NOTE

Chlamydomonas reinhardtii CNX1E Reconstitutes Molybdenum Cofactor Biosynthesis in Escherichia coli Mutants†¶

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We have isolated and characterized the Chlamydomonas reinhardtii genes for molybdenum cofactor biosynthesis, namely, CNX1G and CNX1E, and expressed them and their chimeric fusions in Chlamydomonas and Escherichia coli. In all cases, the wild-type phenotype was restored in individual mutants as well as in a CNX1G CNX1E double mutant. Therefore, CrCNX1E is the first eukaryotic protein able to complement an E. coli moeA mutant.

All eukaryotic Mo enzymes studied have Mo chelated in a prosthetic group named molybdenum cofactor (Moco) (13). In Arabidopsis thaliana, the final step of Moco synthesis, the incorporation of Mo into molybdopterin (MPT), is catalyzed by the two-domain protein CNX1 (14). The CNX1 N terminus (E domain) is homologous to Escherichia coli MoeA, and the C terminus (G domain) is homologous to E. coli MogA. The Arabidopsis CNX1G domain catalyzes the adenylation of MPT in a Mg2+- and ATP-dependent manner (7), and then the MPT-AMP intermediate is transferred to the CNX1E domain, where it is hydrolyzed in a molybdate- and Zn2+-dependent reaction (8), thus making Moco. We have studied the Chlamydomonas reinhardtii CNX1G (CrCNX1G) and CNX1E (CrCNX1E) proteins and found several characteristics that provide new insights into the evolution and function of these proteins.

A search using the Arabidopsis CNX1 cDNA sequence was done in the Chlamydomonas JGI database (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html) and found two nonoverlapping sequences with high similarity to different regions of Arabidopsis CNX1, with each of them localized in different chromosomes. The first sequence matched in scaffold 4 (bp 925,072 to 931,862), corresponding to chromosome VI, with an identity of 51.6% to the Arabidopsis CNX1E domain at the deduced protein level. The other sequence matched in scaffold 26 (bp 467,924 to 466,188), corresponding to chromosome X, with an identity of 43.2% to the Arabidopsis CNX1G domain. Thus, in Chlamydomonas, CNX1 is split into two polypeptides.

By comparing CrCNX1G with the database, the highest identities appeared with prokaryotic MogA proteins, such as those from the cyanobacterium “Gloebacter violaceus” (proposed name) (71%). However, as shown in Fig. 1, the most similar eukaryotic protein to CrCNX1G was the rat gephyrin G domain (44%). Interestingly, in Caenorhabditis elegans, the MogA and MoeA proteins are also split. We have found other eukaryotic organisms with separately expressed CNX1G and CNX1E domains, including the following: Cyanidioschyzon merolae (10), loci CBM143C and CMS449C; the eukaryotic alga Ostreococcus tauri (2), in chromosomes 8 and 6; the diatom alga Thalassiosira pseudonana (9), in scaffolds 11 and 13; and the marine diatom alga Phaeodactylum tricornutum (11), in scaffolds 4 and 7. The orientation of E and G domains is inverted in the Arabidopsis protein compared to the rat protein (14), reflecting separate evolutionary events (Fig. 1A). When CrCNX1E was compared to the database, the most similar proteins were the Arabidopsis CNX1E domain, with 52% identity, and the rat gephyrin E domain, with 48% identity. Two phylogenetic trees have been constructed based on the alignments of CrCNX1G and CrCNX1E with their homologous proteins by the neighbor-joining method (MEGA software [http://www.megasoftware.net/mega.html]). CrCNX1G appears in a common branch with its bacterial homologues (Fig. 1B), far away from the branch of plant homologues. For CrCNX1E, the situation is the opposite (Fig. 1C). This phylogenetic analysis suggests different evolutionary origins of CrCNX1G and CrCNX1E. Any case of lateral gene transfer had been reported in a chlorophyte alga like Chlamydomonas (1). These data led us to propose that CrCNX1G originated not through a lateral gene transfer but by an endosymbiotic gene transfer from the cyanobacterial ancestor of the chloroplast. For instance, for Arabidopsis, 18% of the genome was derived by this mechanism (12).
The CrCNX1G genomic region was amplified by PCR, cloned, and named pCnx1G. By screening a *Chlamydomonas* bacterial artificial chromosome (BAC) library, clone number 33L5 with the entire CrCNX1E genomic DNA was obtained, and the CrCNX1E gene was cloned and named pCnx1E. To overexpress CrCNX1G and CrCNX1E, both genes were cloned into plasmid pSI105a (see the supplemental material) under the control of the *rbcS2-hsp70* chimeric promoter and named pCnx1Gov and pCnx1Eov, respectively. In-frame fusions of both genes were also cloned into pSI105a and named pCnx1GEov and pCnx1EGov, resulting in the chimeric proteins CrCNX1GE (N-terminal CrCNX1G) and CrCNX1EG (N-terminal CrCNX1E), respectively. In addition, similar constructions bearing a C-terminal six-His tag were also generated and named as described above, but with a “-6H” extension.

