The Chloroplast DnaJ Homolog CDJ1 of *Chlamydomonas reinhardtii* Is Part of a Multichaperone Complex Containing HSP70B, CGE1, and HSP90C[1][OA]

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We report on the molecular and biochemical characterization of CDJ1, one of three zinc-finger-containing J-domain proteins encoded by the *Chlamydomonas reinhardtii* genome. Fractionation experiments indicate that CDJ1 is a plastidic protein. In the chloroplast, CDJ1 was localized to the soluble stroma fraction, but also to thylakoids and to low density membranes. Although the *CDJ1* gene was strongly heat shock inducible, CDJ1 protein levels increased only slightly during heat shock. Cellular CDJ1 concentrations were close to those of heat shock protein 70B (HSP70B), the major HSP70 in the *Chlamydomonas* chloroplast. CDJ1 complemented the temperature-sensitive phenotype of an *Escherichia coli* mutant lacking its *dnaJ* gene and interacted with *E. coli* DnaK, hence classifying it as a bona fide DnaJ protein. In soluble cell extracts, CDJ1 was found to organize into stable dimers and into complexes of high molecular mass. Immunoprecipitation experiments revealed that CDJ1 forms common complexes with plastidic HSP90C, HSP70B, and CGE1. In blue native-polyacrylamide gel electrophoresis, all four (co)chaperones migrated at 40% to 90% higher apparent than calculated molecular masses, indicating that greatest care must be taken when molecular masses of protein complexes are estimated from their migration relative to standard native marker proteins. Immunoprecipitation experiments from size-fractioned soluble cell extracts suggested that HSP90C and HSP70B exist as preformed complex that is joined by CDJ1. In summary, CDJ1 and CGE1 are novel cohort proteins of the chloroplast HSP90-HSP70 multichaperone complex. As HSP70B, CDJ1, and CGE1 are derived from the endosymbiont, whereas HSP90C is of eukaryotic origin, we observe in the chloroplast the interaction of two chaperone systems of distinct evolutionary origin.

Molecular chaperones of the heat shock protein 70 (HSP70) family are involved in a variety of different tasks, like the folding of nonnative proteins to the native state (Frydman, 2001; Hartl and Hayer-Hartl, 2002), protein quality control (Bukau et al., 2006), the transport of proteins across membranes (Neupert and Brunner, 2002), the assembly and disassembly of protein complexes (Mayer, 2005), or the regulation of the stress response (Voellmy and Boellmann, 2007). HSP70s function in concert with different cochaperones, which are usually involved in one of the following processes (or a combination of them): they regulate the ATPase activity of their HSP70 partner, supply their HSP70 partner with substrates, or connect it with other proteins involved in protein folding or degradation.

An important class of HSP70 cochaperones is the one of the J-domain proteins (Craig et al., 2006; Qiu et al., 2006). These interact via their J domains with HSP70s in the ATP state (Wittung-Strafshede et al., 2003), stimulate the ATPase activity of their HSP70 partner (Liberek et al., 1991), and lock the latter onto specific substrates (Han and Christen, 2003). By this, J-domain proteins mediate substrate specificity and thereby the function of their HSP70 partner. The J domain has a length of about 70 amino acids and contains a conserved tripeptide of His, Pro, and Asp (HPD motif), which is essential for the stimulation of HSP70's ATPase activity (Wall et al., 1994). J-domain proteins are divided into three groups (Cheetham and Caplan, 1998): type I J-domain proteins, which contain all canonical domains present in DnaJ (the J domain, the Gly/Phe-rich region, the Cys-rich, the zinc-finger domain [ZFD], and the DnaJ C-terminal domain); type II proteins contain a J domain and the Gly/Phe-rich region; and type III proteins only contain a J domain. While type I and II J-domain proteins were shown to function with their HSP70 partner in the folding of (partially) unfolded substrate proteins, type III proteins are thought to recruit their HSP70 partner for highly specific functions (e.g. auxilin recruits Hsc70 for the uncoating of clathrin lattices; Ungewickell et al., 1995). In most organisms studied to date, the number of J-domain proteins exceeds the number of

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Hsp70 chaperones, suggesting that one HSP70 may be recruited by multiple J-domain proteins to different targets within a cellular compartment (Craig et al., 2006). For example, the chloroplast HSP70B protein of *Chlamydomonas reinhardtii* cooperates with at least five different J-domain proteins termed chloroplast DnaJ homologs (CDJ) 1 to 5 (Liu et al., 2005; Schroda and Vallon, 2008). One of these, CDJ2, was shown to recruit HSP70B for the assembly and disassembly of oligomers formed by the VIPP1 protein (Liu et al., 2007).

Another class of HSP70 cochaperones are the GrpE-type nucleotide exchange factors, which regulate the activities of bacterial DnaKs and of the major HSP70s in mitochondria and chloroplasts, where they are called MGE1 (Laloraya et al., 1994) and CGE1 (Schroda et al., 2001), respectively. In *Chlamydomonas*, CGE1 exists as two isoforms, a and b, which differ by the presence in CGE1b of a valine and a glutamine residue at positions 4 and 5 in the N terminus of the mature protein (Schroda et al., 2001). This difference is due to temperature-dependent alternative splicing of *CGE1* transcript, leading to elevated levels of CGE1a at low growth temperatures and CGE1b accumulating to the same levels as CGE1a at temperatures above 30°C. Interestingly, CGE1b had an about 25% higher affinity for HSP70B than CGE1a (Willmund et al., 2007).

Finally, functionality of mitochondrial and chloroplast HSP70s has been shown to depend on specific escort proteins termed HEP (Sichting et al., 2005; Willmund et al., 2008). At least in the case of chloroplast HSP70B, its HEP partner appears to be required for the initial folding of the chaperone, presumably right after its import into the organelle.

We have demonstrated previously that HSP70B interacts with the plastidic HSP90C protein in *Chlamydomonas* chloroplasts (Willmund and Schroda, 2005), suggesting that, like in the eukaryotic cytosol (Pratt and Toft, 2003) and in the endoplasmic reticulum (ER; Meunier et al., 2002), certain chloroplast proteins require both HSP70 and HSP90 chaperone systems for folding. Cytosolic HSP90 interacts with a large set of cohort proteins, which comprise HSP70, HSP40, HOP, p23, CHIP, CDC37, TPR2, AHA1, and several immunophilins (Pratt and Toft, 2003; Wegele et al., 2004). Whereas several cohort proteins have been characterized also for the ER-resident Grp94 (Meunier et al., 2002), none has yet been identified for bacterial or organellar HSP90s. A major function attributed to (cytosolic) HSP90 is a role in the maturation of signal transduction proteins, like hormone receptors and kinases (Richter and Buchner, 2001; Pratt and Toft, 2003; Wegele et al., 2004). However, HSP90 has also been implicated in the general refolding of denatured proteins (Jakob et al., 1995; Wegele et al., 2006) and cytosolic HSP90 also participates in the regulation of the stress response (Ali et al., 1998; Zou et al., 1998). Most information on chloroplast HSP90 functions was gathered from studies with the Arabidopsis (*Arabidopsis thaliana*) *chlorate resistant88* (*cr88*) mutant, which carries a point mutation in the C terminus of an HSP90 targeted to the chloroplast stroma (Lin and Cheng, 1997; Cao et al., 2003). The *cr88* mutant has a yellow-green appearance due to a retarded development of chloroplasts, observed particularly in young leaves. The mutant exhibits reduced light-inducible expression of the nuclear *NR2*, *CAB*, and *RBCS* genes, and also of the plastid-encoded *rbcL* gene. Furthermore, the *cr88* mutant showed retarded deetiolation in red light (Lin and Cheng, 1997; Cao et al., 2000). These findings suggest a role of plastidic HSP90 in the transduction of a plastid-derived signal that is responsible for the regulation of a subset of photosynthesis-related genes.

Here we report on the characterization of the *Chlamydomonas* CDJ1 protein. CDJ1 is one of three ZFD-containing J-domain proteins encoded by the *Chlamydomonas* genome. CDJ1 is a chloroplast DnaJ homolog, since it complements the temperature-sensitive phenotype of an *Escherichia coli* Δ*dnaJ* strain and localizes to the chloroplast stroma and chloroplast membranes. CDJ1 is part of complexes containing HSP90C, HSP70B, and CGE1, and therefore CDJ1 and CGE1 qualify as novel cohort proteins of the plastidic HSP90-HSP70 multichaperone system.

