logical characteristic of *E. dispar* by electron microscopy: some concomitant bacteria cells were free and viable in the cytoplasm of *E. dispar*, without being surrounded by a distinct phagosome membrane. This suggests that some bacterial species can survive in the cytoplasm and exist in a symbiotic relationship with *E. dispar*. If the Entner–Doudoroff pathway does indeed function in glycolysis in *E. dispar*, as reported in *E. histolytica* under xenic conditions (Hilker and White, 1959), the reactions upstream in the EMS pathway (Fig. 3) including a reaction regulating the transformation of fructose-6-phosphate to fructose-1,6-bisphosphate or vice versa, usually catalyzed by 6-phosphofructokinase (6-PFK) and fructose-bis-phosphatase (EC 3.1.3.11.) and affecting the both the glycolysis and glycogenesis pathways, may not function well in *E. dispar*. The regulatory reactions in *E. histolytica* are well known to be regulated by a single unique enzyme (ppi-dependent 6-PFK; EC 2.7.1.90.), and the reaction is reversible and has no apparent regulatory function (Reeves et al., 1974, 1976).

The clear growth-promoting effect of autoclaved *C. fasciculata* (or *P. aeruginosa*) indicated that they contain as yet unidentified heat-stable growth-promoting substances for *E. dispar*. If the substances can be identified, YIGADHA-S medium will be improved. Further analyses of the axenically grown *E. dispar* will enable us to further elucidate the biological properties of *E. dispar* that differ from those of pathogenic *E. histolytica*.

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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 (*Eh*FT) 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 CVLS, 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 *Eh*Ras4, which possesses CVVA at the C terminus, as a sole farnesyl acceptor for *Eh*FT. 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. *Eh*FT 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.

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)[1] (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, *a* 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, when *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-CIIM, and Rap2-CNIQ are known to be farnesylated by FT, while Rap1A-CLLL, 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 abscess 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 data base will help us to comprehensively understand the presence and complexity of these small

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB083372 (FTα of *E. histolytica*), AB083373 (FTβ of *E. histolytica*), AB112425 (*Eh*Ras3), and AB112426 (*Eh*Ras4).

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[1] The abbreviations used are: FT, protein farnesyltransferase; GGT-I, protein geranylgeranyltrasferase type I; FTα, α-subunit of farnesyltransferase; FTβ, β-subunit of protein farnesyltransferase; *Eh*FT, farnesyltransferase of *E. histolytica*; FPP, farnesyl pyrophosphate; NTA, nitrilotriacetic acid; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate, NJ, neighbor-joining.

GTPases in the ameba. The molecular and cellular functions of some of these small GTPases have begun to be unveiled (12, 17, 18, 22). However, the molecular basis of the lipid modification of these small GTPases 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* (*Eh*FT) using recombinant proteins co-expressed in *Escherichia coli*. We also show that only one amebic Ras protein among the many small GTPases tested is farnesylated by *Eh*FT. 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—Trophozoites of *E. histolytica* strain HM-1:IMSS cl6 (23) were cultured axenically in BI-S-33 medium at 35.5 °C (24).

Chemicals—Recombinant human H-Ras-CVLS (wild type), H-Ras-CVLL (mutant type), FPT inhibitor-I {(*E,E*)-2-[(dihydroxyphosphinyl)-methyl]-3-oxo-3-[(3,7,11-trimethyl-2,6,10-dodecatrienyl)-amino]propanic acid, trisodium salt}, FPT inhibitor-II {(*E,E*)-2-[2-oxo-2-[[(3,7,11-trimethyl-2,6,10-dodecatrienyl)oxy]amino]ethyl]phosphonic acid, disodium salt}, Gliotoxin, α-hydroxyl farnesylphosphonic acid, and a peptidomimetic inhibitor FTI-276 {*N*-[2-phenyl-4-*N*-[2(R)-amino-3-mercaptopropylamino]benzoyl]-methionine]} were obtained from EMD Biosciences (San Diego, CA). [^{3}H]FPP (16.1 Ci/mmol) and [^{3}H]geranylgeranyl pyrophosphate (23.0 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA), and [^{35}S]GTPγS (1,173 Ci/mmol) was obtained from Amersham Biosciences (Piscataway, NJ). Restriction endonucleases and modifying enzymes were purchased from Takara Biochemical (Tokyo, Japan). The other chemicals and reagents used were from either Sigma-Aldrich Fine Chemicals (Tokyo, Japan) or Wako Pure Chemical Industries (Osaka, Japan) unless otherwise mentioned and of the highest purity available.

cDNA Library of E. histolytica—A trophozoite cDNA library of *E. histolytica* was constructed using the poly(A)$^+$ RNA and λZAP II phage (Stratagene, La Jolla, CA) as described previously (25).

