Proteolysis of the Caulobacter McpA Chemoreceptor Is Cell Cycle Regulated by a ClpX-Dependent Pathway

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Proteolysis is involved in cell differentiation and the progression through the cell cycle in Caulobacter crescentus. We have constitutively expressed the transmembrane chemoreceptor McpA from a multicopy plasmid to demonstrate that McpA degradation is modulated during the cell cycle. The level of McpA protein starts to decrease only when the swarmer cells differentiate into stalked cells. The reduction in McpA protein levels is maintained until the stalked cells develop into predivisional cells, at which point the level returns to that observed in swarmer cells. The cell-cycle-regulated degradation of McpA does not require the last 12 C-terminal amino acids, but it does require three amino acids (AAL) located 15 residues away from the C terminus. The ClpXP protease is essential in C. crescentus for viability, and thus, we tested McpA degradation in xylene conditional mutants. The effect on McpA degradation occurred within two generations from the start of ClpX depletion. The conditional mutants’ growth rate was only slightly affected, suggesting that ClpX is directly involved in McpA proteolysis.

Proteolysis is an important facet of programmed cellular processes in both eukaryotes and prokaryotes. In Caulobacter crescentus, proteolysis is involved in a number of important biological functions including chromosomal replication, cell division, the generation of asymmetry, and motility (3, 13, 14, 25, 26, 34). One of the first proteins in C. crescentus to be shown to undergo specific proteolysis was McpA, a receptor for the chemotaxis response. McpA, a cytoplasmic membrane protein, is a member of a large family of receptors (18). The cytoplasmic domain of McpA is highly conserved, whereas its transmembrane domains and periplasmic substrate-binding domain show little similarity. McpA is synthesized only in predivisional cells (1), where it is targeted to the cell pole that will form the flagellated pole of the swarmer cell (2). Since McpA is not synthesized in swarmer cells (1), its degradation guarantees that it does not reappear until the sessile stalked cells develop into predivisional cells. The C terminus of McpA is required for its proteolysis (3), but the highly conserved methylation and signaling domains are not (32). In Escherichia coli, the five amino acids at the C terminus in high-abundance chemoreceptors are required for binding the chemoreceptor methyltransferase (CheR) and the chemoreceptor methyltransferase (CheB) (4, 6, 24). The McpA chemoreceptor has very similar amino acids at its C terminus, and since it is also methylated (1), it is conceivable that these residues are involved in binding CheR and CheB, but it is unclear whether they are involved in its proteolysis.

Five ATP-dependent proteases, Lon, ClpXP, ClpAP, ClpYQ, and PtsH, have been identified in the C. crescentus genome (23), but to date, only two have been extensively studied. The Lon protease is required for the degradation of the essential DNA methylase CcrM, but it is not needed for McpA proteolysis (34). The ClpXP protease is required for the degradation of the essential response regulator CtrA, a member of the OmpR family of DNA binding proteins (13). In C. crescentus, unlike in E. coli, the clpX and clpP genes are essential for cell viability (13). The Clp proteases are composed of two subunits, an ATP binding regulatory subunit (ClpA or ClpX) and a proteolytic subunit (ClpP). The regulatory subunit ClpX is responsible for substrate recognition, disassembly, and presentation to ClpP for degradation. Although there are a few exceptions (9, 22), most substrate recognition by ClpX occurs by binding to the disordered C terminus of its substrate (20). Since the extreme C terminus of McpA is required for proteolysis, ClpXP would be a good candidate for the McpA protease. In this study, we have tested the requirement of ClpXP in McpA degradation. Also, we have identified critical C-terminal amino acid residues that are important for the proteolysis of McpA, thus demonstrating that the CheR and CheB docking site is not necessary for degradation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are shown in Table 1. E. coli strains were cultured at 37°C in Terrific broth for liquid medium or Luria-Bertani medium for solid medium supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), or tetracycline (15 μg/ml) as required. C. crescentus strains were grown in PYE medium (0.2% [w/vol] Bacto Peptone [Difco], 0.1% [w/vol] yeast extract [Difco], 1 mM MgSO4, 0.5 mM CaCl2) and incubated at 28°C or at room temperature and supplemented with chloramphenicol (1 μg/ml), tetracycline (1 μg/ml), nalidixic acid (20 μg/ml), or kanamycin (5 μg/ml) as necessary and 20 mM xylose (PYEX) or 20 mM glucose (PYEG), where indicated. C. crescentus strains were also grown in minimal M2 medium supplemented with xylose (8). Plasmids were mobilized into C. crescentus from the E. coli strain S17-1 or from strain DH10B using MT607 as a conjugational helper strain.

