A Context-dependent ClpX Recognition Determinant Located at the C Terminus of Phage Mu Repressor*

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The bacteriophage Mu immunity repressor is a conformationally sensitive sensor that can be interconverted between forms resistant to and sensitive to degradation by ClpXP protease. Protease-sensitive repressor molecules with an altered C-terminal sequence promote rapid degradation of the wild-type repressor by inducing its C-terminal end to become exposed. Here we determined that the last 5 C-terminal residues (CTD5) of the wild-type repressor contain the motif required for recognition by the ClpX molecular chaperone, a motif that is strongly dependent upon the context in which it is presented. Although attachment of the 11-residue ssrA degradation tag to the C terminus of green fluorescent protein (GFP) promoted its rapid degradation by ClpXP, attachment of 5–27 C-terminal residues failed to promote degradation. Disordered peptides derived from 41 and 35 C-terminal residues of CcdA (CcdA41) and thioredoxin (TrxA35), respectively, activated CTD5 when placed as linkers between GFP and repressor C-terminal sequences. However, when the entire thioredoxin sequence was included as a linker to promote an ordered configuration of the TrxA35 peptide, the resulting substrate was not degraded. In addition, a hybrid tag, in which CTD5 replaced the 3-residue recognition motif of the ssrA tag, was inactive when attached directly to GFP but active when attached through the CcdA41 peptide. Thus, CTD5 is sufficient to act as a recognition motif but has requirements for its presentation not shared by the ssrA tag. We suggest that activation of CTD5 may require presentation on a disordered or flexible domain that confers ligand flexibility.

The bacteriophage Mu repressor functions in the establishment of lysogeny by shutting down Mu transposition functions needed for its replication (1, 2). Binding to operator sequences that are ∼1 kb away from the Mu left end, the repressor is able to shut down transcription from an early promoter that drives expression of transposition functions encoded by the MuA and B genes (3–6). It also competes with the MuA protein for binding sites in the operator, which serves to enhance assembly of the MuA transpososome (7, 8). Exit from lysogeny is predominantly by the last 3 C-terminal residues (LAA) (20). This degradation signal is added by ssrA RNA or tmRNA, replication can be induced by inactivation of the repressor, a process potentially brought about by reducing the affinity of the repressor for DNA or by increasing its susceptibility to degradation by ClpXP protease (6, 9–12).

DNA binding affinity and protease sensitivity can both be modulated by the repressor C-terminal domain (CTD), which comprises approximately the last 27 residues (Ile170–Val196) of the repressor (13–15). Deletions within this domain suppress temperature-sensitive mutations in the DNA binding domain (DBD), which makes up approximately the first 50 N-terminal residues. These deletions also promote resistance to ClpXP in the presence of dominant-negative Vir repressors (13, 14). The Vir proteins (Vir3060 and -3061) result from frameshift mutations that alter the C-terminal sequence of the repressor (16). The 6- or 13-residue segments created by the mutation not only cause Vir proteins to be rapidly degraded by ClpXP but also allow Vir to promote rapid degradation of the wild-type repressor (Rep) (9–11).

The movement of the Rep CTD plays a critical role in modulating interactions with DNA and ClpX. As Rep binds to DNA, its CTD moves away from the DBD, whereas the CTD of the temperature-sensitive DBD mutant cts62Rep fails to do so at the elevated temperature (17). The close proximity of the CTD to the DBD is associated with low DNA binding affinity, suggesting that the CTD may sterically inhibit interactions of the DBD with DNA. Vir also induces movement of the Rep CTD such that its C terminus is exposed (18). Biochemical analysis of Rep and repressor CTD mutants has indicated the likelihood that a ClpX recognition motif, required for the recognition of Rep as a protease substrate, is present within the CTD. Although Vir can accelerate Rep degradation, Rep alone can be degraded by ClpXP with a relatively high Michaelis constant (9). Moreover, Rep degradation can be accelerated in the absence of Vir by heat denaturation. In contrast, repressor molecules with a deletion in the CTD or with certain amino acid replacements in the CTD are resistant to ClpXP even upon heat denaturation or in the presence of Vir (14).

