystic development, cysts were exposed to 10 μg/ml aphidicolin in an encystation medium before transfer to a growth medium containing the drug. As shown in Fig. 6, the pretreatment caused no further inhibition of excystation and metacystic development as compared to that without the pretreatment.

To follow the change in the protein profile during excystation and metacystic development in cultures with and without aphidicolin, SDS–PAGE and immunoblotting were conducted. As shown in Fig. 7A, the protein profiles of metacystic amoebae were almost the same as those of cysts and trophozoites, but the protein bands of metacystic amoebae at day 3 in control cultures were weakly stained compared to those of other forms. As shown in Fig. 7B, not only trophozoite proteins but also a number of proteins of metacystic amoebae and cyst proteins were immunostained with rabbit anti-trophozoite serum. Also, the proteins of metacystic amoebae at day 1 in cultures with aphidicolin were the most weakly immunostained.

When nitrocellulose filters were immunostained with rabbit anticyst serum, many metacystic amoeba proteins as well as cyst and trophozoite proteins were also immunostained (Fig. 8B). Also, proteins of metacystic amoebae at day 3 in the control cultures and trophozoites were weakly immunostained compared to the others. When the anticyst serum absorbed with trophozoite proteins was used, neither metacystic amoeba proteins at day 3 in control cultures nor trophozoite proteins were immunostained, whereas the 88- and 66-kDa cyst proteins of metacystic amoebae at day 1 in cultures with and without aphidicolin and those at day 3 in cultures with the drug were strongly immunostained (Fig. 8C).

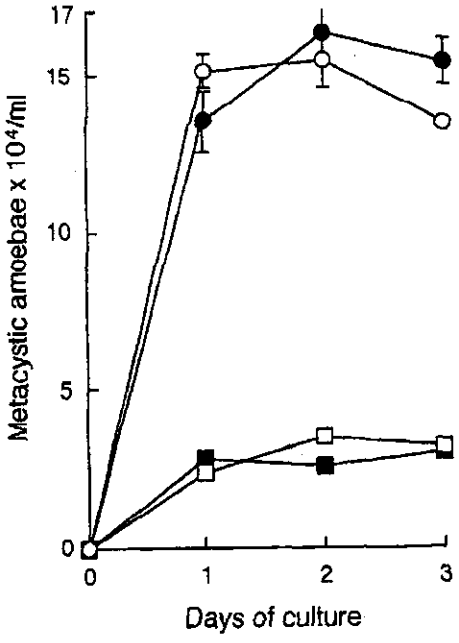

Fig. 6. Effect of pretreatment of aphidicolin on the excystation and metacystic development of *E. invadens*. Cysts were incubated for 30 min in an encystation medium with or without 10 μg/ml aphidicolin before transfer to a growth medium with or without the drug. The mean number ± SE of metacystic amoebae for duplicate cultures is plotted. No pretreatment and no drug in the growth medium (open circles); pretreatment without aphidicolin and growth medium without the drug (solid circles); pretreatment with the drug and growth medium without the drug (open squares); pretreatment without the drug and growth medium with the drug (solid squares); pretreatment with the drug and growth medium with the drug (open triangles).

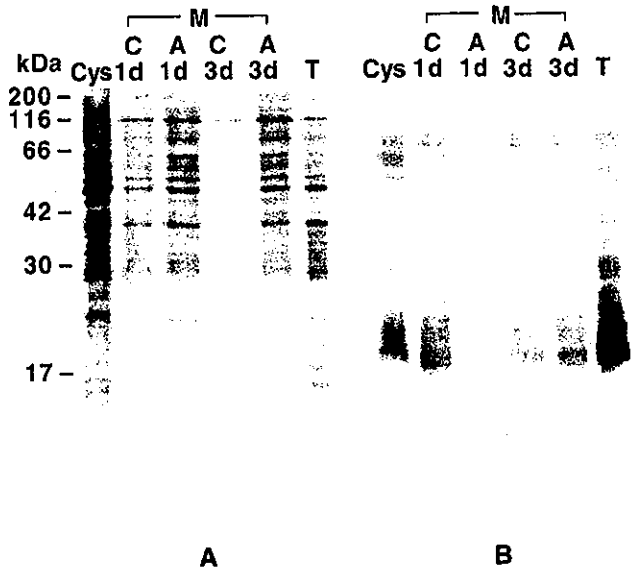

Fig. 7. Immunoblot analysis of cellular proteins that react with rabbit antitrophozoite serum during the metacystic development of *E. invadens*. Metacystic amoebae at days 1 and 3 in cultures with and without aphidicolin as well as cysts and trophozoites were subjected to SDS–PAGE and immunoblotting [Cys, Cysts; M, Metacystic amoebae in cultures with (A) and without (C) aphidicolin; T, Trophozoites]. (A) Protein staining with Coomassie blue; (B) immunostaining with rabbit antitrophozoite serum. The molecular-mass standards are as follows: myosin (200 kDa), β-galactosidase (116 kDa), albumin (66 kDa), aldolase (42 kDa), carbonic anhydrase (30 kDa), and myoglobin (17 kDa).

4. Discussion

The results clearly indicate that aphidicolin inhibits the excystation and metacystic development of *E. invadens*. Although it may be difficult to distinguish excystation completely from metacystic development because it does not occur simultaneously (Cleveland and Sanders, 1930; Dobell, 1928), the number of metacystic amoebae at 5 h in cultures containing aphidicolin, at which time the percentage of 4-nucleate amoebae was more than 50% in cultures with and without the drug, was significantly reduced compared to the controls. This suggests that aphidicolin affected the excystation possibly through its inhibition of the replicative DNA