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 Institute for Genomic Research (www.tigr.org/tdb/). The sense primer for *EhFTα* was 5′-ATGGAAGAAGACGAAGAAATCACATTTG-3′. Sense and antisense primers for *EhFTβ* were 5′-ATGGAAATTGAAGAAGTAGAAGTAGAAACTGTTAC-3′ and 5′-TTAGAGCGAACGGAAATACTCACAACGCTTATC-3′, respectively. Since we did not find a sequence containing the C terminus of FTα in the data base, we used T7 primer, located downstream of the cloning site of our cDNA library, to amplify the FTα-coding region. PCR was performed using a one-hundredth volume of the cDNA phage lysate as template with the following parameters. An initial step of denaturation at 94 °C for 3 min was followed by 30 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 SmaI 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 putative Ras homologues in the *E. histolytica* Genome data base via a TBLASTN search using amebic (*Eh*Ras1and *Eh*Ras2) 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 CVVA-C 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 *Eh*Ras3 and *Eh*Ras4, respectively, and characterized further. A protein coding region of *Eh*Ras1-4, and *Eh*RacC 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 ClustalW version 1.81 (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 EhFT—A plasmid containing the protein-coding regions of FTα (without the stop codon), FTβ (with the stop codon), and the ribosome binding sequence (GAGGAGTTTTAACTT) between them were constructed by three rounds of PCR using the recombinant approach (30, 31). Briefly, two sets of initial PCRs were conducted to amplify the FTα and FTβ protein-coding region using a sense primer, 5′-ATGGAAGAAGACGAAGAAATCACATTTG-3′ and an antisense primer, 5′-ATGATTAGTAATTTTTGTTAAATACCAATCCC-3′ (for FTα); a sense primer, 5′-ATGGAAATTGAAGAAGTAGAAGTAGAAACTGTTAC-3′ and an antisense primer, 5′-TAAGAGCGAACGGAAATACTCACAAGCCTTATC-3′ (for FTβ). Two sets of second 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), flanked by a BamHI site (italicized) and the ribosome binding site (underlined), a sense primer, 5′-GGA*GGATCC*CATGGAAGAAGACGAAGAAATCACATTTG-3′ (primer 1) and an antisense primer, 5′-AAGTTAAAACTCCTCATGATTAGTAATTTTTGTTAAATACCAATCCC-3′, were used. To amplify the FTβ sequence including the stop codon, flanked by the ribosome binding site (underlined) and a HindIII site (italicized), sense, 5′-GAGGAGTTTTAACTTATGGAAATTGAAGAAGTAGAAGTAGAAACTGTTAC-3′ and antisense, 5′-CCAA*AGCTT*TAAGAGCGAACGGAAATACTCACAAGCCTTATC-3′ (primer 2) were used. The third round of PCR was conduced 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-double digested pQE31 to construct pEhFTαβ. In pEhFTαβ, 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 GTPases—A protein-coding region of *Eh*Ras1-4, and *Eh*RacC flanked by additional BamHI and SalI sites (italicized), were amplified by PCR using cDNA as a template and sense and antisense primers: 5′-GGA*GAATCC*CATGACTGCCAATACATATAAATTAGTTATG-3′ and 5′-CC*AGTCGAC*TTAGAACATTATGCATTTCTTTTCTTTCTT-3′ (*Eh*Ras1); 5′-GGA*GAATCC*CATGACTACAAATACTTATAAATTAGTTATGCTTG-3′ and 5′-CCA*GTCGAC*TTATAACAATTCACACTTTGATTTAGAAGG-3′ (*Eh*Ras2); 5′-GGA*GAATCC*CATGAGTTTAAAAAGAATTGTTATGCTTGGA and 5′-CCA*GTCGAC*TTACATAACAGAACATCCAATTTTCTTATA-3′ (*Eh*Ras3); 5′-GGA*GAATCC*CATGAACTCAACAATAAAAAGAATATCTGTT-3′ and 5′-CCA*GTCGAC*TTAAGCAACCACACATGAAGTATTATTCTC-3′ (*Eh*Ras4); 5′-GGA*GAATCC*CATGAGTGAAAAACCCACATCAAT-3′ and 5′-CCA*GTCGAC*TTATAAAAGAGCACACTTTGACCTTTG-3′ (*Eh*RacC), where restriction sites are italicized. PCR products were electrophoresed, purified, and cloned into BamHI- and SalI double-digested pQE31 plasmid to obtain pEhRas1-4 and pEhRacC. The resulting plasmids were designed to contain an N-terminal histidine tag to facilitate purification. Plasmids to express an N-terminal Nus fusion protein of *Eh*Rap2 and a glutathione S-transferase-*Eh*Rab5 or glutathione *S*-transferase-*Eh*Rab7 fusion protein were constructed using pET-43.1a and pGEX4T-1 with appropriate restriction sites included in PCR primers. The characterization of *Eh*Rab5, *Eh*Rab7, and *Eh*Rap2 and details of the construction of these expression plasmids are described elsewhere.

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 after the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside to induce protein expression. The bacteria were harvested by centrifugation 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-

pended in cold lysis buffer, phosphate-buffered saline, pH 8.0, containing 10 mM imidazole and 1% lysozyme, 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 $MgCl_2$, and 2 mM dithiothreitol and stored with 20 and 50% glycerol, respectively, at −80 °C until use. Protein concentrations were determined by the method of Bradford (33) using Protein Assay CBB solution (Nacalai Tesque, Kyoto, Japan). Bovine serum albumin was used as the protein standard.

Protein Analyses—The expression and purity of recombinant proteins were evaluated by standard SDS-PAGE as described (34). To prepare *E. histolytica* extracts, trophozoites were washed three times with ice-cold phosphate-buffered saline, resuspended at 10^7/ml in phosphate-buffered saline containing a proteinase inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 1 nM trypsin inhibitor, 100 μM *trans*-epoxysuccinyl-L-leucylamino-(4-guanidino)butane, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml *N*-α-p-tosyl-L-lysine chloromethyl ketone hydrochloride, and 1 mM benzamidine hydrochloride), and subjected to 3 cycles of freezing and thawing. After centrifugation at 10,000 × *g* for 10 min, the supernatant was subjected to further analyses.

Enzyme Assays—The enzymatic activity of recombinant FT and the whole lysate of *E. histolytica* trophozoites were assayed for incorporation of [^{3}H]farnesyl pyrophosphate or [^{3}H]geranylgeranyl pyrophosphate into the recombinant small GTPases prepared as described above, human H-Ras-CVLS, or H-Ras-CVLL. The assay was performed essentially as described previously (35) with minor modifications. Briefly, in standard assays, the reaction mixture contained, in a total volume of 50 μl, 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 20 μM $ZnCl_2$, 5 mM dithiothreitol, 0.1% polyethylene glycol 20,000, 187 nM [^{3}H]FPP (3 μCi/ml), or [^{3}H]geranylgeranyl pyrophosphate (3 μCi/ml), and 240 to 750 μM of the purified recombinant FT or 1.9 mg/ml of the *E. histolytica* lysate. The reaction was initiated by the addition of either the recombinant enzyme or cell extracts, run at 30 °C for 20 min, and terminated by the addition of 200 μl of 10% HCl in ethanol. The quenched reactions were allowed to stand at room temperature for 15 min. After the addition of 200 μl of 100% ethanol, the reactions were vacuum-filtered through glass filter GF/C (Whatman, Maidstone, UK) using a Sampling Manifold (Millipore Corp., Bedford, MA). The filters were washed with 4 ml of 100% ethanol and then subjected to scintillation counting (LS 6,000IC, Beckman Coulter, Fullerton, CA). The K_m value was calculated from Lineweaver-Burk plots. FT assays were also conducted in the presence of known FT inhibitors: farnesylpyrophosphate analogues (FPT inhibitor-I, FPT inhibitor-II, Gliotoxin, α-hydroxyl farnesylphosphonic acid) and a peptidomimetic inhibitor (FTI-276) under the condition described above.

Guanine Nucleotide Binding Assays—GTP binding activity was measured using [^{35}S]GTPγS (Amersham Biosciences) and a nitrocellulose filter (Millipore HA filter, Millipore Corporation) as previously described (36).

RESULTS

Features of FTα and FTβ of E. histolytica—Nucleotide sequences of *Eh*FTα and *Eh*FTβ obtained by PCR were identical to sequences available from the genome data base (EH02829 and EH04188, respectively). The putative protein-coding region of *Eh*FTα and *Eh*FTβ, consisting of 894 and 1,125 bps, encodes proteins of 298 and 375 amino acids with a calculated molecular mass of 35.6 and 42.6 kDa and a pI of 5.4 and 5.7, respectively. A search for previously identified domains and motifs (37) using the NCBI Conserved Domain Search revealed that both *Eh*FTα and *Eh*FTβ contained well conserved signature domains shared by other organisms. *Eh*FTα contained one BET4 domain and five "protein prenyltransferase α-subunit repeats"; *Eh*FTβ possessed one CAL1 domain and five "prenyltransferase and squalene oxidase repeats" (Fig. 1). The deduced protein sequences of *Eh*FTα and *Eh*FTβ were aligned with those from other organisms using the ClustalW program (Fig. 1). Both *Eh*FTα and *Eh*FTβ were the smallest in size, and lack any recognizable secretory signal sequence, an organelle targeting signal, and any domain implicated in membrane association including the transmembrane domain and myristylation signal. *Eh*FTα and *Eh*FTβ also lack the N-terminal extension of 15–58 amino acids found in these subunits from other organisms. *Eh*FTα showed 29, 30, 27, and 24% positional identity with FTα of human, *Arabidopsis thaliana, Saccharomyces cerevisiae*, and *T. brucei*, respectively; *Eh*FTβ revealed 36, 35, 28, and 31% positional identity with the FTβ of these organisms, respectively. All the residues implicated to be essential for catalysis (Fig. 1, *A* and *B*) (39–41) are conserved in both *Eh*FTα and *Eh*FTβ.

