Molecular Cloning and Characterization of a Protein Farnesyltransferase from the Enteric Protozoan Parasite Entamoeba histolytica*

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Genes encoding α- and β-subunits of a putative protein farnesyltransferase (FT) from the enteric protozoan parasite Entamoeba histolytica were obtained and their biochemical properties were characterized. Deduced amino acid sequences of the α- and β-subunit of E. histolytica FT (EhFT) were 298- and 375-residues long with a molecular mass of 35.6 and 42.6 kDa, and a pI of 5.43 and 5.65, respectively. They showed 24% to 36% identity to and shared common signature domains and repeats with those from other organisms. Recombinant α- and β-subunits, co-expressed in Escherichia coli, formed a heterodimer and showed activity to transfer farnesyl using farnesylpyrophosphate as a donor to human H-Ras possessing a C-terminal CVLxS, but not a mutant H-Ras possessing CVLL. Among a number of small GTPases that belong to the Ras superfamily from this parasite, we identified EhRas4, which possesses CVYA at the C terminus, as a sole farnesyl acceptor for EhFT. This is in contrast to mammalian FT, which utilizes a variety of small GTPases that possess a C-terminal CaaX motif, where X is serine, methionine, glutamine, cysteine, or alanine. EhFT also showed remarkable resistance against a variety of known inhibitors of mammalian FT. These results suggest that remarkable biochemical differences in binding to substrates and inhibitors exist between amebic and mammalian FTs, which highlights this enzyme as a novel target for the development of new chemotherapeutics against amebiasis.

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Ras small GTPases function as a molecular switch of signal transduction in cell proliferation and differentiation (1). Ras small GTPases require a post-translational lipid modification called protein farnesylation in order to become membrane-associated and functional (2). Protein farnesylation, catalyzed by protein farnesyltransferase (FT) (3), which is comprised of two heterologous α- (FTα) and β- (FTβ) subunits, is a major post-translational lipid modification, together with protein geranylgeranylation (3). FT and protein geranylgeranyltransferase type I (GGT-I) catalyze the transfer of the farnesyl and geranylgeranyl group from farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate, respectively, to the cysteine residue of a C-terminal CaaX of small GTPases including Ras, Rac, and Rho, where C is cysteine, α is usually an aliphatic amino acid, and X is any amino acid. Marked differences in substrate specificity have been shown between FT and GGT-I, i.e. FT mainly utilizes, as substrates, small GTPases possessing the terminal CaaX motif, while X is serine, methionine, glutamine, cysteine, or alanine (4), whereas GGT-I prefers proteins with the C-terminal CaaL or CaaF motif (4). Among well-characterized Ras proteins that terminate with a CaaX motif, human H-Ras-CIMF, N-Ras-CVVM, K-RasA-CIM, and Rap2-CNIQ are known to be farnesylated by FT, while Rap1A-CLL, as well as Rho family proteins are geranylgeranylated by GGT-I. It has also been shown that K-RasB-CVIM can be either farnesylated by FT or geranylgeranylated by GGT-I (5). Since constitutively active mutations of Ras proteins have been shown to induce carcinogenesis (6–8), which is suppressed by the inhibition of farnesylation, FT has attracted attention as a target of cancer chemotherapy (9). In addition, several compounds targeting FT have proven promising against African sleeping sickness caused by Trypanosoma brucei and Malaria caused by Plasmodia species (10).

Entamoeba histolytica is an intestinal protozoan parasite, which causes amebic dysentery, colitis, and liver abscesses in humans, and is responsible for an estimated 50 million cases of amebiasis and 40–100 thousand deaths annually (11). A number of small GTPases have been studied including Ras/Rap (12, 13), Rho/Rac (14–18), and Rab (19–21). The completion of the E. histolytica genome database will help us to comprehensively understand the presence and complexity of these small GTPases in this parasite.
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GTases in the ameba. The molecular and cellular functions of some of these small GTases have begun to be unveiled (12, 17, 18, 22). However, the molecular basis of the lipid modification of these small GTases remains largely unknown in this parasite.