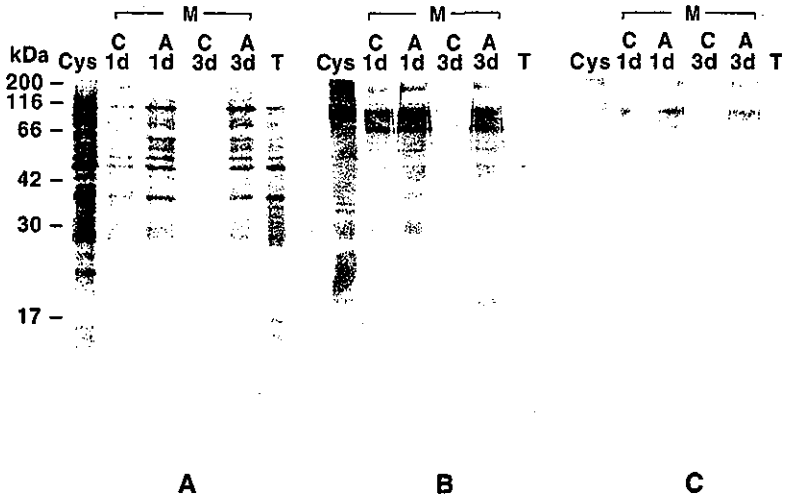

Fig. 8. Immunoblot analysis of cellular proteins that react with rabbit anticyst serum during the metacystic development of *E. invadens*. Conditions for excystation and SDS–PAGE were the same as those described for Fig. 7. (A) Protein staining with Coomassie blue; (B) immunostaining with rabbit anticyst serum; (C) immunostaining with anticyst serum absorbed with trophozoite proteins.

polymerase and thereby, inhibition of DNA synthesis. Since the excystation process does not include nuclear division, it remains unclear how DNA synthesis is related to the process. The results also demonstrated that considerable numbers of the metacystic amoebae with 1–3 nuclei were observed even at 5 h of incubation in cultures with and without 10 μg/ml aphidicolin. This means that those amoebae performed nuclear division even in the presence of aphidicolin. In this regard, although it was originally thought that only mature cysts with 4 nuclei were able to hatch (Cleveland and Sanders, 1930; Dobell, 1928; Geiman and Ratcliffe, 1936), 1- and 2-nucleate cysts, and 4-nucleate cysts with chromatoids of *E. histolytica* were able to hatch (Everritt, 1950; Hegner et al., 1932; Tanabe, 1934; Swartzwelder, 1939). This point may need further study in *E. invadens*.

The hatched 4-nucleate metacystic amoebae grow rapidly and divide to form 8 amoebulae. Since this metacystic development requires DNA synthesis, it is a target of aphidicolin. The inhibition of nuclear division by aphidicolin increased the percentage of 4-nucleate amoebae during incubation. This may become a useful tool for the biochemical characterization of metacystic development. The effect of aphidicolin on excystation and metacystic development of *E. invadens* was reversible, which was the same as that on the growth and encystation as previously demonstrated (Kumagai et al., 1998). The pretreatment of cysts with aphidicolin before transfer to a growth medium containing the drug had no further effect on excystation and metacystic development, suggesting that cysts in the encystation medium are not affected by aphidicolin.

Biochemical characterization of the metacystic amoebae is important to know their biological significance, but it has so far not been conducted. For this, first, an attempt was made to isolate the metacystic amoebae by the Percoll method which was useful for the separation of cysts from trophozoites (Avron et al., 1983), but this was unsuccessful. This suggests that there is a difference in cell density between the metacystic amoebae and trophozoites. Therefore, the lysates of metacystic amoebae were prepared by treatment with sarkosyl, which had no effect on cysts, and the lysates were analyzed by SDS–PAGE and immunoblotting. The protein profile of cysts and trophozoites was almost the same under the conditions of one-dimensional SDS–PAGE and Coomassie blue staining (Makioka et al., 2000). The protein profile of metacystic amoebae was also almost similar to that of cysts and trophozoites, suggesting no occurrence of drastic change in the protein pattern after excystation and during metacystic development. The protein bands of metacystic amoebae at day 3 in the control cultures were weakly stained, suggesting that those amoebae, most of which are 1-nucleate, had less protein content in the amoeba. The antitrophozoite serum immunostained not only trophozoite proteins but also a number of metacystic amoeba and cyst proteins, indicating that these three forms share considerable numbers of proteins. Since the reactivity of metacystic amoeba proteins at day 1 in cultures with aphidicolin was weaker than that in cultures without the drug, those amoebae in which 4-nucleate were predominant, had fewer shared trophozoite proteins due to their early stages of development. The results of the absorbed anticyst serum indicated that cyst-specific proteins were present in the metacystic amoebae with 4 nuclei, but they disappeared in those with 1 nucleus, suggesting change in the expression of proteins during metacystic development.

To the authors' knowledge, the present study is the first analysis of metacystic amoeba proteins by immunoblotting using polyclonal antitrophozoite and anticyst

sera as well as the anticyst serum absorbed with trophozoite proteins. An understanding of excystation and metacystic development will lead to the identification of targets for vaccination and chemotherapy to inhibit *Entamoeba* infection.

In summary, the results show that aphidicolin inhibits both the excystation and metacystic development of *E. invadens* and thereby affected change in the expression of proteins of metacystic amoebae during development.

Acknowledgments

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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*

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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

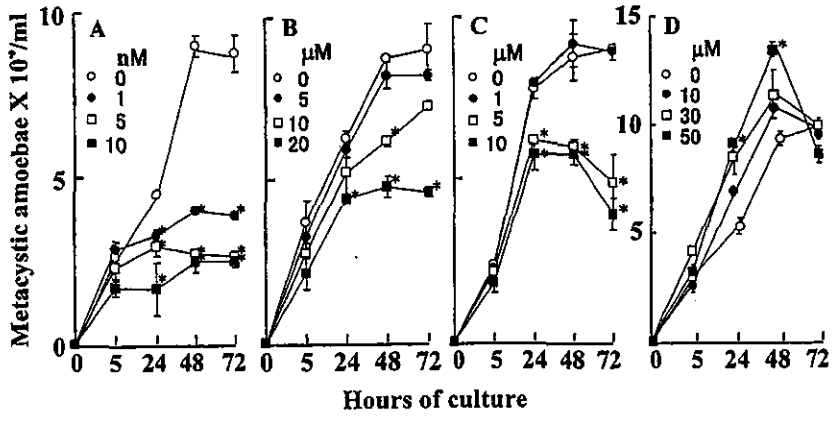

Fig. 1A–D Effect of protein kinase C (PKC) inhibitors on the number of metacystic amoebae of *Entamoeba invadens*. Cysts were transferred to a growth medium containing various concentrations of four PKC inhibitors: staurosporine (A), chelerythrine (B), calphostin C (C) and sphingosine (D). The means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

