Eukaryotic GCP1 is a conserved mitochondrial protein required for progression of embryo development beyond the globular stage in Arabidopsis thaliana

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INTRODUCTION

GCPs (glycoproteases) are members of the HSP70 (heat-shock protein 70)/actin ATPase superfamily that are highly conserved in taxonomically diverse species from bacteria to man, suggesting an essential physiological role. Although originally identified and annotated as putative endopeptidases, a proteolytic activity could not be confirmed for these proteins. Our survey of genome databases revealed that all eukaryotic organisms contain two GCP genes [called GCP1 and GCP2/Kae1 (kinase-associated endopeptidase 1)], whereas prokaryotes have only one, either of the GCP1- (Bacteria) or the GCP2/Kae- (Archaea) type. GCP2/Kae1 is essential for telomere elongation and transcription of essential genes, although little is known about the localization, expression and physiological role of GCP1. In the present study on GCP1-type proteins from eukaryotic organisms we demonstrated that GCP1 is a mitochondrial protein in Homo sapiens [called here GCP1/OSGEPL1 (O-sialoglycoprotein endopeptidase) and Arabidopsis thaliana, which is located/anchored to the mitochondrial inner membrane. Analysis of mRNA and protein levels revealed that the expression of GCP1/OSGEPL1 in A. thaliana and H. sapiens is tissue- and organ-specific and depends on the developmental stage, suggesting a more specialized function for this protein. We showed that homozygous A. thaliana GCP1 T-DNA (transferred DNA) insertion lines were embryonic lethal. Embryos in homozygous seeds were arrested at the globular stage and failed to undergo the transition into the heart stage. On the basis of these data we propose that the mitochondrial GCP1 is essential for embryonic development in plants.

Key words: Arabidopsis thaliana, embryo development, glycoprotease (GCP), mitochondrial localization, phylogeny, tissue-specific expression.

GCPs (glycoproteases) belong to the ASKHA (acetate and sugar kinases, HSP70 and actin) superfamily of phosphotransferases, but differ from other members of this superfamily by the presence of an insertion within the HSP70 (heat-shock protein 70)—actin fold, a protein structural motif which binds ATP in the presence of Ca\(^{2+}\) or Mg\(^{2+}\) ([http://merops.sanger.ac.uk](http://merops.sanger.ac.uk)). It was proposed that during evolution GCPs may have acquired protease function by grafting the metal-binding motif HXEXH on to the structural framework of the HSP70 domain, thus creating a protease-active site [1]. A proteolytic activity against O-sialoglycosylated proteins was reported for a GCP orthologue (Ec 3.4.24.57) in the Gram-negative pathogenic bacterium Mannheimia haemolytica (formerly Pasteurella haemolytica) [2]. However, such a protease activity was never confirmed for other GCP othologues.

In the present study, we have shown that two types of GCP, called GCP1 (found in Bacteria and Eukarya) and GCP2 (found in Archaea and Eukarya), are present in prokaryotic and eukaryotic organisms. It has been reported that GCP2 in yeast, called Kae1 (kinase-associated endopeptidase 1), is a component of the EKC—KEOPS (endopeptidase-like and kinase associated to transcribed chromatin—kinase, endopeptidase and other proteins of small size) complex that is essential for telomere elongation and transcription of essential genes [3,4]. GCP2/Kae1 is also found in a similar complex in Archaea [5,6]. Archaeal GCP2/Kae1 was further reported to bind DNA and exhibit class I apurinic endonuclease activity [7,8]. However, the precise biochemical function and biological role of GCP2/Kae1 still remains to be determined.

It was shown that GCP1 is an essential protein and its knockout is lethal in Escherichia coli [9,10], Staphylococcus aureus [11] and Arabidopsis thaliana (present study). Originally, it was described that GCP1 from M. haemolytica is secreted outside the cell and might be involved in the induction of an immune response [2]. E. coli contains two GCP1 orthologues, called YgjD and YeaZ [10]. Although the amino-acid sequence of YgjD resembles that of plant GCP1, yeast (Saccharomyces cerevisiae) and the gorilla (Gorilla gorilla) have their orthologue GCP1 (Genbank accession no. NM_001173).