RESULTS

Three Genes in the *Chlamydomonas* Genome Encode Zinc-Finger-Containing J-Domain Proteins

Our goal was to identify zinc-finger-containing DnaJ proteins in *Chlamydomonas* that are potentially targeted to the chloroplast. To this end we searched version 3.0 of the *Chlamydomonas* genome (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html) for genes that encode proteins harboring a J domain. Among the 63 genes found to encode J-domain proteins only three coded for DnaJ proteins harboring the four CxxCxGxG repeats typical for DnaJ ZFDs (Fig. 1A; Cheetham and Caplan, 1998; Craig et al., 2006). To get a hint of the evolutionary origin of these three *Chlamydomonas* DnaJ proteins, we performed phylogenetic analyses based on alignments with ZFD-containing DnaJ proteins from (cyano)bacteria and from the cytoplasmic, mitochondrial, and chloroplast compartments of eukaryotic cells. As becomes evident from the phylogram presented in Figure 1B, ZFD-containing DnaJ proteins from cytosol, mitochondria, and chloroplasts/cyanobacteria separate into three distinct clades. Each of the three *Chlamydomonas* ZFD-containing DnaJ proteins was found in one of these three clades, which suggested that they are localized to cytoplasm, mitochondria, and chloroplasts. Accordingly, only the two *Chlamydomonas* proteins within the mitochondrial and chloroplast clades contained N-terminal extensions that by the TargetP program (Emanuelsson et al., 2000) were predicted to qualify as organellar targeting signals. Moreover, the *Chlamydomonas* protein within the mitochondrial clade, like its Arabidopsis homolog

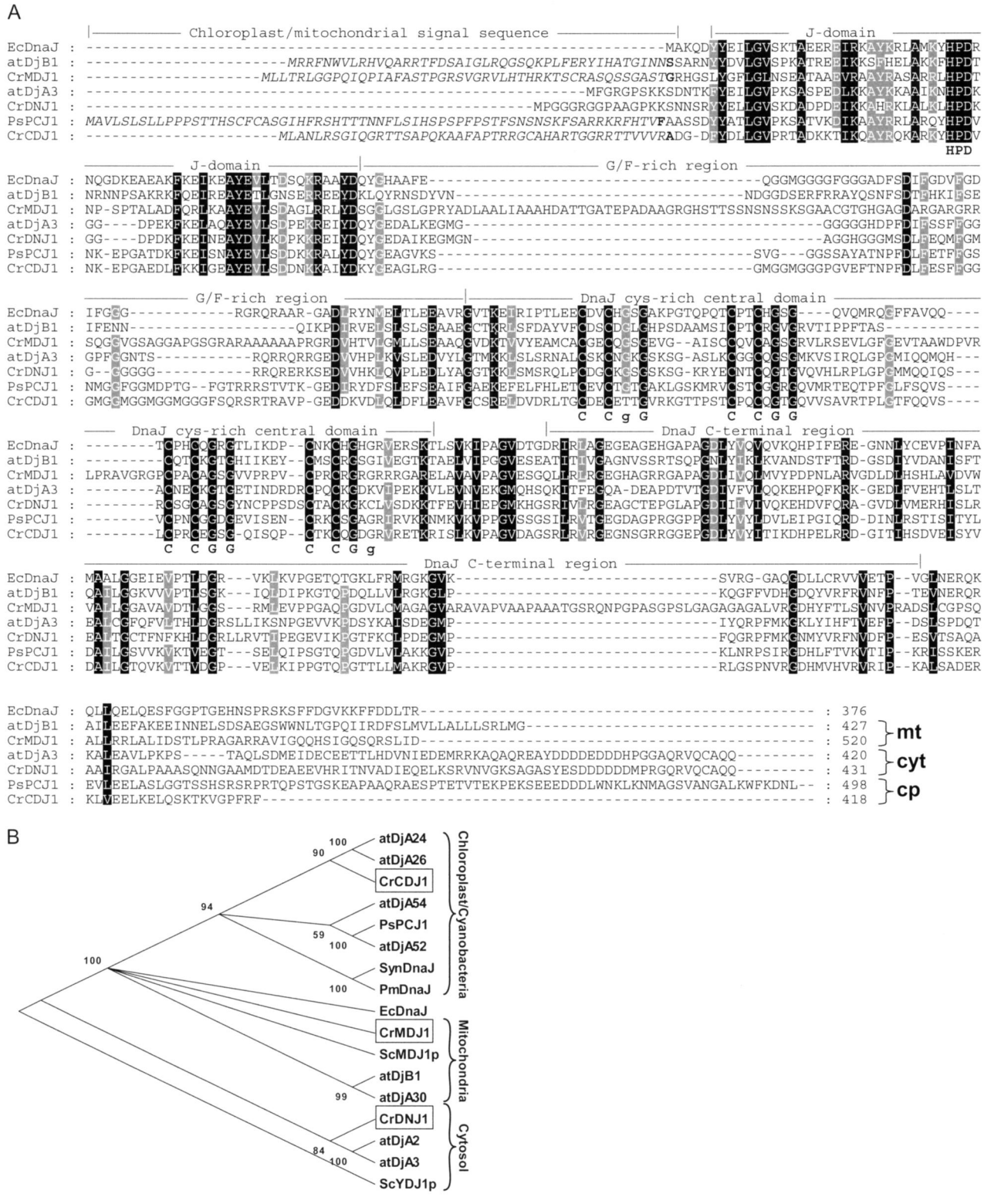

Figure 1. Alignment and phylogenetic tree of DnaJ homologs. A, Alignment of amino acid sequences of DnaJ homologs. Sequences aligned are from *E. coli* DnaJ (EcDnaJ, accession AAA23693); from Arabidopsis mitochondrial and cytosolic DnaJ homologs (atDjB1 and atDjA3, accessions NP_174142 and AAB49030, respectively); from *Chlamydomonas* cytosolic, chloroplast, and mitochondrial DnaJ homologs (CrDNJ1, CrCDJ1, and CrMDJ1, accessions EDP04706, AAU06580, and gene model estExt_fgenesh1_pg.C_870033 at http://genome.jgi-psf.org/Chlre3/Chlre3.home.html, respectively); and from a pea chloroplast DnaJ homolog (PsPCJ1, accession CAA96305). Amino acids highlighted in black are conserved in all seven proteins;

atDjB1 in that clade, lacked the G/F-rich domain normally situated between J domain and ZFD (Kroczynska et al., 1996). Hence, the *Chlamydomonas* protein in the cytoplasmic clade was annotated as DNJ1, that in the mitochondrial clade mitochondrial DnaJ 1 (MDJ1), and that in the chloroplast/cyanobacterial clade CDJ1 (Fig. 1).

CDJ1 Is the Chloroplast DnaJ Homolog

To further characterize CDJ1 biochemically, we expressed CDJ1 in *E. coli* as a C-terminal fusion to the yeast (*Saccharomyces cerevisiae*) VMA intein/chitin-binding domain. As the fusion protein was found in inclusion bodies, we used purified inclusion bodies to raise an antiserum against CDJ1. Significant amounts of soluble protein eventually were recovered when CDJ1 was expressed by means of the pCDF expression vector, with an N-terminal hexa-His tag replacing the predicted transit peptide (Fig. 1A). As shown in Figure 2A, the CDJ1 antiserum detected a single protein band with a molecular mass of approximately 40 kD in *Chlamydomonas* whole-cell extracts, which corresponds well to the 40.3 kD calculated for CDJ1 that had been processed at the VR/A cleavage site predicted by TargetP (Fig. 1A). The CDJ1 antiserum also recognized the recombinant hexa-His-tagged protein at a slightly higher molecular mass, which corresponds well with the 42.1 kD calculated for the recombinant protein. As the CDJ1 precursor has a calculated molecular mass of 45 kD, these data suggest that the CDJ1 precursor is indeed processed, presumably at the predicted VR/A cleavage site to its mature form of 40.3 kD.