In this report, we describe the molecular and biochemical characterization of the α- and β-subunits of FT of E. histolytica (EHFT) using recombinant proteins co-expressed in Esche-

richia coli. We also show that only one amebic Ras protein among the many small GTases tested is farnesylated by EHFT. In addition, we show that the amebic FT exhibits marked resistance to a variety of compounds that are known to inhibit mammalian FT, indicating that the amebic FT possesses distinct biochemical properties from the mammalian FT.

EXPERIMENTAL PROCEDURES

Parasite—Trypanosomes of E. histolytica strain HM-1:IMSS c6 (23) were cultured axenically in BLS-33 medium at 35.5 °C (24).

Chemicals—Recombinant human H-Ras-CVL5 (wild type), H-Ras-CVL6 (mutant type), FPT inhibitor-1 ([E,E]-[2-(diiodophosphoryl)ethyl]-3-oxo-3-[3-(3,11,13-trimethyl-2,6,10-dodecatrienyl)-amino]pro

panic acid, trisodium salt), FPT inhibitor-2 ([E,E]-2-[2-(oxo-2-[3,3,11,13-trimethyl-2,6,10-dodecatrienyl]oxy)amino[ethyl]phosphonic acid, diiodoglutamic acid, glycogen, α-hydroxypropylphosphonic acid, and peptidomimetic inhibitor FTI-276 [N-[2-phenyl-4-N-[2-R-amino-3-mercaptopropylamino][benzoyl]-methionine]} were obtained from EMD Biosciences (San Diego, CA). [3H]FPP (16.1 Ci/mmol) and [3H]geranylgeranylation reaction mixture (23.0 Ci/mmol) were purchased from Amersham Biosciences (Piscataway, NJ).

Identification and Cloning of FTα and FTβ of E. histolytica—We designed oligonucleotide primers to amplify FTα and FTβ protein-coding regions from E. histolytica by PCR based on a homology search using yeast and mammalian FT in the E. histolytica genome data base available at the National Center for Biotechnology Information (www.ncbi.nih.gov/).

The sense primer for EhFTα was 5′-ATGGAAGAAGAAGAAGAATCACATTG-3′ and an antisense primer, 5′-ATGATTGAATTAATTTTTGTTAATATACCC-3′ (for FTα); a sense primer, 5′-ATGGAAATTTGGAAGAGTAGAAGATTAGAACATTTG-3′ and an antisense primer, 5′-TAAAGGCCCAGGGAATCATCAGGCTTAC-3′ (for FTβ). Two sets of initial PCRs were conducted using the respective product of the first reaction as a template. To amplify the FTα protein-coding region (excluding the stop codon) site (italized) and the farnesyl binding site (underlined), a sense primer, 5′-GGGAATCTGGGACAGAAGATCACTGAGTTATTGTTAATGTTAAT-3′, and an antisense primer, 5′-GAAGTTAAACACTCCTGATTTGATTTTTGTTATTAAACCTAACCC-3′, were used. To amplify the FTβ sequence including the stop codon, flanked by the farnesyl binding site (underlined) and a HindIII site (italized), sense, 5′-GGGAATCTGGGACAGAAGATCACTGAGTTATTGTTAATGTTAAT-3′ and antisense, 5′-CCAGTTGGGACAGAAGATCACTGAGTTATTGTTAATGTTAAT-3′ were used. The third round of PCR was conducted using a mixture of the products of the second round, and primers 1 and 2. The resulting 2.1-kb PCR product was digested with BamHI and HindIII and ligated into BamHI- and HindIII-digested pQE10 to construct pEhFTα.

In pEhFTβ, the FTα and FTβ protein-coding regions placed in tandem were presumably translationally coupled, facilitating co-expression of these two subunits at similar levels. An N-terminal histidine tag was also engineered in EhFTα to facilitate purification.

