Paramaecium bursaria Chlorella Virus 1 Encodes a Polyamine Acetyltransferase

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Background: PBCV-1 gene a654l encodes a protein with sequence similarity to GCN5 histone acetyltransferases.

Results: A crystal structure of A654L bound to coenzyme A reveals how A654L acetylates polyamines, not histone lysines.

Conclusion: A654L functions as a polyamine acetyltransferase.

Significance: As the first viral polyamine acetyltransferase, A654L has a possible role in host polyamine catabolism in viral replication.

Paramaecium bursaria chlorella virus 1 (PBCV-1), a large DNA virus that infects green algae, encodes a histone H3 lysine 27-specific methyltransferase that functions in global transcriptional silencing of the host. PBCV-1 has another gene a654l that encodes a protein with sequence similarity to the GCN5 family histone acetyltransferases. In this study, we report a 1.5 Å crystal structure of PBCV-1 A654L in a complex with coenzyme A. The structure reveals a unique feature of A654L that precludes its acetylation of histone peptide substrates. We demonstrate that A654L, hence named viral polyamine acetyltransferase (vPAT), acetylates polyamines such as putrescine, spermidine, cadaverine, and homospermidine present in both PBCV-1 and its host through a reaction dependent upon a conserved glutamate 27. Our study suggests that as the first virally encoded polyamine acetyltransferase, vPAT plays a possible key role in the regulation of polyamine catabolism in the host during viral replication.

Post-translational modifications of DNA-packaging histones provide an attractive mechanism for viral manipulation of the host genome as well as host immune response to viral infection (1, 2). To date, the best studied virus-encoded chromatin-modifying enzyme, vSET (viral SET domain protein), comes from the chlorella virus PBCV-1,2 a DNA virus that consists of an ~330-kb genome and encodes about 400 proteins, among which are a number of other specialized proteins including the smallest functional potassium channel (3, 4); the enzymes involved in the complete biosynthetic pathway for the rare polyamine homospermidine (5, 6); as well as the enzymes for endoplasmic reticulum- and Golgi-independent protein glycosylation (7). Our recent studies have shown that vSET functions to specifically methylate histone H3 at lysine 27 with the functional consequence of silencing the host genome; in heterologous expression systems, vSET suppresses host transcription within 1 h (8, 9). In addition to histone lysine methylation, lysine acetylation also has strong regulatory capability on gene transcription in chromatin. Remarkably, the PBCV-1 genome has a gene, a654l, that encodes a protein with sequence similarity to the GCN5 family of histone acetyltransferases (HATs) that are known for their role in chromatin remodeling and gene transcriptional activation through hyperacetylation of histone lysines (10, 11). To understand the structure and function of A654L, in this study, we determined a 1.5 Å resolution crystal structure of A654L bound to CoA and discovered and performed structure-guided characterization of the acetyl transfer reaction of A654L with an unexpected class of substrates.

EXPERIMENTAL PROCEDURES

Protein Preparation—The a654l gene from the PBCV-1 viral genome was cloned into a pET-15b vector using PCR primers incorporating Ndel and BglII restriction sites. The N-terminal His6-tagged fusion protein was expressed in Escherichia coli (strain BL21 DE3) cells by overnight induction at 18 °C using 0.4 mM isopropyl-1-thio-β-D-galactopyranoside. Cell pellets were washed and lysed using a Microfluidizer in an ice-cold lysis buffer of 100 mM NaCl, 1 mM EDTA, 10 mM DTT, and 0.5% Nonidet P-40. A654L protein was purified by nickle-nitrilotriacetic acid affinity chromatography followed by size-exclusion chromatography on a Superdex 75 column. Overnight cleavage of the His6 tag was followed by flow over a SOURCE Q ion exchange column to a final buffer of 20 mM Tris, pH 8.5, containing 200 mM NaCl.

Site-directed Mutagenesis—A654L point mutants were generated by pfu PCR amplification of wild-type DNA with mutation-containing primers designed by PrimerX. PCR mixtures were digested with Dpn1 and transformed into DH5α E. coli cells. The presence of appropriate mutations was verified by DNA sequencing.

