A Novel Polyubiquitin Structure in Cercozoa and Foraminifera: Evidence for a New Eukaryotic Supergroup

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Ubiquitin is a 76 amino acid protein with a remarkable degree of evolutionary conservation. Ubiquitin plays an essential role in a large number of eukaryotic cellular processes by targeting proteins for proteasome-mediated degradation. Most ubiquitin genes are found as head-to-tail polymers whose products are posttranslationally processed to ubiquitin monomers. We have characterized polyubuiquitin genes from the photosynthetic amoeboflagellate *Chlorarachnion* sp. CCMP 621 (also known as *Bigelowiella natans*) and found that they deviate from the canonical polyubiquitin structure in having an amino acid insertion at the junction between each monomer, suggesting that polyubiquitin processing in this organism is unique among eukaryotes. The gene structure indicates that processing likely cleaves monomers at the amino terminus of the insertion. We examined the phylogenetic distribution of the insertion by sequencing polyubiquitin genes from several other eukaryotic groups and found it to be confined to Cercozoa (including *Chlorarachnion*, *Lotharella*, *Cercomonas*, and *Euglypha*) and Foraminifera (including *Reticulomyxa* and *Haynesina*). This character strongly suggests that Cercozoa and Foraminifera are close relatives and form a new "supergroup" of eukaryotes.

Introduction

Ubiquitin-mediated protein degradation is an important biochemical process in eukaryotic cells. Through its covalent attachment to other proteins, ubiquitin plays a major role in a large number of basic processes such as apoptosis, signal transduction, endocytosis, and cell cycle regulation (Hershko and Ciechanover 1998). Ubiquitin genes are typically part of a large multigene family whose members are arranged in several different ways. They occasionally exist as stand-alone open reading frames (e.g., Krebber, Wostmann, and Bakker-Grunwald 1994) but are more often found fused to ribosomal protein genes or as multimers of head-to-tail ubiquitin coding regions or polyubiquitin genes. Polyubiquitin genes are transcribed and translated into polyproteins that are cleaved into monomers by specific proteases (Baker, Tobias, and Varshavsky 1992). Free 76 amino acid ubiquitin monomers are then conjugated to proteins through the sequential action of a set of conjugating enzymes (reviewed in Hershko and Ciechanover 1998; Pickart 2001). Ubiquitination acts as a flag, targeting a protein for degradation by the multisubunit ATP-dependent protease, the proteasome (Baumeister et al. 1998). Ubiquitination is also known to target cell-surface proteins for endocytosis and subsequent lysosomal degradation (Hicke 1997; Hershko and Ciechanover 1998).

We have examined the structure and evolution of polyubiquitin genes from two important, yet evolutionarily enigmatic, protist lineages, Cercozoa and Foraminifera. The Foraminifera are a diverse group of extraordinarily abundant marine and freshwater protists, which are characterized by granulose, reticulating pseudopodia and organic or mineralized tests (shells) (Lee 1990). The evolution of Foraminifera has been extensively studied, and they have perhaps the best-characterized fossil record of any protist lineage. However, their evolutionary origin and relationships to other eukaryotes remains controversial because foraminiferan ribosomal RNA gene sequences are generally divergent, show dramatic fluctuations in evolutionary rates, and conflict with fossil evidence (Pawlowski et al. 1996, 1997). The Cercozoa are another very large and diverse group of eukaryotes that includes euglyphid amoebae, cercomonad amoeboflagellates, thaumatomonads, and chlorarachniophyte algae, among others. These organisms are morphologically so diverse that they were only recently recognized as being related through phylogenetic analysis (e.g., Bhattacharya, Helmchen, and Melkonian 1995; Cavalier-Smith and Chao 1997; Cavalier-Smith 1998; Keeling, Deane, and McFadden 1998; Keeling 2001). As is the case with Foraminifera, the relationships of Cercozoa to other eukaryotes has largely been a matter of speculation, as different gene trees conflict in their placement of Cercozoa relative to other eukaryotic groups (e.g., Bhattacharya, Helmchen, and Melkonian 1995; Cavalier-Smith and Chao 1997; Keeling, Deane, and McFadden 1998; Keeling et al. 1999; Keeling 2001). Here we show that cercozoan and foraminiferan polyubiquitin genes contain a unique insertion with important functional implications for polyubiquitin processing. All evidence indicates that the insertion is a shared derived character and that Foraminifera and Cercozoa share a common origin. These two groups represent a significant fraction of eukaryotic biodiversity, and their union marks the emergence of a new eukaryotic supergroup.