Recognition motifs of a number of ClpX substrates can be attached to heterologous proteins to promote their degradation by ClpXP. For example, 10 C-terminal residues of the MnA protein can be attached to the C terminus of phage P22 Arc repressor to promote its degradation (19). The 11-residue ssrA degradation tag includes a ClpX recognition motif specified predominantly by the last 3 C-terminal residues (LAA) (20). This degradation signal is added by ssrA RNA or tmRNA, 1

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1 The abbreviations used are: CTD, C-terminal domain; DBD, DNA binding domain; CcdA41, peptide containing 41 C-terminal residues of F plasmid CcdA protein; GFP, green fluorescent protein; TrxA, full-length thioredoxin protein; TrxA35, peptide containing the last 35 C-terminal residues of thioredoxin; Vir, repressor encoded by dominant-negative virulent mutants of Mu; Rep, wild-type repressor.
which has characteristics of both tRNA and mRNA and serves to rescue stalled ribosomes while attaching the degradation tag encoded within its open reading frame to the C terminus of the stalled peptide (21–24). ClpX recognition motifs have been found both at the N and C termini (19, 20, 25, 26), and although there are considerable variations in the sequences of these motifs, some general rules for the sequences of these motifs have begun to emerge (25). ClpX substrates with motifs at their C-terminal ends have been found to have C-terminal sequences similar to that of MuA or that of the ssrA tag (25). The last 6 residues of the Mu repressor (EVKKKAV) most resemble that of MuA (RRKKAI). The C-terminal motifs present on the various ClpX substrates have suggested that a 3–6 residue sequence is sufficient to promote ClpX recognition. However, cysteine-scanning mutagenesis of the Rep CTD has indicated that 8 single-replacement mutations of the Rep CTD has indicated that 8 single-replacement mutations known to confer ClpXP resistance span 16 residues of the CTD, the Val196 segment of the CTD at high levels without using protease-deficient cells.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Proteins—**ClpX (27), ClpP (28), Rep, and Vir3060 proteins (17, 18) were purified as described previously. Green fluorescent peptide (GFP) fusion proteins, which contain an N-terminal His tag, were overproduced in strain BL21(DE3) (Novagen) from plasmid vectors described below.

Overproducing plasmids for GFPmut3 (29) fusion proteins were all derivatives of pYH2, which was a gift from Drs. Yanzhu Yang and Elliott Crooke (Georgetown University Medical University). Coding sequences for attachments and linkers were fused to GFP through a NheI tag were attached directly to the NheI site as double-stranded oligonucleotides with cohesive ends. All constructs were verified by DNA sequencing. The mixture was fractionated by centrifugation in a Beckman Ti-50 rotor at 40,000 rpm for 45 min. The purified protein could be purified by affinity chromatography using ClpX, ClpP, and all protease substrate concentrations were determined by the method of Pace (30) and are given in equivalents of protease activity. Concentration of ClpX was determined by the method of Pace (30) and are given in equivalents of protease activity. Concentration of ClpX was determined by the method of Pace (30).

**GFP Fusion Proteins—**GFP fusion proteins in the pellet were solubilized in 5–10 ml of Buffer B (25 mM HEPES-NaOH, pH 7.5, 500 mM NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol, 7 mM urea, 10% w/v glycerol), dispersed with a Dounce homogenizer, and then placed on ice for 1 h. Insoluble materials were removed by centrifugation (Beckman Ti 50 Ti rotor; 40,000 rpm for 30 min at 4 °C), and the supernatant was mixed with Superflow nickel-nitrilotriacetic acid resin (Qiagen, 2.5 ml of bed volume). Batch adsorption of GFP proteins to the resin was allowed to proceed with gentle rocking at 4 °C for 1 h. The mixture was then washed twice in batch with 10–12 ml of Buffer B. The mixture was then washed twice in batch with 10–12 ml of Buffer B. The purified protein was then dialyzed extensively in Buffer C for storage at 80 °C.

**ClpX, ClpP, and all protease substrate concentrations were determined by the method of Pace (30) and are given in equivalents of protein monomers.**

**RESULTS**

**The Last 5 C-terminal Residues of the Repressor Promote Degradation of GFP When Attached to Its C Terminus via the CcdA41 Peptide Linker.—**To determine whether a ClpX recognition motif is contained within the Rep CTD (Ile<sup>170</sup>–Val<sup>199</sup>), the last 27 (CTD27), 15 (CTD15), 10 (CTD10), or 5 (CTD5) C-terminal residues were attached to the C terminus of GFP (Fig. 1). Because ClpX recognition motifs found at the C-terminal end of protein substrates have been found to be as small as 3–6 residues (20, 25), a fully functional recognition motif was likely to be contained within the CTD. However, the GFP could not be degraded by ClpXP when any of the CTD

