J|A|C|S COMMUNICATIONS

Published on Web 01/11/2010

Analysis of p300/CBP Histone Acetyltransferase Regulation Using Circular Permutation and Semisynthesis

Kannan R. Karukurichi, Ling Wang, Lerna Uzasci, Cara Marie Manlandro, Qing Wang, and Philip A. Cole*

Department of Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, Baltimore, Maryland 21205

Received November 7, 2009; E-mail: pcole@jhmi.edu

Post-translational modifications (PTMs) by phosphorylation, acetylation, methylation, ubiquitylation, and glycosylation are major mechanisms for regulating protein function.[1] As has been well-established in protein kinase signaling cascades, enzymatic activities can be activated or inhibited by reversible, site-specific covalent modification of protein side chains.[1,2] Several years ago, the transcriptional coactivator p300/CBP histone acetyltransferase (HAT) was shown to be densely autoacetylated on lysines in an apparent regulatory loop.[3] Partial loop deletion or autoacetylation of up to 17 sites in p300 HAT leads to an increase in catalytic activity by 4−10-fold.[3] In general, it is difficult to assess the specific functional contributions of individual Lys acetylation events using site-directed mutagenesis. In classical mutagenesis, Lys is usually replaced with Arg and Gln, which are crude structural mimics of the unmodified and acetylated Lys, respectively. Recent advances in nonsense suppression mutagenesis to incorporate acetyl-Lys are promising[4] but difficult to apply to cases where multiple sites are modified. When PTMs are near protein termini, expressed protein ligation (EPL) offers the potential for installing multiple PTMs.[5] However, in the case of p300/CBP, the ∼40 amino acid (aa) autoacetylation loop is in the middle of the catalytic domain (Figure 1) and is a difficult candidate for conventional EPL since multiple peptide ligation steps would be needed.

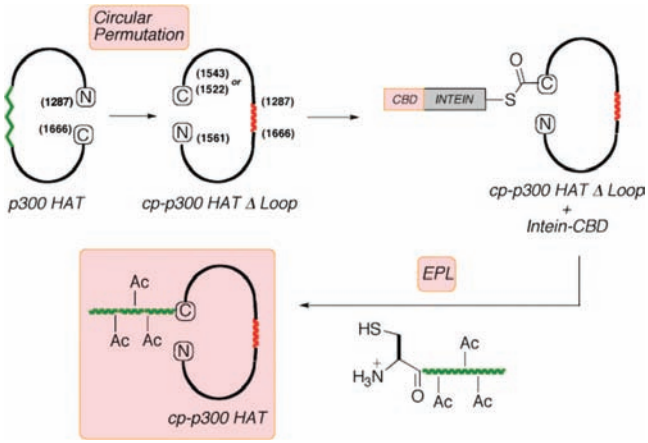

Figure 1. Approach to circularly permuted p300 HAT containing site-specific acetylations via expressed protein ligation (EPL). CBD = chitin binding domain.

We considered an alternative EPL approach to study the autoacetylation loop in p300/CBP HAT that would involve generating a circularly permuted (cp) enzyme. In cp proteins, the natural N- and C-termini are fused, and novel N- and C-termini are created in an alternative location (Figure 1).[6] Examples where cp proteins have been successfully generated include green fluorescent protein, ribonuclease A, and beta-lactamase.[6] If circular permutation were feasible for p300 HAT, designer regulatory loops containing stoichiometric, site-specific acetylations could be installed and ligated to the C-terminus of the cp-p300 HAT construct (Figure 1). An X-ray crystal structure of the p300 HAT domain revealed that the natural N- and C-termini (aas 1287 and 1666) are close in space (∼15 Å apart),[7] which is favorable for circular permutation since in principle only a short linker might be required. Our strategy with respect to cp-p300 HAT involved genetically inserting a 7 aa flexible spacer (TGGGSGG) between the natural N- and C-termini and making residue 1543 the new C-terminus fused to an intein (Figure 1 and Figure S1 in the Supporting Information). Ligation via the intein-generated C-terminal thioester would allow us to explore specific acetylations in an artificial 17 residue regulatory segment prepared with an N-Cys by solid-phase peptide synthesis (Figure S2).

