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Identification of the Hybrid Cluster Protein, HCP, from Amitochondriate Eukaryotes and Its Phylogenetic Implications

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Abstract Hybrid cluster protein (HCP) was investigated because of its unique iron-sulfur clusters, which have been found in bacteria and archaea. Here, HCP homologous proteins from the third domain, "eukarya" (3 amitochondriate protozoans, *Giardia lamblia*, *Entamoeba histolytica*, and *Trichomonas vaginalis*), were identified. All three amitochondriate protozoan HCPs (GlHCP, EhHCP, and TvHCP) belonged to Class I on the basis of two key characters, the cysteine spacing, Cys-$(Xaa)_2$-Cys$(Xaa)_{7-8}$-Cys$(Xaa)_5$-Cys, and the absence of N-terminal deletion characteristic to the Class III. In phylogenetic analysis performed with amino acid sequences of 3 eukaryal, 5 bacterial, and 4 archaeal HCPs, the maximum likelihood (ML) tree indicated that TvHCP was clustered with Class I HCPs, whereas the other two HCPs (GlHCP and EhHCP) formed an independent clade with a high bootstrapping value (96%) not belonging to any previously recognized HCP class. In spite of the relatively lower bootstrapping value (61%), the position of the new eukaryal GlHCP-EhHCP clade was close to Class I, including the TvHCP, and Classes II and III were closely related with each other. The finding of eukaryal HCPs would help to understand the evolutionary history of HCP.

Key words: Hybrid cluster protein, amitochodriate protozoa, *Giardia lamblia*, *Trichomonas vaginais*, *Entamoeba histolytica*

Hybrid cluster protein (HCP) has been investigated mainly because of the unusual properties of its iron-sulfur clusters. X-ray crystallography of *Desulfovibrio vulgaris* HCP showed that this protein contains two different types of Fe-S clusters [2]: Cluster 1 in the N-terminus of the protein is a [4Fe-4S] cubane type, whereas Cluster 2 is a novel hybrid 4 Fe cluster with two μ_2-sulfido bridges and two μ_2-oxo bridges, and is referred to as "hybrid cluster protein".

Two signatures in the predicted amino acid sequences of HCPs allowed the groupings of putative HCPs into three classes [13]. Class I genes have been found only in strict anaerobic bacteria and in the methanogenic archeon *Methanococcus jannaschii*. Class I HCPs have the typical spacing of the N-terminal cysteine ligands in Cluster 1 as Cys-$(Xaa)_2$-Cys-$(Xaa)_{7,8}$-Cys-$(Xaa)_5$-Cys, where Xaa indicates any amino acid. The spacing of the N-terminal cysteines in Class II proteins is Cys-$(Xaa)_2$-Cys-$(Xaa)_{11}$-Cys-$(Xaa)_6$-Cys, which are found in facultative anaerobic bacteria. Class III HCPs are associated with hyperthermophilic and other archaeal bacteria. They have the same spacing of the N-terminal cysteines as Class I proteins, but have 116 amino acid residues deleted downstream of the N-terminal cysteine cluster. These *hcp* homologous genes have not been reported in all of the strains of which the genomes have been completely sequenced; for example, they are not found in the genomes of *Bacillus subtilis*, *Archaeoglobus fulgidus*, or other eukaryotic organisms.

Despite extensive investigation of the structure of HCPs, the number of studies on the biological function of these proteins has been limited. In *Clostridium perfringens*, HCP was identified as one of the proteins involved in resistance to oxidative stress [3], and HCP purified *from Escherichia coli* was found to have a hydroxylamine reductase activity [14].

The *hcp* homologous gene was found to be one of the genes induced during *in vitro* encystation of *Giardia lamblia*, an amitochondriate protozoan, which was the first identification of an eukaryal HCP. This discovery prompted us to search the distribution of this gene in other protozoans, such as *Entamoeba histolytica* and *Trichomonas vaginalis*, and also analyze phylogenetic positions of newly defined eukaryal HCPs.