In fact, in a screen for cDNAs potentially encoding chloroplast-targeted J-domain proteins we had already identified the *CDJ1* cDNA and had elucidated that the *CDJ1* gene is slightly inducible by light and strongly inducible by heat shock (Liu et al., 2005). To test whether the strong induction of *CDJ1* mRNA after heat shock also resulted in elevated CDJ1 protein levels, we subjected *Chlamydomonas* cells to a 2-h heat stress treatment at 40°C. Surprisingly, whereas HSP90C protein levels as observed previously (Willmund and Schroda, 2005) were strongly increased by this treatment, CDJ1 protein levels increased only slightly (Fig. 2B). Presumably, CDJ1 protein levels under nonstress conditions were already so high that additional protein synthesized upon stress did not significantly alter bulk CDJ1 levels. To test this idea we estimated cellular CDJ1 protein levels by quantitative western blotting (Fig. 2C). Four independent experiments revealed that CDJ1 represented roughly 0.1% of *Chlamydomonas* total cellular proteins. For comparison, cellular concentrations of HSP70B and CGE1 were 0.19% and 0.01%, respectively (Liu et al., 2007). Hence, when taking into account the different molecular masses of the mature proteins (67.9 kD for HSP70B; 40.3 kD for CDJ1; 23.8 kD for CGE1), we obtain a molar ratio for HSP70B:CDJ1:CGE1 of about 6.7:5.9:1.

To verify the predicted chloroplast localization of CDJ1 and to determine its suborganellar distribution, mitochondria and chloroplasts were isolated from *Chlamydomonas* cells; chloroplasts were subsequently subfractioned into stroma, thylakoids, and low-density membranes, which are considered to consist of inner envelopes and of transitory membranes between inner envelope and thylakoids (Zerges and Rochaix, 1998). The purity of the fractions was tested with antibodies against mitochondrial carbonic anhydrase, stromal CGE1, and the integral thylakoid membrane protein cytochrome *f*. As judged from the signals obtained with these antibodies (Fig. 3), chloroplasts were contaminated with mitochondria; stroma fractions were free from thylakoid and mitochondrial contaminations; low density membranes were free from mitochondrial contaminations; thylakoids were free from stromal contaminations, but contained some mitochondrial matter; and mitochondria were slightly contaminated with thylakoids. CDJ1 was detected in whole cells and chloroplasts; no CDJ1 was found in the mitochondrial fraction. Within chloroplasts, CDJ1 was localized mainly in the stroma and low density membrane fractions but little CDJ1 was also detected in the thylakoid fraction. Hence, CDJ1 displayed the same localization pattern as its presumed chaperone partner HSP70B. Note that also the pea (*Pisum sativum*) chloroplast DnaJ homolog PCJ1 localized to stroma and chloroplast membrane fractions (Schlicher and Soll, 1997). Note also that the strong signal for HSP90C in mitochondrial fractions is due to its unspecific association with mitochondrial membranes after cell disruption (Willmund and Schroda, 2005).

Figure 1. (*Continued.*)
those highlighted in gray are conserved in at least six of them. Cys and Gly of the four conserved CXXCXGXG motifs are given below the sequences in bold letters. Italicized sequences represent organellar transit peptides as predicted by the TargetP program (Emanuelsson et al., 2000), with the residue in boldface corresponding to the first amino acid of the mature protein. Domain designations are according to Cheetham and Caplan (1998). B, Phylogram based on an alignment of the amino acid sequences of ZFD-containing J-domain proteins from *Chlamydomonas*, Arabidopsis, yeast, pea, *E. coli*, and two cyanobacteria. Sequences were from the same sources as in A, but also included additional sequences from Arabidopsis (atDjA30, atDjA2, atDjA54, atDjA24, atDjA26, and atDjA52, accessions BAB11067, NP_568412, NP_188410, NP_568076, NP_565533, and AAD55483, respectively), from yeast (ScYDJ1p and ScMDJ1p, accessions NP_014335 and NP_116638, respectively), and from the cyanobacteria *Synechococcus* sp. WH5701 (SynDnaJ, accession ZP_01084411) and *Prochlorococcus marinus* (PmDnaJ, accession YP_001013845). Phylogenetic analysis was conducted using version 4 of the MEGA program (Tamura et al., 2007) on the basis of alignments made by version 1.8 of the ClustalW program.

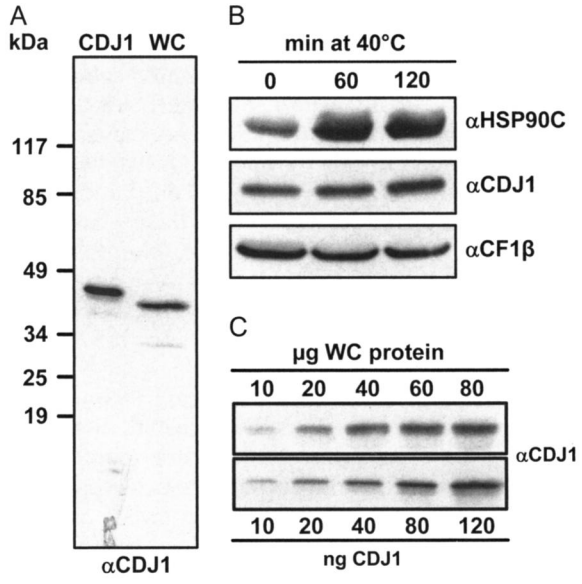

Figure 2. Accumulation of CDJ1 in *Chlamydomonas*. A, Test of the specificity of the CDJ1 antiserum. Fifty micrograms of *Chlamydomonas* whole-cell proteins (WC) and 50 ng of purified hexa-His-tagged CDJ1 were separated on a 7.5% to 15% SDS-PAGE and analyzed by immunoblotting. B, Analysis of the heat shock inducibility of CDJ1. Fifty micrograms of whole-cell proteins from *Chlamydomonas* cells grown at 25°C or heat shocked at 40°C for 1 or 2 h were separated on a 7.5% to 15% SDS-PAGE and analyzed by immunoblotting. CF1β served as loading control. C, Quantification of cellular CDJ1 concentrations. Recombinant CDJ1 purified from *E. coli* and *Chlamydomonas* whole-cell proteins at the amounts indicated were separated on a 7.5% to 15% SDS-PAGE and analyzed by immunoblotting. WC, Whole cell.

In summary, we can conclude that CDJ1 is a rather abundant chloroplast protein that partitions between soluble stroma and chloroplast membranes. Although strongly induced by heat shock at the mRNA level, CDJ1 protein levels increased only slightly upon heat shock. The CDJ1 precursor upon import into chloroplasts appears to be processed, presumably at the predicted VR/A cleavage site.

CDJ1 Complements the Temperature-Sensitive Phenotype of an *E. coli* Δ*dnaJ* Mutant Strain

It was reported previously that the yeast mitochondrial DnaJ homolog Mdj1p and the Arabidopsis mitochondrial DnaJ homolog AtJ1 (now termed atDjB1) could at least partially complement the temperature-sensitive phenotype of an *E. coli* Δ*dnaJ* mutant strain (Kroczynska et al., 1996; Deloche et al., 1997). To test whether also CDJ1 was able to functionally replace *E. coli* DnaJ, we introduced the pCDF expression vector alone, or the same vector encoding CDJ1 with an N-terminal hexa-His tag into *E. coli* strain OD259 carrying a deletion of the *dnaJ* gene (Deloche et al., 1997). As shown in Figure 4, OD259 containing the empty vector grew well at 23°C, but growth was impaired at 37°C and abolished at 40°C. In contrast, OD259 containing the CDJ1 expression vector grew well at all three temperatures, indicating that CDJ1 could indeed functionally replace *E. coli* DnaJ. Immunoblotting verified weak, leaky CDJ1 expression in OD259 cells containing the CDJ1 expression vector (data not shown). The ability of CDJ1 to functionally replace *E. coli* DnaJ implied that CDJ1 was able to interact with *E. coli* DnaK. As expected, precipitation of CDJ1 by nickel beads, taking advantage of the hexa-His tag at its N terminus, led to the coprecipitation of DnaK (data not shown). Taken together, CDJ1 can functionally replace *E. coli* DnaJ and this ability correlated with the ability of CDJ1 to physically interact with *E. coli* DnaK.