Abbreviations used: ASKHA, acetate and sugar kinases, HSP70 and actin; COXII, subunit II of cytochrome c oxidase; Cy3, indocarbocyanine; EEC, endopeptidase-like and kinase associated to transcribed chromatin; F1α, F1β, subunit of ATP synthase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCP, glycoprotease; GDC-H, subunit H of glycine decarboxylase; HSP70, heat-shock protein 70; Kae1, kinase-associated endopeptidase 1; KEOPS, kinase, endopeptidase and other proteins of small size; LHCBI, the light-harvesting chlorophyll a/b-binding protein of photosystem II; OSGEPL, O-sialoglycoprotein endopeptidase; qPCR, quantitative PCR; sin2-1, short integuments 2-1; T-DNA, transferred DNA; TCA, trichloroacetic acid; WT, wild-type.

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The nucleotide sequence data reported will appear in Genbank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession numberAY024338.
cerevisiae) Qri7 or worm (Caenorhabditis elegans) and human (Homo sapiens) OSGEPL (O-sialoglycoprotein endopeptidase), the YeaZ sequence is truncated and restricted to Bacteria. It was reported that the repression of YeaZ expression results in cells with highly condensed nucleoids, whereas the repression of YgiD results in a large cell with unusual peripheral distribution of nucleoids [10]. Recently, the involvement of yeast Qri7 and worm OSGEPL in mitochondrial genome maintenance was proposed [12].

We selected GCP1 orthologues from A. thaliana and human (GCP1/OSGEPL1) for detailed analysis in order to gain insights into the function of these conserved proteins in higher eukaryotes. We demonstrated that GCP1/OSGEPL1 is located in mitochondria of both organisms. In plants, GCP1 is located anchored to the mitochondrial inner membrane and is expressed in young developing organs. Human GCP1/OSGEPL1 is also expressed in certain organs and tissue types, with the maximum expression measured in pituitary gland, prostate, rectum and uterus. Furthermore, homozygous gcpl-knockout mutants of A. thaliana are lethal at the early stages of embryo development, indicating that GCP1 is essential for plant viability. Our results suggest an unexpected connection between mitochondrial functions and the progression of embryo development beyond the globular stage in A. thaliana.

EXPERIMENTAL

Plant material and growth conditions

A. thaliana ecotype Columbia (Col-0) were grown in a growth chamber on soil at 20°C at a photon-flux density of 100 μmol·m⁻²·s⁻¹ under a light/dark cycle of 16 h light/8 h dark. The T-DNA (transferred DNA) insertion mutants GABI-Kat 322F06 (gcpl-1) and GABI-Kat 158E07 (gcpl-2) were generated under the GABI-Kat programme (http://www.gabi-kat.de) and provided by Dr Brnd Weisshaar (Max Planck Institute for Plant Breeding Research, Cologne, Germany) [13]. T-DNA insertion sites were verified by PCR using gene-specific primers that annealed upstream and downstream of the insertion sites of 322F06 (5'-CTT TCA TGC CAT TGC TTA TAT CAC G-3' and 5'-GTT CTA ATG CCC AGT CTA TTG CTC-3'), 158E07 (5'-GTT AGA GGT AAT GGT GAA ACT TTC-3' and 5'-AAA CCT AAT CCC TAC GTG GAA ACT-3') and a T-DNA-specific primer (5'-CCC ATT TGG ACG TGA TAG ATG CAC A-3'). The PCR products obtained were purified with a QiaEx kit (Qiagen) and sequenced (GATC Biotech AG, Konstanz, Germany). T3 generation mutant plants were selected by resistance against sulfadiazine on agar plates and the GCP1 band excised from the blot prior to raising polyclonal antibodies. SDS/PAGE, the proteins transferred on to a nitrocellulose membrane and the GCP1-containing fractions were separated by chromatography with Ni²⁺-NTA (nitrilotriacetate)–agarose. The GCP1 was purified under denaturing conditions by affinity chromatography with Ni²⁺-NTA (nitrilotriacetate)–agarose (Qiagen) and eluted from the column with 250 mM imidazole. The GCP1-containing fractions were separated by SDS/PAGE, the proteins transferred on to a nitrocellulose membrane and the GCP1 band excised from the blot prior to raising polyclonal antibodies (BioGenes).

Gene cloning and sequencing

On the basis of the A. thaliana GCP1 genomic sequence (GenBank® accession number, AA828263; locus, Atg45270), a pair of primers (5'-ATG GTT CGT CTG TTT CTT ACA CCT TTC-3' and 5'-GTT GAA GAG ATT CTC TAA-3') were designed and used for amplification of the GCP1 open-reading frame from the l-ZAP (Stratagene) cDNA library [10⁵ pfu (plaque-forming units)] prepared from A. thaliana seedlings. PCR (30 cycles) was carried out as follows: denaturation at 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 2 min. The amplified 1443 bp cDNA fragment was purified (QiaEx; Qiagen) and cloned into the pCR2.1 cloning vector (Invitrogen) prior to sequencing of both DNA strands (CyberGene).