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MATERIALS AND METHODS

Protozoan Cultivations

G. lamblia WB (ATCC 30957) was grown in a modified TYI-S-33 medium supplemented with bovine bile (0.5 mg/ml) [6], *E. histolytica* HM-1: IMSS (ATCC 30459) was maintained in TYI-S-33 medium [5], and TYM medium [4] was used to cultivate *T. vaginalis* strain KT4 [10].

Preparation of DNA

Genomic DNAs of *E. histolytica* and *G. lamblia* were prepared by lysis of 10^7 trophozoites in 1% SDS, phenol/chloroform extraction, and ethanol precipitation. Since *T. vaginalis* showed higher nuclease activities, its genomic DNA was extracted in the presence of DEPC, a nuclease inhibitor, as described elsewhere [9].

Differential Display

Differential display was performed on RNA samples of *G. lamblia* at various stages of differentiation, trophozoite, and 6 h, 12 h, and 48 h post-induction of encystations, as previously described [15].

Degenerate PCR

To obtain the *hcp* gene of *E. histolytica*, a pair of degenerate primers, HCP-F and HCP-R, were designed based on the conserved regions of putative HCPs of nine species of archaea and bacteria; namely *M. thermobacterium thermoautotrophium*, *Thermotoga maritima*, *Pyrococcus abyssi*, *E. coli*, *Acidithiobacillus ferroxidans*, *D. desulfuricans*, *D. vulgaris*, *C. acetobutylium*, and *M. jannaschii* (Table 1).

Another set of degenerate primers, THCP-F and THCP-R, encoding the conserved regions between EhHCP and GlHCP, were used to clone a major part of the *Tvhcp* gene (DNA fragment of 1,527 bp corresponding to the HCP from amino acid no. 1 to amino acid no. 509). The 3' end of the *Tvhcp* gene encoding the remaining carboxyl-terminus of TvHCP was identified using a Universal GenomeWalker™ kit (Clontech, Palo Alto, CA, U.S.A.).

Library Screenings

Using the cDNA isolated by the differential display as a probe, the full sequence of the *hcp* gene was identified from the *G. lamblia* cDNA library, a λZAP®II-based library. cDNA clones hybridized with the ^{32}P-labeled *Glhcp* probe were purified until homogeneity, and then rescued as plasmids, as directed by the manufacturer (Stratagene, La Jolla, CA, U.S.A.). Identities of these cDNA clones were verified by sequencing of the double strand plasmids.

The *Ehhcp* DNA fragment obtained by degenerate PCR was labeled with α-[^{32}P]dATP using a Random Primer DNA Labeling kit. In the same manner, the full *hcp* gene of *E. histolytica* was obtained by screening a λZAP^{express}-based *E. histolytica* cDNA library that was previously constructed in our laboratory.

Phylogenetic Analysis

A phylogenetic tree was reconstructed with 12 putative HCP amino acid sequences from five bacteria (*E. coli*, *A. ferroxidans*, *D. desulfuricans*, *D. vulgaris*, and *C. acetobutylium*), four archaea (*M. thermobacterium thermoautotrophium*, *T. maritime*, *P. abyssi*, and *M. jannaschii*), and three eukarya (*G. lamblia*, *E. histolytica*, and *T. vaginalis*) by the maximum-likelihood (ML)-based quartet puzzling method of Tree-Puzzle 5.0 [7, 11, 16]. To estimate appropriate parameters for the ML tree reconstruction, the quartet sampling (for substitution process)+Neighbor-Joining (NJ) tree (for rate variation) option was used, and number of quartet puzzling was 1,000.

Formation of GlHCP-Specific Polyclonal Antibodies and Western Blot Analysis

The full sequence of the *Glhcp* gene was amplified from *G. lamblia* genomic DNA using the Glhcp-F and Glhcp-R primers, and then cloned to pET21b expression plasmid (Novagen, Darmstadt, Germany). Recombinant GlHCP was overexpressed in *E. coli* BL21 (DE3) by adding 1 mM IPTG, and purified by inclusion body preparation and subsequent gel electrophoresis. Eluted protein was used to produce GlHCP-specific polyclonal antibodies by three consecutive immunizations of Sprague-Dawley rats (3-week intervals; 200 μg for each immunization).