Since expression of wt p300 HAT in *Escherichia coli* is greatly hampered by promiscuous acetyltransferase activity leading to host toxicity, we initially focused on the preparation of Y1467F cp-p300 HAT (cp-p300-F). Tyr1467 appears to serve as a general acid protonating the departing coenzyme A sulfur, and Y1467F shows a 150-fold reduction in catalytic activity relative to wild-type enzyme.[7] Significant soluble expression (>1 mg/L) of cp-p300-F protein was confirmed, so we performed EPL with synthetic peptides (17-mers) to generate cp-p300-F-*n*Ac containing $n = 0$, 3, or 6 acetyl-Lys residues. Prior mass spectrometry and mutagenesis studies had implicated several of these acetylation positions as early sites in autoacetylation reactions and potentially contributory to catalytic regulation.[3] The EPL proceeded smoothly, and these semisynthetic cp-p300-F HAT proteins were obtained in high purity as determined by SDS−PAGE and mass spectrometry (Figure 2 and Figure S3).

To examine whether these cp-p300-F HAT proteins were still properly folded, we analyzed their affinities for acetonyl-CoA, a high-affinity CoA analogue for p300, using a fluorescence binding assay (Figure 2 and Figure S4). In this way, it was shown that each of these semisynthetic proteins [unacetylated (0Ac), triacetylated (3Ac), and hexaacetylated (6Ac) cp-p300-F] retained a high affinity for acetonyl-CoA binding ($K_d = 140-320$ nM), within a factor of 4 of that of standard Y1467F partially loop-deleted p300 HAT ($K_d = 470$ nM). These studies suggest that circular permutation of p300 does not dramatically alter the basic fold of the enzyme, although it may be relevant that the hexaacetylated form shows the K_d closest to that of the standard, loop-deleted enzyme.

Next, we chose to prepare catalytically active semisynthetic cp-p300s. Because of the aforementioned acetyltransferase cytotoxicity, the corresponding cp-HAT-intein fusion was coexpressed with the histone deacetylase sirtuin Hst2.[3] Unfortunately, this failed to generate a significant amount of protein. However, we found that

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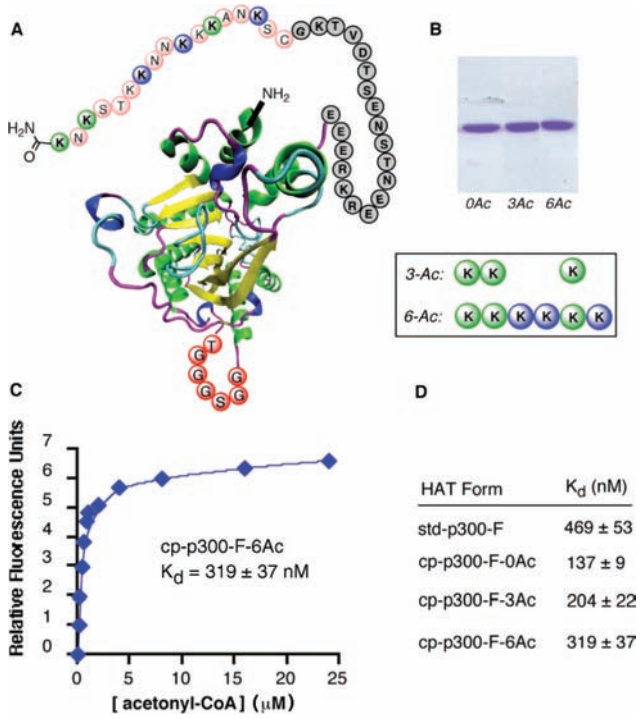

Figure 2. (A) Schematic of cp-p300-F HAT (circularly permuted Y1467F) showing site-specific acetylations (blue, green) on the engineered loop (colored circles). The 7 aa flexible spacer is shown in red, and the gray circles are the C-terminus of the recombinant moiety. (B) SDS−PAGE of cp-p300 HATs. (C) Representative fluorescence plot of acetonyl-CoA binding to cp-p300-F-6Ac. (D) Dissociation constants of acetonyl-CoA with cp-p300-F-*n*Ac.

shortening the recombinant fragment to terminate at residue 1522 gave workable soluble protein yields (∼0.2 mg/L) with the intein system. The corresponding thioester was successfully ligated to 38-mer synthetic peptides comprising residues 1523−1560 to generate the desired semisynthetic 0Ac, 3Ac, and 6Ac cp-p300 forms (Figure 3). While these proteins are likely to be autoacetylated on several Lys residues in the recombinant moiety despite the presence of Hst2,[3] these nonloop acetylation sites appear to be less important for regulation[3] and in any case should be identical among the semisynthetic species, which were all generated with the same parent thioester.