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**Fig. 1B** with or without C-terminal attachments was constructed in an analogous fashion using GAGCTGCTAGGAGTTAATCGGAGGACGTTG-TCTTGC as the first (N-terminal) primer. All constructs were verified by DNA sequencing.
peptides were attached, even the entire CTD (GFP-CTD27), whereas the 11-residue ssrA tag (GFP-ssrA) promoted rapid GFP degradation (Fig. 2). GFP-Rep, which has the entire Rep sequence attached to GFP, could be degraded at a low level. The presence of Vir could accelerate the degradation of GFP-Rep but not of GFP-CTD27 (data not shown). We therefore examined whether a linker placed between GFP and Rep CTD residues would promote substrate degradation. We chose as a linker the C-terminal residues of the CcdA protein, which can be converted to a ClpXP substrate by the addition of a recognition motif to its C-terminal end. CcdA is the antidote protein for the postsegregational killing system of the F plasmid that eliminates plasmid-free cells (31, 32). The CcdA protein is a substrate of the lon protease (33, 34), but not of ClpXP, but it is degraded by ClpXP in vivo when 7 C-terminal residues (FMNRLKVL) of the ClpXP-sensitive Vir protein are attached to the CcdA C terminus (35). When 41 C-terminal residues of the CcdA protein were attached as linkers (CcdA41) between the GFP C terminus and the CTD5 peptide, the GFP substrate (GFP-CcdA41-CTD5) could then be degraded by ClpXP (Fig. 3). No intermediates in degradation, such as products of aborted degradation after proteolysis of the CcdA41-CTD5 moiety, were observed (Fig. 3A).

It should be noted that differences in protease sensitivity between GFP-CTD5 and GFP-CcdA41-CTD5 are not likely caused by differential effects on GFP folding caused by the different peptide appendages. As was demonstrated previously for the ssrA tag (36), these appendages do not interfere with the fluorescent properties of the GFP protein. In addition, the stability of the GFP portion is not affected by these appendages. Fluorescence quantum yield and emission spectrum for GFP, GFP-CcdA41, GFP-CTD5, and GFP-CcdA41-CTD5 were essentially the same in the presence of 6 M urea (data not shown).2

The control GFP protein with the attached CcdA41 peptide (GFP-CcdA41) but without Rep CTD peptides could not be degraded. Attachment of more than 5 residues of the CTD did not increase the rate of degradation. GFP-CcdA41-CTD10, -CTD15 (data not shown), and -CTD27 could be degraded but more slowly than GFP-CcdA-CTD5 (Fig. 3B). The results indicate that the CcdA41 protein linker allows the C-terminal motif of Rep to be properly displayed for recognition by ClpXP. Together with additional data described later, the results also indicate that CTD5 is sufficient to serve as a ClpX recognition determinant. Thus, the CTD residues outside of CTD5 are not likely to be part of the ClpX recognition motif and may play a key role in the proper display of CTD5 when Rep is in the ClpXP-sensitive form.

The ClpX Recognition Motif Attached to the C Terminus of Thioredoxin Promotes Degradation in the Context of the Disordered Peptide but Not in the Context of the Native Full-length Protein—An important question is why attachment of even 27 C-terminal residues of Rep directly to GFP fails to promote

2 K. Marshall-Batty and H. Nakai, unpublished results.
GFP degradation whereas attachment of the 11-residue ssrA tag readily can. The CcdA protein apparently has an extended or partially unfolded conformation (34), which may serve not only to expose the attached motif but also promote conformational flexibility of a recognition motif attached to the C terminus. We chose to use the CcdA41 peptide as the linker on the GFP substrate because it lacks the /H9251-helical structure that is present in the full-length, 72-residue CcdA protein (34), a property that may confer conformational disorder to the attached Rep peptide. Ligand flexibility (37–40) conferred by presentation of the motif on a disordered peptide linker can potentially enhance recognition by ClpX. We therefore wished to determine whether the remaining 8 residues can generally help display or expose ClpX recognition motifs when attached directly to the GFP. To evaluate whether the CTD5 of Rep can be as active as a recognition motif as the LAA motif of ssrA when attached to these 8 residues, we replaced these 3 residues of the ssrA tag with CTD5 and constructed a GFP with this hybrid tag (ssrA/H90043-CTD5) attached directly to its C-terminal end. Although GFP-ssrA is very rapidly degraded, GFP-ssrA/H90043-CTD5 could not be degraded even after prolonged incubation for 60 min (Fig. 5). We verified that ssrA/H90043-CTD5 can provide an active ClpX recognition determinant. When the CcdA41 linker was placed between GFP and this hybrid tag (GFP-CcdA41-ssrA/H90043-CTD5), the resulting substrate was degraded by ClpXP (Fig. 5). The rate of degradation of this substrate was considerably slower than the rate of degradation of GFP-ssrA; it was comparable with the rate of degradation of GFP-CcdA41-CTD27 (Fig. 3B). GFP-CcdA41-CTD5 is degraded more rapidly than either GFP-CcdA41-CTD27 or GFP-CcdA41-ssrA/H90043-CTD5; it appears that the extra residues between the CcdA41 linker and CTD5 slow down rather than enhance GFP degradation, regardless of whether the residues are derived from the ssrA tag or the Rep CTD. The results indicate that CTD5 as a
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**DISCUSSION**