RNA isolation and analysis

Total plant RNA was extracted from material frozen in liquid nitrogen using an RNeasy mini kit (Qiagen), according to the manufacturer’s protocol. After separation of 10 μg of RNA in 1.2% agarose gel, RNA was transferred on to a Hybond-N+ membrane (Amersham Biosciences) prior to the hybridization. The full-length GCP1 cDNA probe was labelled with [α-³²P]dCTP using a megaprime DNA labelling kit (Amersham Biosciences). The hybridization was performed as described previously [15].

For measurement of human tissue expression a cDNA panel representing 48 different human tissues [TissueScan Human Major Tissue qPCR (quantitative PCR) Array HMRT102; Origene] was used. The cDNA content of a single sample in the panel was normalized to equal levels of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) by the manufacturer. PCR was performed in a real-time 7900HT PCR system (Applied Biosystems) using Power SybrGreen Master Mix with ROX (Applied Biosystems) in 96-well system. Melting curves were recorded for single wells for verification of amplification specificity. Results of the qPCR (Ct values) were analysed with SDS 2.2 software (Applied Biosystems). The following primers were used for the amplification of human GCP1/OSGEPL1 (GenBank® accession number, AAH11904): forward 5'-CTGCC-ACAGTACGACCA-C-3' and reverse 5'-GGAGGAGGACA-CAAAAGTG-3'. Their specificity was verified with a dilution series of HeLa cell line cDNA. The resulting signals of each tissue were normalized against the GAPDH signals. The qPCR results (Ct values) are given as 1/exp(ΔΔCt) for the difference between the GCP1/OSGEPL1 and GAPDH Ct values. The values represent the Ct of GCP1/OSGEPL1–ct of GAPDH, recalculated as reciprocal values 1/Ct of GCP1/OSGEPL1–ct of GAPDH. We used further exponent function to facilitate presentation of differences. The specificity of amplification was shown by sequencing of the resulting PCR products.

Recombinant protein overexpression, purification and polyclonal antibody sources

The A. thaliana GCP1 cDNA was amplified using a pair of PCR primers (5'-GTT GTT CGT CTG TTT CTT ACA CCT TTG-3' and 5'-TTG AAG AGA ATC TGC TCT AAT G-3') and a full-length GCP1 cDNA clone (see above) as template. GCP1 was expressed in E. coli as a fusion protein with thioredoxin attached to the N-terminus and the His₆ tag attached to the C-terminus using the ThioFusion™ Expression System (Invitrogen), according to the manufacturer’s protocol. Recombinant A. thaliana GCP1 was purified under denaturing conditions by affinity chromatography with Ni²⁺-NTA (nitrilotriacetate)–agarose (Qiagen) and eluted from the column with 250 mM imidazole. The GCP1-containing fractions were separated by SDS/PAGE, the proteins transferred on to a nitrocellulose membrane and the GCP1 band excised from the blot prior to raising polyclonal antibodies (BioGenes).

The polyclonal antibodies against plant proteins, such as COXII (subunit II of cytochrome c oxidase), GDC-H (subunit H of the glycine decarboxylase) and LHCb2 (light-harvesting chlorophyll a/b-binding protein of photosystem II), were purchased from Agrisera AB (Vännäs, Sweden).

Protein analysis

Total plant proteins were isolated as described previously [16]. Total or mitochondrial proteins (see below) were separated
by SDS/PAGE [17] generally using 12% polyacrylamide minigels (Hoefer mini-gel system). The gels were loaded on an equal protein basis (5 μg). Immunoblotting was carried out as described previously [18] using a nitrocellulose membrane with 45-μm pores (Hybond-P; Amersham Biosciences) and an enhanced chemiluminescence assay (ECL; Amersham Biosciences) as the detection system.