Plasmid constructions. Six primers were designed for PCR: BstXI (5′-ATC ATC GGG TTC ATC GAC AAC ATC TTC GTC TCC-3′), CTD5 (5′-GGG GCC TGG CGG AGA ATT CAC GAA CCG CTG-3′), CTD7 (5′-TCC GAC CCG GAC GAA TTC AGG TGT TCA GAC-3′), CTD11 (5′-ATA TCG AAT TCC
TCT TAG ACA TCA GGC GGC-3'), CTA 3' and ID4 (5'-CTC CCT TCC AGC TGG GCT CCG GTT CGT-3') were used as a template to generate site-directed mutations (ID2* and ID4*) by following the manufacturer's instructions (Promega). These mutant plasmid DNAs (ID2*, ID3*, ID4*, and ID5*) were then subcloned into plasmid pXCP3 to construct plasmids pXCP305, pXCP307, and pXCP308, respectively. The latter two plasmids contained the mcpl deletion constructs pXCP382 and pXCP384. The amplified products were subcloned into pXCP3 to construct plasmids pXCP38, pXCP307, pXCP305, pXCP303, and pXCP301 (Table 2), respectively. A further two primers, ID2 (5'-CTG CCC TTC A-3') and ID4 (5'-CTG CTC-3'), were used with plasmid pCHE22 DNA as a template in PCR to amplify products (ID8*, CTD7*, CTD5*, CTD13*, and CTD11*). These PCR products were de- signed to create the internal deletion constructs pXCP30 and pXCP38, respectively. The PmlI site was included in the CTD5, -7, -11, and -13 primers; and the SacI site was included in the CTD3, -7, and -13 primers; and the BstEII site was included in the BstXI primer for subcloning the amplified products. These primers were used with plasmid pCHE22 as a template in PCR to amplify products (ID8*, CTD7*, CTD5*, CTD13*, and CTD11*). These PCR products were subcloned into pXCP3 to generate site-directed mutations (ID2*, ID3*, ID4*, and ID5*) were then subcloned into pXCP3 to construct plasmids pXCP302 and pXCP34, respectively. The PmlI site was included in the ID2 and ID4 primers for subcloning the amplified products. These primers were used with plasmid pCHE22 DNA as a template to generate site-directed mutations (ID2* and ID4*) by following the manufacturer's instructions (Promega). These mutant plasmid DNAs (ID2* and ID4*) were then subcloned into pXCP3 to construct plasmids pXCP302 and pXCP34, respectively. The 0.4-kb BstXI fragment of plasmid pXCP30 was cloned into plasmids pXCP302 and pXCP34 to generate plasmids pXCP302 and pXCP34 (Table 2), respectively. The latter two plasmids contained the mcpl gene with internal deletions. The following primers were used to mutagenize pXCP3 to generate pXCP3ELD: 5'-CTG AGC AGC GGT TCG GTC GGT GCT CCG GGT CCG GTT CCT GCT GTC-3' and 5'-GCC TGG GTC GGA CCC GGG CTG CTC CTC C-3'. All mutants were confirmed by DNA sequencing.