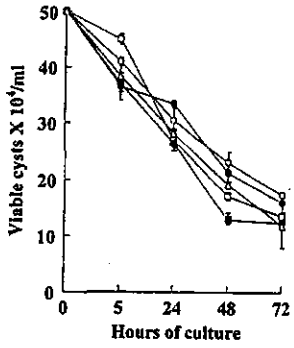

Fig. 2 Effect of PKC inhibitors on cyst viability of *E. invadens* in the growth medium. Experimental conditions were the same as those for Fig. 1. The means ± SE of viable cysts for duplicate cultures are plotted. *White circles* Control, *black circles* staurosporine (10 n*M*), *white squares* chelerythrine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

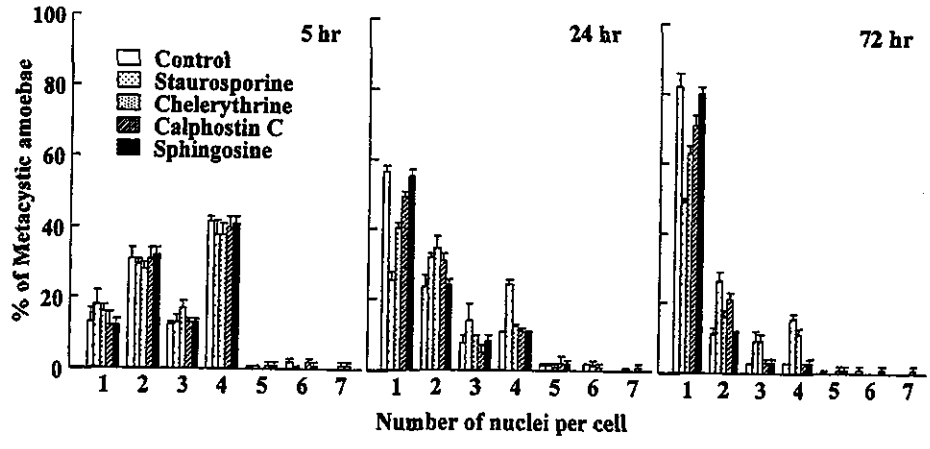

Fig. 3 Effect of PKC inhibitors on the metacystic development of *E. invadens*. Cysts were transferred to a growth medium with or without 10 n*M* staurosporine, 20 μ*M* chelerythrine, 10 μ*M* calphostin C or 50 μ*M* sphingosine. The numbers of nuclei per metacystic amoeba stained with modified Kohn at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

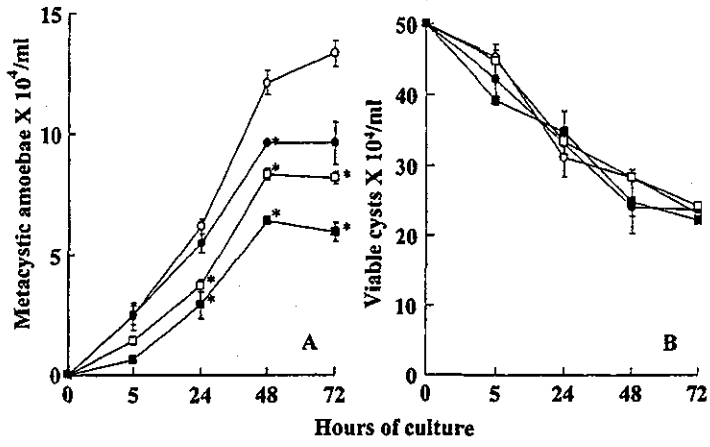

Fig. 4A, B Effect of wortmannin on the number of metacystic amoebae and cyst viability of *E. invadens*. Cysts were transferred to a growth medium containing various concentrations of wortmannin. The means ± SE of metacystic amoebae (A) and viable cysts (B) for duplicate cultures are plotted (*each asterisk*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

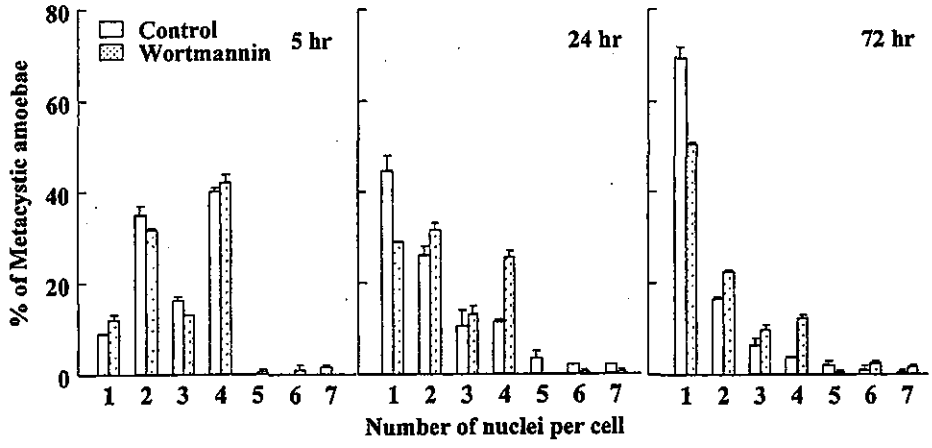

Fig. 5 Effect of wortmannin on the metacystic development of *E. invadens*. Cysts were transferred to a growth medium *with or without* 1 μ*M* wortmannin. The numbers of nuclei per metacystic amoeba stained with modified Kohn at 5, 24 and 72 h of incubation were counted and the percentage of amoebae 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.*

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Molecular and structural characterization of NADPH-dependent D-glycerate dehydrogenase from the enteric parasitic protist *Entamoeba histolytica*