Key words: Cercozoa, eukaryotes, Foraminifera, polyubiquitin genes.

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Materials and Methods

Strains, Culture Conditions, and DNA Isolation

Cultures of *Lotharella amoeboformis* (strain CCMP 2058) and *L. globosa* (strain CCMP 1729) were kindly provided by K. Ishida and were maintained in f/2-Si medium at 20°C under a 16-h light/8-h dark cycle. *Cercomonas* sp. 18 (strain RS/18A, ATCC 50316) was grown in ATCC medium 1967 at 25°C. *Haynesina germanica* was collected in Dorum Neufeld, Germany (foraminiferan DNA collection # 2569). One hundred seventy specimens were individually cleaned with a

paintbrush and washed in several baths of sterile sea water. *Reticulomyxa filosa* was maintained at 20°C using Volvic brand table water as medium and fed twice monthly with prewetted wheat germ flakes. Finally, *Euglypha rotunda* (strain CCAP 1520/1) was cultivated on a biphasic medium using Prescott's and James's solution as per CCAP instructions (liquid phase, 1 g/L of Hima la vie brand wheat grass powder as food; solid phase, 0.1 g/L wheat grass powder and 15 g/L BactoAgar [Difco]). All total DNA extractions were performed with the DNeasy Plant Mini Kit (Qiagen). DNAs from *Cercomonas* sp. and *Cercomonas* sp. 22 (strain RS/22, ATCC 50318) were generously provided by T. Cavalier-Smith and E. E. Chao. A *Chlorarachnion* sp. (strain CCMP 621; also known as *Bigelowiella natans*) lambda Zap II cDNA library was kindly provided by G. I. McFadden and P. Gilson.

Cloning and Sequencing of Polyubiquitin Genes

Multiple cDNAs encoding polyubiquitin gene fragments from *Chlorarachnion* sp. CCMP 621 were sequenced in the course of an ongoing EST sequencing project. The fragments were assembled to form three complete and distinct polyubiquitin genes, the sizes of which were confirmed by PCR using the universal primer sites flanking the multiple cloning site of the vector. Polyubiquitin gene fragments were amplified from *Lotharella amoeboformis*, *L. globosa*, *Cercomonas* sp., *Cercomonas* sp. 18, *Cercomonas* sp. 22, *Euglypha rotunda*, *Reticulomyxa filosa*, and *Haynesina germanica* using the following primers: UBIQ1: 5′-GGCCATGCARATHTTYGTNAARAC-3′; IUB2: 5′-GATGCCYTCYTTRTCYTGDATYTT-3′. The UBIQ1/IUB2 primer pair generates a ladder of ubiquitin gene products ranging from a half-monomer fragment to increasing numbers of tandem repeats of the polyubiquitin tract. Polyubiquitin fragments between 1.5 and 3.5 repeat units were isolated and cloned into pCR2.1 using the Topo TA cloning kit (Invitrogen). Multiple independent clones were sequenced from each species. Spliceosomal introns were present in several of the cercomonad polyubiquitins. For a given organism, amino acid sequences inferred from independent clones were generally identical, although synonymous substitutions were often observed between clones. For the foraminiferan *Haynesina germanica*, nonforaminiferan polyubiquitins were also sequenced, likely corresponding to genes amplified from food organisms. The foraminiferan sequences determined here were found to share several unique amino acid substitutions with ubiquitin monomers sequenced from other foraminiferans in a previous study (Wray and DeSalle 1994). New ubiquitin sequences were deposited in GenBank under the accession numbers AY099115–AY099148 and AY101385.

Results and Discussion

Atypical Polyubiquitin Genes in *Chlorarachnion*

We sequenced three full-length polyubiquitin genes (*pub1*, *pub2*, and *pub3*) from the chlorarachniophyte alga *Chlorarachnion* sp. CCMP 621. The *pub1* gene comprises four complete ubiquitin coding regions, while *pub2* and *pub3* each contain three complete monomers. The sequences of the ubiquitin monomers in the three genes are extremely similar to one another, differing only at silent sites. The last full-length monomer of all three genes is followed by a truncated ubiquitin: *pub1* terminates with the first 10 codons of a fifth ubiquitin monomer, whereas *pub2* and *pub3* terminate with extensions that are three codons short of a full repeat. This is in contrast to the polyubiquitins characterized from other organisms, which possess nonubiquitin C-terminal extensions of only one or a few amino acids (e.g., Keeling and Doolittle 1995; Guerreiro and Rodrigues-Pousada 1996). Another unique feature of the *Chlorarachnion* polyubiquitins is that all three genes are predicted to initiate two codons upstream of the first monomer, resulting in a methionine-serine (MS) amino-terminal extension (fig. 1*A*).