Cell extracts of *G. lamblia*, *E. histolytica*, and *T. vaginalis* were prepared and fractionated by SDS-PAGE. After transferring these proteins to a nitrocellulose filter, western blot analysis was performed by serially incubating the filter with GlHCP-specific polyclonal antibodies (1:500 dilution) and alkaline phosphatase (AP)-conjugated rabbit anti-rat IgG (1:1,000 dilution; Sigma, St. Louis, MO, U.S.A.). The HCP bands were visualized using the NBT-BCIP system (Promega, Madison, WI, U.S.A.).

Immunolocalization

G. lamblia trophozoites that had attached to chamber slides were fixed in 3.7% formaldehyde/PBS (150 mM NaCl, 17 mM $NaPO_4$, 1 mM EGTA, pH 7.0) for 30 min [8]. The fixed cells were permeabilized with 0.5% Triton X-100 for 9 min, and then blocked for 1 h in blocking solution (PBS containing 10 mg/ml of BSA). The prepared cells were reacted with GlHCP antibodies, and subsequently incubated with goat anti-rat IgG conjugated to TRITC (Jackson Immuno Research Labs Inc., West Grove, PA, U.S.A.). After mounting on slides with 90% glycerol/PBS/1 mM p-phenylene diamine, the cells were examined under an OLYMPUS fluorescence microscope (U-LH100HG, Nagano, Japan) at 510-550 nm.

RESULTS AND DISCUSSION

Isolation of *hcp* Homologous Genes in *G. lamblia*, *E. histolytica*, and *T. vaginalis*

In a differential display experiment, a cDNA clone of 400 bp, encoding a protein homologous to a HCP which had previously been found only in bacteria and archaea, was isolated. Based on the cDNA clones obtained by library screening, the complete ORF of *Glhcp* was found to be of 1,671 bp. Amino acid sequence similarities of complete GlHCP and those of bacterial and archaeal HCP ranged from 33 to 35% (Fig. 1). It had two clusters, 1 and 2, which are typical ligands for [4Fe-4S] or [4Fe-2S-2O] and present in all the reported HCPs. Since the spacing of the N-terminal cysteine ligand in Cluster 1 is Cys-Xaa$_2$-Cys-Xaa$_8$-Cys-Xaa$_5$-Cys, GlHCP belonged to either Class I or III. The absence of a large deletion downstream of its N-terminal cysteine cluster, compared to the Class III HCP of *D. vulgaris*, suggested that GlHCP was a member of Class I rather than that of Class III. Interestingly, GlHCP had an insertion from amino acid residue #359 to #378, which was absent in other HCPs.

Two degenerate primers were designed based on the conserved regions of archeal and bacterial HCPs. PCR with these two primers produced a DNA fragment of 680 bps encoding a part of EhHCP (from amino acid #272 to amino acid #496). *Entamoeba histolytica* cDNA library screening with this DNA fragment provided a clone containing the full sequence of the *hcp* ORF of 1,620 bps. Putative amino acid sequence of HCP deduced from this ORF showed an identity of 37% with GlHCP and 39 to 44% with other bacterial and archaeal HCPs. EhHCP also had two clusters, 1 and 2, for [4Fe-4S] and [4Fe-2S-2O], respectively. The spacing of the N-terminal cysteine ligand in Cluster 1 (Cys-Xaa$_2$-Cys-Xaa$_7$-Cys-Xaa$_5$-Cys) and the absence of the large deletion downstream of this N-