Isolation and fractionation of organelles

Intact chloroplasts of A. thaliana were purified on Percoll gradients as described by Cline [19]. Mitochondria from rat liver and HeLa cells were isolated as described previously [20,21]. Intact mitochondria from A. thaliana were isolated either from 2-3-week-old seedlings (developing leaves) or from 4-6-week-old (mature) leaves of A. thaliana using a density-step gradient centrifugation as described previously [22]. For fractionation into soluble and membrane fractions, mitochondria were osmotically lysed for 10 min on ice in 10 mM Tris/HCl, pH 7.5, and centrifuged for 1 h at 4 °C at 130000 g. The pellet (total mitochondrial membranes) and supernatant (total soluble fractions) were collected, proteins in the supernatant precipitated with 5% (v/v, final concentration) TCA (trichloroacetic acid) on ice for 30 min and collected by centrifugation at 14000 g at 4 °C for 10 min. Both subfractions were solubilized in equal volumes of Laemmli sample buffer [17]. For isolation of peripheral and integral membrane proteins, isolated mitochondrial membranes were resuspended in 25 mM Mes buffer, pH 6.5, at a protein concentration of 0.1 mg · ml⁻¹, and subjected to washes with salt and chaotropic agents as described previously [23]. For the protease protection assay, intact or osmotically lysed mitochondria were resuspended in 50 mM Hepes, pH 8.8, and 330 mM sorbitol at a protein concentration of 1 mg · ml⁻¹ and incubated for 30 min at 4 °C in the absence or the presence of 50 μg · ml⁻¹ trypsin. After TCA precipitation (5% (v/v, final concentration) for 30 min on ice, proteins were pelleted by centrifugation at 14000 g at 4 °C for 10 min and used for immunoblotting as described above.

Immunohistochemistry

Human HeLa cells were grown under humidified standard conditions (37 °C, 5% CO₂) in high-glucose DMEM (Dulbecco’s modified Eagle’s medium) (Invitrogen) supplemented with 10% (v/v) FBS (fetal bovine serum) (Biochrom AG, Berlin, Germany), 2 mM GlutaMAX™ (Invitrogen) and 100 units · ml⁻¹ penicillin/100 μg · ml⁻¹ streptomycin (Invitrogen). The cells were incubated for 30 min with a fresh staining medium containing minimum essential medium with 10% (v/v) FBS and 300 nM MitoTracker Orange. After removal of medium, cells were washed twice with PBS and fixed with 3.7% (v/v) formaldehyde for 10 min at 37 °C. To enable binding of specific antibodies, fixed cells were permeabilized with 0.5% (v/v) Triton X-100 for 5 min at 25 °C. To prevent unspecific binding of proteins, blocking with salt and chaotropic agents as described previously [23]. For the protease protection assay, intact or osmotically lysed mitochondria were resuspended in 50 mM Hepes, pH 8.8, and 330 mM sorbitol at a protein concentration of 1 mg · ml⁻¹ and incubated for 30 min at 4 °C in the absence or the presence of 50 μg · ml⁻¹ trypsin. After TCA precipitation (5% (v/v, final concentration) for 30 min on ice, proteins were pelleted by centrifugation at 14000 g at 4 °C for 10 min and used for immunoblotting as described above.

Phenotypic analysis of A. thaliana mutants

Developing siliques of heterozygous mutants and WT (wild-type) plants were sliced longitudinally and analysed under a stereomicroscope (Carl Zeiss). Seeds were counted and classified as normal (green seeds) or abnormal (white seeds). For microscopical analysis, developing siliques of heterozygous mutants were fixed and cleared essentially as described previously [24], with three additional washing steps of 50% and 25% (v/v) ethanol and distilled water before the addition of the clearing solution. The seeds were dissected before viewing by transmitted light microscopy with Nomarski’s differential interference contrast illumination using an BX51 epifluorescence microscope (Olympus) equipped with a Dxm1200 digital camera system (Nikon). Digital pictures were acquired with the ACT-1 software (Nikon).

Bioinformatics

Similarity searches were performed using the Advanced BLAST program. The transmembrane regions were predicted using the dense-alignment surface method and the hydropathy plot was drawn as described in [25]. Prediction of subcellular location and determination of the processing site were analysed by the TargetP v.1.1 and MitoProt II 1.0a4 programs. The prediction of protein pattern and motifs were performed using sequence motif search and protein motif fingerprint databases. All software programs used are accessible on the Internet (http://www.expasy.org/tools and http://www.genome.ad.jp). The phylogenetic tree was prepared using full-length amino-acid sequences of the GCP1 and GCP2/Kae1 orthologues from selected organisms. The sequences were aligned with T-Coffee software [26] and the neighbour-joining tree was reconstructed from this alignment with the MEGA software package, version 3.1 [27].

Accession numbers

The GenBank accession number for the nucleotide sequence (A. thaliana Col-0) described in this article is AY024338 (GCP1 cDNA).