The most striking feature of the *Chlorarachnion* polyubiquitin sequences, however, is the presence of an S residue inserted at each of the monomer-monomer junctions such that the inferred monomer is 77 amino acids in length (fig. 1*B*). This deviation from the canonical 76 amino acid ubiquitin protein is extremely significant as the insertion occurs at perhaps the most functionally important position in the molecule. The monomer-monomer junction region is (by definition) involved in polyubiquitin processing, and the C-terminal glycine (G76) residue is known to be critical for conjugation of the ubiquitin monomer to other proteins (reviewed in Hershko and Ciechanover 1998; Pickart 2001). This process involves the formation of an isopeptide bond between G76 and the ϵ-amino group of a lysine residue of the target protein or another ubiquitin (Hershko and Ciechanover 1998). The presence of an S at the C-terminus would seem to inhibit ubiquitin conjugation. This suggests that the residue is removed in a novel processing step, that polyubiquitin cleavage takes place upstream of the insertion (leaving G76 as the C-terminal residue), or that both processes occur. The initiation of each polymer with an MS dipeptide extension (fig. 1*A*) seems to suggest that amino-terminal extensions are either tolerated in *Chlorarachnion* ubiquitins or that a mechanism of amino-terminal postcleavage "trimming" exists. It is significant that the sequence of the amino-terminal extension has an S reside in the same context as the junction-insertion S. In any case, the processing of ubiquitin polyproteins in this organism appears to be unlike other eukaryotes, and the polyubiquitin structure is unique.

Polyubiquitin Genes in Cercozoa and Foraminifera

Chlorarachnion possesses a previously uncharacterized insertion in an otherwise invariant region of the ubiquitin molecule, which has important functional implications. Characteristics such as this are rare and can be powerful indicators of evolutionary relationships. We therefore isolated polyubiquitin genes from other members of the Cercozoa in an attempt to determine the phylogenetic distribution of this novel feature. We amplified and sequenced polyubiquitin gene fragments from two additional chlorarachniophytes that are distantly related to

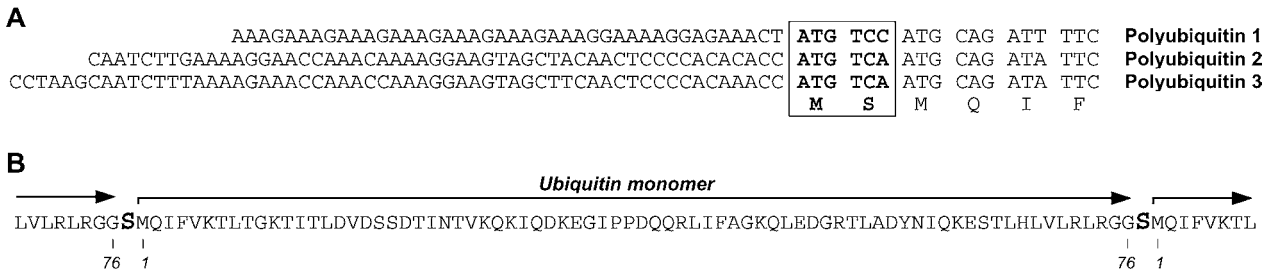

FIG. 1.—Atypical polyubiquitin genes in *Chlorarachnion*. *A*, Alignment of the 5′-untranslated region and first six codons of the three *Chlorarachnion* sp. CCMP 621 polyubiquitin genes. The putative start and serine codons upstream of the canonical start codon are highlighted. *B*, Inferred amino acid sequence of an individual ubiquitin monomer and flanking regions from the polyubiquitin 1 (*pub1*) gene in *Chlorarachnion* sp. CCMP 621. The canonical methionine (M) residue at position one and the C-terminal glycine (G) residue at position 76 are indicated and the extra serine (S) residues between adjacent monomers are highlighted. Both of these characters are unique among eukaryotes and have significant functional implications (see text).