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Putative NADPH-dependent GDH (D-glycerate dehydrogenase) of the protozoan parasite *Entamoeba histolytica* (EhGDH) has been characterized. The *EhGDH* gene encodes a protein of 318 amino acids with a calculated isoelectric point of 6.29 and a molecular mass of 35.8 kDa. EhGDH showed highest identities with GDH from ε-proteobacteria. This close kinship was also supported by phylogenetic analyses, suggesting possible lateral transfer of the gene from ε-proteobacteria to *E. histolytica*. In contrast with the implications from protein alignment and phylogenetic analysis, kinetic studies revealed that the amoebic GDH showed biochemical properties similar to those of mammalian GDH, i.e. a preference for NADPH as cofactor and higher affinities towards NADPH and β-hydroxypyruvate than towards $NADP^+$ and D-glycerate. Whereas the amino acids involved in nucleotide binding and catalysis are totally conserved in EhGDH, substitution of a negatively charged amino acid with a non-charged hydroxy-group-containing amino acid is probably responsible for the observed high affinity of EhGDH for $NADP^+$/NADPH. In addition, the amoebic GDH, dissimilar to the bacterial and mammalian GDHs, lacks glyoxylate reductase activity. Native and recombinant EhGDH showed comparable subunit structure, kinetic parameters and elution profiles on anion-exchange chromatography. We propose that the GDH enzyme is likely to be involved in regulation of the intracellular concentration of serine, and, thus, also in controlling cysteine biosynthesis located downstream of serine metabolic pathways in this protist.

Key words: anaerobic protist, cysteine biosynthesis, *Entamoeba histolytica*, gluconeogenesis, glycerate dehydrogenase, serine biosynthesis.

INTRODUCTION

D-Glycerate dehydrogenase (GDH; EC 1.1.1.29) catalyses the NADH- or NADPH-dependent reduction of HP (β-hydroxypyruvate) as a committed step of serine degradation in mammals [1]. The product of this reaction, D-glycerate, is channelled into the gluconeogenic pathway [2]. Therefore GDH has a critical role to link serine metabolism and gluconeogenesis in mammalian organisms. GDH also catalyses the NAD^+- or $NADP^+$-linked oxidation of D-glycerate in the direction of serine biosynthesis in plants [3]. Therefore the plant GDH apparently functions in both the forward and reverse orientations, and consequently has two roles, i.e. degradation of serine leading to gluconeogenesis and serine biosynthesis [3]. In humans, GDH is expressed in the various organs, but the highest GDH activity and mRNA level were found in the liver [4,5], suggesting GDH is involved primarily in serine degradation, leading to gluconeogenesis in mammals. The physiological importance of *GDH* has been demonstrated by its deficiency in humans. Primary hyperoxaluria type 2 is a genetic metabolic disease attributable to a deficiency in GDH activity, accompanied with compensatory high lactate dehydrogenase activity, which causes excretion of excessive L-glycerate and oxalate in the urine leading to kidney dysfunction [4,6,7]. Although GDH has been shown to be present in a wide variety of organisms from bacteria to mammals and plants, and its physiological importance in higher eukaryotes is well understood, neither its presence nor its biochemical properties has been demonstrated in unicellular eukaryotes.

Entamoeba histolytica, the causative agent of human amoebiasis, is an enteric protozoan parasite and causes amoebic colitis and extraintestinal abscesses in approximately 50 000 000 inhabitants of endemic areas [8]. Sulphur-containing amino acid metabolism in *E. histolytica* is unique in a variety of aspects, including: (1) a lack of both forward and reverse trans-sulphuration pathways; (2) a lack of enzymes responsible for cysteine and homocysteine degradation in mammals, including cysteine dioxygenase and phosphopanthotenylcysteine synthase; and (3) the presence of the *de novo* sulphur-assimilatory cysteine-biosynthetic pathway [9–11a]. Together with unique metabolism of sulphur amino acids in this parasite, a physiological requirement of cysteine has also been shown [12,13]. The major, and probably sole, route of cysteine biosynthesis is the condensation of OAS (*O*-acetylserine) with sulphide, mediated by the *de novo* cysteine-biosynthetic pathway. OAS is produced by a transacetylation reaction (the addition of an acetyl moiety to serine), which is probably obtained via *de novo* serine biosynthesis. To understand better sulphur-containing amino acid metabolism and cysteine biosynthesis in protozoan parasites, we attempted to identify and characterize putative serine metabolic pathways. We have identified in the *E. histolytica* genome database genes encoding GDH, GK (glycerate kinase), PGDH (phosphoglycerate dehydrogenase) and PSAT (phosphoserine aminotransferase)

Abbreviations used: DTT, dithiothreitol; (Eh)GDH, (*Entamoeba histolytica*) D-glycerate dehydrogenase; GK, glycerate kinase; HP, β-hydroxypyruvate; OAS, *O*-acetylserine; ORF, open reading frame; PGDH, phosphoglycerate dehydrogenase; PSAT, phosphoserine aminotransferase; rEhGDH, recombinant EhGDH.

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The nucleotide sequence data reported for *EhGDH* will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AB091511.

(V. Ali and T. Nozaki, unpublished work). The presence of these genes indicates that *E. histolytica* possesses both phosphorylated and non-phosphorylated pathways for serine metabolism. In addition, we found that cysteine biosynthesis is co-ordinately regulated with serine metabolism: serine is a precursor for cysteine synthesis, and cysteine biosynthesis is inhibited by intermediates of both glycolysis and serine metabolism (results not shown). The non-phosphorylated pathway of serine metabolism is composed of the sequential reactions catalysed by L-serine:pyruvate aminotransferase, D-glycerate dehydrogenase and D-glycerate kinase [1,3]. In the present work, we describe cloning and enzymological characterization of a gene encoding GDH from *E. histolytica*. As far as we are aware, this is the first report on GDH in unicellular eukaryotes.

EXPERIMENTAL

Chemicals

All chemicals of analytical grade were purchased from Wako (Tokyo, Japan) or Sigma–Aldrich (Tokyo, Japan), unless stated otherwise. Pre-packed Mono Q 5/5 HR and Sephacryl S-300 HR Hiprep columns were purchased from Amersham Biosciences (Tokyo, Japan).

Parasite culture

Trophozoites of the *E. histolytica* clonal strain HM1:IMSS cl6 [14] were cultured axenically in TYI-S33 medium at 35 °C, as described previously [15].