Acetyltransferase assays were performed with catalytically active 0Ac, 3Ac, and 6Ac cp-p300 using a synthetic histone H4 tail peptide substrate under conditions where autoacetylation is minimal, as previously described.[3] These assays revealed that cp-p300-6Ac had an ∼5-fold greater activity compared with the cp-p300-0Ac form, whereas the cp-p300-3Ac species showed intermediate activity. The HAT rate of cp-p300-6Ac proved to be similar to standard, loop-deleted (constitutively active[3a]) p300 HAT under the same assay conditions (Figure 3). The enhanced rate of cp-p300-6Ac vs cp-p300-0Ac is noteworthy since, unlike in prior studies,[3] these cp-p300 proteins have well-defined acetylation modifications on specific sites rather than a more heterogeneous distribution, which is a natural consequence of promiscuous autoacetylation. These findings further suggest that functional aspects of the regulatory loop can be recapitulated in this circularly permuted system, even though the regulatory loop is only covalently anchored at one side in cp-p300 HAT.

In addition to providing new insights into p300 regulation, this study suggests a more general strategy for applying EPL to protein systems in which a flexible segment of interest lies in the middle

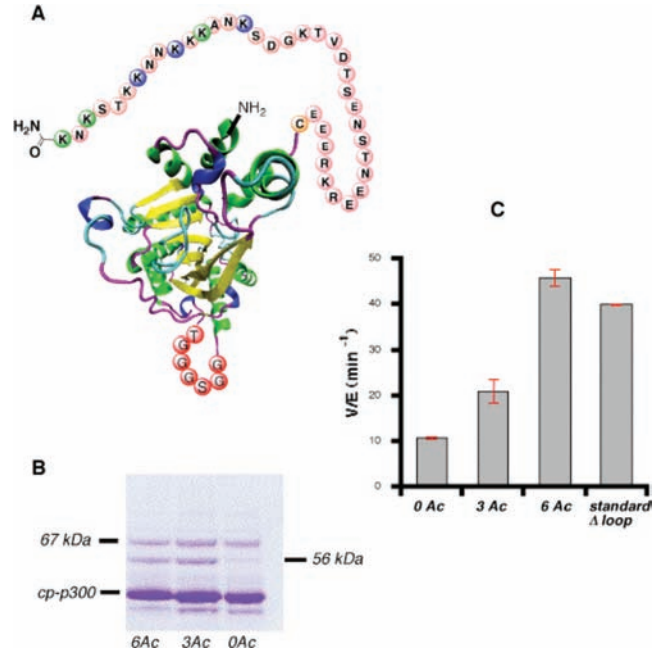

Figure 3. (A) Schematic of catalytically active cp-p300 HAT. Color scheme as in Figure 2; 38-mer peptide used for the loop. (B) SDS−PAGE of cp-p300 HATs; contaminants at 67 and 56 kDa are likely GroEL and DNAK, respectively. (C) HAT activity of cp-p300s and standard, unacetylated p300 HAT containing partial loop deletion.[3a] [H4−15-mer substrate] = 500 μM; [acetyl-CoA] = 20 μM. The error bars reflect duplicate measurements.

of a large recombinant protein sequence. By judicious selection of novel N- and C-termini for a protein, circular permutation coupled with EPL can offer the opportunity to install unnatural amino acids, biophysical probes, or PTMs and their mimics in functionally internal protein regions. Such an integration of methods can thus extend the protein chemistry toolbox to previously difficult-to-approach biochemical problems.

Acknowledgment. We are grateful to R. Cotter and his group for assistance with mass spectrometry and R. Marmorstein and X. Liu for helpful discussions. We are thankful for support from the NIH.

Supporting Information Available: Experimental methods and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA909466D