**Cell synchronization.** C. crescentus strains were grown at 28°C in PYE medium containing antibiotics, xylose (20 mM), or glucose (20 mM) when required. The strains were synchronized by first concentrating them by centrifugation at 6,000 × g for 5 min at room temperature, the loose pellet was discarded, and the hard pellet was resuspended in 10 ml of 10 mM phosphate buffer, pH 7.0, to which 10 ml of Percoll (Pharmacia) was added. Cells were then centrifuged at 10,000 × g for 20 min at room temperature, and the lower swarmer band was removed. Swarmer cells were washed twice by resuspending them in 10 mM phosphate buffer, pH 7.0, and centrifuging them at 6,000 × g for 5 min at room temperature. Finally, the swarmer cells were resuspended in PYEX and allowed to progress through the cell cycle. Swarmer cells were resuspended in PYE supplemented with xylose or glucose and allowed to proceed through the cell cycle at 28°C.

**ClpX and ClpP deletion experiments.** The strains UI200 and UI199 were grown first in permissive conditions, PYE containing 20 mM xylose (PYEX), to an A600 of 0.8 to 1.0. The cells were washed twice with PYE and then grown in PYE containing 20 mM glucose (PYEG) for 0, 3, 6, 9, and 12 h. At these times, swarmer cells were isolated and allowed to proceed through the cell cycle in PYEG.

**Immunoblotting.** Immunoblotting was carried out as described previously (3). Polyclonal antiserum against MepA was used at a 1:5,000 dilution. Secondary antibody (horse-radish peroxidase-conjugated anti-rabbit) (Roche) was diluted 1:3,000, and ECL (Amersham) was used as the detection system.
pXCP1, which has the modulated during the cell cycle. We constructed the plasmid McpA constitutively to show that the McpA protease activity is sized in swarmer cells. Therefore, it is necessary to express McpA is present prior to cell division (1). McpA is not synthe-

gene is expressed only in predivisional cells, and thus, all the synchronized culture was labeled with 20

analysis. Equal amounts of protein were loaded in each lane. (C) A the cell cycle in M2X medium. The numbers above the cell cycle immunoblot represent the time in minutes at which samples were taken, and the C. crescentus drawings denote the progression through the cell cycle. Cell cycle progression was monitored by microscopic analysis. Equal amounts of protein were loaded in each lane. (C) A synchronized culture was labeled with 20 μCi of Tran35S-label (ICN)/ml for 4 min at 0 min (open boxes) or at 40 min (filled boxes) and then chased with 0.2% (wt/vol) Bacto Peptone, 0.1% (wt/vol) Bacto yeast extract, 0.5 mM methionine, and 0.05 mM cysteine. Samples were taken every 10 min except for the last time point. Equal counts were immunoprecipitated with McpA1 antisera. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were visualized by a Molecular Dynamics phosphorimager and quantified with IPLabScan software.

RESULTS

McpA proteolysis is cell cycle regulated. The wild-type mcpA gene is expressed only in predivisional cells, and thus, all the McpA is present prior to cell division (1). McpA is not synthesized in swarmer cells. Therefore, it is necessary to express McpA constitutively to show that the McpA protease activity is modulated during the cell cycle. We constructed the plasmid pXCP1, which has the mcpA gene under the control of the inducible xylose promoter on a multicopy plasmid. When the xyL promoter is induced in the presence of xylose, it is expressed constitutively throughout the cell cycle (21). The plasmid pXCP1 was introduced into strain MRKA828, which overexpresses McpA constitutively throughout the cell cycle in M2X medium. The numbers above the cell cycle immunoblot represent the time in minutes at which samples were taken, and the C. crescentus drawings denote the progression through the cell cycle. Cell cycle progression was monitored by microscopic analysis. Equal amounts of protein were loaded in each lane. (C) A synchronized culture was labeled with 20 μCi of Tran35S-label (ICN)/ml for 4 min at 0 min (open boxes) or at 40 min (filled boxes) and then chased with 0.2% (wt/vol) Bacto Peptone, 0.1% (wt/vol) Bacto yeast extract, 0.5 mM methionine, and 0.05 mM cysteine. Samples were taken every 10 min except for the last time point. Equal counts were immunoprecipitated with McpA1 antisera. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were visualized by a Molecular Dynamics phosphorimager and quantified with IPLabScan software.