Phylogenetic Analysis of EhFTα and FTβ—Phylogenetic trees of *Eh*FTα and *Eh*FTβ were constructed (Fig. 2). Neither the α- nor β-subunit of the amebic FT showed statistically significant (*i.e.* supported with high bootstrap values) affinity to those from other organisms while trypanosomal, mammalian, and plant proteins encoding α- and β-subunits formed well supported independent clades, representing distinct groups. These results were compatible with the notion that both subunits of *Eh*FT developed independently from other eukaryotes, suggestive of the presence of unique biochemical properties of *Eh*FT (see below).

Expression of EhFT in E. coli—A complex consisting of *Eh*FTα and *Eh*FTβ was expressed and purified as described under "Experimental Procedures." Purified proteins revealed two major proteins with an equal intensity of an apparent molecular mass of 38 and 43 kDa (Fig. 3), which likely correspond to *Eh*FTα and *Eh*FTβ, respectively. The apparent molecular mass of α- and β-subunits agreed well with a theoretical value of 37.6 kDa with the N- and C-terminal addition of MRGSHHHHHHTDP and EEF, respectively, for the recombinant *Eh*FTα and 42.6 kDa for *Eh*FTβ. When histidine-tagged *Eh*FTα or *Eh*FTβ were expressed separately, the apparent molecular mass of these proteins agreed well with their identity (data not shown). Densitometric quantitation of these two bands also supported that they contain an equal number of protein molecules (data not shown). Gel filtration chromatography of the recombinant *Eh*FT using Sephacryl S-300 revealed a molecular mass of about 80 kDa (data not shown). Thus, the recombinant *Eh*FTα and *Eh*FTβ were present as a stable dimer during the process of purification by Ni-NTA agarose and Q Sepharose Fast Flow chromatography (Fig. 3). After the purification, recombinant *Eh*FT was estimated to be >95% pure by densitometric quantitation (data not shown), and further utilized for enzymatic assays.

Demonstration of FT Activity of the Recombinant EhFT against Human Ras Proteins—When assayed for incorporation of [^{3}H]farnesyl pyrophosphate, the recombinant *Eh*FT showed FT activity [1.03 ± 0.012 nmol of FPP/mg of protein (mean ± S.E.)] against human recombinant wild-type H-Ras-CVLS, whereas it showed ~20-fold less activity against mutant H-Ras-CVLL (0.05 ± 0.01 nmol of FPP/mg of protein) (Fig. 4), which was previously shown to be predominantly geranylgeranylated by human GGT-I. The addition of EDTA (10 mM) to the reaction mixtures completely abolished the enzymatic activity of the recombinant *Eh*FT (data not shown), suggesting, as shown for mammalian and yeast FT, that *Eh*FT also requires Zn^{2+} and Mg^{2+} for its activity.

Identification and Phylogenetic Analysis of the Novel Ras Proteins in E. histolytica—To identify the protein substrates of *Eh*FT in the parasite, we searched for putative Ras proteins in the genome data base based on homology to the *Eh*Ras1 and human H-Ras. In addition to *Eh*Ras1, *Eh*Ras2, *Eh*Rap1, and *Eh*Rap2, which have already been reported (12), we found 8 additional putative ras proteins previously uncharacterized, with the C-terminal *CaaX* motif. Of these 8 proteins, 6 possess