The dominant-negative properties of Vir proteins and the way they promote degradation of Rep by ClpXP have raised questions about the mechanism of trans targeting of Rep and whether it can play an important role in the regulation of Mu transposition with respect to cell physiology. Inactivation of Rep to induce Mu transposition functions is promoted by starvation and stationary phase conditions, a process termed S derepression (47), and Rep is generally thought to be a sensor for cell physiology. Repressor peptides that include at least the DBD and that are marked with the ssrA tag confer ClpX recognition determinant properties from the LAA motif when attached to the ssrA3 peptide linker, still requiring attachment to the flexible linker CcdA41 for its activation.

Rep (VKKAV or CTD5) can act as a ClpX recognition motif, being able to promote degradation of certain heterologous substrates when attached to its C terminus. This indicates that Rep has a ClpX recognition determinant no larger than those in the MuA-type class of motifs. All 5 residues may not necessarily be essential for ClpX recognition. Replacement of lysine at the second position or valine at the fifth position promotes ClpXP resistance in the context of the full-length repressor; however, replacement of valine, lysine, or alanine at the first, third, and fourth positions, respectively, with cysteine does not promote protease resistance (14). The CTD5 motif was especially dependent upon its context to be active, and this property was consistent with the ability of Rep conformational changes to activate the motif. CTD5 was active when attached to the GFP substrate via the flexible or disordered peptide linkers CcdA41 or TrxA35, whereas it was dormant when attached directly to GFP or to the Trx linker, both of which have very stable and ordered conformations (36, 48). The flexibility of the CcdA41 and TrxA35 linkers may activate the CTD5 motif by allowing it to be fully exposed. The local conformation at the C terminus of GFP or Trx could occlude the motif, sterically inhibiting its binding by ClpX. However, we think this is a less likely explanation for why CTD5 attached to GFP or Trx remains inactive. The ssrA tag attached to GFP or to the Trx linker readily promoted substrate degradation. Although it is possible that the ssrA tag sequence allows its recognition motif to be better exposed, the CTD5 motif was inactive even when it replaced the LAA motif in GFP-ssrA. It seems unlikely that the sequence of CTD5 causes it to be inaccessible in three different contexts (the C terminus of GFP, Trx, and ssrA3), which allow the LAA motif or ssrA tag sequence to be highly accessible.

The stability of substrate structure can also influence the rate at which the protein is unfolded and degraded by ClpXP (49, 50). Destabilization of its structure can apparently reduce the repeated application of mechanical force by ClpXP required to unfold it. One of our major reasons for choosing GFP as test substrate in this work is its very stable conformation, resistant to denaturation even in the presence of 6 M urea (36). The peptide appendages CcdA41-CTD5 and TrxA35-CTD5 that allowed GFP degradation by ClpXP promoted no apparent change in the stability of the GFP moiety, although the fluorescent properties were not significantly changed even in the presence of 6 M urea. Thus, these appendages cannot be accelerating the substrate degradation by destabilizing overall substrate structure. It has been proposed that the local structure next to a degradation tag influences the rate of degradation by affecting the initiation of protein unfolding by ATP-dependent proteases pulling at this site (51). Instability of the protein structure at this site would aid in initiating the collapse of cooperative interactions that are involved in protein folding. Using the titin-I27 domain as a test substrate, Kenniston et al. (49) have demonstrated that destabilizing the protein conformation near the ssrA attachment site accelerates its degradation by ClpXP and reduces the demand for ATP in this process. The CcdA41 and TrxA35 spacers may enhance the activity of the CTD5 recognition motif by aiding in initiation of protein unfolding at this site. Nevertheless, it is clear that these spacers are essential for CTD5 to act as a recognition motif on GFP but not for the ssrA tag. Moreover, the activity of the ssrA tag was essentially equivalent whether the spacer was native Trx or the disordered TrxA35, whereas only TrxA35 activated CTD5 (Fig. 4). That is, the disordered spacer plays a more critical part with CTD5 than with the ssrA tag in allowing ClpX to get a tight grip of the substrate C terminus. In other words,
the activity of CTD5 is more readily modulated by local protein conformation than the activity of the ssrA tag.