The N-terminal extent of the requirement for McpA degradation. We have already shown that the deletion of the methylation site, and thus, all the McpA is not synthesized in swarmer cells. Therefore, it is necessary to express McpA constitutively to show that the McpA protease activity is modulated during the cell cycle. We constructed the plasmid pXCP1, which has the mcpA gene under the control of the inducible xylose promoter on a multicopy plasmid. When the xyL promoter is induced in the presence of xylose, it is expressed constitutively throughout the cell cycle (21). The plasmid pXCP1 was introduced into strain MRKA828, which overexpresses McpA constitutively throughout the cell cycle in M2X medium. The numbers above the cell cycle immunoblot represent the time in minutes at which samples were taken, and the C. crescentus drawings denote the progression through the cell cycle. Cell cycle progression was monitored by microscopic analysis. Equal amounts of protein were loaded in each lane. (C) A synchronized culture was labeled with 20 μCi of Tran35S-label (ICN)/ml for 4 min at 0 min (open boxes) or at 40 min (filled boxes) and then chased with 0.2% (wt/vol) Bacto Peptone, 0.1% (wt/vol) Bacto yeast extract, 0.5 mM methionine, and 0.05 mM cysteine. Samples were taken every 10 min except for the last time point. Equal counts were immunoprecipitated with McpA1 antisera. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were visualized by a Molecular Dynamics phosphorimager and quantified with IPLabScan software.

The N-terminal extent of the requirement for McpA degradation. We have already shown that the deletion of the methylation and signaling domains of McpA (Fig. 2) is not required for its degradation (32). Therefore, we constructed internal deletions from the methylation domain (R1) to the C terminus (Fig. 2) in order to determine the N-terminal extent of the requirement for degradation. An internal deletion of the amino acid residues 61 to 70 from the C-terminal end, located immediately downstream of the R1 methylation domain, resulted in the strain MRKA843 (Fig. 2), which also degraded this McpA deletion during the swarmer-to-stalked-cell differentiation event (Fig. 3). Since it has been reported previously that proteins with nonpolar C termini are more vulnerable to proteolysis (12, 30), we decided to delete the next three-amino-acid sequence which contained three sequential hydrophobic amino acids (AAL), pXCP305 (Fig. 2). The plasmid pXCP305 was inserted into MRKA850 to make MRKA852, and the McpA deletion in this strain was not degraded (Fig. 3), suggesting that these nonpolar amino acids in the C-terminal region are required for McpA proteolysis. A further deletion of 25 C-terminal amino acids from McpA resulted in the strain MRKA843 (Fig. 2), and this McpA deletion was not degraded (Fig. 3).

The extreme C terminus is not required for McpA proteolysis. It has been previously shown using M2 epitope tags that the extreme C terminus of McpA is essential for its degradation (3). Because epitopes can perturb the localized structure of a protein, we decided to introduce ochre and amber mutations into mcpA in order to determine the C-terminal extent of the McpA degradation signal. Several C-terminal truncations of McpA were constructed (Fig. 2) and were fused to the xyL inducible promoter and inserted into the chromosome of MRKA850 at the xyL locus. Deletion of the four C-terminal amino acids (WEEF) from McpA resulted in the strain MRKA801 (Fig. 2). The mutant McpA in MRKA801 was still degraded (Fig. 3), like the full-length McpA in the control strain MRKA800 (Fig. 3). The deletion of the last 12 C-terminal amino acids from McpA resulted in the strain MRKA803 (Fig. 2), which also degraded this McpA deletion during the swarmer-to-stalked-cell differentiation event (Fig. 3). Since it has been reported previously that proteins with nonpolar C termini are more vulnerable to proteolysis (12, 30), we decided to delete the next three-amino-acid sequence which contained three sequential hydrophobic amino acids (AAL), pXCP305 (Fig. 2). The plasmid pXCP305 was inserted into MRKA850 to make MRKA852, and the McpA deletion in this strain was not degraded (Fig. 3), suggesting that these nonpolar amino acids in the C-terminal region are required for McpA proteolysis. A further deletion of 25 C-terminal amino acids from McpA resulted in the strain MRKA843 (Fig. 2), and this McpA deletion was not degraded (Fig. 3).