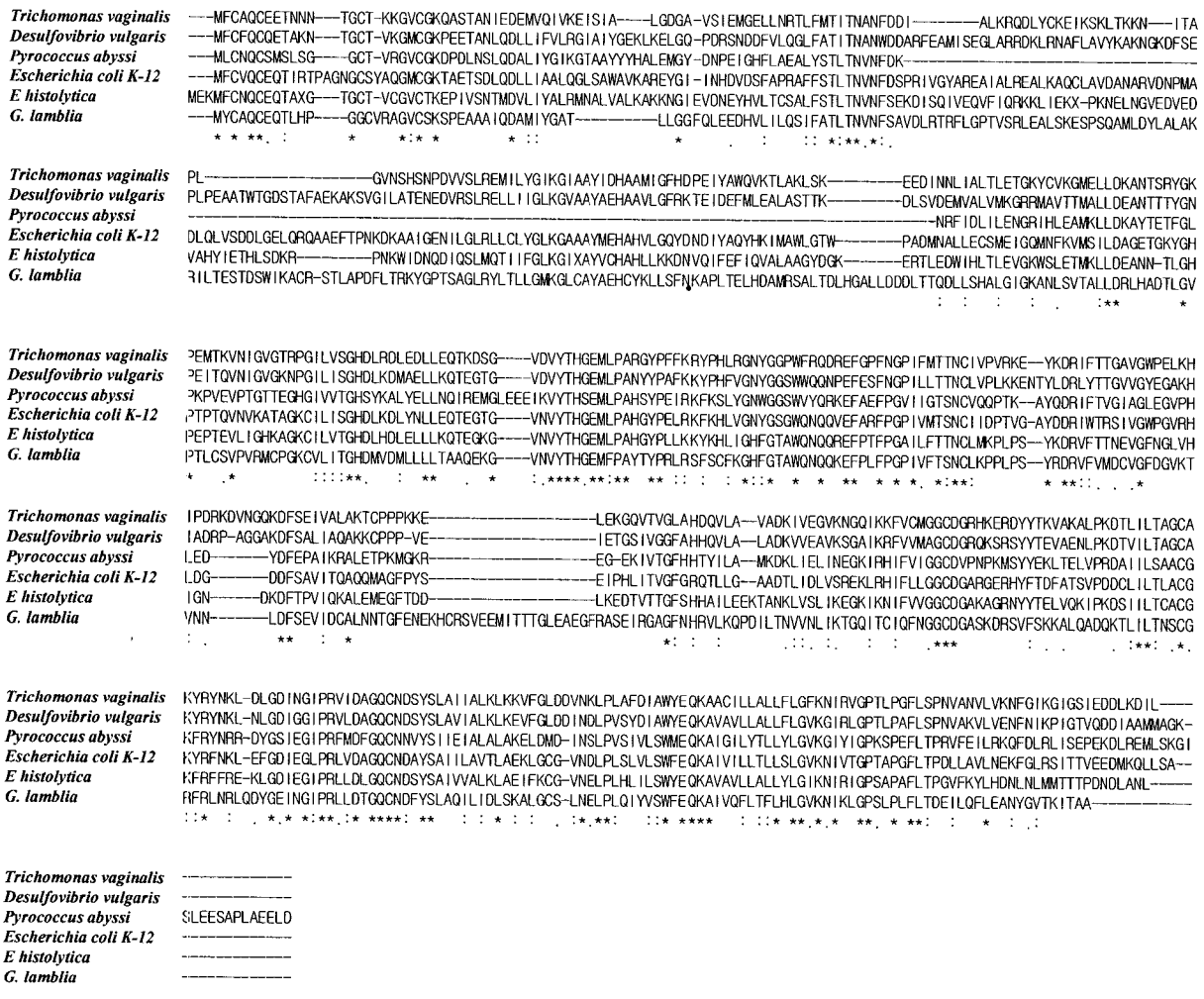

Fig. 1. Alignment of the deduced amino acid sequences of HCPs (Hybrid Cluster Protein) of *Giardia lamblia*, *Enamoeba histolytica*, and *Trichomonas vaginalis* with selected homologs of other organisms.
*, fully conserved residue; :, strong conservation; and ., weak conservation.

terminal cysteine cluster suggested that EhHCP belongs to Class I HCPs.