Chlorarachnion (*Lotharella amoeboformis* and *L. globosa*), as well as from three cercomonads (*Cercomonas* sp., *Cercomonas* sp. 18, and *Cercomonas* sp. 22) and from a euglyphid (*Euglypha rotunda*). All six were found to contain insertions at the same position as *Chlorarachnion*. The *L. amoeboformis* and *L. globosa* polyubiquitins have a single alanine (A) or S insertion at their monomer-monomer junctions, while the cercomonads and *E. rotunda* contain an SG or SA doublet (fig. 2). This character provides strong evidence—independent of phylogenetic analysis—for the sisterhood of cercomonads and euglyphids, consistent with small subunit ribosomal RNA (SSU rRNA) phylogenies (Cavalier-Smith and Chao 1997; Wylezich et al. 2002). More generally, the insertion supports a specific relationship between chlorarachniophytes and cercomonads, as has been observed in analyses of SSU rRNA, tubulins, and actin (Bhattacharya, Helmchen, and Melkonian 1995; Cavalier-Smith and Chao 1997; Keeling, Deane, and McFadden 1998; Keeling et al. 1999; Keeling 2001).

While the Cercozoa are now beginning to be recognized as a major taxonomic group (Cavalier-Smith 1998), it is far from clear how they fit into the larger picture of eukaryotic evolution, as different gene phylogenies conflict in their placement of Cercozoa on the tree of eukaryotes. However, a recent analysis of actin protein sequences suggested a relationship between Cercozoa and Foraminifera (Keeling 2001). We therefore sequenced polyubiquitin fragments from two Foraminifera, *Reticulomyxa filosa* and *Haynesina germanica*. Significantly, the monomer-monomer junctions from both species were found to contain a single A insertion (fig. 2). This strongly suggests that Foraminifera and Cercozoa share a more recent common ancestor with each other than with any other known eukaryotic group.

The absolute conservation of length and high degree of amino acid sequence conservation characteristic of the ubiquitin molecule across the full breadth of eukaryotic diversity (fig. 2) makes the possibility of independent insertions at such a functionally critical position of polyubiquitins from cercomonads/euglyphids, chlorarachniophytes, and Foraminifera extremely improbable. The cercozoan/foraminiferan ubiquitin insertion is thus very likely a synapomorphy uniting the two groups. Nevertheless, the fact that there are both single and double amino acid insertions in the polyubiquitins from these organisms indicates that multiple insertion/deletion events have occurred. Several scenarios could explain the variation in insertion size within the Cercozoa/Foraminifera lineage, but the significance of this insertion is in its presence. An insertion of any length at this location indicates a unique tolerance of size heterogeneity or, perhaps more likely, the presence of a novel processing pathway that removes the extra residues. Either way, the result is an increased tolerance for variation at this site of the polygene.

Indeed, this tolerance is also suggested by our observation of two separate instances of variability in amino acid sequence within the polyubiquitin insertions of a given organism (fig. 2). In both *Lotharella amoeboformis* and *Cercomonas* sp. 18, sequence heterogeneity was found between different insertions within the same polyubiquitin gene. This is somewhat unexpected, given the high degree of conservation of the amino acid sequence flanking the junctions. Combined with the heterogeneity in insertion length between cercomonads and *Euglypha* versus Foraminifera and chlorarachniophytes, this is consistent with the possibility (discussed above) that the extra amino acids are in fact removed during polyubiquitin processing and are thus under somewhat reduced evolutionary constraints.

Concluding Remarks

The origins of both Cercozoa and Foraminifera have been evolutionary puzzles, but for very different reasons. On one hand, the evolution and systematics of Foraminifera have been extensively studied using morphological, paleontological, and molecular approaches, and various suggestions for their evolutionary position have been made. On morphological grounds, they have been suggested to be related to various amoebae or heterokonts (Lee 1990). Molecular data from Foraminifera have also generated conflicting conclusions; rRNA gene trees have suggested that Foraminifera are closely related to slime moulds and amoebae (Pawlowski et al. 1994) or, alternatively, that they are an extremely ancient eukaryotic lineage (Pawlowski et al. 1996). Detailed analyses of SSU rRNA have led to the conclusion that the rate of substitution in foraminiferan sequences is very high, confounding any conclusions as to the position of