RESULTS AND DISCUSSION

Conservation of GCP1 in Bacteria and Eukarya

Two highly conserved GCP paralogues, called GCP1 and GCP2/Kae1 in the present study, are present in all Archaea, Bacteria and Eukarya investigated so far (Figure 1A) [12]. The only two exceptions are the highly reduced genomes of the endosymbiotic bacteria Carsonella ruddii and Sulcia muelleri [7]. Phylogenetic analysis revealed that GCP1 and GCP2/Kae1 form two distinct clades (Figure 1A). The GCP1 clade contains orthologues present in Bacteria and Eukarya, whereas the GCP2/Kae1 clade clusters orthologues present in Archaea and Eukarya (Figure 1A). We focused our present study on the eukaryotic GCP1 orthologues from A. thaliana (NCBI Gene ID, 819135) and H. sapiens (NCBI Gene ID, 64172).

Recently, the atomic structures of the KEOPS complex [6] and GCP2/Kae1 protein from Archaea [5,8] were solved. On the basis of these structures GCP2/Kae1 is a metalloprotein containing an iron ion directly linked to ATP via the γ-phosphate.
Figure 1 Conservation of GCPs in prokaryotic and eukaryotic organisms

(A) Phylogenetic analysis of GCP1 and GCP2/Kae1 paralogues from various prokaryotic and eukaryotic organisms. A non-rooted phylogenetic tree was generated as described in the Experimental section. Bootstrap percentage supports are indicated. Ath, A. thaliana; Bme, Brucella melitensis; BsU, Bacillus subtilis; Dme, Drosophila melanogaster; Dre, Danio rerio; Eco, E. coli; Hin, H. influenzae; Hsa, H. sapiens; Mmu, Mus musculus; Mth, Methanobacterium thermoautotrophicum; Osa, Oryza sativa; Plu, Pyrococcus furiosus; Rco, Rickettsia conorii; Spp, Schizosaccharomyces pombe; Tak, Kaempferia galanga. The accession numbers of sequences are listed in Supplementary Table S1 (http://www.BiochemJ.org/bj/423/bj4230333add.htm). A highly conserved histidine residue present +23 to +25 amino acids downstream from the histidine dyad might provide a fourth metal ligand-binding residue in GCP1 orthologues (Supplementary Figure S1). Furthermore, eukaryotic GCP1 orthologues possesses an N-terminal transit peptide that is present in proteins targeted to mitochondria (predicted for human) and to chloroplasts (predicted for plants) (Supplementary Figure S1).

Orthologous GCP1 from M. haemolytica has been reported previously to exhibit protease activity [2]. Later, the authors of the original annotation suggested that the identification of GCP1 as a glycoprotease might have been misleading [28]. Similarly, we could not detect proteolytic activity when we tested recombinant refolded A. thaliana GCP1 in vitro against several universal protease substrates, such as β-casein, gelatine, haemoglobin, azocoll, albumin or specific glycosylated substrates, such as glycophorin A or bovine milk κ-casein (results not shown). Also the GCP2/Kae1 from Archaea did not exhibit endopeptidase activity in vitro [8]. This suggests that purified GCP1 (and possibly also GCP2/Kae1) might not be correctly folded, has a narrow substrate specificity, requires specific interacting partners, cofactors or post-translational modification for its activity, or finally, and most probably, has a function other than proteolysis. It was further reported that mutation of the two conserved histidine residues in yeast GCP2/Kae1 completely inactivated its function and led to several defects in cell-cycle progression and polarized growth, which were ascribed to a defective transcriptional response [3]. This is in agreement with a previous study [6] showing that the ATPase catalytic function of GCP2/Kae1 in Archaea depends on bound metal ions. This further implies a similar importance of the conserved histidine residues for GCP1 function.

The crystal structure of YeaZ from Salmonella typhimurium revealed the lack of the conserved histidine dyad involved in metal binding in GCP2/Kae1, although it was suggested that both paralogous YeaZ and YgjD might bind the same ligands due to their conserved pocket [29]. It was further suggested that YeaZ requires interaction with a partner protein to adopt an active conformation in order to bind its ligand. Interestingly, an interaction between YeaZ and YgjD in E. coli has been reported recently [10].

Localization and expression of GCP1 in A. thaliana

In order to perform localization and expression studies at the protein level we expressed A. thaliana GCP1 in E. coli. The recombinant protein accumulated in inclusion bodies and was purified under denaturing conditions prior to raising polyclonal antibodies. The obtained anti-GCP1 antibody cross-reacted with two distinct bands in A. thaliana membrane protein extracts (Figure 2).

We assume that the lower band may have resulted either from post-translational modifications or limited proteolysis of GCP1, since it appeared in various ratios, depending on sample storage and treatment.

To determine the cellular location of GCP1, we performed Western blot analysis using total cell extracts, isolated intact mitochondria and chloroplasts. GCP1 was detected in the total cell extract and mitochondria, but not in chloroplasts (Figure 2A).