Construction of Plasmids to Express Recombinant Small GTases—A protein-coding region of EhRas1, and EhRacC flanked by additional BamHI and SalI sites (italized), were amplified by PCR using cDNA as a template with the following parameters. An initial step of denaturation at 94 °C for 5 min was followed by 20 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 2 min. A final step at 72 °C for 10 min was used to complete the extension. The amplified DNA fragments were electrophoresed, purified using a GeneClean II kit (BIO101, La Jolla, CA), and cloned into the SacI site of pUC18 using a SureClone Ligation Kit (Amersham Biosciences). Nucleotide sequences were confirmed with an ABI Prism BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA) on an ABI Prism 310 Genetic Analyzer.

Identification and Cloning of Ras Small G Proteins of E. histolytica—To identify substrates for the amebic FT, we searched for puta-
tive Ras homologues in the E. histolytica Genome data base via a TBLASTN search using amebic (EhRas1and EhRas2) and mammalian Ras as inquiry sequences. We identified 8 previously uncharacterized putative full-length Ras genes. The C terminus of these Ras proteins ended with Phe (four genes), Leu (two), Met (one), or Ala (one). The two latter genes encoding putative Ras proteins containing the CSVM- or CVVCLA terminus (identical to EH02830 and EH01021 in the E. histolytica genome data base) were assumed to be good candidates to be farnesylated by the amebic FT, designated EhRas3 and EhRas4, respectively, and characterized further. A protein coding region of EhRas1-4, and EhRacC were amplified by PCR using cDNA as template and appropriate primers based on the sequences in the genome data base.

Sequence Analysis—FTα and FTβ protein sequences from E. histolytica and 9 other organisms, and 20 putative Ras and Ras-related proteins of E. histolytica were retrieved from the TIGR and the National Center for Biotechnology Information data bases (www.ncbi.nih.gov/) using the BLASTP and TBLASTN algorithms. The protein alignment and phylogenetic analyses were performed with the ClustalW version 1.81 (25) (26) using the neighbor-joining (NJ) method (27) with the Blosum matrix created using the ClustalW program (26). Unrooted NJ trees were drawn with Treeview ver.1.6.0 (28). Branch lengths and bootstrap values (1000 replicates) (29) were derived from the NJ analysis.

Construction of a Plasmid to Express Recombinant EhFTα—A plasmid containing the protein-coding regions of FTα (with stop codon), and the biochemical binding sequence (GAGAGTTTAAAATC-3′ and an antisense primer, 5′-ATGATTTAAATTTTTGTTAATATACTACCC-3′ (for FTα); a sense primer, 5′-ATGGAAATTTGGAAGAGTAGAAGATTAGAACATTTG-3′ and an antisense primer, 5′-TAAAGGCCCAGGGAATCATCAGGCTTAC-3′ (for FTβ). Two sets of initial PCRs were conducted using the respective product of the first reaction as a template. To amplify the FTα protein-coding region (excluding the stop codon) site (italized) and the farnesyl binding site (underlined), a sense primer, 5′-GGGAATCTGGGACAGAAGATCACTGAGTTATTGTTAATGTTAAT-3′, and an antisense primer, 5′-CCAGTTGGGACAGAAGATCACTGAGTTATTGTTAATGTTAAT-3′ were used. The third round of PCR was conducted using a mixture of the products of the second round, and primers 1 and 2. The resulting 2.1-kb PCR product was digested with BamHI and HindIII and ligated into BamHI- and HindIII-digested pQE10 to construct pEhFTα.

In pEhFTβ, the FTα and FTβ protein-coding regions placed in tandem were presumably translationally coupled, facilitating co-expression of these two subunits at similar levels. An N-terminal histidine tag was also engineered in EhFTα to facilitate purification.

Construction of Plasmids to Express Recombinant Small GTases—A protein-coding region of EhRas1, and EhRacC flanked by additional BamHI and SalI sites (italized), were amplified by PCR using cDNA as a template and sense and antisense primers: 5′-GAAGTTAAACACTCCTGATTTGATTTTTGTTATTAAACCTAACCC-3′ and 5′-GGGAATCTGGGACAGAAGATCACTGAGTTATTGTTAATGTTAAT-3′.