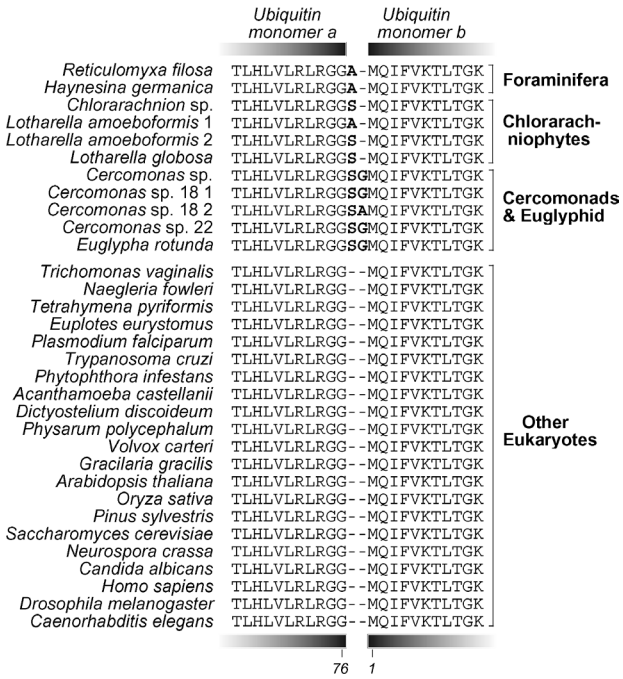

FIG. 2.—Evidence from polyubiquitin gene structure that Foraminifera are related to Cercozoa. Figure shows a junction between two ubiquitin monomers in polyubiquitins from Foraminifera and Cercozoa (chlorarachniophytes, cercomonads, and a euglyphid) aligned with those of animals, fungi, plants, algae, and other protists. The canonical C-terminal glycine residue (G76) of the ubiquitin monomer is indicated and the extra amino acids present in the foraminiferan and cercozoan sequences are in bold. Note that, with the exception of the insertions themselves, the sequence of this region is extremely highly conserved across the entire spectrum of eukaryotes. Amino acid substitutions are very rare and, when present, exist in only one or two of the repeat units present in the polyubiquitin gene of a given organism (data not shown).

Foraminifera in rRNA trees (Pawlowski et al. 1997). Cercozoa, on the other hand, have presented a very different puzzle since the group has only recently been recognized. Prior to the recognition of the Cercozoa, the evolutionary origin of each of its members was naturally considered independently. The cercomonads have been hypothesized to be related to bodonids (e.g., Hollande 1952), whereas thaumatomonads were thought to be related to heterokonts (e.g., Hollande 1952; Beech and Moestrup 1986). Chlorarachniophytes have been allied with heterokonts (Geitler 1930) and even tentatively with forams (Grell 1990), and various cercozoan amoebae have been considered most closely related to other amoeboid groups (e.g., Lee et al. 2000). The recognition that these morphologically diverse lineages were in fact related did little to suggest how they fit into the larger picture of eukaryotic evolution. Molecular phylogenies have been largely inconclusive, suggesting that Cercozoa (or some of its members) might be related to heterokonts (Van de Peer et al. 1996; Cavalier-Smith and Chao 1997) or revealing no stable position whatsoever (e.g., Keeling, Deane, and McFadden 1998; Dacks et al. 2002). Most recently, analyses of actin genes showed Cercozoa branching with Foraminifera (Keeling 2001), and now the shared presence of a unique insertion in a functionally critical position of their polyubiquitins significantly reinforces this conclusion.

It is now generally recognized that single-gene phylogenetic analyses often fail to correctly infer the relationships among the major groups of eukaryotes. This has led to the analysis of large concatenated sequence data sets (e.g., Martin et al. 1998; Baldauf et al. 2000; Bapteste et al. 2002). These analyses are helping to reshape eukaryotic diversity into a relatively small number of very diverse lineages, dubbed "supergroups." Unfortunately, many key eukaryotic lineages are still extremely poorly sampled from a molecular perspective, resulting in their exclusion from combined data analyses. In such cases, molecular markers that are independent of phylogenetic reconstruction, such as the polyubiquitin insertion characterized in this report, can be useful predictors of large-scale evolutionary relationships. The cercozoan/foraminiferan supergroup proposed here unites two large and diverse eukaryotic groups and represents a major advance towards a comprehensive and realistic picture of eukaryotic phylogeny.

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