Interestingly, a strong GCP1 signal was obtained only in mitochondria isolated from very young seedlings and not in those isolated from mature leaves (Figure 2A). Immunoblots with

As predicted by Aravind and Koonin [1] the archaeal GCP2/Kae1 contains the ASKHA fold that consists of two subdomains with the active-site HHEXH motif residing in the cleft region between these subdomains [5,6,8]. The ferric ion in GCP2/Kae1 from the archaeon Pyrococcus abyssi is tetrahedrally liganded by His-107, His-111, Tyr-127 and Asp-285 [8]. A comparison of the conserved domains of GCP1 orthologues from various organisms (alternative names are given on the left of Figure 1C) revealed the conservation of two histidine residues (Figures 1B and 1C). Although both histidine (His-194 and His-198 in A. thaliana, and His-147 and His-151 in H. sapiens) and aspartate (Asp-402 in A. thaliana and Asp-358 in H. sapiens) residues are conserved in GCP1 orthologues the tyrosine residue ligand is missing (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/423/bj4230333add.htm). A highly conserved histidine residue present +23 to +25 amino acids downstream from the histidine dyad might provide a fourth metal ligand-binding residue in GCP1 orthologues (Supplementary Figure S1). Furthermore, eukaryotic GCP1 orthologues possesses an N-terminal transit peptide that is present in proteins targeted to mitochondria (predicted for human) and to chloroplasts (predicted for plants) (Supplementary Figure S1).
Figure 2 Localization of GCP1 in *A. thaliana*

(A) Localization of GCP1 in total cell extract (Total), isolated intact chloroplasts (Chloro) and mitochondria from young developing seedlings (Mito-D) and mature leaves (Mito-M) assayed by immunoblotting. LHCB2 and F1β were used as markers for chloroplast and mitochondria respectively. The protein pattern of isolated fractions was analysed on SDS gels stained with Coomassie. (B) Isolated intact mitochondria were lysed osmotically and separated into soluble and membrane (Memb) fractions followed by immunoblotting with the anti-GCP1 antibody. As references, the distribution of the inner-membrane-located COXII and the matrix-located GDC-H were analysed by immunoblotting. (C) Isolated mitochondrial membranes were incubated in the absence (Control) or in the presence of various salt and chaotropic agents to remove peripheral membrane proteins. The membrane pellets (M) containing integral/anchored membrane proteins and the supernatants (S) containing extracted peripheral membrane proteins were used for immunoblotting with the anti-GCP1 antibody. As a reference for the distribution of the peripheral membrane proteins the presence of F1β is shown by immunoblotting. (D) A protease-protection assay carried out in the absence (−) or the presence (+) of trypsin added to intact or osmotically lysed mitochondria prior to immunoblotting with the anti-GCP1 antibody.

Immunoblots using antibodies against the inner-membrane-located COXII [32] and the matrix-located GDC-H [33] confirmed the purity of the obtained fractions.

According to the hydropathy plot, GCP1 contains two hydrophobic stretches that might theoretically provide membrane attachment sites (results not shown). However, on the basis of the GCP2/Kae1 structure these α-helices form a hydrophobic core of the protein [5,6,8]. To determine whether GCP1 is a peripheral protein or a membrane-anchored protein, we washed isolated mitochondrial membranes with various salt and chaotropic agents to release extrinsic membrane proteins [23]. The pellet fraction, containing integral and membrane-anchored proteins, and the supernatant fraction, containing peripheral membrane proteins, were used for immunoblotting. The presence of GCP1 in the pellet fraction indicated its membrane location (Figure 2C). In contrast, F1β, which is known to be a peripheral membrane protein [30], was partially or completely released from the membrane, depending of the stringency of washes.

To investigate whether GCP1 is located in the mitochondrial outer or inner membrane, we performed protease-protection assays with intact or osmotically lysed mitochondria. The protection of GCP1 in intact mitochondria and its susceptibility to trypsin digestion in osmotically broken organelles suggested that GCP1 is located/anchored in the mitochondrial inner membrane (Figure 2D).

Expression studies revealed that the GCP1 transcript and protein accumulated transiently at the early stages of seedling development (Figure 3A).