CDJ1 in *Chlamydomonas* Soluble Cell Extracts Forms Dimers and Complexes of Higher Molecular Mass

Next, we wanted to determine whether CDJ1 forms oligomers and complexes of higher molecular mass in *Chlamydomonas*. For this we subjected lysed *Chlamydomonas* cells to crosslinking with the homobifunctional thiol-cleavable crosslinker dithio-bis(succinimidyl propionate) (DSP). To achieve crosslinking as rapidly as possible, we added DSP right before cell lysis. To cope with the higher concentrations of proteins in the lysate caused by the presence of membrane proteins, we used DSP at twice the concentration normally employed for soluble proteins (Liu et al., 2007). We first assayed the quality of this crosslinking approach by testing whether it would reveal complexes known to be formed by other chloroplast (co)chaperones. As shown in Figure 5, we could indeed detect CGE1 crosslinks migrating at approximately 55 kD and ap-

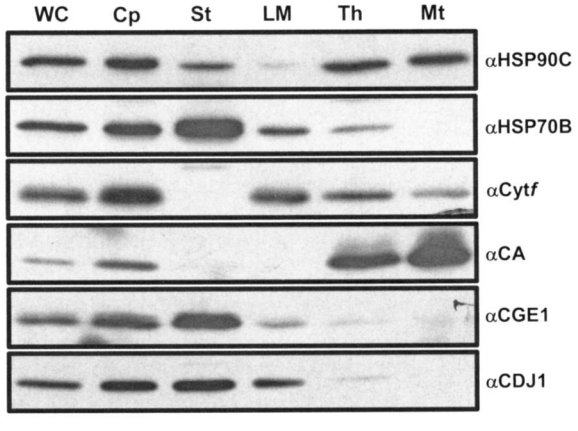

Figure 3. Intracellular localization of CDJ1. *Chlamydomonas* chloroplasts (Cp) were isolated, lysed by hypoosmotic shock, and separated into stroma (St), low density membranes (LM), and thylakoid membranes (Th). Mitochondria (Mt) were isolated from the same strain. Whole cells (WC) and fractions (7 μg protein each) were separated on a 7.5% to 15% SDS-PAGE, transferred to nitrocellulose, and immunodecorated with antibodies against HSP90C, HSP70B, thylakoidal cytochrome *f* (Cyt*f*), mitochondrial carbonic anhydrase, stromal CGE1, and CDJ1.

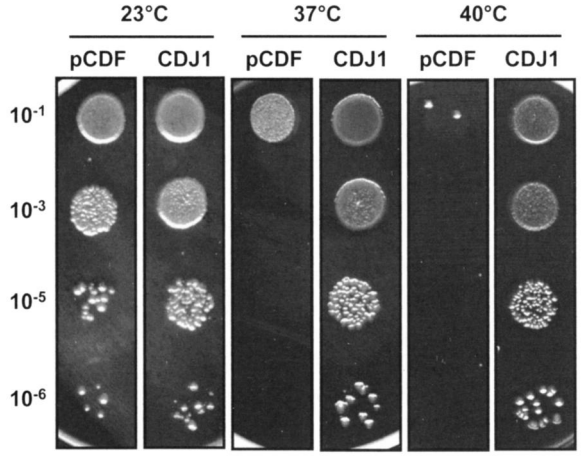

Figure 4. Test for functional complementation of *E. coli* DnaJ by CDJ1. Temperature-sensitive *E. coli* strain OD259 carrying a deletion of its *dnaJ* gene (Deloche et al., 1997) was transformed with a plasmid vector for the expression of mature, hexa-His-tagged CDJ1 (pCDJ1, pMS458) or the empty expression vector (pCDF). Dilutions of transformant cultures were spotted onto Luria-Bertani plates and incubated overnight at 23°C, 37°C, or 40°C.

proximately 120 kD, which correspond to CGE1 dimers and dimers interacting with HSP70B, respectively (Schroda et al., 2001; Liu et al., 2007; Willmund et al., 2007). Several prominent HSP70B crosslinks migrating above approximately 160 kD were detected, suggesting that HSP70B was not forming dimers but rather was interacting with several other proteins. Finally, prominent HSP90C crosslinks were detected at approximately 170 kD and above, which correspond to HSP90C dimers and interactions of HSP90C with several other proteins (Willmund and Schroda, 2005). Since DSP crosslinking indeed revealed (co)chaperone complexes known from previous studies, we turned to the analysis of CDJ1 complexes. As shown in Figure 5, the most prominent crosslinking product of CDJ1 migrated in SDS-PAGE at approximately 80 kD, indicating that CDJ1 might form dimers. Other prominent crosslinking products migrating at approximately 45 kD and approximately 105 kD are likely to represent intramolecular crosslinks that result in retarded gel migration properties of CDJ1 monomers and dimers, respectively. Less prominent CDJ1 crosslinking products migrating above 105 kD suggest that CDJ1 might interact with other proteins as complexes of high molecular mass.

DSP-crosslinked protein complexes were well resolved and detected up to a molecular mass of approximately 230 kD. However, presumably because of insufficient transfer of the SDS-denatured, crosslinked polypeptides from the gel, we could not detect larger complexes. A more suitable method for the separation of high molecular mass complexes is blue native (BN)-PAGE, where compact, native proteins are resolved. When we used BN-PAGE to separate *Chlamydomonas* soluble proteins, we found CDJ1 and HSP90C to comigrate with the same punctuate pattern at very high molecular mass (Fig. 6A). Longer exposure of the HSP70B signal revealed the same punctuate pattern (data not shown). This suggested that HSP90C, HSP70B, and CDJ1 might form common complexes of very high molecular mass. Bulk CDJ1 migrated between bulk HSP90C and HSP70B (Fig. 6A). This was consistent with CDJ1 dimers of approximately 80 kD migrating between HSP90C and HSP70B monomers of 83 kD and 68 kD, respectively (Fig. 5). However, in BN-PAGE the bulk of all three proteins roughly comigrated with the native 140-kD marker protein, suggesting that HSP90C and HSP70B were forming dimers and CDJ1 was forming trimers. Also bulk CGE1 comigrated with the 66-kD native marker protein (Fig. 6A), although bulk CGE1 was expected to be present as dimers of approximately 50 kD (Fig. 5).

To solve this apparent contradiction, we separated the purified recombinant (co)chaperones by BN-PAGE, but additionally subjected them to DSP crosslinking to stabilize their oligomeric states, and to SDS denaturation to destroy their oligomeric states (Vinothkumar et al., 2005). As shown in Figure 6B, the bulk of the untreated recombinant proteins displayed the same migration pattern as the native proteins, i.e. HSP90C, HSP70B, and CDJ1 roughly comigrated with the 140-kD marker protein and CGE1 with the 66-kD

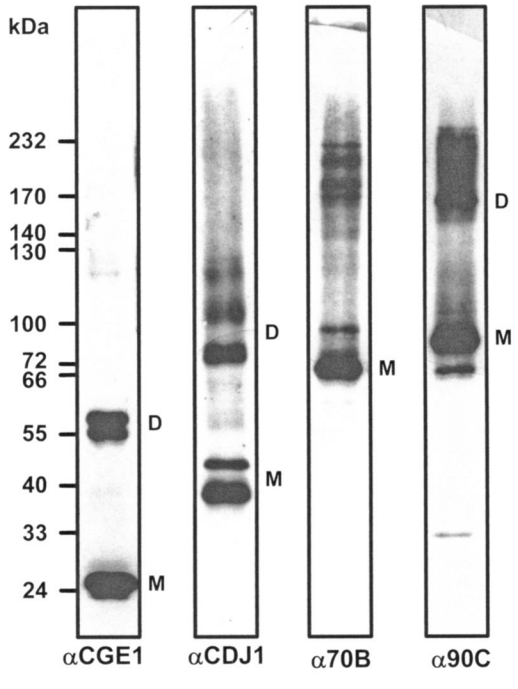

Figure 5. Analysis of chloroplast chaperone complexes by crosslinking. *Chlamydomonas* total proteins from about 1.5×10^7 cells were subjected to crosslinking with 4 mM DSP, separated on a 4% to 15% nonreducing SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting. The positions of monomers (M) and dimers (D) potentially formed by the (co)chaperones are indicated.