Although exposure of the motif is most likely important for recognition of the motif, the CcdA41 and TrxA35 linkers may endow the motif with ligand flexibility. Conformational disorder or flexibility endowed to CTD5 by these linkers may promote recognition by ClpX by enhancing its interactions with the substrate binding site in several ways (37–40). Flexibility can allow the recognition motif to assume a number of ligand conformations, a subset of which may be recognized with high affinity. The disordered configuration can allow more rapid sampling of ligand conformations by the ClpX active site. And if the conformational disorder at and around the recognition motif increases the exposure of hydrophobic residues to water, substrate binding may be favored by net entropic gain that would result from displacement of ordered water molecules as ClpX and substrate interact. The unfolded structure of the linkers may also help ClpX achieve tighter binding to the substrate by allowing it to more readily initiate a productive reaction cycle in which it pulls the substrate into the ClpP proteolytic chamber. That is, the initial unfolding process may expose sites at which ClpX establishes a tight grip on the substrate for translocation, and this may be more difficult to achieve if the protein is stably folded at the site of the recognition motif. The ClpX recognition determinant in the ssrA tag may be composed of a sequence that may be a more ideal ligand for ClpX than CTD5. That is, it may have a higher affinity for ClpX, rendering it less dependent upon presentation on a disordered linker to be active.

Recognition of substrates is a key step for regulating proteolysis by ClpXP, and even the degradation of ssrA-tagged substrates is influenced by a trans-acting factor. The SspB protein, which serves as a substrate specificity factor for ClpX, can enhance the degradation of ssrA-tagged substrates (52), increasing affinity and cooperativity of their binding to the ClpX hexamer (53). SspB is bound by the ClpX hexamer without being unfolded and degraded and promotes a very stable association of the tagged substrate to ClpX (53). The mutant Vir repressor also enhances recognition of Rep (9), although it is unlike SspB in that it is also a ClpXP substrate. The self-associating property of the repressor (5, 54) would lead to the formation of mixed Rep-Vir oligomers. Vir in the oligomer would not only provide a ligand for binding of the oligomer by ClpX but also help expose and activate the ClpX recognition motifs in Rep protomers (18). A ClpX hexamer has the potential to bind more than one repressor protomer, and this could stabilize its binding to a repressor oligomer. The unfolding of a single substrate by ClpX has been determined to be an inefficient process that requires the hydrolysis of as many as 150 ATP molecules for every ssrA-tagged Arc repressor substrate, implying that there may be many unsuccessful catalytic cycles before a single substrate is unfolded (50). Thus, it has been suggested that the stabilization of substrate binding (such as the effect of SspB on ssrA-tagged substrates) might enhance the probability that the bound substrate is unfolded and degraded rather than being dissociated from ClpX (53).

We have characterized previously repressor mutations promoting ClpXP recognition resistance such as V183C, which is in the CTD but outside the CTD5 motif (14). Although CTD residues outside CTD5 may not be part of the ClpX recognition motif, they most likely play an important role in displaying the CTD5 motif when Rep undergoes the conformational change to the ClpXP-sensitive form. Thus, CTD mutations like V183C could prevent repressor conformational changes that make CTD5 accessible to ClpXP. They could also prevent the display of CTD5 with the conformational flexibility needed to activate the motif. Yet another possibility is that it affects the oligomeric structure of the repressor, which may influence its properties as a ClpXP substrate. As discussed above, binding of more than one repressor protomer by the ClpX hexamer could, for example, increase the probability of a successful reaction cycle in which the substrate is unfolded. Although the oligomeric structure of ClpX substrates may potentially enhance their binding and degradation by ClpXP, the CTD5 motif does not necessarily have to be attached to oligomeric proteins to be active. GFP (55) and Trx (45) are predominantly monomeric proteins, and the TrxA35 peptide exists as a disordered monomer (56).

Conformational flexibility may be an important property that activates ClpX recognition motifs in general. Nuclear magnetic resonance analysis of a ClpXP substrate composed of phage P22 Arc repressor with 19 C-terminal residues of MuA attached at the C terminus indicates that the MuA residues are displayed as a disordered peptide in the context of a properly folded Arc protein (57). The motif at the C terminus of MuA protein is still active when GFP is attached to its C terminus such that the motif is in the interior of the protein, but it is recognized with reduced efficiency (58). At an internal position, segmental motions of the motif may be reduced in the conformation of the motifs acquired through LexA cleavage and conformational changes can generally be an important mechanism in regulating proteolysis, providing a means of selectively degrading unwanted proteins as well as inducing a response to conditions of stress.

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