Figure 3 Expression of GCP1 in *A. thaliana*

(A) Expression of GCP1 transcript and GCP1 protein during 1–4 weeks of seedling development, as assayed by Northern (upper two panels) and Western blotting (lower two panels) respectively. All Northern blots contained 10 μg of total RNA and were hybridized to a 32P-labelled GCP1 cDNA probe. As a reference, the rRNA pattern in the gel stained by ethidium bromide is shown. An equal loading of proteins (5 μg) is shown by Coomassie staining of a constantly expressed protein of unknown identity (Loading control). (B) Accumulation of GCP1 in various organs. D, developing; M, mature; Md, mature dried; Mi, mature imbibed. An equal loading of proteins (5 μg) is shown by Coomassie staining of a constantly expressed protein of unknown identity (Loading control).

two marker proteins, mitochondria-located F1β (F1β subunit of the ATP synthase) [30] and LHCB2 [31], confirmed the purity of preparations. Also, the pattern of proteins, as visualized by Coomassie staining, differed for each fraction (Figure 2A). These results are in agreement with the mitochondrial location predicted for the GCP1 orthologue from *S. cerevisiae* and experimentally shown for *C. elegans* [12].

To test whether GCP1 is a membrane or a soluble protein, we fractionated purified mitochondria into the membrane fraction, containing mitochondrial inner and outer membranes, and the soluble fraction, containing the mitochondrial matrix and intramembrane space. Immunoblot analysis with the anti-GCP1 antibody found GCP1 exclusively in the membrane fraction (Figure 2B).
development (Figure 3A). The maximal transcript level of GCP1 in total seedlings was reached during week 3 after seed germination, as shown by Northern blotting (Figure 3A). During weeks 1, 2 and 4 only traces of GCP1 transcripts were detected. In contrast, a significant amount of GCP1 protein accumulated already during week 1 after seed germination and this amount increased 3- and 5-fold during week 2 and 3 respectively. During week 4 the amount of GCP1 decreased again, reaching the level present after week 1 (Figure 3A).

We also investigated GCP1 expression during development of various organs. Immunoblotting detected GCP1 in young developing leaves, roots, flowers and siliques (Figure 3B). Much lower amounts of GCP1 were found in mature roots, flowers and siliques, and no immunoblot signals were visible in mature leaves or mature dried or imbibed seeds.

Localization and expression of GCP1/OSGEPL1 in H. sapiens

The high conservation of GCP1 among various taxa prompted us to test the A. thaliana anti-GCP1 antibody for its cross-reactivity with mitochondria isolated from yeast, human and rat. Although no positive reaction was obtained using yeast samples (results not shown), a strong specific GCP1/OSGEPL1 signal was observed in human and rat mitochondrial extracts (Figure 4A). Three protein bands were recognized by the anti-GCP1/OSGEPL1 antibody in human mitochondria samples, which might correspond to different GCP1/OSGEPL1 isoforms. At least nine isoforms have been reported for human GCP1/OSGEPL1 [34].

Taking advantage of the cross-reactivity of A. thaliana anti-GCP1 antibody with its human orthologue, we investigated the localization of GCP1/OSGEPL1 in HeLa cells by immunocytochemistry. Co-localization of a fluorescent mitochondrial marker MitoTracker Orange and signals from labelled anti-GCP1/OSGEPL1 antibodies demonstrated the presence of GCP1/OSGEPL1 in mitochondria of HeLa cells (Figure 4B).

A cDNA panel representing 48 human tissues was used to investigate GCP1/OSGEPL1 expression. Similar to A. thaliana the expression of human GCP1/OSGEPL1 transcripts was tissue-specific (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/423/bj4230333add.htm). The maximum expression level was measured in prostate, pituitary glands, uterus and rectum. No or very low expression of GCP1/OSGEPL1 transcripts was observed in bone marrow, liver, mammary gland, muscle, nasal mucosa and oviduct (see Supplementary Figure S2). This suggests that, in contrast with GCP2/Kae1, the physiological function of GCP1/OSGEPL1 is less general and connected to certain organs and tissues.