Crystallization—To optimize protein solubility, we followed the protocol of Jancarik et al. (12) and found that A654L yielded
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the most homogeneous light-scattering profile in Tris buffer of pH 8.5 containing 200 mM NaCl and 1 mM DTT, which was then used as our standard buffer. For initial crystallization trials of A654L, we used 96-well sitting-drop Intelli-Plates (Hampton Research) and screened against the Hampton Crystal, Index, and Wizard screening conditions. Mother liquor of 1 μL was mixed with 1 μL of A654L immediately prior to the addition of 5 molar eq acetyl-CoA. Final protein concentration in the drops was 9 mg/mL. Optimization of pH, salt, and cryoprotectant yielded crystals in a buffer of 0.9 M sodium citrate, 100 mM glycine-NaOH, 100 mM imidazole, 150 mM NaCl, and 15% glycerol. Data were collected with the X6A beam-line at the Brookhaven National Laboratory. Diffraction data to 1.5 Å was collected from these crystals. Absent a highly homologous structure, we also collected SAD data using sulfur as the anomalous diffractor. Sulfur-SAD data were collected at a 1.77 Å wavelength for 1,000° of rotation at a final resolution of 1.9 Å. Diffraction data were scaled and integrated using HKL2000.

Data were processed using the Phenix suite of crystallographic tools (13). Anomalous data from the Sulfur-SAD were input into AutoSolve, with instructions to use the SAD data to provide initial phase information and merge the native 1.5 Å data set for refinement. Further refinement was iterated between Coot (14), PyMOL, and Phenix. Ambiguous density in the binding pocket led us to try co-crystallization of A654L with CoA, as well as both CoA and spermine. Crystal conditions were those used above, but the structure was solved using molecular replacement from our original structure. The protein was crystallized in the presence of CoA and spermine, although electron density was visible only for CoA. X-ray data collection and refinement statistics are listed in Table 1.

Mass Spectrometry—To test for the presence of acetylated species using mass spectrometry, we set up 20-μL HAT reactions with the following composition: 100 μM Hepes buffer of pH 7.4, containing 100 μM acetyl-CoA, 20 μM histone peptide substrate, and 50 μM A654L or histone acetyltransferase 1 (HAT1) (gift from A. P. Woster). Reactions were incubated for 30 min at room temperature. Samples were diluted to 50 μL with water and submitted for HPLC-MS analysis with a loading volume of 5 μL. Raw data were exported as a text file, and traces were generated using mMass (15). Anomalous data from the Sulfur-SAD were input —To test for the presence of acetylated spermine ranging from 15 μM to 3 mM. Reaction mixtures of 50 μL contained polyamine, 50 μM acetyl-CoA, and 50 μM Tris buffer of pH 8. Reactions were initiated by adding 5 μL of A654L for a final concentration of 50 nM. Absorbance data were converted to velocity, and Michaelis-Menten parameters were obtained using GraphPad Prism.

Enzyme Kinetics Study Using Fluorescent Assay—To characterize the enzyme kinetics of A654L, we used a fluorescent assay that uses a thiol-reactive dye, 7-diethylamino-3-(4’-maleimidylphenyl)-4-methylcoumarin, to monitor acetylation reaction progression. Following the protocol reported by Treivel et al. (16), we set up 50-μL reactions with buffer, substrate, acetyl-CoA, and the enzyme. Reactions were stopped with 50 μL of ice-cold propanol after 2 min. 100 μL of 25 mM 7-diethylamino-3-(4’-maleimidylphenyl)-4-methylcoumarin was added, allowed to incubate for 10 min in the dark, and then measured for fluorescent absorption/emission at 390/450 nm using the Tecan Safire plate reader (Tecan) with a gain setting of 45. Data for all experiments were collected in triplicate, and background signal, which constitutes the reaction mixture without enzyme, was subtracted. Raw data were imported and processed in GraphPad Prism using the Michaelis-Menten parameters. Conversion from intensity to units of CoA was obtained by the use of a control plate consisting of a serial dilution of CoA. Data for all experiments were limited to the linear absorbance range. Acetyl-CoA for enzymatic reactions was pretreated with acetic anhydride before freezing aliquots of 6 mM. To study the enzyme kinetics of A654L for each of the polyamine substrates including spermine, spermidine, putrescine, cadaverine, and sym-homospermidine (kind gift of P. Woster), we set up triplicate serial dilutions of each polyamine ranging from 15 μM to 3 mM. Reaction mixtures of 50 μL contained polyamine, 50 μM acetyl-CoA, and 50 μM Tris buffer of pH 8. Reactions were initiated by adding 5 μL of A654L for a final concentration of 50 nM. Absorbance data were converted to velocity, and Michaelis-Menten parameters were obtained using GraphPad Prism.