Bacterial expression and purification of recombinant EhGDH (rEhGDH)

A plasmid was constructed to produce rEhGDH possessing the N-terminal histidine tag. A fragment corresponding to an ORF (open reading frame) of EhGDH was amplified by PCR using a cDNA library [9] as the template and the following oligonucleotide primers: 5′-caGGATCCaagatagttgtattagacgca-3′ and 5′-caCTCGAGttagactattctatttctattttc-3′, where capital letters indicate *Bam*HI or *Xho*I restriction sites. The cycling parameters were: (1) denaturation at 94 °C for 30 s; (2) annealing at 55 °C for 30 s; (3) elongation at 72 °C for 60 s; and (4) 30 cycles. An approx. 1.0 kb PCR fragment was digested with *Bam*HI and *Xho*I, electrophoresed, purified with Geneclean kit II (BIO 101; Vista, CA, U.S.A.), and cloned into *Bam*HI- and *Xho*I-double-digested pET-15b (Novagen) to produce pET–EhGDH. The nucleotide sequence of the amplified EhGDH ORF was verified by sequencing, and was found to be identical with that of contig 317757 in the *E. histolytica* genome database (nt 11 110–12 066). The pET–EhGDH construct was introduced into *Escherichia coli* BL21(DE3) cells (Novagen). Expression of the rEhGDH protein was induced with 0.4 mM IPTG (isopropyl β-D-thiogalactoside) for 4–5 h at 30 °C. The bacterial cells were harvested, washed, lysed in 50 mM Tris/HCl, pH 8.0/300 mM NaCl containing 10 mM imidazole, 0.1 % (v/v) Triton X-100, 100 μg/ml lysozyme and complete mini EDTA-free protease inhibitor cocktail (Roche, Tokyo, Japan), and then sonicated. The rEhGDH protein was purified from the supernatant fraction using an Ni^{2+}-nitrilotriacetate column (Novagen) according to the manufacturer's instructions. The eluted rEhGDH protein was dialysed extensively in 50 mM Tris/HCl, pH 8.0/300 mM NaCl containing 10 % (v/v) glycerol and the protease inhibitors described above overnight at 4 °C, before storage at − 80 °C with 50 % glycerol. Enzyme remained active for more than 1 month when stored at − 80 °C under these conditions.

Enzyme assays

The enzymic activity of GDH was assayed in both the forward and reverse directions using either a spectrophotometer or a fluorimeter. The GDH activity in the forward reaction was measured spectrophotometrically using a Beckman DU530 spectrophotometer by following the decrease in absorbance at 340 nm due to HP-dependent oxidation of NADPH or NADH for 2–4 min at 25 °C. The reaction mixture contained 50 mM sodium phosphate, pH 6.5, 300 mM NaCl, 0.2 mM NADPH or NADH, 0.2 mM DTT (dithiothreitol), 500 μM HP and the enzyme. Kinetic parameters for NADPH in the forward direction were also estimated fluorimetrically (using a Fluorimeter F-2500; Hitachi, Tokyo, Japan) by measuring the rate of change in fluorescence (emission wavelength 470 nm; excitation wavelength 340 nm). D-Glycerate-dependent production of NADPH in the reverse reaction was measured fluorimetrically. Since the reverse reaction showed an optimum pH of 8.5, all reactions were allowed to proceed at this pH. The assay mixture contained 50 mM Tris/HCl, pH 8.5, 300 mM NaCl, 0.1 mM DTT, 0.2 mM $NADP^+$, 1.5 mM glycerate and 1.0 μg of the purified rEhGDH. K_m and V_{max} values were estimated with Hanes–Woolf and Lineweaver–Burk plots.

Chromatographic separation of native EhGDH from *E. histolytica* lysate

E. histolytica trophozoites (≈ 10^7; 200 mg wet weight) resuspended in 1.0 ml of 100 mM Tris/HCl, pH 8.0, 1.0 mM EDTA, 2.0 mM DTT and 2.0 M glycerol containing 10 μg/ml E64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane] and complete mini-EDTA-free protease inhibitor cocktail were subjected to three cycles of freezing and thawing and sonication. After centrifugation at 45 000 *g* for 15 min at 4 °C, the supernatant was filtered through a 0.45 μm cellulose acetate membrane and applied on to a Mono Q 5/5 HR column that had been pre-equilibrated with binding buffer [100 mM Tris/HCl (pH 8.0) containing 1.0 mM EDTA, 2.0 mM DTT, 2.0 M glycerol and 1 μg/ml E64] on AKTA Explorer 10S system (Amersham Biosciences, Tokyo, Japan). After extensive washing, bound proteins were eluted with a linear gradient of 0–1 M NaCl with a flow rate of 1 ml/min. Each fraction (0.5 ml) was analysed for GDH activity. The rEhGDH was fractionated on the same column under identical conditions; native EhGDH and rEhGDH were also separated by gel-filtration chromatography using a 60-cm-long, 1.6-cm-diam. Sephacryl S-300 HR Hiprep pre-packed column. The column was pre-equilibrated, loaded, washed and eluted with the gel-filtration buffer [0.1 M Tris/HCl (pH 8.0)/0.1 M NaCl] at a flow rate of 0.5 ml/min.

Amino acid comparison and phylogenetic analysis

Amino acid sequences that showed significant similarity to EhGDH were obtained from the DDBJ/GenBank/EBI databases by using a BLASTP search. These sequences included GDH, PGDH, HP reductase, glyoxylate reductase and putative D-2-hydroxyacid dehydrogenase from various organisms. Sequence alignments were generated using the program CLUSTAL W version 1.81 [16] with the BLOSUM matrix. Phylogenetic analysis using the Neighbor-Joining method with Kimura's correction was also performed using CLUSTAL W. Phylogenetic

trees were drawn using the Tree View PPC program. The branch lengths in these trees were obtained from the PHYLIP analysis with bootstrap values in 1000 replicates.

RESULTS

Features of the deduced protein primary structure of EhGDH

We obtained a contig sequence (contig 317757) by a homology search of the *E. histolytica* genome database with GDH from bacteria, plants and mammals. The putative *GDH* gene contained a 957 bp ORF, which encodes a protein of 318 amino acids, with a predicted molecular mass of 35.8 kDa and a pI (isoelectric point) of 6.29. No other independent contig was found to contain the *GDH* gene (results not shown), suggesting that this *GDH* gene is present as a single copy. We searched thoroughly for other possible *GDH* genes using this amoebic *GDH* gene in the *E. histolytica* genome database. However, no other possible GDH-related sequence was found, except for a putative *PGDH* gene, which we will report separately.