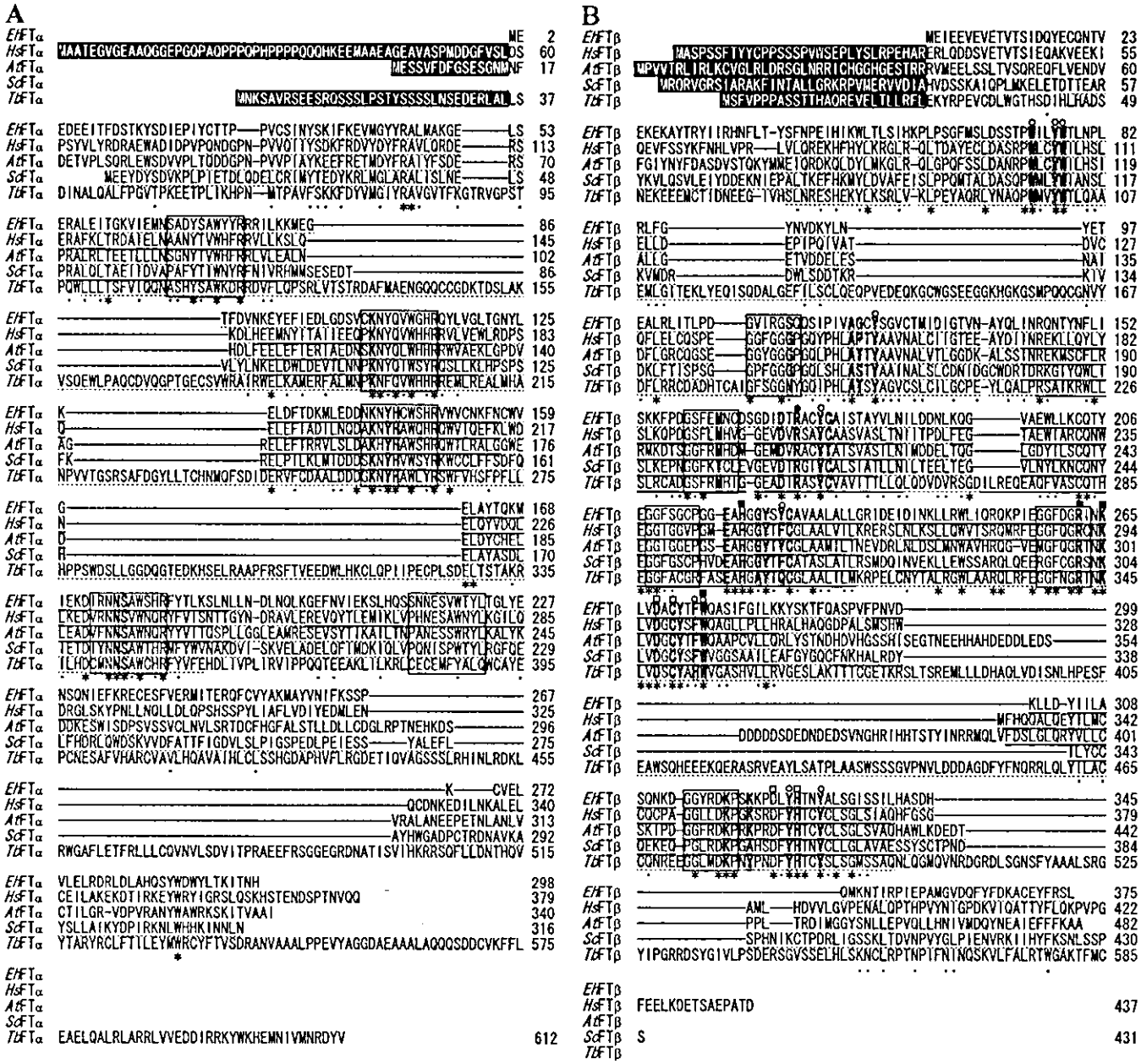

FIG. 1. **Alignment of the deduced amino acid sequences of α- (*A*) and β- (*B*) subunits of protein farnesyltransferase of *E. histolytica* with those from other organisms.** *Eh, Entamoeba histolytica*; *Hs, Homo sapiens*; *At, A. thaliana*; *Sc, S. cerevisiae*; *Tb, T. brucei*. *Asterisks* (*) under the alignments indicate identical amino acid residues and *dots* (.) indicate conserved amino acid substitutions. BET4 (in *A*) and CAL1 (in *B*) domains detected by the NCBI Conserved Domain Search are *dotted-underlined* (partially *solid underlined*). The prenyltransferase α-subunit repeats and the prenyltransferase/squalene oxidase repeats, also detected by the search, are indicated by *solid underline* in *A* and *B*, respectively. The regions corresponding to the motif P*X*N*Y**XX*WYR (37), previously found in the turns connecting two helices of the coiled-coil, in FTα and a glycine-rich motif GGF*XGXXP* (37), corresponding to the loop regions that connect the C termini of the peripheral helices with the N termini of the core helices in the barrel (38) in FTβ are *boxed*. All amino acids implicated in catalysis by crystallographic and mutational studies of mammalian FTs (38–41) are shaded. Aromatic amino acids located in the hydrophobic cleft at the center of the α-α barrel implicated in the interaction with the farnesyl residue are marked with *open circles* (38). Arg^{202} implicated in the binding of the essential C-terminal carboxylate of the protein substrate is marked with a *filled circle* (39). Amino acids implicated in the coordination of zinc are marked with *open squares* (40). Amino acids implicated in the binding and utilization of FPP, shown by a mutational analysis (39) are marked with *filled squares*. N-terminal extensions absent in *Eh*FTα and *Eh*FTβ are also *boxed*. DDBJ/EMBL/GenBank™ accession numbers are given in Fig. 2.

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 *Eh*Ras3 or *Eh*Ras4, and the other proteins as *Eh*Ras5–10. The nucleotide sequence of the *Eh*Ras1 cDNA we cloned was identical to that previously reported; the nucleotide sequence of our *Eh*Ras2 cDNA differed at one nucleotide (A368G) from the sequence previously reported (12), resulting in a Y123C substitution. *Eh*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 *Eh*Ras3 and 4, together with the previously identified *Eh*Ras1, *Eh*Ras2, *Eh*Rap1, and *Eh*Rap2, share conserved GTP binding consensus sequences (42) and also, at a moderate level, the effector binding region (42) (Fig. 5*A*).

Percent identity among the *Eh*Ras1-4 and *Eh*Rap1-2 (Fig. 5*B*) also indicates that *Eh*Ras3 is, together with *Eh*Ras5 and *Eh*Ras6 (*Eh*Ras5 *versus Eh*Ras1-2, 62–66%; *Eh*Ras6 *versus Eh*Ras1-2, 39–41%; *Eh*Ras5 and *Eh*Ras6 were not studied further in the present work) closely associated with *Eh*Ras1

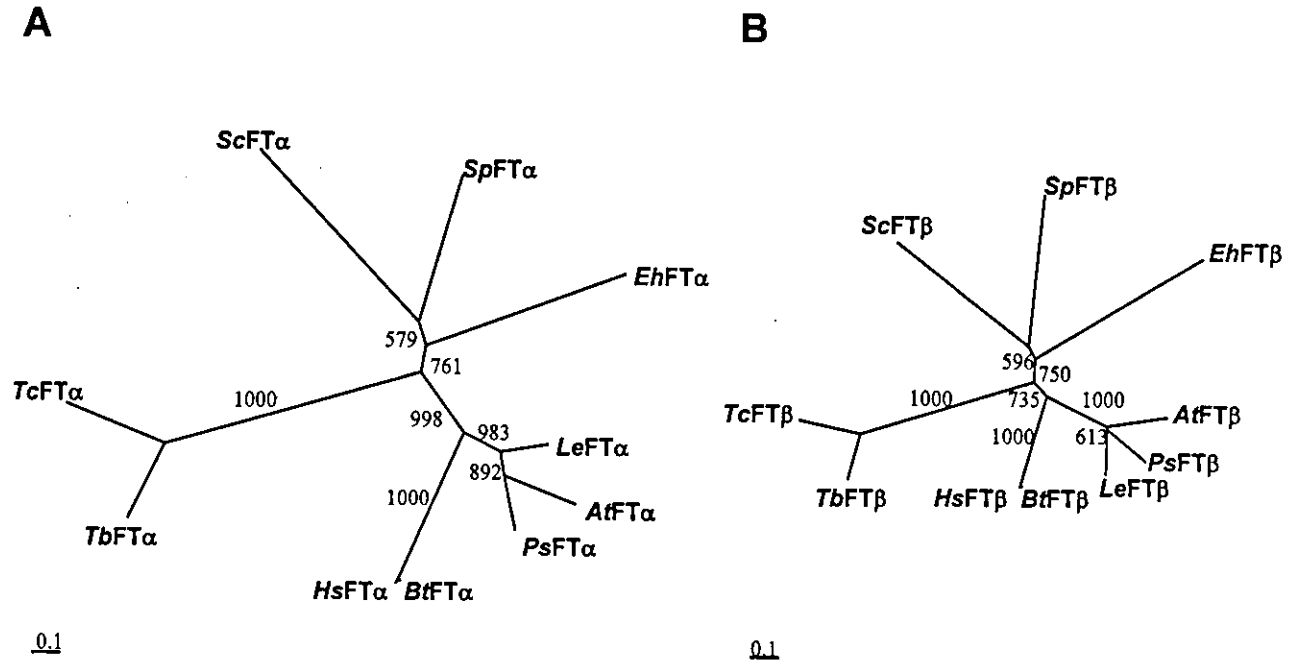

FIG. 2. **Phylogenetic trees of FTα (*A*) and FTβ (*B*).** Only FTα and FTβ protein sequences that were previously characterized enzymologically were included. The *trees* were constructed by Neighbor-joining distance analysis using the ClustalW and TreeView programs. *Line lengths* indicate distances between nodes. The *bar* represents a distance of 0.1 amino acid changes per site. Bootstrap values for 1,000 replicates are shown at nodes. Abbreviations are: *Eh*, *E. histolytica* (DDBJ/EMBL/GenBank™ number of FTα and FTβ, AB083372, AB083373); *Sc*, *S. cerevisiae* (P29703, P22007); *Sp*, *Schizosaccharomyces pombe* (O60052, O13782); *Tb*, *T. brucei* (AAF73919, AAF73920); *Tc*, *T. cruzi* (AAL69904, AAL69905); *At*, *A. thaliana* (Q9LX33, AAF74564); *Ps*, *Pisum sativum* (O24304, Q04903); *Le*, *Lycopersicon esculentum* (P93277, AAC49666); *Bt*, *Bos taurus* (NP_803464, P49355). *Hs*, *Homo sapiens* (NP_002018, NP_002019).