Since Moco mutants are defective in nitrate reductase (NR), the mutants unable to grow on nitrate from a *Chlamydomonas* ordered mutant library (4) were screened by restriction enzyme site-directed amplification–PCR for mutations in *CNX1* genes. We found in mutant 1.78 an insertion located within the *CNX1G* gene and in mutant 70.9 an insertion located 7 kb upstream of the *CNX1E* gene, with a deletion towards this.

**FIG. 1.** Comparison of CrCNX1G and CrCNX1E with their homologous proteins. (A) Domain structure and homology of *Chlamydomonas* proteins CrCNX1G and CrCNX1E to *Gloeobacter violaceus* proteins, *Caenorhabditis elegans* MoeA and MogA, the CNX1 proteins (*Arabidopsis*), and gephyrin (*Rattus*). Homologies are given in the lower center of each box as percentages of amino acids (identical, similar, and divergent, respectively) compared with the corresponding region of CrCNX1G or CrCNX1E. Phylogenetic trees of (B) CrCNX1G and (C) CrCNX1E with selected homologous proteins from the three kingdoms of life are also shown. In both trees, only the conserved domains of each protein were aligned. GenBank accession numbers for CrCNX1G homologous proteins are as follows: *Haloarcula marismortui*, AAV46137; *Methanosarcina acetivorans*, NP615160; *Thermofilum pendens*, ZP01393969; *Pyrococcus horikoshii*, NP142491; *Escherichia coli* MoeB, NP415303; *Escherichia coli*, AAC73120; *Gloeobacter violaceus*, BAC88607; *Shewanella amazonensis*, ZP00587276; *Photobacterium profundum*, ZP01221987; *Vibrio fischeri*, YP206525; *Arabidopsis thaliana*, Q39054; *Oryza sativa*, CAE54543; *Zea mays*, ABB30174; *Hordeum vulgare*, AAF73075; *Chlamydomonas reinhardtii*, ABC42491; *Caenorhabditis elegans*, AAB04971; *Neurospora crassa*, XP064448; *Gibberella zeae*, XP358475; *Aspergillus oryzae*, BAE60479; *Rattus norvegicus*, AAH30016; *Drosophila melanogaster*, S47896; and *Nematostella vectensis*, 171664. For CrCNX1E homologous proteins, GenBank accession numbers are as follows: *Pyrococcus horikoshii*, NP143497; *Methanosarcina acetivorans*, NP618955; *Haloarcula marismortui*, YP135940; *Thermofilum pendens*, EAT67638; *Photobacterium profundum*, EAS41634; *Escherichia coli*, NP415348; *Vibrio fischeri*, YP204991; *Shewanella amazonensis*, EAN40969; *Gloeobacter violaceus*, BAC91680; *Chlamydomonas reinhardtii*, ABC42492; and *Caenorhabditis elegans*, CAA90060. Bootstrap support values above 50%, based on 1,000 replicates, are shown. Branch lengths are proportional to numbers of amino acid substitutions, which are indicated by the scale bar below the tree.