The amino acid sequence of the *E. histolytica* GDH showed 24–40 % identities with those from bacteria, mammals and plants. The *E. histolytica* GDH showed the highest amino acid identities (38–40 %) with GDH from ε-proteobacteria, including *Campylobacter jejuni* and *Helicobacter pylori*, and the lowest identities (24–26 %) with GDH from higher eukaryotes, including plants and humans (specifically, EhGDH showed identities of 40 % with *C. jejuni* GDH, 38 % with *H. pylori* GDH, 34 % with *Neisseria meningitidis* GDH, 32 % with *Methylobacterium extorquens* GDH, 31 % with *Archaeoglobus fulgidus* GDH, 28 % with mouse and *Schizosaccharomyces pombe* GDHs, 27 % with *Bacillus subtilis* and *E. coli* GDHs, 26 % with human GDH and 24 % with *Hyphomicrobium methylovorum* and cucumber GDHs). The amoebic GDH, similar to GDH from ε- and β-proteobacteria, possesses a 5-amino-acid insertion in the central region (amino acids 130–134 of EhGDH) that is absent in other members of GDH, but lacks two internal insertions (between amino acids 33 and 34 and between amino acids 49 and 50 of EhGDH) that are found in some of other organisms (Figure 1). EhGDH also lacks both an internal 17-amino-acid insertion at the centre and an approximately 27-amino-acid C-terminal extension found in the plant GDH. We also searched for putative *GDH* genes in the genome and expressed sequence tag databases of other parasitic protozoa, including *Leishmania*, *Plasmodium*, *Giardia*, *Trypanosoma* and *Trichomonas*, and the non-parasitic protozoan *Dictyostellium discoideum*, but did not find orthologues in these databases, suggesting that GDH is exclusively present only in this anaerobic enteric parasite among the protists. The consensus sequence Gly-Xaa-Gly-Xaa_2-Gly-Xaa_{17}-Asp (where 'Xaa' denotes 'any amino acid'), involved in the binding of the adenosine portion of NAD^+ [17], was located at residues 154–177 of EhGDH. Asp^{177}, which was conserved among *A. fulgidus*, *H. methylovorum* and cucumber GDHs, was replaced with serine in EhGDH, as observed in GDH from *C. jejuni* and *H. pylori*. All important residues implicated in pairing in the active-site-histidine–carboxylate couple, as predicted from the crystal structure of *H. methylovorum* GDH (Arg^{241}, Glu^{270}, His^{288} and Asp^{265}) [18], and also an arginine residue (Arg^{241}) involved in substrate orientation, were totally conserved in EhGDH (Arg^{235}, Glu^{264}, His^{285} and Asp^{259}). This type of catalytic arrangement is also found in the other enzymes possessing 2-hydroxyacid dehydrogenase activities, i.e. lactate dehydrogenase and malate dehydrogenase, and other D-isomer-specific dehydrogenases (but not formate dehydrogenase) [18,19], serine proteases, thermolysin [20] and phospholipase A_2 [21].

Phylogenetic analysis

Phylogenetic reconstruction was performed with 16 GDH and three PGDH protein primary structures, which were used as the 'out-group', from various organisms using the CLUSTAL W program. The phylogenetic tree (Figure 2) demonstrates that two major groups of GDH represent individual clades, which are well supported by high bootstrap proportions at the nodes (98–99 %). One group comprises *E. histolytica* and proteobacteria, including α-, γ- and ε-proteobacteria; the other group comprises mammals, plants, *B. subtilis*, *E. coli* and *H. methylovorum*. Within the first clade, a group including *E. histolytica* and ε-proteobacteria forms a sister group with α- and γ-proteobacteria; both clades were statistically well supported (96–97 %). A close phylogenetic association between EhGDH and GDHs from ε-proteobacteria, together with the shared insertions and deletions of amino acids described above among these GDHs, suggest that amoebic GDH was probably obtained from an ancestor of modern ε-proteobacteria by lateral transfer, as suggested for other metabolic enzymes in this parasite [22,23].

Purification and characterization of rEhGDH

The rEhGDH protein revealed an apparently homogeneous band of 38 kDa on SDS/PAGE analysis (results not shown), which is consistent with the predicted size of the deduced *EhGDH* protein primary structure with an extra 20 amino acids added at the N-terminus. The purified rEhGDH protein was evaluated as being > 95 % pure, as determined from the Coomassie-Blue-stained SDS/PAGE gel. We first optimized the conditions for enzymic assay, i.e. pH, salt concentrations, requirement for co-factors, bivalent metal ions, DTT and stabilizing reagents. rEhGDH was found to be unstable: the enzyme was totally inactivated when stored without any preservative or additive at room temperature, 4 °C or − 20 °C overnight. When rEhGDH was stored in 50 mM Tris/HCl buffer, pH 8.0, containing 50 % (v/v) glycerol at − 80 °C, rEhGDH remained fully active for more than 1 month. The maximum activity of rEhGDH for the forward reaction was observed at pH 6.0–6.5, which decreased substantially at higher measurements of pH (results not shown). The GDH activity in the reverse reaction was less affected by variations in pH; the activity was found to be highest at a slightly basic pH (pH 8.0–8.5). Substrate inhibition by HP (at 0.2 mM and higher concentrations) was alleviated by the addition of salt (100–400 mM NaCl), as reported for bovine liver GDH [24,25]. Substrate inhibition by HP was more pronounced with NADPH than with NADH for mammalian GDH [7]. In contrast, inhibition of rEhGDH by HP (1.0 mM) was not observed in the presence of higher NaCl concentrations (e.g. 300 mM) at a wide range of NADPH/NADH concentrations (between 40 and 200 μM). The maximum stimulatory effect (2–3-fold) was observed with 250–300 mM NaCl or KCl. The univalent salts, i.e. Na^+ and K^+, were found to be more effective than bivalent salts, such as Mg^{2+} and Ca^{2+} (results not shown), for rEhGDH. rEhGDH showed a 2–3-fold-higher activity with NADPH as compared with NADH when 0.1–0.5 mM HP, 0.2 mM cofactors and 300 mM salt were added, as shown for the mammalian GDH. The addition of neither DTT nor EDTA resulted in any significant change in the activity of rEhGDH.