The N-terminal extent of the requirement for McpA degradation. We have already shown that the deletion of the methylation and signaling domains of McpA (Fig. 2) is not required for its degradation (32). Therefore, we constructed internal deletions from the methylation domain (R1) to the C terminus (Fig. 2) in order to determine the N-terminal extent of the requirement for degradation. An internal deletion of the amino acid residues 61 to 70 from the C-terminal end, located immediately downstream of the R1 methylation domain, resulted in the strain MRKA849 (Fig. 2). This McpA deletion was still degraded, as shown by the change in levels of McpA during the cell cycle (Fig. 3). However, the drop in McpA levels was not as obvious as that seen in the wild-type strain MRKA800 (Fig. 3), which suggests that it is degraded at a much lower rate. A further internal deletion including the amino acid residues 19 to 70 from the C terminus resulted in the strain MRKA841 (Fig. 2), and this McpA deletion was not degraded (Fig. 3). These data would suggest that the requirements for degradation are located after the conserved R1 methylation domain.
The C-terminal AAL amino acids are required for degradation. The comparison of the McpA degradation data from strains MRKA852 and MRKA803 (Fig. 3) would suggest that the amino acids AAL are required for McpA degradation. Thus, these three amino acid residues in McpA were mutated to the amino acids ELD to create pXCP3ELD. The suicide plasmid pXCP3ELD was then conjugated into MRKA580, which created the strain MRKA930. Swarmer cells were isolated from the MRKA930 strain and were allowed to progress through the cell cycle. The immunoblot of cell cycle extracts from strain MRKA930 (Fig. 3) showed that the ELD derivative of McpA was not degraded. This suggests that the amino acid residues AAL at positions 643 to 645 in McpA are required for its degradation.

McpA proteolysis is dependent on ClpX. In the search for the McpA protease, we have shown elsewhere that the Lon (34) and ClpYQ (M. R. K. Alley, unpublished data) proteases are not required for McpA proteolysis. Since C-terminal hydrophobic amino acid residues are required for McpA proteolysis and hydrophobic amino acid residues at the extreme C terminus have been shown previously to be required for degradation of proteins via ClpXP (20), we decided to test the requirement for ClpXP in the degradation of McpA. The clpX gene is essential in C. crescentus (13); therefore, we had to use a conditional strain. In the clpX conditional strain UJ200, the expression of ClpX is under the tight control of the xylose-inducible promoter xylXp. After four generations, most of the ClpX protein in the cell has been removed (13). As described for UJ200, we set up a series of xylose depletion experiments by growing UJ200 for various times in the absence of xylose. At set times (0, 3, 6, 9, and 12 h), we isolated swarmer cells and performed cell cycle immunoblotting. At time zero, the degradation of McpA proceeded in a cell-cycle-dependent manner (Fig. 4A). The swarmer cells progressed through the cell cycle (Fig. 4D), with cell division taking place at 120 min. After 3 h of xylose depletion, or after approximately two generation times, McpA degradation had been virtually abolished (Fig. 4B). However, the swarmer cells still progressed through the cell cycle, with cell division occurring at 135 min, which is only slightly later than that for time zero (Fig. 4D). This would suggest that the effects on McpA degradation were due to ClpX depletion. After 6 h of xylose depletion, McpA was no longer degraded (Fig. 4C). Although the swarmer cells still progressed through the cell cycle, cell division occurred at 180 min, which was much later than that for 0 and 3 h of xylose depletion. Similarly, after 9 and 12 h of xylose depletion, no degradation of McpA was observed (data not shown), but the cells became filamentous and grew much more slowly (Fig. 4D).