Through degenerate PCR and genome walking, the full sequence of *Tvhcp* was identified. The resultant ORF of the *Tvhcp* gene was composed of 1,545 bp, which showed significant identities with other known HCPs (39–56%). TvHCP also contained the two conserved clusters and the required spacing of the N-terminal cysteine ligand, Cys-Xaa$_2$-Cys-Xaa$_8$-Cys-Xaa$_5$-Cys. Again, no large deletion downstream of this N-terminal cysteine cluster was present. Thus, TvHCP could be considered as a Class I HCP like the HCPs of the other two amitochondriates examined in this study.

The isolated genes from amitochondriates were confirmed as *hcp* homologous genes using the BLASTX program on the SDSC Bio-work bench [1]. It was found that these genes were recognized with high expectation probability values ($E>1e^{-86}$) as the prokaryotic HCPs in the GenBank database. The nucleotide sequences of the *hcp* homologous genes of *G. lamblia, E. histolytica,* and *T. vaginalis* have been deposited to the GenBank under accession numbers AF458335, AF458334, and AF458336, respectively.

Phylogenetic Analyses of *hcp* Homologous Genes in *G. lamblia, E. histolytica,* and *T. vagnialis*

The putative amino acid sequences of the HCPs of *G. lamblia*, *E. histolytica*, and *T. vaginalis* were aligned to homologous sequences of nine species from bacteria and archea using the Clustal X program [12]. With the alignment of these 12 amino acid sequences, a phylogenetic analysis was performed using the maximum likelihood method (ML) of Tree-Puzzle 5.0 [11]. The resultant ML tree (Fig. 2) revealed that the TvHCP was strongly grouped with the Class I members (methane-producing archaea, *Methanococcus*, and strict anaerobes, *Desulfovibrio* and *Clostridium*) with high confidence value (bootstrapping value 99%), in concordance with the classification using key signatures such as cysteine spacing pattern in Cluster 1 and presence/absence of a large deletion downstream of Cluster 1. Contrary to the expectation, however, the other two HCPs (GlHCP and EhHCP) formed an independent clade (possibly Class IV suggested here first) with high bootstrapping value (96%), not belonging to any previously recognized HCP class. In spite of the relatively lower bootstrapping value (61%), the position of the new eukaryal clade was near Class I including the TvHCP, and Classes II and III were closely related with each other. All four of the classes, including Class IV, were robustly supported with high bootstrapping values over 94%.

Therefore, it is likely that HCPs identified from eukarya, bacteria, and archaea, were grouped into four different classes, as shown in Fig. 2. When protozoan HCPs were grouped in terms of key features, such as Cluster 1 and size of the deletion downstream of Cluster 1, all turned out to

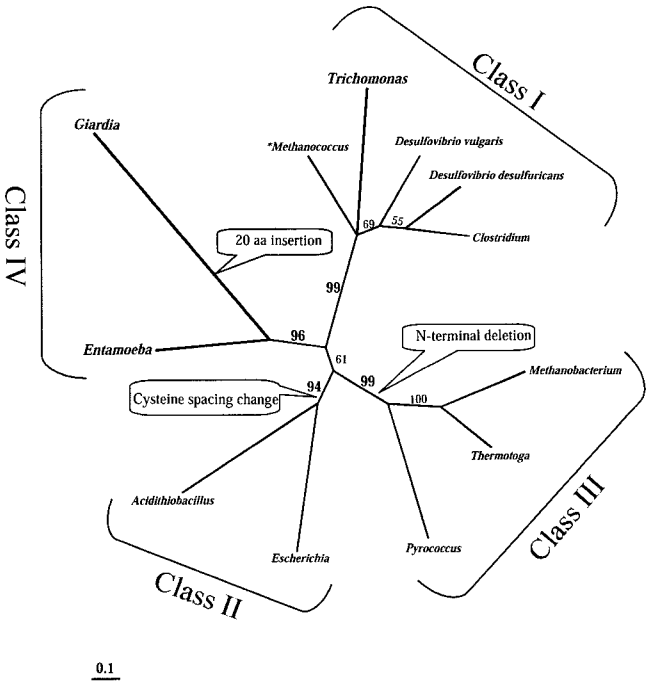

Fig. 2. Unrooted phylogram of a maximum likelihood tree reconstructed with 12 putative HCP amino acid sequences from 5 Bacteria, 4 Archaea, and 3 Eukarya (log likelihood: -11671.33).