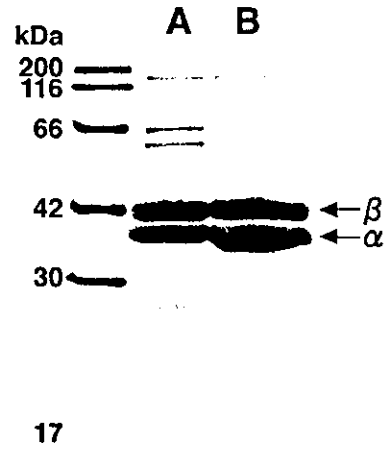

FIG. 3. **SDS-PAGE analysis of the purified recombinant FT of *E. histolytica*.** Both *Eh*FT subunits were coexpressed in *E. coli*, copurified on Ni-NTA agarose (*lane A*), or further purified with anion exchange Q Sepharose Fast Flow (*lane B*). Samples were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue.

and *Eh*Ras2, showing 48–51% identities, whereas *Eh*Ras4 showed only 26–30% identities to *Eh*Ras1, *Eh*Ras2, *Eh*Rap1, and *Eh*Rap2. Phylogenetic reconstructions (Fig. 6) confirmed the results of the protein alignment: both *Eh*Ras3 and *Eh*Ras4, together with *Eh*Ras5 and *Eh*Ras6, 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 *Eh*Ras1-4. We chose *Eh*Ras3-4, together with *Eh*Ras1-2, 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 *Eh*Ras4 (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 *Eh*Ras1-3. We also tested if *Eh*Rab, *Eh*Rac, or *Eh*Rap are farnesylated by *Eh*FT. The recombinant *Eh*FT did not transfer farnesyl to *Eh*Rab7, *Eh*RacC, or *Eh*Rap2 (data not shown). Furthermore, the recombinant *Eh*FT did not utilize geranylgeranyl pyrophosphate as a isoprenyl donor to transfer the geranylgeranyl residue to *Eh*Ras1, *Eh*Ras2, *Eh*Ras3, *Eh*Ras4, *Eh*Rap2, *Eh*RacC, or *Eh*Rab7 (data not shown). To confirm that *Eh*Ras3 and 4 are capable of binding GTP, a GTP binding assay was conducted. Both *Eh*Ras3 and 4 showed comparable GTP-binding activity to *Eh*Ras2 and *Eh*Rab7 (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 *Eh*Ras4 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 EhFT—Lineweaver-Burk plots showed the K_m of recombinant *Eh*FT for *Eh*Ras4 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

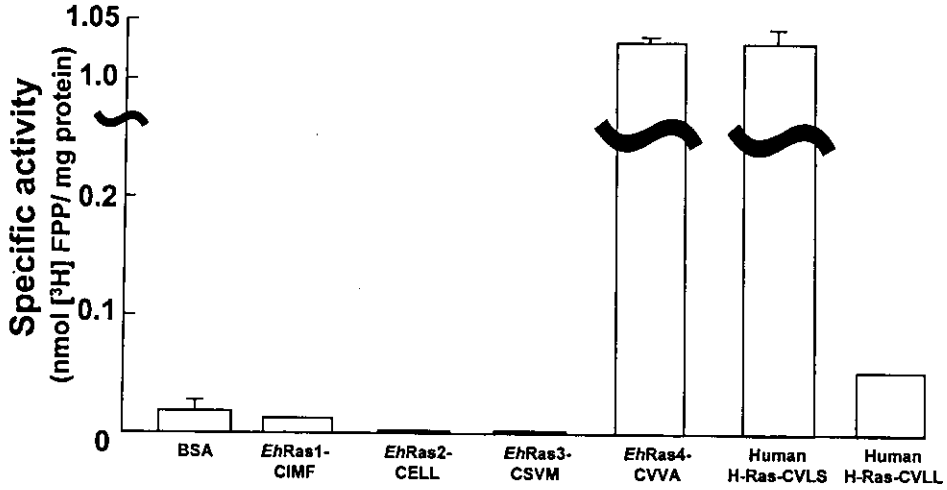

FIG. 4. **Substrate specificity of recombinant FT of *E. histolytica*.** The specific activity of the recombinant *Eh*FT was determined from the incorporation of [^{3}H]farnesyl pyrophosphate into the recombinant *Eh*Ras1, human wild type H-Ras-CVLS, and mutant type H-Ras-CVLL. Means ± S.E. of quadruplicates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

A

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                    GXXXXGKS                DXT
EhRas1   MTANTYKLVMLGGGAVGKSAITVQLVSGHFVQIYDPTIEDSYRTSISVDGEMVSLDIL  58
EhRas2   MTTNTYKLVMLGGGAVGKSAITVQLVSGHFVQIYDPTIEDSYRTSISVDGEMVSLDIL  58
EhRas3     MSLKRIVMLGTGAVGKSAITLQYVSHYFASDYNPTIEDSFRTSITIGDDNYPIEIL  56
EhRas4   MNSTIKRISVVLLGDHGVGKSSVAIRFATNVFMSEYSIHPKQIQVSRVSVDGTDYDIGVI  60
EhRap1     MPVKDYKIVVLGSGADGKSSITVRFVQGIFLVKYDPTIEDSYRKQLDLDGQQYVLEIL  58
EhRap2     MPVKDYKIVVLGSGAVGKSSITVRFVQGIFLVKYDPTIEDSYRKQLDLDGQQYVLEIL  58
                 DXAG
EhRas1   DTAGQEEYSALRDQYMRSGDGYVIVYSITSTTSFLEANGFREQLYRVLDKDVSEHVSIAL 118
EhRas2   DTAGQEEYSALRDQYMRSGDGYVIVYSITSTTSFLEANGFREQLYRVLDKDVSEHVSIAL 118
EhRas3   DTAGQEEFDALKDTYIRSGDGFAIVYSITSLNTFLEANEIRERIYRVLDKDPSQHIPICL 116
EhRas4   DTN—ATMTIQNDDLIMKGDAFVVMYSVTNKNSFLHLPLYLNRIYELRK-DHSKTIPIIL 117
EhRap1   DTAGTEQFTAMRDLYMKTGQGFVLVYSIIAQSTYNDLDPIHDQIVRVRD—TEDVPIIV 115
EhRap2   DTAGTEQFTAMRDLYMKTGQGFVLVYSIMAQSTYNDLDPIHDQIVRVRD—TENVPIIV 115
                NKXD                EXSAX
EhRas1   CGNKCDLESERQVQTNEAKNLAEQWKVLFFETSAKAKINITETFQALVKDIKANRAATEP 178
EhRas2   CGNKYDLENERQVETAEAKKLADEWKVLFFETSAKAKINITETFQALVKDIKVNRVSSEP 178
EhRas3   VGNKSDLESERIVSKEQAEELACLWGISFIETSAKKKSNINELYQIIMKDIIDTKTKEDE 176
EhRas4   VGNKCDEK-RREVIYESGQLFSDSISCLFMETSALDYTNINHLFDIIIREMVSFHPVENN 176
EhRap1   VGNKCDLESQRIVSQDDGKALADKYGADFLEVSAKAETRISDIFTTLIKRINSSNGNKKP 175
EhRap2   VGNKCDLESQRIVSQDDGKALADKYGAEFLEVSAKAETRISDIFTTLIKRINAMNQTKPK 175