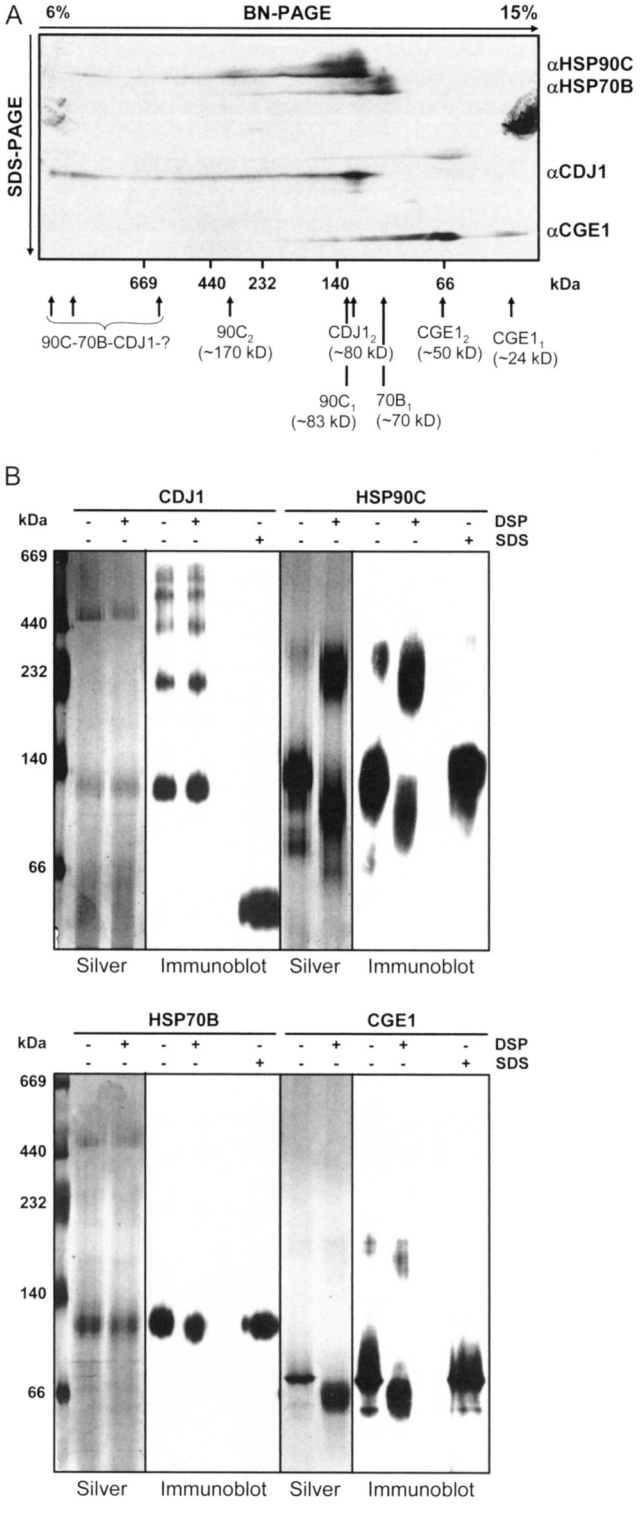

Figure 6. Analysis of chloroplast chaperone complexes by BN-PAGE. A, BN-PAGE on cellular proteins. *Chlamydomonas* total soluble proteins were separated on a 6% to 15% BN-PAGE, separated in the second dimension on a 10% SDS-PAGE, and analyzed by immunoblotting. Molecular weights of oligomers formed by HSP90C, HSP70B, CDJ1, and CGE1 were inferred from the results from Figure 5. B, BN-PAGE on recombinant proteins. Two micrograms of purified, recombinant HSP90C, HSP70B, CDJ1, and CGE1b were left untreated, subjected to DSP crosslinking, or incubated with 2% SDS. Proteins were then separated on 6% to 18% polyacrylamide gradient BN gels and stained with silver or transferred to polyvinylidene difluoride membranes and analyzed by immunoblotting. HSP90C and CGE1b contained no additional protein tags; HSP70B and CDJ1 contained hexahistidine tags at their C and N termini, respectively.

marker protein. Part of recombinant CDJ1 displayed a ladder-like migration pattern, suggesting successive oligomer formation. Whereas oligomer formation was not increased by DSP crosslinking, it was completely abolished by SDS denaturation, after which the entire CDJ1 protein became monomeric and migrated below the 66-kD marker protein (Fig. 6B). Some recombinant HSP90C migrated between the 232- and 440-kD marker proteins. This HSP90C fraction became the most prominent after DSP crosslinking, but was virtually absent after SDS denaturation. SDS denatured HSP90C comigrated quantitatively with the 140-kD marker protein (Fig. 6B). This suggested that HSP90C monomers comigrated with the 140-kD marker protein and HSP90C dimers between the 232- and 440-kD marker proteins. Comigration of recombinant HSP70B with the 140-kD marker protein was unaffected by DSP crosslinking or SDS denaturation, suggesting that HSP70B monomers migrated at that position. Comigration of recombinant CGE1b with the 66-kD marker protein was unaffected by DSP crosslinking and after SDS denaturation only a tiny amount of CGE1b migrated at the bottom of the gel. The latter fraction increased when recombinant CGE1b was treated at 65°C with 2% SDS for 10 min instead of simply adding 2% SDS and loading directly, suggesting that CGE1b comigrating with the 66-kD marker protein was dimeric. Apparently, CGE1 rapidly refolded to the native, dimeric state after dilution of SDS during the run, which is consistent with the ability of this protein to rapidly refold and dimerize after thermal denaturation (Willmund et al., 2007).

We conclude that CDJ1 appears to form stable dimers and that it might form complexes of high molecular mass with HSP90C and HSP70B. Presumably because of their nonglobular shapes (Harrison et al., 1997; Shi et al., 2005; Ali et al., 2006; Chang et al., 2008), HSP90C, HSP70B, CDJ1, and CGE1 all migrate in BN-PAGE with 40% to 90% higher apparent than calculated molecular masses.

CDJ1 Is Part of Multichaperone Complexes Containing HSP90C, HSP70B, and CGE1

The observation that CDJ1, HSP70B, and HSP90C in BN-PAGE comigrated in distinct complexes of high molecular mass suggested that they might form common complexes; and since HSP70B interacts with CGE1 (Schroda et al., 2001; Liu et al., 2007; Willmund et al., 2007, 2008), it seemed possible that also CGE1 was part of them. Immunoprecipitation analyses from *Chlamydomonas* soluble cell extracts were used to test

this idea. As the composition of (co)chaperone complexes may differ in the presence and absence of ATP (Schroda et al., 2001; Liu et al., 2007), cell extracts were supplemented with ATP and an ATP regenerating system, or with apyrase for ATP depletion. Moreover, protein complexes in cell extracts were stabilized by the addition of DSP. While none of the four chloroplast (co)chaperones was precipitated by preimmune serum, each (co)chaperone coprecipitated with the three others from ATP-depleted cell extracts (Fig. 7A). The same was true also for immunoprecipitations performed from ATP-containing cell extracts, with the exception of CGE1: In the presence of ATP, very little CGE1 was coprecipitated with the three other (co)chaperones, and very little of the other (co)chaperones coprecipitated with CGE1. These results suggest that the interaction of HSP90C, HSP70B, and CDJ1 is stable in the presence of ATP, whereas CGE1 appears to be part of the HSP90C-HSP70B-CDJ1 complex mainly in the absence of ATP. The ATP dependence of CGE1 coprecipitation, on the one hand, underscores the specificity of the DSP crosslinker for truly interacting proteins. The much higher amounts of HSP70B that coprecipitated with HSP90C from DSP-treated cell extracts compared to untreated cell extracts, on the other hand, demonstrates the stabilizing effect of the crosslinker on protein complexes (Fig. 7B).