The CrCNX1G genomic region was amplified by PCR, cloned, and named pCnx1G. By screening a *Chlamydomonas* bacterial artificial chromosome (BAC) library, clone number 33L5 with the entire CrCNX1E genomic DNA was obtained, and the CrCNX1E gene was cloned and named pCnx1E. To overexpress CrCNX1G and CrCNX1E, both genes were cloned into plasmid pSI105a (see the supplemental material) under the control of the *rbcS2-hsp70* chimeric promoter and named pCnx1Gov and pCnx1Eov, respectively. In-frame fusions of both genes were also cloned into pSI105a and named pCnx1GEov and pCnx1Eov, resulting in the chimeric proteins CrCNX1GE (N-terminal CrCNX1G) and CrCNX1EG (N-terminal CrCNX1E), respectively. In addition, similar constructions bearing a C-terminal six-His tag were also generated and named as described above, but with a “-6H” extension.

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gene. These findings show the validity of this mutant library available for Chlamydomonas. A Chlamydomonas double mutant affected at both CNXIG and CNXIE was obtained by genetic crosses (3) and was named Dm.

Table 1 shows the transformation efficiencies determined by the glass bead method (5) for strains 1.78, 70.9, and Dm, using the indicated DNAs. A large number of transformants able to grow on nitrate were recovered in each case. This result confirms that the mutations in CNXIG and CNXIE were indeed responsible for the nitrate utilization deficiency phenotype due to a defect in Moco biosynthesis. Upon exchange of the native promoter with the rbcS2-hsp70 chimeric promoter, the transformation efficiencies increased about 25 to 30%. The constructions expressing His-tagged CrCNX1G and CrCNX1E proteins were also highly efficient in transformations, which suggests that the His epitope does not affect the protein functionality. The double and single mutants were successfully reconstituted with the chimeric genes and the His-tagged variants. The low transformation efficiency found for the Dm strain is probably due to the presence of a wild-type cell wall in this mutant. These results indicate that the chimeric proteins are functionally independent of their domain orientation. By real-time PCR, it was detected that the CNXIG and CNXIE transcripts were indeed overexpressed upon transformation, about 100 times more than in the wild type (data not shown). The correct expression of the His-tagged proteins in the Chlamydomonas strains bearing these genes was confirmed by Western blotting using His tag antibodies (data not shown).

Besides phenotypic repair, we also determined the MPT and MPT-AMP contents in Chlamydomonas mutants and reconstituted strains by FormA high-performance liquid chromatography (HPLC) analysis (7). As shown in Fig. 2A, the wild-type strain 704 showed only MPT, suggesting that it is efficiently converted into Moco without accumulation of the MPT-AMP intermediate. The MPT contents in mutants 1.78 and 70.9 were two to three times lower than that in the wild type. There was no MPT-AMP in the 70.9 strain, probably due to the low expression levels of the endogenous CNXIG gene. Meanwhile, strain 1.78+Gov, which overexpressed CrCNX1G, accumulated a large amount of MPT-AMP intermediate, which is the first report of MPT-AMP accumulation in a eukaryotic organism. In strain 70.9+Eov, which expressed CrCNX1E, significant MPT and MPT-AMP accumulation was detected. However, in strains Dm+GEov and Dm+EGov, which expressed the CrCNX1GE and CrCNX1GE chimeric proteins, respectively, the main intermediate accumulated was MPT, indicating that in these strains, the CrCNX1E protein efficiently converts MPT-AMP to Moco, which is detected as FormA in HPLC analysis. These results confirm that the disposition of the CrCNX1G and CrCNX1E moieties in the chimeric proteins does not affect their in vivo functionality.

The Moco content was measured by the nit-l assay (6) (Fig. 2B). No Moco was detected in mutants 1.78, 70.9, and Dm, but strains 1.78+G and 70.0+E had wild-type Moco levels. Meanwhile, in all strains that expressed CrCNX1 proteins, the Moco content was about three to four times higher than that in the wild type. These data correlate with the high pterin levels in these strains and demonstrate the functionality of the chimeric proteins with respect to their ability to synthesize active Moco.