EhRas1   SATTAEANDSKGKKDKKEKKEKKCIMF                                  205
EhRas2   -TASIEPTDTKGKKERKPSKSKCELL                                   203
EhRas3   EKKRLEEHESITKKSSKKDRDGKKFYKKIGCSVM                          210
EhRas4   ———T———SCVVA                                                182
EhRap1   ———D———HAHHCSLL                                             184
EhRap2   ———K———KSGGCSLF                                             184
```

B

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

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

and 0.13 ± 0.03 μM, respectively (35).

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

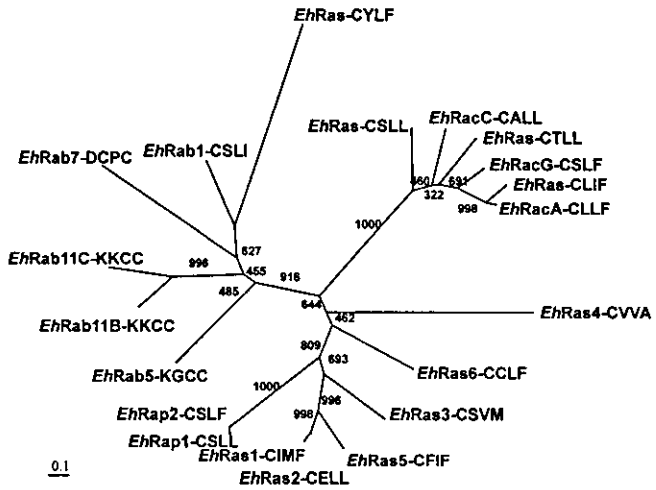

FIG. 6. **Phylogenetic tree of representative members of small GTPases of *E. histolytica* constructed by the Neighbor-joining method.** DDBJ/EMBL/GenBank™ accession numbers of these proteins are: *Eh*Ras1, AAA21446; *Eh*Ras2, AAA21447; *Eh*Ras3, AB112425; *Eh*Ras4, AB112426; *Eh*Rap1, AAA21444; *Eh*Rap2,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

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

DISCUSSION

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

TABLE I
Inhibition of recombinant EhFT by inhibitors of human FT

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

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

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

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

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

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

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

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

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

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Evaluation of Recombinant Fragments of *Entamoeba histolytica* Gal/GalNAc Lectin Intermediate Subunit for Serodiagnosis of Amebiasis

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

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

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

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MATERIALS AND METHODS

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

TABLE 1. List of oligonucleotide primers used in this study

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

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

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

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

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

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

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

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

RESULTS

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

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

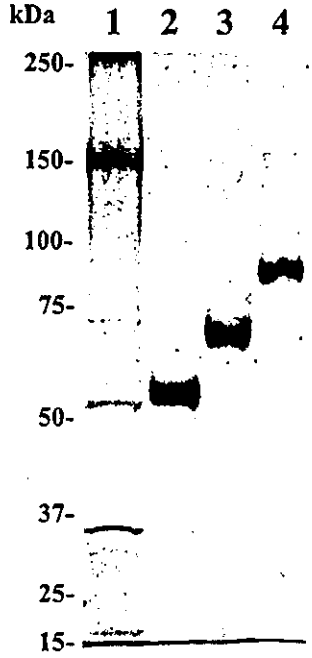

FIG. 1. SDS-PAGE analyses of recombinant Igl. A total of 4 μg of refolded proteins was electrophoresed in a 7.5% gel under reducing conditions. Protein bands were visualized with Coomassie brilliant blue. Lane 1, F-Igl; lane 2, N-Igl; lane 3, M-Igl; lane 4, C-Igl. The numbers to the left indicate molecular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

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

DISCUSSION

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

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

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

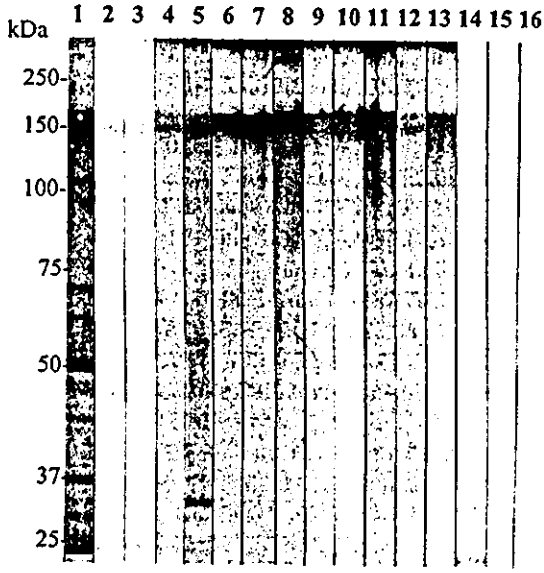

FIG. 2. Western immunoblot analysis of human sera reactivity with F-Igl. Purified F-Igl was subjected to SDS-PAGE in a 7.5% gel under reducing conditions and then transferred to polyvinylidene difluoride membranes. Protein bands of lane 1 were stained with Coomassie brilliant blue. Lanes 2 to 5, sera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

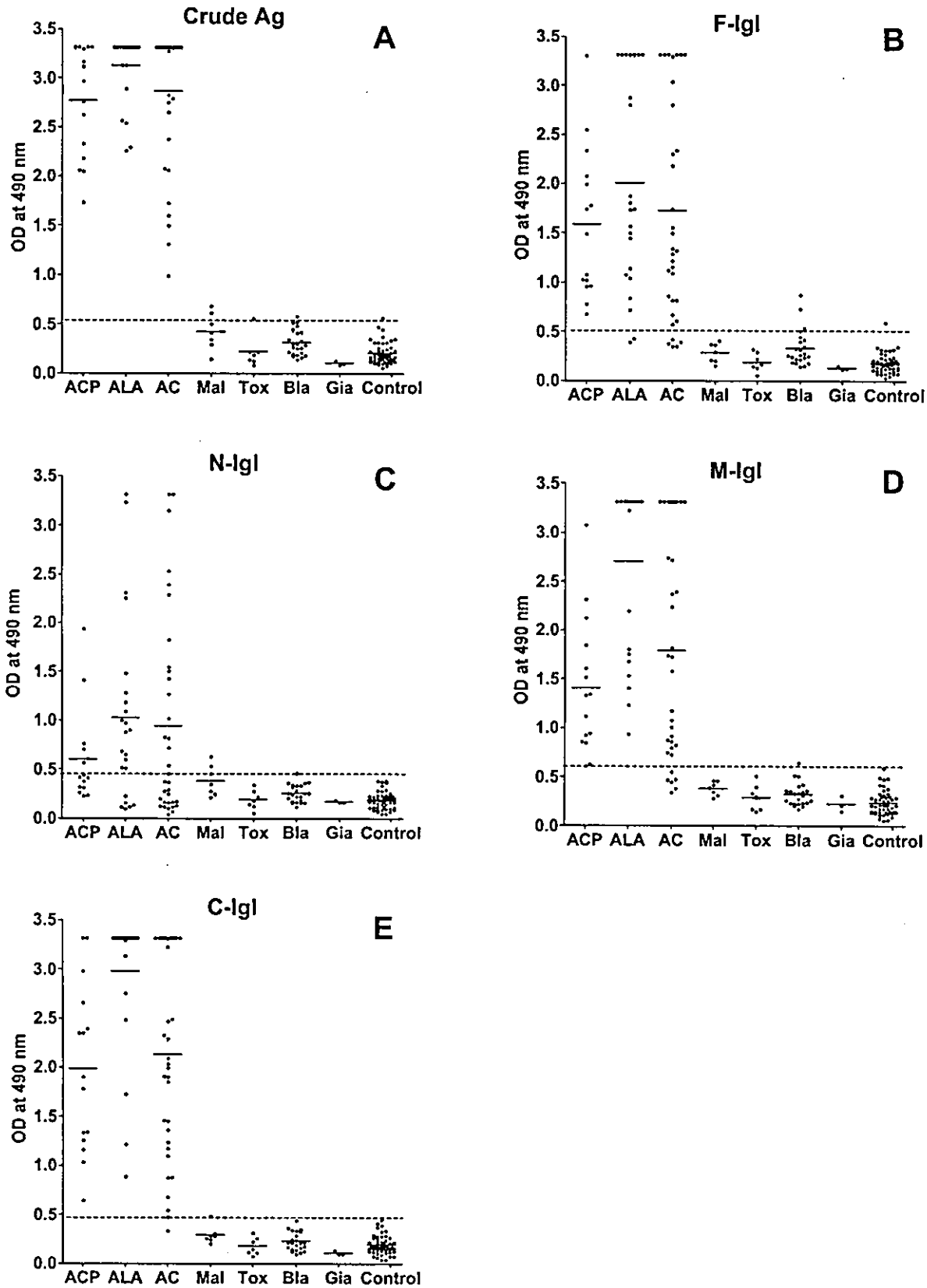

FIG. 3. ELISA reactivities of crude antigen and recombinant Igls from *E. histolytica* with sera from various patients. ELISA plates were coated with 1 μg per well of crude antigen (A) or 100 ng per well of F-Igl (B), N-Igl (C), M-Igl (D), or C-Igl (E). Serum samples used were as follows: ACP, asymptomatic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

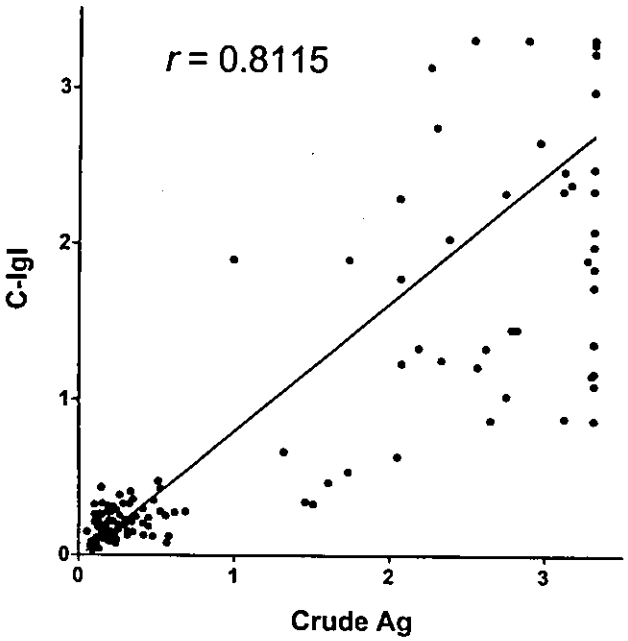

FIG. 4. Correlation between ELISA reactivities to C-Igl and crude antigen in sera from *E. histolytica*-infected individuals and controls. The assay was as described in Fig. 3.

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

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

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

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

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研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	編集者名	書籍名	出版社名	出版地	ページ	出版年
K. Kawakami	Innate immunity in the lungs to cryptococcal infection	G. B. Haffnagle, P. Fidel	Fungal Immunology Book	Kulwer Publishers		印刷中	2005
K. Kawakami	Possible immunotherapy with interleukin-18 in intractable infectious diseases	K. Kawakami, D. A. Stevens	Immunomodulators as promising therapeutic agents against infectious diseases	Research Signpos	Trivandrum	89-104	2004