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RESULTS AND DISCUSSION

The cloned full-length PBCV-1 A654L was purified to homogeneity as described under “Experimental Procedures.” Because we predicted acetyl-CoA would be a cofactor, we co-crystallized A654L in the presence of either acetyl-CoA or CoA and obtained crystals of the binary complex to a resolution of 1.5 Å (supplemental Fig. 1). Taking advantage of the 11 sulfur atoms in A654L, we obtained the phases of the A654L–CoA diffraction pattern by collecting a second anomalous dataset and refined the structure with the native dataset (Table 1). The

| TABLE 1 Crystallographic data collection and refinement statistics |
|---------------------------------------------------------------|
| **Sulfur-SAD** | **Native** |
| **Data collection** | | |
| Space group | P31 |
| Cell dimensions | | |
| a, b, c (Å) | 65.4, 65.4, 112.6 |
| a, b, c (Å) | 65.4, 65.4, 112.6 |
| Resolution (Å) | 30.1-1.9 |
| Resolution (Å) | 28.2-1.36 |
| Completeness (%) | 50 (33) |
| Completeness (%) | 98.6 (85.9) |
| Rmerge or Rmerge (%) | 8 (50) |
| Rmerge or Rmerge (%) | 6 (37) |
| I/σI | 64.4 (8.7) |
| I/σI | 32.1 (16) |
| Redundancy | 76.6 (7.7) |
| Redundancy | 5.1 (4.9) |

*Numbers in parentheses refer to the highest resolution shell.

* Rmerge = ∑|Fobs - Fcalc|/∑Fo, where I is the integrated intensity of a given intensity.

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* Rmerge = calculated using 10% random data omitted from the refinement.

* c.m.s., root mean square.
structure of A654L shows the canonical GNAT fold of a six-stranded β-sheet with interspersed helices (Fig. 1). The conserved acetyl-CoA binding features are present including the bulge between β3 and β4 that serves as the Ac-CoA-binding cavity; the short loop of Gly-121-Leu-124 below α6 comprises the conserved GXGX motif where the backbone amides form hydrogen bonds with CoA phosphates. The carbonyl oxygen of the CoA pantethine arm is also hydrogen-bonded to backbone hydrogen bonds with CoA phosphates. The carbonyl oxygen of the CoA pantethine arm is also hydrogen-bonded to backbone amides of residues in β4, and the amide of conserved asparagine, Asn-150, coordinates two CoA carbonyl oxygen atoms.

Although the A654L-CoA structure has conserved CoA binding features, it contains a secondary structural element comprising α5 and flanking residues that is incompatible with a peptide substrate (Fig. 1, red; supplemental Fig. 2). Superposition with the GCN5-H3 peptide complex structure (supplemental Fig. 3) shows that the conserved A654L insert is situated where an incoming substrate would sit. Instead of having an open substrate-binding cleft like the histone-acetylating GCN5, the catalytic site of A654L is completely enclosed within a tunnel running through the interior of the protein, which is visualized by the enclosed surface volume (Fig. 1, green). Furthermore, mapping of the electrostatic potential reveals that the channel is negatively charged (supplemental Fig. 4), suggesting an unbranched and positively charged substrate such as a polyamine. Subjecting the A654L structure to a Dali structure similarity search yielded several structures with unknown functions along with a polyamine acetyltransferase from Bacillus subtilis (Protein Data Bank (PDB): 1TIQ) with a moderate Dali Z-score (17) of 15.1, strengthening the likelihood that A654L might acetylate polyamines.

We investigated the acetyltransferase activity of A654L using a mass spectrometry method. In contrast to the well-characterized human HAT1 (18), which effectively monoacetylates histone H4 peptide, A654L showed no acetyltransferase activity with peptides derived from histone 3 or 4 (Fig. 2a). However, A654L acetylated two common polyamines, spermine and spermidine, in a concentration-dependent manner (Fig. 2b). As PBCV-1 and its host, Chlorella NC64A, contain several polyamines including putrescine, spermidine, cadaverine, and homospermidine (5), we measured the catalytic activity of A654L against each of these substrates as well as the common mammalian polyamine spermine. Using a fluorescence assay that indirectly measures CoASH production, we discovered that A654L acetylates tetra-amines (spermine), triamines (spermidine and sym-homospermidine), as well as diamines (cadaverine and putrescine) (Fig. 2, b and c; also see supplemental Table 1). Although spermine is the preferred substrate, it was not previously detected in either the viral particle or Chlorella NC64A. Of the four polyamines detected in both the host and the virus, A654L prefers cadaverine 6-fold over the next polyamine, homospermidine. Given its acetyltransferase activity against polyamines, we gave A654L the name vPAT.

Structural analysis of the vPAT catalytic site suggests that either Cys-148 or Glu-27 may be important for catalysis that may proceed through one of two plausible mechanisms. Transfer of the acetyl group from acetyl-CoA to a substrate could occur either through an acetyl enzyme intermediate or by direct nucleophilic attack of an incoming polyamine on the acetyl group of the cofactor followed by formation of a tetrahedral intermediate and its conversion to the product of acetylated polyamine. The latter mechanism is assisted by a nearby residue that acts as a general base. Glu-27 is the only acidic residue that is close enough to the substrate at the active site (Fig. 2d).