Disruption of the GCP1 gene in A. thaliana

The conservation of GCP1 orthologues across taxonomically diverse species implies an essential physiological function. To elucidate this function we analysed two A. thaliana mutant lines from the GABI-Kat collection carrying T-DNA insertions within the GCP1 gene [13]. PCR analysis showed that the insertion in the gcp1-1 (GABI-Kat line 322F06) mutant is located in the third intron and that the gcp1-2 (GABI-Kat line 158E07) mutant carries an inverted tandem repeat T-DNA insertion in the seventh intron (Figure 5A). The PCR screening of T3 and T4 seedling generations grown on selective media detected only heterozygous plants. This suggested that the disruption of the GCP1 gene might be lethal at the early stages of seed formation and embryo development. Therefore, we investigated individual immature siliques of heterozygous plants for abnormal seeds [35]. Whereas WT siliques showed consistently maturing seeds, abnormal seeds were present beside normally formed and developed seeds in young siliques of heterozygous plants. Out of the 794 and 736 seeds analysed for heterozygous gcp1-1 and gcp1-2 mutant plants, 183 (23 %) and 188 (26 %) respectively were abnormally formed (Table 1). According to statistical analysis with the χ² test, these numbers are in agreement with a recessive lethal segregation of seeds homozygous for the insertion [24,35]. We further followed embryo development in immature siliques of heterozygous plants by interference contrast light microscopy. We noticed that whereas embryos in WT and heterozygous seeds underwent the well-defined developmental stages, such as globular, heart, torpedo, walking-stick or curled, embryos in, presumably, homozygous seeds were arrested at the globular stage and did not undergo transition into the heart stage (Figure 5B). Both GCP1 alleles conferred the same embryo phenotypes.

It was reported that a number of critical developmental events occur during the globular–heart transition period, including the differentiation of primary embryonic tissue layers and the initiation of embryonic organs, which make this period particularly sensitive to mutations [36]. Thus we expected that A. thaliana GCP1 is essential for one of these events. This is
Recent studies demonstrated that Qri7 in *S. cerevisiae* and OSGEPL in *C. elegans* are involved in mitochondrial genome maintenance [12]. The *gri7Δ* mutant of yeast contained highly fragmented mitochondria that lost their mtDNA (mitochondrial DNA) [12]. The *osgl-1Δ* mutant of *C. elegans* has enhanced longevity, showed increased resistance to oxidative stress and the mitochondrial network was disorganized [12]. Although all these reports imply that GCP1 is important for mitochondrial physiology, the exact role of this protein still remains to be elucidated.

**Conclusions**

On the basis on their conservation throughout the genomes, GCPs were included into a set of approx. 60 universal proteins that were probably present in the last universal cellular ancestor of all extant life [40]. Therefore understanding the biological role of the universal GCPs remains a major challenge.

Localization and expression studies suggest that the physiological function of GCP1 in Eukarya and Bacteria might differ from that reported for GCP2/Kae1 in yeast and Archaea [3,7,8]. First, GCP2/Kae1 is part of the EKC–KEOPS transcription complex and is associated with serine/threonine kinase Bud32/PRPK (p53-related protein kinase) in Archaea [6], yeast [41,42] and humans [43]. No subunits of EKC–KEOPS complex have been identified in bacteria [6,8] or in mitochondria that are of bacterial origin [44]. Secondly, the expression of GCP1/OSGEPL1 in plants and humans is restricted to certain developmental stages, tissues and organs, which suggests a more specific function of GCP1/OSGEPL1. The putative protease activity of GCP proteins...
can still not be completely dismissed, although neither the crystal structure of GCP2/Kae1 [5,6] nor biochemical activity tests with recombinant GCP1 (present work) and GCP2/Kae1 [8] provided evidence for an endopeptidase activity.

AUTHOR CONTRIBUTION

Kirsten Haussuehl and Pitter Huesgen performed research on plant GCP1, analysed data and wrote the draft of the paper. Marc Meier performed experiments on GCP1/OSGEPL1 expression in H. sapiens, and analysed and interpreted data. Patrick Dessi performed the localization study in plants and interpreted data. Elzbieta Glaser provided suggestions and advice on mitochondrial analysis. Jerzy Adamski performed research on GCP1/OSGEPL1 localization in H. sapiens, analysed and interpreted data and contributed to the manuscript draft. Iwona Adamska performed research on plant GCP1, co-conceived the research and edited the manuscript.

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SUPPLEMENTARY ONLINE DATA

Eukaryotic GCP1 is a conserved mitochondrial protein required for progression of embryo development beyond the globular stage in Arabidopsis thaliana

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Figure S1  Amino acid alignment of GCP1 orthologues from prokaryotic and eukaryotic organisms

Abbreviations of organism names are explained in Figure 1 of the main paper. Three confirmed metal ligand-binding residues in the GCP2/Kae1 structure [1–3] that are conserved in GCP1 are marked in red, and the predicted metal ligand-binding residues in black. Arrows show the predicted mitochondrial processing sites (mTP).

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Expression of GCP1/OSGEPL1 transcripts in 48 human tissues was analysed as described in the Experimental section. The expression levels are given as comparative Ct values on the basis of real-time PCR. The qPCR results corresponding to Ct values are given as 1/exp(ΔCt) for the difference between Ct for GCP1/OSGEPL1 and GAPDH respectively.

### Table S1  GCP1 and GCP2/Kae1 orthologues in different organisms

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