Y. Kinjo, K. Kawakami	α-Galactosylceramide: NKT cell-based immunotherapy in intractable infectious diseases	K. Kawakami, D. A. Stevens	Immunomodulators as promising therapeutic agents against infectious diseases	Research Signpos	Trivandrum	105-122	2004

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
K. Miyagi, K. Kawakami, et al.	CpG oligodeoxy nucleotides promote the host protective response against infection with Cryptococcus neoformans through induction of interferon-gamma production by CD4+ T cells	Clinical and Experimental Immunology	139	印刷中	2005
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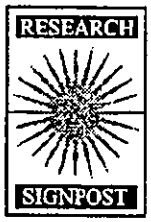

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5 Possible immunotherapy with Interleukin-18 in intractable infectious diseases

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Introduction

Interleukin (IL)-18, originally discovered as an interferon (IFN)-γ-inducing cytokine [1], is produced by macrophages, dendritic cells, Kupfer cells and keratinocytes upon stimulation with microbial products [2-4]. This cytokine is originally synthesized in a premature 26kD form and needs cleavage by caspase-1 for converting to the bioactive 18kD form [2-4]. IL-18 induces IFN-γ production by natural killer (NK) and T cells [1-4] and potentiates IL-12-mediated Th1 cell development, although it does not exhibit such an effect alone [5]. In collaboration with IL-12, IL-18 also induces IFN-γ secretion by NK cells, NKT cells,

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T cells, B cells and even macrophages and dendritic cells [2-4,6-10]. Surprisingly, IL-18 has recently been reported to induce synthesis of Th2 cytokines such as IL-4 and IL-13 by NK, T cells and basophils when administered with IL-2 and IL-3, respectively [4,11,12] and to cause Th2-type immune response, including IgE production and eosinophilic accumulation [4,13-15]. To date, many investigators have addressed the question of how IL-18 is involved in the development and regulation of host resistance against a variety of pathogenic microorganisms, including bacteria, fungi, protozoa and viruses.

In this review, I first focus on the role of IL-18 in the development and regulation of host resistance against infectious pathogens, with an emphasis on cryptococcal infection as determined by recent studies from our laboratory, and then on the possible application of this cytokine to the therapy of intractable infectious diseases.

Role of IL-18 in host defense against infectious pathogens

The role of IL-18 in host protection against various infectious pathogens, including extracellular and intracellular bacteria, fungi, protozoa and viruses, has so far been investigated by many investigators. Most studies reported the beneficial effects of this cytokine. The major findings are summarized in Table.

Table. IL-18 and infectious diseases

Microorganisms	recombinant IL-18	anti-IL-18 Ab	IL-18-/- mice	References
Extracellular bacteria				
Yersinia enterocolitica	Improved	Exacerbated	-	Bohn et al., 1998 (18)
Staphylococcus aureus	-	-	Not exacerbated	Wei et al., 1999 (19)
Streptococcus pneumoniae	-	Exacerbated	Exacerbated	Lauw et al., 2002 (21)
Intracellular bacteria				
Mycobacterium tuberculosis	-	-	Exacerbated	Sugawara et al., 1999 (22)
	-	-	Exacerbated	Kinjo et al., 2002 (23)
Salmonella typhymurium	Improved	Exacerbated	-	Mastroeni et al., 1999 (28)
Legionella pneumophila	-	Not exacerbated	-	Brieland et al., 2000 (31)
Fungi				
Candida albicans	Improved	-	-	Mencacci et al., 2000 (33)
Cryptococcus neoformans	Improved	Exacerbated	-	Kawakami et al., 1997 (55)
	-	-	Exacerbated	Kawakami et al., 2000 (45)
	-	Exacerbated	Exacerbated	Kawakami et al., 2000 (46)
Protozoa				
Leishmania major	-	-	Exacerbated	Wei et al., 1999 (19)
	Improved (with IL-12)	-	Exacerbated	Ohkusu et al., 2000 (47)
	-	-	Not exacerbated	Monteforte et al., 2000 (48)
Toxoplasma gondii	Improved	Not exacerbated	-	Cai et al., 2000 (50)
Plasmodium berghei	Improved (with IL-12)	Exacerbated	-	Okamura et al., 1998 (2)
	Improved	Exacerbated	Exacerbated	Singh et al., 2002 (51)
Viruses				
Herpes simplex	Improved	-	-	Fujioka et al., 1999 (52)
Murine cytomegalovirus	-	-	Not exacerbated	Pien et al., 2000 (54)

-: Not examined.

Extracellular bacteria

Host protection against extracellular bacteria is primarily mediated by neutrophil-dependent killing mechanism. IL-18 indirectly causes the production of IL-8, a major chemokine for neutrophils, by peripheral blood monocytes by inducing the secretion of tumor necrosis factor (TNF)-α from T and NK cells [16]. In addition, neutralizing anti-IL-18 antibody reduces the accumulation of neutrophils in mice challenged with *Escherichia coli* and *Salmonella typhimurium* endotoxin [17]. Thus, IL-18 may contribute to neutrophil-dependent host defenses.