We wondered whether CDJ1 in the chloroplast was preferentially interacting with HSP70B and only occasionally, for the folding of specific proteins, was in complex also with HSP90C; or whether CDJ1 was interacting with (and presumably transferring substrates to) preformed HSP90C-HSP70B complexes. To distinguish between these two possibilities we immunoprecipitated HSP90C and CDJ1 from size-fractioned soluble cell extracts in which protein complexes had been stabilized by DSP crosslinking. Immunoprecipitates were analyzed by immunoblotting for the presence of HSP90C, HSP70B, CDJ1, and CGE1. In Figure 8A the distribution of the four (co)chaperones in the size-fractioned soluble cell extracts used as input for the immunoprecipitations is shown. Whereas CGE1 was detected mainly in fractions 6 to 13, HSP90C, HSP70B, and CDJ1 were detected in all fractions of the gel filtration eluate. The tendency of these three (co)chaperones to smear over a large size range was also observed in native PAGE analyses of *Chlamydomonas* soluble cell extracts (Schroda et al., 2001; Willmund and Schroda, 2005; Fig. 6A). As this behavior was not observed with the purified proteins (Fig. 6B), it is most likely due to the interaction of the (co)chaperones with other proteins during the run. Similar to what we have observed in BN-PAGE (Fig. 6), the bulk of the (co)chaperones eluted at higher apparent than calculated molecular masses (Fig. 8A). For example, most of CGE1 was detected in fractions 8 to 11, corresponding to a molecular mass of approximately 200 kD. Note that a similar elution profile was also observed for GrpE, the bacterial homolog of

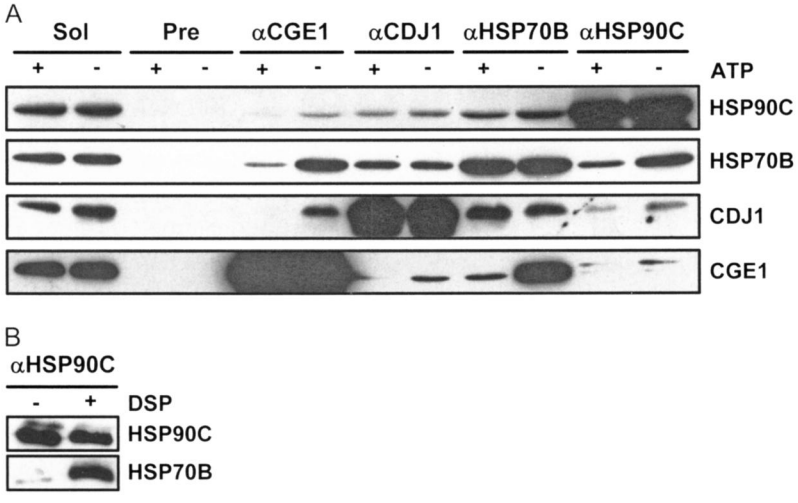

Figure 7. Immunoprecipitation of chloroplast (co)chaperones from soluble cell extracts. A, Immunoprecipitation of HSP90C, HSP70B, CDJ1, and CGE1. Soluble proteins were extracted from *Chlamydomonas*, supplemented with 2.5 mM ATP and an ATP-regenerating system (+), or depleted of ATP with apyrase (−). After stabilization of protein complexes by crosslinking with 2 mM DSP, proteins were incubated with protein-A sepharose coupled to antibodies of preimmune (Pre), anti-HSP90C, anti-HSP70B, anti-CDJ1, or anti-CGE1 serum. Precipitated proteins and an aliquot of the soluble cell extract (Sol; corresponding to 1.5% of the input for each immunoprecipitation) were separated on a 7.5% to 15% SDS-PAGE and analyzed by immunoblotting. B, Analysis of the stabilizing effect of DSP on HSP90C-HSP70B complexes. Soluble proteins were extracted from *Chlamydomonas* and half of them were subjected to crosslinking with 2 mM DSP. Extracts were incubated with protein-A sepharose coupled to anti-HSP90C and anti-HSP70B antibodies. Precipitated proteins were separated on a 7.5% to 15% SDS-PAGE and analyzed by immunoblotting.

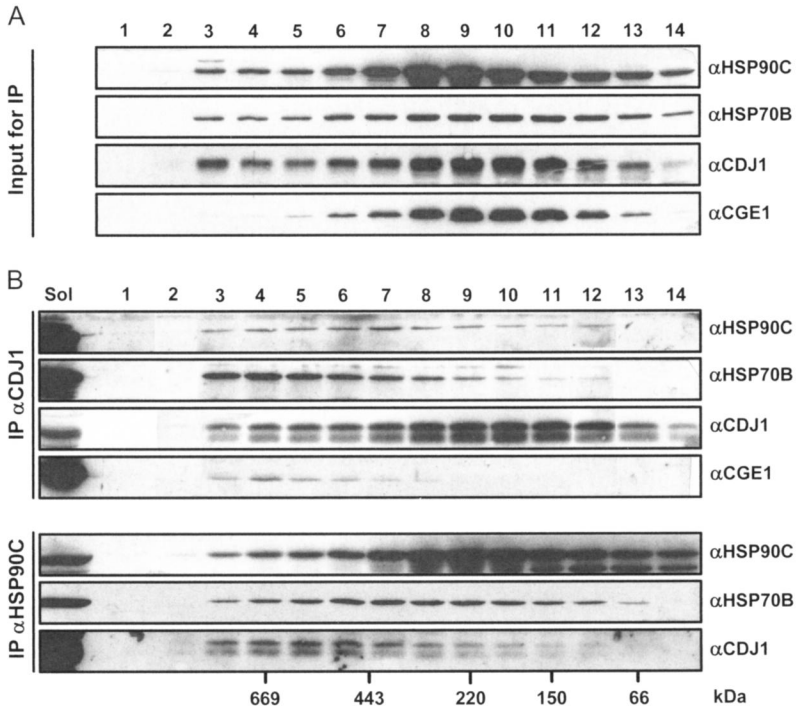

Figure 8. Analysis of complexes formed by HSP90C, HSP70B, CDJ1, and CGE1. A, Size distribution of HSP90C, HSP70B, CDJ1, and CGE1 complexes. Soluble proteins were extracted from *Chlamydomonas* and protein complexes, after stabilization by crosslinking with 2 mM DSP, were separated by gel filtration. Aliquots of collected fractions were separated on a 7.5% to15% SDS-PAGE and analyzed by immunoblotting using antibodies against HSP90C, HSP70B, CDJ1, and CGE1. B, Analysis of HSP90C- and CDJ1-containing complexes. Gel filtration fractions containing DSP-crosslinked protein complexes were incubated with protein-A sepharose coupled to antibodies against CDJ1 and HSP90C. An aliquot of soluble proteins (representing 3.8% of the input to gel filtration) and precipitated proteins were analyzed by immunoblotting as in A.

CGE1, and was attributed to the elongated shape of the GrpE dimers (Schönfeld et al., 1995).

As expected, the same distribution as observed for the size-fractioned cell extracts (Fig. 8A) was observed for immunoprecipitated CDJ1 (Fig. 8B), suggesting that CDJ1 was quantitatively immunoprecipitated from the fractions. Interestingly, whereas most of CDJ1 was immunoprecipitated from fractions 8 to 11, HSP90C, HSP70B, and CGE1 were mainly coprecipitated with CDJ1 from fractions 3 to 8. This suggested that most of CDJ1 predominantly existed as lower molecular mass forms (presumably as dimers), and a smaller fraction of CDJ1 was in complex with HSP90C, HSP70B, and CGE1. The almost equal distribution of coprecipitated HSP90C, HSP70B, and CGE1 is more supportive for the idea that CDJ1 was interacting with a preformed HSP90C-HSP70B complex rather than preferentially with HSP70B and only occasionally with both chaperones. Accordingly, HSP70B coprecipitated with HSP90C mainly from fractions containing protein complexes of intermediate molecular mass, whereas CDJ1 coprecipitated with HSP90C mainly from fractions containing high molecular mass complexes (Fig. 8B). The amounts of CGE1 expected to coprecipitate with HSP90C were below the detection limit. Similar results were obtained when size fractionation of soluble cellular proteins prior to immunoprecipitation was done by Suc gradient centrifugation instead of gel filtration (data not shown).

We conclude that CDJ1 is part of complexes containing HSP90C, HSP70B, and CGE1. It appears likely that CDJ1 was interacting with preformed HSP90C-HSP70B complexes.