We generated C148A and E27Q mutants to investigate the effects of the mutation on enzyme kinetics. The C148A mutant had no significant effect on $k_{cat}$, whereas the E27Q mutation resulted in a 26-fold reduction in $k_{cat}$ and 126-fold reduction in $k_{cat}/K_m$. These results indicate that Glu-27 is likely a key residue for catalysis. Plotting log$K_m$ versus pH (supplemental Fig. 5) reveals pH dependence of the enzyme activity associated with two $pK_a$ values of 7.12 and 9.37, likely representing ionizable groups involved in catalysis. The lower $pK_a$ could be accounted for by Glu-27, whereas the higher $pK_a$ might be a base such as Tyr-84, Tyr-79, or Tyr-76 that functions to donate a proton to the tetrahedral intermediate via a network of water molecules. It is worth noting that when one compares the vPAT sequences...
of the 44 chlorella viruses that have been sequenced (supplemental Fig. 6), Tyr-76 is the only one of the three sites that is conserved in all the viruses, whereas Tyr-79 is a Phe in about half of the viral sequences and Tyr-84 is an Asn in five of the viral vPAT proteins. Therefore, we propose the reaction mechanism (Fig. 2), which resembles the described catalytic mechanism for lysine acetyltransferases (19).

Polyamines are abundant organic cations with diverse cellular functions whose cellular concentrations are highly regulated (20–23). As efficient inducers of chromatin compaction (24), polyamines are used by some viruses for packaging DNA into viral particles (25, 26). However, the four polyamines detected in the PBCV-1 virion (putrescine, cadaverine, spermidine, and homospermidine) are unlikely to be important in neutralizing its DNA because the number of polyamine molecules per virion could neutralize only \( \frac{1}{1000} \) of the DNA phosphate residues (5). Presumably, polyamines play an essential role in the PBCV-1 replication cycle because PBCV-1 encodes four functional polyamine biosynthetic enzymes, arginine/ornithine decarboxylase, agmatine iminohydrolase, N-carbamoylpur- trecine amidohydrolase, and homospermidine synthase (HSS) (5, 6, 27). The first three enzymes can convert arginine to putrescine, which is an important intermediate in the synthesis of spermidine, spermine, and homospermidine (supplemental Fig. 7). With the exception of the hss gene, all of the genes, including vpat, are expressed prior to initiation of PBCV-1 DNA synthesis (which occurs 60–90 min after PBCV-1 infection), and thus they are classified as early genes. Therefore, one might expect the polyamine levels to increase during the first 60 min of PBCV-1 infection. However, little change occurs in either the polyamine concentration or its composition during the first 60 min of virus infection (5). By 240 min after infection, the concentration of putrescine increases about 3.5 times, whereas the other polyamines decrease during this time. The net result is that the total cellular polyamine concentration decreases slightly during virus replication, possibly due to the action of vPAT. The fact that PBCV-1 encodes enzymes involved in both polyamine biosynthesis and polyamine catalysis suggests that the virus needs to tightly regulate polyamine concentrations in its host during its replication.

In mammalian cells, polyamine acetylation is the first rate-limiting step in polyamine catabolism, and the human acetyltransferase, spermine/spermidine \( N^1 \)-acetyltransferase, is highly regulated due to the association of polyamine abundance with cellular proliferation (19, 20, 28). Through our newly determined crystal structure of vPAT and characterization of its catalytic activity as a polyamine acetyltransferase, we describe for the first time a virally encoded polyamine acetyltransferase with a catalytic efficiency (\( K_{\text{cat}} = 21 \text{ M}^{-1}\text{s}^{-1} \)) that is similar to that of the human spermine acetyltransferase.
acetyltransferase spermidine/spermine N\(^1\)-acetyltransferase (\(K_m = 5.7 \mu M\) and \(k_{\text{cat}} = 155 \text{ min}^{-1}\)) (10) and more efficient than that of a bacterial spermidine acetyltransferase PaiA (\(K_m = 76 \mu M\) and \(k_{\text{cat}} = 19 \text{ min}^{-1}\)) (19, 32). Therefore, our study suggests a new basic molecular mechanism by which a eukaryotic virus depletes polyamines in the host, presumably for its own advantage for replication. Support for the importance of vPAT in chlorella virus replication is the finding that the vpat gene is present in all 44 of the chlorella viruses that have been sequenced to date (29–31).  

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