Bohn and co-workers [18] reported that hepatic expression of IL-18 mRNA in mice after infection with *Yersinia enterocolitica*, was higher in resistant mice than in susceptible mice. Neutralization of endogenous IL-18 by specific antibody markedly reduced resistance to this infection without affecting the synthesis of IFN-γ, invoking an IFN-γ-independent pathway of resistance. Exogenous administration of IL-18, however, failed to increase the protective host response against the same infection. Thus, endogenously synthesized IL-18 apparently contributed to at least the clearance of *Yersinia* infection, although the precise mechanism remains to be elucidated.

IL-18-/- mice were used to investigate the role of IL-18 in septicemia and septic arthritis induced by intravenous injection of *Staphylococcus aureus* [19]. Septicemia was less profound in these mice than in similarly infected wild-type mice, while the opposite results were obtained in septic arthritis. The IL-18-/- mice developed a reduced synthesis of Th1-type cytokines such as IFN-γ and TNF-α. These data suggested that IL-18 played a pro-inflammatory role in the development of septicemia, although its role in septic arthritis remains to be further elucidated. In contrast, Hochholzer *et al.* [20] revealed IL-18-independent IFN-γ synthesis and lethal shock caused by *S. aureus* enterotoxin B.

Lauw and co-workers elucidated the role of IL-18 in host protective responses to *Streptococcus pneumoniae* using mice genetically lacking the synthesis of IL-18 [21]. These mice were more susceptible to this infection, as indicated by the enhanced number of live colonies in the infected organs than control mice. Similar results were obtained in mice which received neutralizing anti-IL-18 antibody. Interestingly, the clearance of pneumococci from lungs of IL-12-/- mice was not affected, when compared with control mice. Their works clearly indicated the important role of IL-18, but not IL-12, in the antibacterial host responses during pneumococcal pneumonia.

Intracellular bacteria

Most intracellular bacteria possess survival mechanisms that enable them to evade the host defense system and reside in phagocyte cells. Hosts therefore need to promote cell-mediated immunity to attack such pathogens. The role of

IL-12 in this process has been extensively studied by many investigators. Recently, several investigations have addressed the role of IL-18 in defense against intracellular bacteria.

Sugawara and co-workers [22] used IL-18-/- mice to investigate the role of IL-18 in host resistance and cytokine responses to mycobacterial infection. IL-18-/- mice were more susceptible to *M. tuberculosis* infection than control, wild-type mice, as indicated by the increased number of live bacteria in their lungs. Although such impairment of host defenses was associated with reduced IFN-γ production by bacille Calmette-Guérin (BCG)-stimulated spleen cells, the magnitude of the impairment was not as pronounced as that observed in IFN-γ-/- mice. Defective IL-18 synthesis led to the development of large granulomatous lesions in the lung and spleen, which were significantly reduced by administration of exogenous IL-18.

Our investigation compared the role of IL-18 in host defense against *M. tuberculosis* infection more critically with that of IL-12 using IL-12p40-/-, IL-18-/- and IL-12p40-/-IL-18-/- mice [23]. IL-18-/- mice were more prone to this infection than control mice, and in addition, mice lacking both IL-12p40 and IL-18 died earlier than IL-12p40 mice. This increased mortality under IL-18 deficient condition was correlated with the reduced production of IFN-γ. Recently, IL-23 has been discovered as a novel IFN-γ-inducing cytokine [24]. Interestingly, this cytokine is formed by combination of p19 protein with IL-12p40 to constitute the active heterodimer. Thus, our data indicated that IL-18 played a considerable role in the host defense to mycobacterial infection even in the absence of both IL-12 and IL-23, two other IFN-γ-inducing cytokines. Compatible with this conclusion, mice over-expressing IL-18 gene produced higher Th1 responses and were more resistant to this infection than control littermate mice [23].

Th1 cytokine expression has been demonstrated to predominate in lesions of patients with resistant tuberculoid leprosy, while Th2 cytokines predominated in lesions from patients with susceptible lepromatous leprosy [25]. The protective role of IL-18 in host defense to *M. leprae* infection was demonstrated by Kobayashi *et al.* using an animal model of leprosy [26]. Consistent with this study, Garcia and colleagues [27] observed higher IL-18 mRNA expression in tuberculoid than lepromatous leprosy lesions. Similar results were obtained using peripheral blood mononuclear cells (PBMC) from the patients after stimulation with microbial antigens; exogenous IL-18 augmented IFN-γ production by PBMC in tuberculoid, but not in lepromatous patients. Thus, IL-18 was identified as a cytokine that acts to limit leprosy lesions by inducing IFN-γ synthesis. In a sharp contrast, Yoshimoto *et al.* [15] documented higher production of IL-18 in the serum of lepromatous than tuberculoid leprosy patients. The explanation for these discrepant results is not yet known.