The CrCNXIG and CrCNXIE cDNAs were isolated, sequenced, cloned into the bacterial expression vector pQ80 to produce six-His-tagged proteins, and named pQ80Cnx1G and pQ80Cnx1E, respectively. Two chimeric cDNAs were constructed by in-frame fusion of both cDNAs in both orientations, cloned into pQ80, and named pQ80Cnx1GE (N-terminal CrCNX1G) and pQ80Cnx1EG (N-terminal CrCNX1E) (see the supplemental material). The E. coli mogA mutant strain RK5206 and the mogA mutant strain SE1581 were transformed with the above-mentioned plasmids. All constructs resulted in high protein expression, and the molecular weights observed in sodium dodecyl sulfate-polyacrylamide gels were in agreement with the predictions (data not shown).

The ability of CrCNX1G, CrCNX1E, and their chimeric proteins to synthesize active Moco in E. coli was studied by analyzing their ability to restore NR activity to the mogA and mogA mutants in vivo. NR activity was measured as indicated previously (15). As shown in Fig. 2C, the NR activity was fully restored in the mogA mutant expressing CrCNX1G and the chimeric proteins. As expected, CrCNX1E failed to restore the mogA mutant. The E. coli mogA mutant cannot be complemented by the Arabidopsis CNXIE domain (14). Interestingly, CrCNX1E and the chimeric proteins were able to restore NR activity to about 20%. This is the first eukaryotic MoeA homologue found to be able to restore prokaryotic Moco biosynthesis. Therefore, we studied the effects of CrCNX1 proteins on E. coli Moco synthesis by measuring the MPT and MPT-AMP contents upon copurification with CNX1 proteins (8). As shown in Fig. 2D, when CrCNX1G and the chimeric proteins were expressed in strain RK5206, MPT and MPT-AMP saturation was around 80%. However, purified CrCNX1E protein expressed in the mogA mutant was free of MPT and MPT-AMP. When CrCNX1G and the chimeric proteins were expressed in the E. coli mogA mutant, which accumulates MPT-AMP (8), they were purified with six times more MPT-AMP than MPT. Interestingly, CrCNX1E expressed in this mutant was able to copurify bound MPT-AMP, although with only low saturation (0.71%), but MPT was undetectable.

The capacity of MPT and MPT-AMP bound to CNX1 proteins to be converted into Moco was determined by nit-l reconstitution in the presence of Mg$^{2+}$ (6). Under these
conditions, MPT adenylation by CNX1G and/or molybdenum insertion coupled to MPT-AMP hydrolysis by CNX1E is catalyzed. The Moco activities obtained were normalized either to the total pterin amounts bound to the proteins or to the protein content (Fig. 2E). Moco synthetic activities were very similar for CrCNX1G and the chimeric proteins isolated from the \textit{mogA} and \textit{moeA} mutants. In addition, the Moco activity measured for CrCNX1E from the \textit{moeA} mutant was high relative to the pterin content. This result confirms that MPT-AMP copurified with CrCNX1E, although scarce, is fully able to be converted into active Moco. Therefore, the ability of CrCNX1E to receive MPT-AMP in \textit{E. coli} in vivo might explain the partial restoration of NR activity in the \textit{E. coli moeA} mutant after CrCNX1E expression. Since in vivo protein-protein interactions among MogA and MoeA have been reported, CrCNX1E should interact with MogA in order to receive MPT-AMP. Since \textit{Arabidopsis} CNX1E does not reconstitute the \textit{moeA} mutant, \textit{Chlamydomonas} CrCNX1E should interact more efficiently than \textit{Arabidopsis} CNX1E with MogA. We propose that a critical difference in the efficiencies of these interactions could be that CrCNX1G, in contrast to \textit{Arabidopsis} CNX1G, is closely related to bacterial MogA proteins (Fig. 1B). Therefore, CrCNX1E has retained its ability to interact with
prokaryotic MogA proteins, and other CNX1E proteins might have lost this ability due to their domain fusions. Further experiments are needed to uncover these specific differences among eukaryotic CNX1 proteins.

**Nucleotide sequence accession numbers.** The CrCNX1G and CrCNX1E cDNA sequences are available in GenBank under accession numbers DQ311645 and DQ311646, respectively.

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