The resulting plasmids were designed to contain an N-terminal histidine tag to facilitate purification. Plasmids to express an N-terminally fused protein of E. histolytica Rab7 fusion protein were constructed by digesting pQE31-rab2 and a glutathione S-transferase-EhRab5 or glutathione S-transferase-EhRab7 fusion protein were constructed by using pET-43.1a and pGEX-4T-1 with appropriate restriction sites included in PCR primers. The characterization of EhRab5, EhRab7, and EhRab2 and details of the construction of these expression plasmids are described elsewhere (34). Expression and Purification of Recombinant Proteins—Plasmids constructed as described above were introduced into E. coli M15 cells. A 12-ml seed culture was grown overnight at 37 °C in LB medium containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin. The overnight culture was then inoculated into 250 ml of fresh medium containing the antibiotics. The bacteria were grown for 1 h, and then another 4 h in the addition of 1 mM isopropyl-1-thio-

β-D-galactoside to induce protein expression. The bacteria were harvested by centrifuga-
tion at 4,000 × g for 20 min, and the pellet was stored at −20 °C until purification. The recombinant proteins were purified according to the manufacturer’s instructions. Briefly, the bacterial cells were resus-

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...pended in cold lysis buffer, phosphate-buffered saline, pH 8.0, containing 10 mM imidazole and 1% [y-sonicate, sonicated, and centrifuged at 10,000 × g for 20 min. The supernatant was applied to a Ni-NTA-agarose column (Qiagen, Hilden, Germany), washed extensively with the wash buffer containing 20 mM imidazole, and eluted with the lysis buffer containing 250 mM imidazole. The recombinant FT proteins were further purified with Q Sepharose Fast Flow (Amersham Biosciences) at a flow rate of 0.5 ml/min as described (32). The purified recombinant FT and Ras proteins were then dialyzed against the enzyme assay buffer described below and 40 mM Tris-HCl, pH 8, containing 90 mM NaCl, 10 mM MgCl

Protein Analyses—The expression and purity of recombinant protein...
Phe or Leu (4 with Phe, 2 with Leu) at the C terminus, while 1 each has Met or Ala. We tentatively designated proteins possessing Met or Ala as \( \text{Eh} \text{Ras3} \) or \( \text{Eh} \text{Ras4} \), and the other proteins as \( \text{Eh} \text{Ras5} \)–10. The nucleotide sequence of the \( \text{Eh} \text{Ras1} \) cDNA we cloned was identical to that previously reported; the nucleotide sequence of our \( \text{Eh} \text{Ras2} \) cDNA differed at one nucleotide (A368G) from the sequence previously reported (12), resulting in a Y123C substitution.

\( \text{Eh} \text{Ras3} \) and 4 consisted of 210 and 182 amino acids with a calculated molecular mass of 23.9 and 20.6 kDa and a pI of 5.5 and 5.8, respectively. The ClustalW multiple alignment showed that \( \text{Eh} \text{Ras3} \) and 4, together with the previously identified \( \text{Eh} \text{Ras1} \), \( \text{Eh} \text{Ras2} \), \( \text{Eh} \text{Rap1} \), and \( \text{Eh} \text{Rap2} \), share conserved GTP binding consensus sequences (42) and also, at a moderate level, the effector binding region (42) (Fig. 5A).

Percent identity among the \( \text{Eh} \text{Ras1-4} \) and \( \text{Eh} \text{Rap1-2} \) (Fig. 5B) also indicates that \( \text{Eh} \text{Ras3} \) is, together with \( \text{Eh} \text{Ras5} \) and \( \text{Eh} \text{Ras6} \) (\( \text{Eh} \text{Ras5} \) versus \( \text{Eh} \text{Ras1-2} \), 62–66%; \( \text{Eh} \text{Ras6} \) versus \( \text{Eh} \text{Ras1-2} \), 39–41%; \( \text{Eh} \text{Ras5} \) and \( \text{Eh} \text{Ras6} \) were not studied further in the present work) closely associated with \( \text{Eh} \text{Ras1} \).
and EhRas2, showing 48–51% identities, whereas EhRas4 showed only 26–30% identities to EhRas1, EhRas2, EhRap1, and EhRap2. Phylogenetic reconstructions (Fig. 6) confirmed the results of the protein alignment: both EhRas3 and EhRas4, together with EhRas5 and EhRas6, represent new members of the Ras/Rap family. Rac and Rab proteins were categorized to independent clades, whose association was well supported by moderate to high bootstrap values (only representative Rac and Rab proteins were included in this analysis).