Kinetic properties of rEhGDH

Owing to the apparent stimulatory effect of salt on rEhGDH activity, as described above, we conducted further kinetic studies

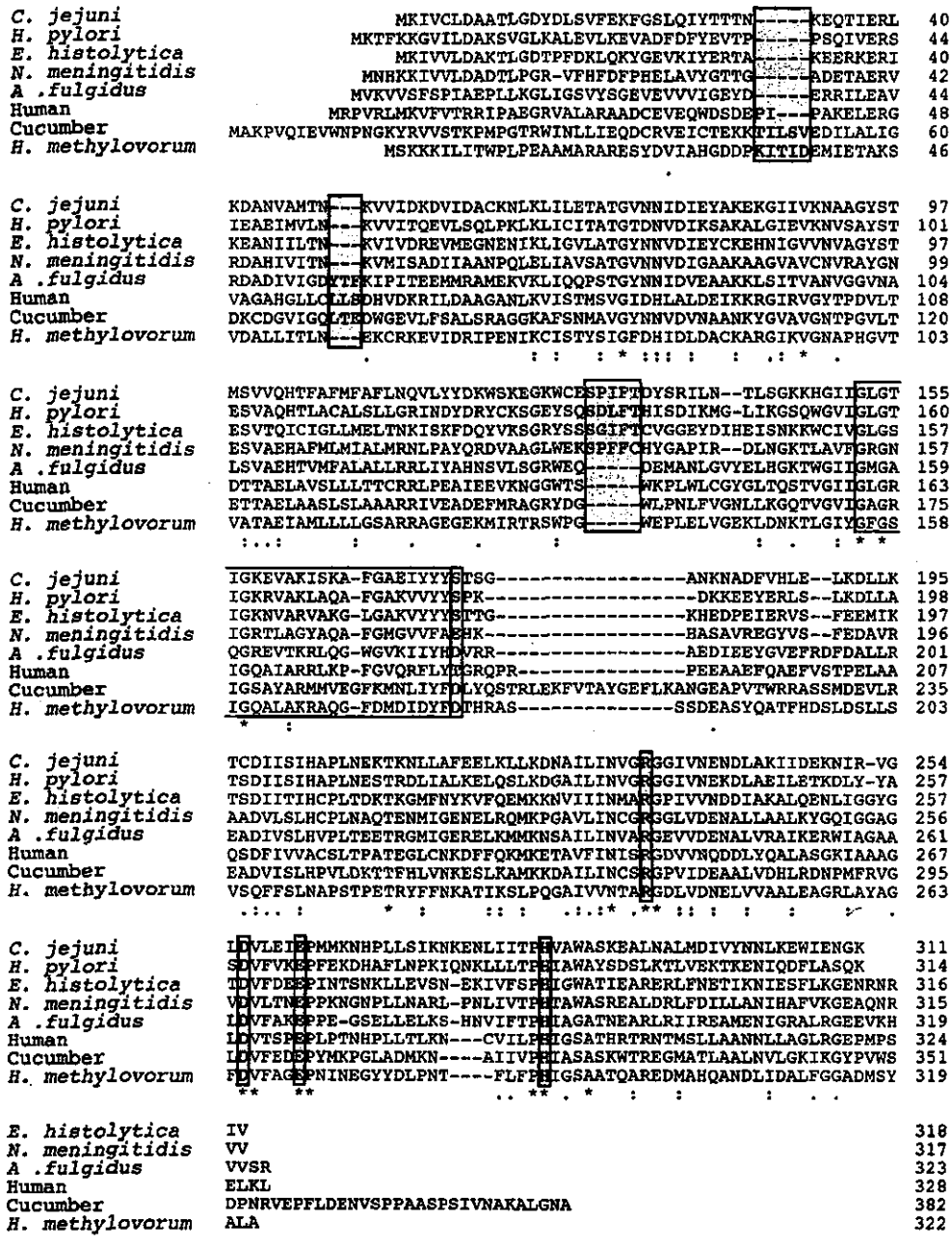

Figure 1 Multiple alignments of deduced amino acid sequences of GDH from *E. histolytica* and other organisms

Protein primary structures were aligned using the CLUSTAL W program (www.ebi.ac.uk/clustalw/). Sequences are *C. jejuni* (accession no. CAB74209), *H. pylori* (AAD07165), *E. histolytica* (AB091511), *N. meningitidis* (AAF40500), *A. fulgidus* (AAB89467), human (NP_036335), cucumber (DEKVG) and *H. methylovorum* (P36234). Asterisks indicate identical amino acids. Dots and colons indicate conserved amino acids substitutions. Dashes indicate computer-generated gaps. An open box indicates the conserved sequence for the NAD-binding domain (Gly-Xaa-Gly-Xaa$_2$-Gly-Xaa$_{17}$-Asp). A shaded rectangle next to an open box depicts the aspartate substitution, as described in the text. Other shaded rectangles indicate either the conserved amino acid that participates in substrate binding (shaded single amino acids) or insertion and deletion found in *E. histolytica* (shaded multiple amino acids).

in the presence of 300 mM NaCl. At saturating concentrations of the substrate, rEhGDH showed a two orders of magnitude (≈100-fold) higher affinity for NADPH than for NADH (Table 1). However, the specific activity was 3.9-fold higher with NADH than with NADPH. The K_m values for D-glycerate and $NADP^+$ in the reverse reaction were calculated to be one order of magnitude higher than those for HP and NADPH in the forward reaction. We did not observe utilization of NAD^+ as a substrate in the reverse reaction, even in the presence of high concentrations of NAD^+ (0.3 mM) and D-glycerate (5–10 mM). Although GDH from mammals and plants was shown to utilize glyoxylate as a substrate to produce glycolate, the amoebic enzyme did not catalyse this reaction (results not shown), and thus appears to be specific for the conversion of HP into glycerate. Both serine and cysteine at 5 mM inhibited recombinant GDH activity by 20–25 %, with a maximum inhibition of approx. 70–80 % observed in the presence of these amino acids at concentrations of 20–25 mM. Other structurally related amino acids (alanine, glycine, valine, methionine and threonine) did not show any inhibition up to 10 mM.