DISCUSSION

We report here on the molecular and biochemical characterization of CDJ1, one of three zinc-finger-containing J-domain proteins encoded by the *Chlamydomonas* genome. We provide the following evidence that CDJ1 is a bona fide chloroplast-targeted DnaJ homolog and cochaperone of chloroplast HSP70B. (1) CDJ1 contains all domains characteristic for type I J-domain proteins, i.e. the J domain, the G/F-rich domain, the ZFD with the four conserved CXXCXGXG motifs, and the DnaJ C-terminal domain (Cheetham and Caplan, 1998; Craig et al., 2006; Fig. 1A). Moreover, CDJ1 like other type I J-domain proteins appears to form homodimers (Sha et al., 2000; Shi et al., 2005; Figs. 5 and 6). (2) CDJ1 complemented the temperature-sensitive phenotype of an *E. coli* Δ*dnaJ* mutant and interacted physically with *E. coli* DnaK (Fig. 4). (3) CDJ1 in a phylogenetic tree is in the same clade as cyanobacterial DnaJ homologs, pea chloroplast PCJ1 (Schlicher and Soll, 1997), and Arabidopsis J-domain proteins that by TargetP are predicted to be localized to the chloroplast (Fig. 1B). (4) CDJ1 contains an N-terminal extension, which by the TargetP program (Emanuelsson et al., 2000) is predicted to qualify as a chloroplast transit peptide and that appears to be processed (Fig. 2A). Accordingly, fractionation experiments revealed that mature CDJ1 is indeed targeted to the chloroplast, where it localized to the stroma, low density membranes, and thylakoids (Fig. 3). This localization pattern is identical with that observed for chloroplast HSP70B (Schroda et al., 2001; Liu et al., 2005; Willmund and Schroda, 2005; Fig. 3). (5) In *Chlamydomonas*, CDJ1 appears to be about as abundant

as HSP70B and therefore roughly 17 times more abundant in chloroplasts than the specialized J-domain protein CDJ2 (Liu et al., 2007; Fig. 2C). This suggests that CDJ1 is the major J-domain protein in the chloroplast and thus likely to cooperate with HSP70B, the most abundant chloroplast HSP70 (Schroda, 2004). Accordingly, CDJ1 was shown to interact physically with HSP70B (Figs. 7 and 8).

Of the 63 genes identified in the *Chlamydomonas* genome sequence (Merchant et al., 2007) only three encode zinc-finger-containing J-domain proteins, which by phylogenetic analyses appear to be targeted to the cytosol (DNJ1), to the chloroplast (CDJ1), and to mitochondria (MDJ1; Fig. 1B). In contrast, in the Arabidopsis genome 89 genes were identified that encode J-domain proteins; eight of these encode zinc-finger-containing J-domain proteins of which, as judged by prediction programs and phylogenetic analyses, two appear to be targeted to the cytosol, four to chloroplasts, and another two to mitochondria (Miernyk, 2001; Fig. 1B). Thus, the tendency that chaperone gene families in Arabidopsis are larger and more complex than in *Chlamydomonas* is also reflected at the level of genes encoding DnaJ homologs. Note that the members of the five major chaperone systems, HSP100, HSP90, HSP70, HSP60, and sHSPs, are encoded by 74 genes in Arabidopsis and only by 39 genes in *Chlamydomonas* (34 have been identified in version 1.0 of the *Chlamydomonas* genome sequence [Schroda, 2004], five more have been added by version 3.0 [Schroda and Vallon, 2008]). Hence, regarding the chaperone genes, *Chlamydomonas* is more minimalistic than Arabidopsis, which underscores the suitability of *Chlamydomonas* as a model for studying fundamental chaperone functions in photosynthetic eukaryotes.

Most interesting is our finding that CDJ1 forms common complexes with HSP90C, HSP70B, and CGE1 (Figs. 6–8) because of the following two aspects. First, whereas HSP70B, CDJ1, and CGE1 are derived from the cyanobacterial endosymbiont (Schroda, 2004; Fig. 1B), HSP90C appears to be of eukaryotic origin. Specifically, phylogenetic analyses on HSP90 sequences from various organisms had recently allowed the conclusion that the gene encoding chloroplast-targeted HSP90 was not derived from that encoding cyanobacterial HSP90 (*htpG*). Rather, it stemmed from a gene originally encoding an ER-targeted HSP90 that had duplicated and in a secondary event had acquired a sequence encoding a chloroplast transit peptide (Emelyanov, 2002; Stechmann and Cavalier-Smith, 2004; Willmund and Schroda, 2005; Chen et al., 2006). Hence, two chaperone systems of distinct evolutionary origin interact in chloroplasts.

Second, the organization of cytosolic HSP90 and HSP70 and the ER-lumenal family members Grp94 and BiP into common complexes has been well documented (Csermely et al., 1998; Pratt and Toft, 2003) and only recently has been extended also to plastidic HSP70 and HSP90 family members (Willmund and Schroda, 2005). In the cytosol, a complex consisting of HSP90, HSP70, HSP40, and HOP, also referred to as the foldosome, has been shown to preexist even in the absence of substrates and to form spontaneously upon mixing of the purified components in the presence of ATP (Hutchison et al., 1994; Dittmar et al., 1998; Pratt and Toft, 2003). HSP40 appears to dynamically cycle in and out of this complex (Dittmar et al., 1998) and appears to be essential for the productive transfer of substrates from HSP70 to HSP90 (Cintron and Toft, 2006; Wegele et al., 2006). Similarly, a multichaperone complex in the ER, containing Grp94, BiP, a protein disulfide isomerase and several other cohort proteins, but not necessarily the ER HSP40 homolog ERdj3, also has been shown to preexist in the absence of substrates (Meunier et al., 2002). We have detected a complex comprising chloroplast HSP90C, HSP70B, and CDJ1 that existed in the presence of ATP and remained stable after ATP depletion (Fig. 7A). Immunoprecipitation from size-fractioned cell extracts suggested that CDJ1 was joining preformed HSP90C-HSP70B complexes (Fig. 8B). These findings suggest that, similar to the situation in the cytosol, CDJ1 appears to have a high affinity for the HSP90C-HSP70B complex also in the presence of ATP, but might likewise dynamically cycle in and out of the complex. In contrast, CGE1 was found associated with the HSP90C-HSP70B-CDJ1 complex mainly after depletion of ATP (Fig. 7A). Hence, CGE1 also appears to dynamically cycle in and out of the HSP90C-HSP70B-CDJ1 complex, but apparently has a significantly lower affinity for the complex in the presence of ATP. A low affinity of CGE1 for HSP70B in the presence of ATP has been demonstrated earlier (Schroda et al., 2001; Willmund et al., 2008).

When using BN-PAGE to analyze the stoichiometry of complexes formed by HSP90C, HSP70B, CDJ1, and CGE1 we observed that all four (co)chaperones migrated with 40% to 90% higher apparent molecular masses than calculated from the migration of standard native marker proteins (Fig. 6). Hence, greatest care has to be taken when trying to interpret the stoichiometry of native protein complexes on the basis of their migration properties in BN-PAGE. Because of this problem, we have in previous work (Willmund and Schroda, 2005) misinterpreted HSP90C migrating with an apparent molecular mass of approximately 140 kD as dimers. We demonstrate here that the use of crosslinking reagents and SDS to stabilize and to destroy protein complexes, respectively, represents a valuable tool to better interpret the stoichiometry of protein complexes in BN-PAGE (Fig. 6B).

In conclusion, we have been able to identify two cohort proteins of the chloroplast HSP90-HSP70 systems, CDJ1 and CGE1. As in Arabidopsis a mutation in the C terminus of plastidic Hsp90 impaired plastid-to-nucleus signal transduction (Lin and Cheng, 1997; Cao et al., 2000, 2003), it seems possible that the HSP90-HSP70-CDJ1-CGE plastidic multichaperone complex might play a role in the maturation of components of plastid-to-nucleus signal transduction pathways.

Chlorophyll biogenesis intermediates have been shown to serve as chloroplast signaling molecules for the induction of nuclear genes by light (Beck, 2005; Woodson and Chory, 2008). As cyanobacterial HSP90 has been shown to control chlorophyll biogenesis by regulating the activity of the HemE protein (Watanabe et al., 2007), this might also be the mechanism by which light induction of nuclear genes is influenced by a chloroplast HSP90-HSP70-CDJ1-CGE1 multichaperone complex. Clearly, the future challenge will lie in the identification of the substrates of this multichaperone complex.

MATERIALS AND METHODS

Cells and Culture Conditions

Chlamydomonas reinhardtii strains were grown photomixotrophically in TAP medium (Harris, 1989) on a rotary shaker at 25°C and an illumination of approximately 30 μE $m^{-2}s^{-1}$. For chloroplast isolation, cells were grown in TAP medium supplemented with 0.5% peptone. cw15 strain CF185 (Schroda et al., 1999) was used for all experiments.