This tree was reconstructed by the maximum-likelihood-based quartet puzzling method of Tree-Puzzle 5.0. Numbers with branches indicate node confidence values (bootstrapping values obtained with 1,000 replicates). To estimate appropriate parameters for ML tree reconstruction, the quartet sampling (for substitution process)+NJ tree (for rate variation) option was used. Bar represents 0.1 substitutions per site. Eukarya: *Giardia lamblia* (abbreviation, *Giardia*), *Entamoeba histolytica* (*Entamoeba*), and *Trichomonas vaginalis* (*Trichomonas*); Archaea: *Methanobacterium thermobacterium thermoautotrophium* (*Methanobacterium*), *Thermotoga maritime* (*Thermotoga*), *Pyrococcus abyssi* (*Thermotoga*), *and Methanococcus jannaschii* (*Methanococcus*); Facultative anaerobic bacteria: *Escherichia coli* (*Escherichia*), and *Acidithiobacillus ferroxidans* (*Acidithiobacillus*); Strict anaerobic bacteria: *Desulfovibrio desulfuricans*, *D. vulgaris*, and *Clostridium acetobutylium* (*Clostridium*). "*" shows that a methane-producing bacteria *Methanococcus* taxonomically belonging to archaea is positioned in Class I with strict anaerobes and *Trichomonas*.

be Class I HCPs. However, phylogenetic analysis using complete amino acid sequences of putative HCPs revealed that only the TvHCP belongs to Class I, as expected by the two signature features, and that the two remaining GlHCP and EhHCP form an independent clade quite far apart from the Class I branch, although the relationship between the Class I and the GlHCP-EhHCP clade is weakly supported by bootstrapping value. Such an independent phylogenetic position of the GlHCP-EhHCP clade (Fig. 2) makes it possible to suggest a new Class IV HCP.

Expression and Localization of GlHCP in *G. lamblia*

Verification of these *hcp* genes in the genomes of these three protozoans did not indicate that these genes were

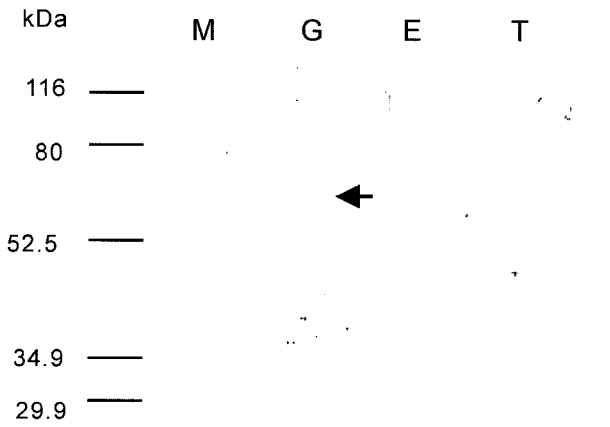

Fig. 3. Western blot analysis using GlHCP-specific antibodies. The expression of HCPs was examined in amitochondriate protozoans. Respective lanes were loaded with 10 μg of the cellular extract of *Giardia lamblia* (G), *Entamoeba histolytica* (E), and *Trichomonas vaginalis* (T).

expressed in these organisms. Most of all, the expression of the *hcp* gene during encystations of *G. lamblia* was confirmed by Northern blot analysis (data not shown). Whereas a significant amount of transcripts of this gene were present in trophozoites, its expression increased slightly as the cells underwent encystation. The expression of these HCPs was examined in these protozoans using GlHCP-specific polyclonal antibodies (Fig. 3). A protein band interacting with anti-GlHCP antibodies was observed only in the extract of *G. lamblia* at the expected size of 60.5 kDa, but not in crude extracts of the other two protozoans. However, this result does not indicate that HCP is not expressed in the other two protozoans, since GlHCP antibodies may not cross-react with other protozoan HCPs.