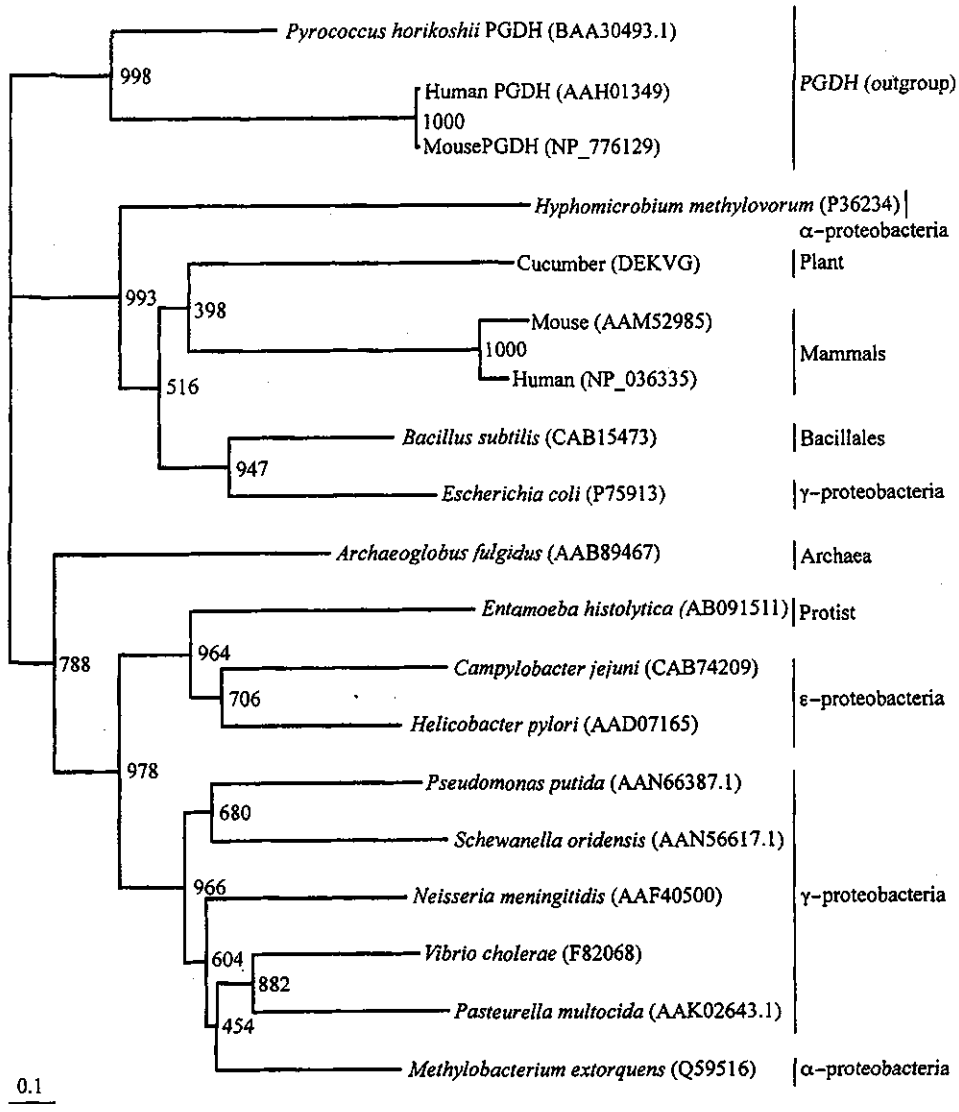

Figure 2 Phylogenetic analysis of GDH proteins from a variety of organisms

A phytogenetic tree was constructed using the CLUSTAL W program and drawn using the Treeview PPC program. A rooted tree with three PGDH sequences used as the out-group is shown. Numbers at the nodes represent bootstrap values of 1000 replicates. Species names and accession numbers of these sequences are indicated in the tree. The scale bar indicates 0.1 substitutions per each amino acid position.

Table 1 Kinetic properties of recombinant EhGDH

Mean values ± S.D. for independent measurements ($n = 3$–5) are shown. ND, not detected.

Substrate/cofactor	pH	K_m (mM)	Specific activity (mmol/min per mg of protein)
Hydroxypyruvate	6.5	61.1 ± 4.86*	15.6 ± 2.28*
NADPH	6.5	1.55 ± 0.43†	1.69 ± 1.29†
NADH	6.5	147 ± 4.95‡	11.4 ± 2.34‡
D-Glyceric acid	8.5	483 ± 58.4§	64.1 ± 26.8§
$NADP^+$	8.5	27.5 ± 2.12‖	45.6 ± 3.06‖
NAD^+	8.5	ND¶	ND¶

* 0.2 mM NADPH was used.
† 0.1 mM HP was used.
‡ 0.5 mM HP was used.
§ 0.2 mM $NADP^+$ was used.
‖ 5 mM D-glyceric acid was used.
¶ 0.3 mM NAD^+ and 5–10 mM D-glyceric acid was used.

Chromatographic separation of the native and recombinant EhGDH activities

In order to correlate native GDH activity in the *E. histolytica* lysate with that of the recombinant enzyme, the lysate from the trophozoites and rEhGDH were subjected to chromatographic separation on a Mono Q anion-exchange column (Figure 3). The *E. histolytica* lysate showed a GDH activity of approx. 3.94 ± 0.25 nmol/min per mg of lysate protein. Thus native GDH represents 0.14–0.2% of the total soluble protein, assuming that native EhGDH and rEhGDH possess comparable specific activities. GDH activity was eluted as a single peak at an identical salt concentration for both native EhGFH and rEhGDH. This finding, together with the fact that the *GDH* gene is present as a single copy, indicates that the *EhGDH* gene we have cloned represents at least the dominant, and probably the sole, gene responsible for GDH activity in the axenic trophozoites. In order to gain an insight into its multimeric composition, the recombinant GDH enzyme was subjected to gel-filtration chromatography.