Cloning, Expression, and Purification of CDJ1

Expression of the CDJ1 fusion protein: The coding region for the N-terminal part of CDJ1 was amplified by reverse transcription-PCR from *Chlamydomonas* total RNA with 5′ primer 5′-GGACTAGTGCTCTTCGAACGACGGTGACTTCTACGAC-3′ and 3′ primer 5′-CAGCTCGCGACTGCACCCGAACAC-3′. The 460-bp PCR product was digested with *Nru*I and *Spe*I and cloned into *Nru*I-*Spe*I-digested cDNA clone AV626034, which contains the coding region for CDJ1 lacking the N-terminal part. The obtained construct was digested with *Sap*I and *Xho*I and the resulting 2-kb fragment was cloned into *Sap*I-*Xho*I digested pTYB11 (NEB), giving pMS377. pMS377 was expressed in *Escherichia coli* ER2566 cells (NEB). As the obtained fusion protein was insoluble, inclusion bodies were purified as follows: Following induction with 0.4 mM isopropylthio-β-galactoside for 4 h, cells were harvested and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, 0.1% Na-azide, 0.5% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Cells were sonicated, 10 mM $MgSO_4$, 0.1 mg/mL lysozyme, 0.01 mg/mL DNase I were added, and samples were incubated for 20 min at room temperature. The mixture was centrifuged for 15 min at 4,500*g* and the pellet was resuspended in lysis buffer by sonication. This procedure was repeated two more times with lysis buffer and once with lysis buffer lacking Triton and Na-azide. The final protein pellet was resuspended in 10 mL of a buffer containing 6 M urea, 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl. For the generation of antibodies a rabbit was immunized with 1 mg of purified CDJ1 inclusion bodies.

Expression of hexa-His-tagged CDJ1: A PCR was performed on pMS377 with 5′ primer 5′-CGTGGGATCCCATGGCCGACGGTGACTTCTAC-3′ and the 3′ primer used above. The approximately 450-bp fragment after restriction with *Bam*HI and *Eco*47III was cloned into *Bam*HI-*Eco*47III-digested AV626034 giving pMS437. pMS437 was cut with *Bam*HI and *Kpn*I and the 2,030-bp fragment cloned into the *Bam*HI-*Kpn*I-digested pCDF expression vector, giving pMS458. pMS458 was expressed in BL21(DE3) cells (Novagen) and proteins were purified by nickel-nitrilotriacetic acid agarose according to the manufacturer's instructions (Qiagen). CDJ1 was eluted with KMH buffer (80 mM KCl, 20 mM HEPES-KOH pH 7.2, 2.5 mM $MgCl_2$) containing 200 mM imidazole.

PAGE and Gel-Blot Analyses

SDS-PAGE was performed as described earlier (Laemmli, 1970). For cell fractionation experiments, one volume of 2× sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.005% bromphenol blue) was added to the samples and protein concentrations were determined by amido black (Popov et al., 1975). BN-PAGE was carried out according to published protocols (Schägger and von Jagow, 1991; Schägger et al., 1994). Soluble proteins were prepared as described previously (Schroda et al., 2001). The native high molecular mass marker (66–669 kD) was purchased from GE-Healthcare. Proteins in gels were stained with Coomassie Brilliant Blue R250, silver nitrate, or transferred to nitrocellulose or polyvinylidene difluoride membranes (GE-Healthcare) by semidry blotting using a discontinuous transfer system. Blocking and immunodecorations were performed in phosphate-buffered saline containing 3% nonfat dry milk. Immunodetection was done by enhanced chemiluminescence. Antisera described previously were against HSP90C (Willmund and Schroda, 2005), HSP70B (Schroda et al., 1999), CGE1 (Schroda et al., 2001), mitochondrial carbonic anhydrase (Eriksson et al., 1996), CF1β (Lemaire and Wollman, 1989), and cytochrome *f* (Pierre and Popot, 1993).

Complementation Assay

pMS458 and the empty pCDF vector were transformed into temperature-sensitive *E. coli* strain OD259 containing a deletion of its *dnaJ* gene (Deloche et al., 1997). For gene expression from the T7 promoter of pCDF, an additional vector encoding T7 polymerase (Gentaur) was cotransformed.

Crosslinking

Chlamydomonas was grown in 500 mL TAP medium to a density of about 5×10^6 cells mL^{-1}, harvested by centrifugation, and resuspended in 3 mL KH buffer (125 mM KCl, 20 mM HEPES-KOH pH 7.5) supplemented with 0.25× protease inhibitor cocktail (Roche Diagnostics) and 4 mM DSP (Perbio Science). Cells were lysed by sonication on ice for 90 s and incubated under agitation for 90 min at 10°C. DSP was quenched by the addition of Gly to a final concentration of 100 mM and incubation for 15 min at 10°C. Subsequently, the lysate was loaded onto a Suc cushion (20 mM HEPES-KOH pH 7.2, 0.6 M Suc) and centrifuged in a TI50 rotor (Beckman) for 30 min at 152,000*g* and 4°C. One volume of 2× sample buffer lacking β-mercaptoethanol was added to the supernatant and proteins were solubilized by a 2-h incubation at 25°C.

For in vitro crosslinking, 2 μg of purified, recombinant proteins in 50 mM Bis-Tris, 15% glycerol, 1 mM $MgCl_2$, 80 mM KCl were supplemented with 200 μM DSP and incubated for 30 min at 25°C. Crosslinking was quenched by adding ε-aminocaproic acid at a final concentration of 0.4 M and incubating for 15 min at 25°C.

Immunoprecipitations

Chlamydomonas was grown to a density of approximately 8×10^6 cells mL^{-1}, harvested, washed twice with KH buffer, and resuspended in lysis buffer (20 mM HEPES-KOH, pH 7.2, 10 mM KCl, 1 mM $MgCl_2$, 154 mM NaCl, 0.25× protease inhibitor cocktail). Cells were split into two equal fractions, 10 units apyrase were added to one fraction (−ATP), 2.5 mM ATP, 80 mM creatine phosphate, and 0.125 $\mu g/\mu L$ creatine phosphate kinase were added to the other (+ATP). For crosslinking, DSP was added to the cells at a final concentration of 2 mM. Cell lysis, crosslinking, quenching, and centrifugation on Suc cushions were done as described above. Antibody coupling and immunoprecipitations were carried out as described previously (Schroda et al., 2001; Liu et al., 2005). Proteins were eluted from the beads by the addition of one volume of 2× sample buffer and boiling for 5 min at 95°C.

Gel Filtration and Coimmunoprecipitations

Crosslinking and extraction of soluble proteins was performed as described above. Samples were filtered through a 0.22-μm filter and loaded onto a Superdex 200 gel filtration column. The column was equilibrated with 100 mM Tris-HCl pH 8.0, 100 mM NaCl, and calibrated with the following M_r standards: blue dextran (2,000,000 D), thyroglobulin (669,000 D), apoferritin (443,000 D), β-amylase (220,000 D), alcohol dehydrogenase (150,000 D), bovine serum albumin (66,000 D), and riboflavin 5′-P (478 D). Three runs were performed with a flow rate of 0.5 mL/min. Fractions of 0.5 mL were collected and pooled accordingly. Fractions were supplemented with 0.5% Triton X-100 and divided into equal portions for immunoprecipitation with antibodies against HSP90C and CDJ1. Proteins were eluted by boiling for 5 min at 95°C with 2× SDS sample buffer.

Miscellaneous

Isolation of chloroplasts and fractionation into stroma, thylakoids, and low-density membranes was done as described previously (Zerges and

Rochaix, 1998). Mitochondria were isolated following a published protocol (Eriksson et al., 1995), but using a Nebulizer as outlined in Willmund and Schroda (2005). Quantification of cellular CDJ1 protein concentrations was carried out as described previously (Willmund and Schroda, 2005; Liu et al., 2007). Heat shock kinetics were performed according to Liu et al. (2005). Recombinant HSP90C and CGE1b were expressed from plasmids pMS335 and pMS301, respectively, and purified by chitin affinity chromatography (Willmund and Schroda, 2005; Willmund et al., 2007). Recombinant HSP70B was expressed from plasmid pMS462 and purified by nickel-affinity chromatography (Willmund et al., 2008).

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