The intracellular location of HCP within *G. lamblia* was investigated using the same antibodies. Serial incubation of *G. lamblia* trophozoites with GlHCP antibodies and fluorescent dye-conjugated antibodies as secondary antibodies revealed an immunostaining of the entire cytoplasm of *Giardia* trophozoites, as shown in Fig. 4. Staining of trophozoite with pre-immune serum instead of GlHCP-specific antibodies served as a negative control (Fig. 4C). Trophozoites incubated only with the fluorescent secondary antibodies did not show any signal (data not shown).

The first identification of the *hcp* genes in eukarya (amitochondriates) and establishment of Class IV HCP provide several speculations on the understanding of the evolution of HCP. 1) With respect to the cysteine spacing pattern found in HCPs, there are two types; Class I-type Cys-$(Xaa)_2$-Cys$(Xaa)_{7,8}$-Cys$(Xaa)_5$-Cys and Class II-type Cys-$(Xaa)_2$-Cys-$(Xaa)_{11}$-Cys-$(Xaa)_6$-Cys. Considering that the Class I-type cysteine spacing appears in Classes I, III, and IV HCPs in common, it is possible to deduce that Class I-type is major- and ancestral-form cysteine spacing of HCP under the parsimony criterion. Therefore, Class II-type spacing seems to be a synapomorphy characteristic to Class II, arising recently in HCP evolution. 2) The large N-terminal deletion is found only in Class III (archaeal group excluding the methane-producing archaea), but not in the other three HCP Classes (I, II, and IV), meaning that this N-terminal deletion is a synapomorphy characteristic to Class III. 3) GlHCP has a unique insertion located in #359-#378 (according to GlHCP numbering), which is about 20 amino acid residues in length. This insertion seems to be an autapomorphic character of *G. lamblia*.

At present, it is too premature to draw any conclusion on the biological function of HCPs. HCP was suggested to be involved in bacterial response to oxidative stresses from limited investigations on HCPs [3, 14]. However, these studies cannot rule out a possible metabolic role of HCP, because of its unique iron-sulfur centers. The presence of *hcp* genes in amitochondriate protozoan raises a possibility of its presence in other eukaryotic organisms. General evolutionary history of HCP, including those from archaea,

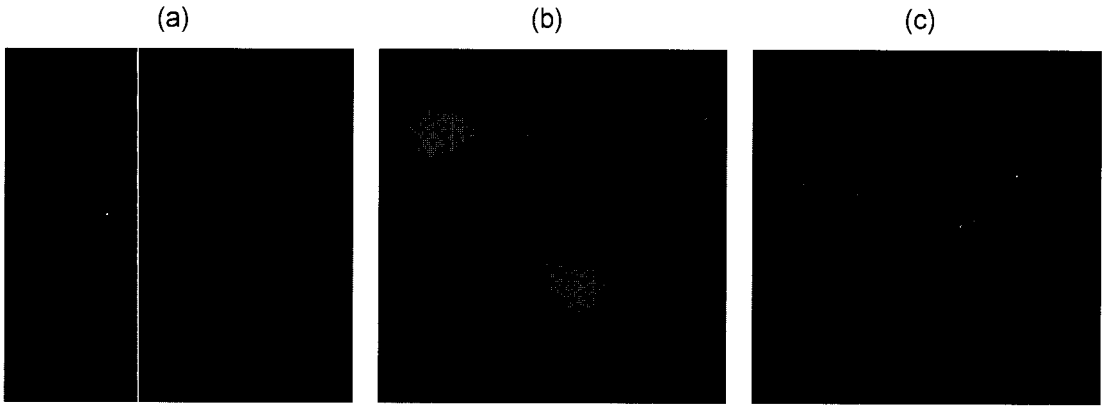

Fig. 4. Immunolocalization of HCP within *Giardia lamblia* trophozoites.
(a) DAPI staining for visualization of nuclei; (b) staining with GlHCP-specific antibodies; and (c) staining with normal serum as a negative control.

bacteria, and eukarya, can be established through further studies on eukaryotic HCPs.

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