Identification of EhRas4 as a Substrate of Eh FT—We tested substrate specificity of Eh FT toward EhRas1-4. We chose EhRas3-4, together with EhRas1-2, as possible candidates for Eh FT substrates because it was previously shown that mammalian and yeast small GTPases with a C-terminal Ser, Met, Gln, Cys, or Ala have a tendency to be farnesylated whereas those containing Phe or Leu at the C-terminal end tend to be geranylgeranylated (4). The recombinant Eh FT showed farnesyltransferase activity toward EhRas4 (1.03 ± 0.005 nmol of FPP/mg of protein), which was comparable to the activity toward human H-Ras-CVLS (Fig. 4). In contrast, Eh FT showed no detectable or only minimal activity toward EhRas1-3. We also tested if EhRab, EhRac, or EhRap are farnesylated by Eh FT. The recombinant Eh FT did not transfer farnesyl to EhRab7, EhRacC, or EhRap2 (data not shown). Furthermore, the recombinant Eh FT did not utilize geranylgeranyl pyrophosphate as a isoprenyl donor to transfer the geranylgeranyl residue to EhRas1, EhRas2, EhRas3, EhRas4, EhRap2, EhRacC, or EhRab7 (data not shown). To confirm that EhRas3 and 4 are capable of binding GTP, a GTP binding assay was conducted. Both EhRas3 and 4 showed comparable GTP-binding activity to EhRas2 and EhRab7 (data not shown). We also assayed for the FT activity in the whole lysate of the E. histolytica trophozoites. Among the 2 human H-Ras and 4 E. histolytica Ras homologues described above, FT activity was detected only against human H-Ras-CVLS and EhRas4 in the whole lysate (data not shown), which excluded the possibility that some other Eh FT protein (or proteins) exists to farnesylate these small GTPase in the amebic lysate.

Kinetic Properties of Eh FT—Lineweaver-Burk plots showed the $K_m$ of recombinant Eh FT for EhRas4 to be 5.13 ± 0.02 μM (plots not shown), significantly higher than that of bovine FT, the $K_m$ of which for Ras-CVLS and Ras-CVIM is 0.63 ± 0.05.
Sensitivity of Recombinant EhFT to Human FT Inhibitors

We examined the sensitivity of EhFT to known FT inhibitors. As shown in Table I, EhFT was virtually insensitive at up to 30 μM except to FPT inhibitor-II and FPT 276 when recombinant EhRas4 was used as substrate. The lack of sensitivity of EhFT to FT inhibitors was not dependent on the substrate; EhFT 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 FT and FT contained well-conserved signature domains such as BET4 and CAL1 domains, and the repeats, i.e. protein prenyltransferase-subunit repeats and prenyltransferase/squalene oxidase repeats (Fig. 1). EhFT forms a heterodimeric

FIG. 4. Substrate specificity of recombinant FT of E. histolytica. The specific activity of the recombinant EhFT was determined from the incorporation of [3H]farnesyl pyrophosphate into the recombinant EhRas1, human wild type H-Ras-CVLS, and mutant type H-Ras-CVLL. Means ± S.E. of quadruplicates are shown.