The requirement for ClpP in McpA degradation. Since ClpX and ClpP form a protease complex, we tested whether ClpP is required for McpA degradation. We used the conditional clpP strain UJ199, because clpP is essential in C. crescentus (13). The expression of the essential clpP gene in UJ199 is under the tight control of the xylose-inducible promoter xylXp. As in the UJ200 strain, growth of UJ199 on glucose medium represses ClpP synthesis. After four generations, most of the ClpP protein in the cell has been removed (13). As described for UJ200, we set up a series of xylose depletion experiments by growing UJ199 in glucose-containing medium for various times to examine the effect on the cell cycle degradation of McpA. At zero time (Fig. 5A) and at 3 h (Fig. 5B) and 6 h (Fig. 5C) of xylose depletion, McpA was slowly degraded. As shown in Fig. 5F, the...
effect of xylose depletion on the growth of UJ199 went from cell division occurring at 120 min for time zero, to division occurring at 135 min for 3 h, to division occurring at 150 min for 6 h of xylose depletion. After 9 h of xylose depletion, which is equivalent to four generations, McpA was still degraded (Fig. 5D). After 12 h of xylose depletion, which is equivalent to five generations, McpA degradation was affected (Fig. 5E).

**DISCUSSION**

A large number of cell-cycle-regulated proteins are specifically degraded during the *C. crescentus* life cycle (11). These include not only the motility proteins FliF (14), McpA (3), CheYI (11), and CheD (11) but also proteins required for progression through the cell cycle, CtrA (7), FtsZ (16), and CcrM (34). Although a large number of protease substrates have been identified, cognate proteases are known for only two substrates, CcrM and CtrA. The Lon protease degrades CcrM (34), and ClpXP degrades CtrA (13). The CtrA response regulator is degraded at the same time as McpA and like McpA has a C-terminal requirement for its degradation (7). In this study, we have added McpA to the list of *C. crescentus* proteins that are degraded by ClpXP.
to be exposed at its extreme C terminus (7), while McpA is still degraded even when β-lactamase is fused to its C terminus (Alley, unpublished). In *E. coli*, ClpXP is involved in the degradation of a number of different substrates including, for example, the stationary-phase sigma factor RpoS (27), λO replication protein (33), and SsrA-tagged proteins (10). RpoS degradation requires the response regulator RssB (5), while the N terminus of the λO replication protein plays a critical role in its degradation (9). The ClpXP-mediated SsrA tagging system requires SspB, a ribosome-associated protein, which is regulated by nutrient stress (19). These examples demonstrate that ClpXP-mediated proteolysis can be regulated in various ways in *E. coli*, which would suggest that there are likely to be alternative ClpXP degradation pathways in *C. crescentus*. Therefore, showing that CtrA and McpA are degraded by the same protease does not explicitly guarantee that both proteins are regulated identically.

**ClpP requirement.** Although the data for ClpP involvement are less strong than those for ClpX, the absence of any evidence for ClpX involvement with any other protease strongly suggests that the McpA protease is ClpXP. The best explanation for the weak effect observed with ClpP depletion is that in the absence of ClpP other proteases might be able to compensate for its loss. For example, in *E. coli* overproduction of ClpQY can compensate for the absence of Lon, ClpXP proteases, or low levels of FtsH protease in the degradation of the heat shock sigma factor σ^32* (15).

**The McpA degradation signal.** The crystal structure of the cytoplasmic domain of the *E. coli* chemoreceptor Tsr (17) suggests that the sequences C terminal to the R1 methylation domain in chemoreceptors form a surface-exposed flexible structure separate from the α-helices. The extreme C terminus contains the CheRB binding site (4, 35), which is exposed to allow CheR and CheB to bind the chemoreceptor. We have shown that the sequences located seven amino acids from this CheRB site (Fig. 2) are important for proteolysis, and therefore, we predict that this region, like the CheRB binding site, will be exposed. Our internal deletion data would suggest an upper limit on the size of the domain required for degradation of approximately 46 amino acids. However, not all these residues might be involved in McpA proteolysis, and most of the residues might be there just to form a linker to allow the critical residues to be exposed.

The identification of the McpA protease and the critical residues in McpA required for its degradation should enable us to dissect the processes involved in cell-cycle-regulated proteolysis.

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