FIG. 5. Similarity among EhRas1-4 and EhRap1-2. A, sequence alignment of EhRas1-4 and EhRap1-2 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. GXXXGKS and DXXT depict GXXXXGK(S/T) and DXX(T) consensus sequences, respectively. DDBJ/EMBL/GenBank™ accession numbers of these proteins are: EhRas1, AAA21446; EhRas2, AAA21447; EhRas3, AB112425; EhRas4, AB112426; EhRap1, AAA21444; EhRap2, AAA21445; EhRasA, Q24814; EhRasC, Q24816; EhRacG, O76321; EhRab1, BAB40689; EhRab5, BAB40673; EhRab7, BAB40674; EhRab11b, BAB40678; EhRab11c, BAB40679. The other proteins without accession numbers shown in the tree are putative Ras superfamily small GTPases found in the E. histolytica genome database. EhRas7-10 are labeled as EhRas-XXXX, because there is no sufficient evidence to support that these proteins are Ras homologues.

and 0.13 ± 0.03 μm, respectively (35).

Sensitivity of Recombinant EhFT to Human FT Inhibitors— We examined the sensitivity of EhFT to known FT inhibitors. As shown in Table I, EhFT was virtually insensitive at up to 30 μM except to FPT inhibitor-II and FPT 276 when recombinant EhRas4 was used as substrate. The lack of sensitivity of EhFT to FT inhibitors was not dependent on the substrate; EhFT was also insensitive to these inhibitors when H-Ras-CVLS was used as a substrate.

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: EhRas1, AAA21446; EhRas2, AAA21447; EhRas3, AB112425; EhRas4, AB112426; EhRap1, AAA21444; EhRap2, AAA21445; EhRasA, Q24814; EhRasC, Q24816; EhRacG, O76321; EhRab1, BAB40689; EhRab5, BAB40673; EhRab7, BAB40674; EhRab11b, BAB40678; EhRab11c, BAB40679. The other proteins without accession numbers shown in the tree are putative Ras superfamily small GTPases found in the E. histolytica genome database. EhRas7-10 are labeled as EhRas-XXXX, because there is no sufficient evidence to support that these proteins are Ras homologues.

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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 EhFTα and EhFTβ 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 EhRas4-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 EhRas4 as a farnesyl acceptor. In contrast, recombinant EhRas1-3, 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 EhRas4, but not other 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 EhRas3, which terminates with CSV, 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 unrelated 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 EhRas (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, EhRas3–6 were the only ones that contained a terminal CaaX and also showed a closer kinship to EhRas1 and EhRas2 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 EhRas1–3, Rap2, RacC, and Rab7, indicates that EhRas4 protein is the sole Ras protein farnesylated by *Eh*FT. It is also conceivable that EhRas1–3 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 EhRas2-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. EhRas1-2 and EhRap1-2) (12) was presumed to be geranylgeranylated, a study using rabbit reticulocytes lysates (as a source of enzyme) and recombinant EhRas1 and EhRap2 showed that these proteins were not geranylgeranylated, but farnesylated (13). Considering that recombinant *Eh*FT neither farnesylates nor geranylgeranylates EhRas1 and EhRap2, we have to conclude that the results of the previous report (13) are likely a consequence of artificial 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 EhRas4, this protein shares all the conserved domains characteristic of Ras (42) except for incomplete DXAG and DXX(T,G) consensus sequences, and showed a close kinship to other Ras proteins in the phylogenetic reconstruction (Fig. 6). We demonstrated that EhRas4 was capable of binding GTP (data not shown), verifying its identity as a small GTP-binding protein. EhRas1-4 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, EhRas4, in contrast to EhRas1-3, also lacks the so-called polybasic region (Fig. 5A), which was found in K-Ras 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 EhRas1-3 with the polybasic region and high affinity toward EhRas4 without it, possesses a number of substitutions of negatively charged with either positively-charged or neutral amino acids particularly in helices 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. EhRas4-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 (Trp102, Tyr105, Trp108, Tyr130, Tyr205, Phe253, Phe260, Trp119, Tyr361, and Tyr365 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
whereas human FT is equally sensitive to these inhibitors agrees well with the notion that EhFT 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 EhFT is not sensitive to the CaaM peptidomimetic FTI-276 (>1000-fold less than human FT) since EhRas3-CSVM was not a substrate of EhFT. 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 EhFT, leading to the development of new chemotherapeutics against amebiasis.

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