Characterization of the Histone Acetyltransferase (HAT) Domain of a Bifunctional Protein with Activable O-GlcNAcase and HAT Activities* ♦

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Histones and transcription factors are regulated by a number of post-translational modifications that in turn regulate the transcriptional activity of genes. These modifications occur in large, multisubunit complexes. We have reported previously that mSin3A can recruit O-GlcNAc transferase (OGT) along with histone deacetylase into such a corepressor complex. This physical association allows OGT to act cooperatively with histone deacetylation in gene repression by catalyzing the O-GlcNAc modification on specific transcription factors to inhibit their activity. For rapid, reversible gene regulation, the enzymes responsible for the converse reactions must be present. Here, we report that O-GlcNAcase, which is responsible for the removal of O-GlcNAc additions on nuclear and cytosolic proteins, possesses intrinsic histone acetyltransferase (HAT) activity in vitro. Free as well as reconstituted nucleosomal histones are substrates of this bifunctional enzyme. This protein, now termed NCOAT (nuclear cytoplasmic O-GlcNAcase and acetyltransferase) has a typical HAT domain that has both active and inactive states. This finding demonstrates that NCOAT may be regulated to reduce the state of glycosylation of transcriptional activators while increasing the acetylation of histones to allow for the concerted activation of eukaryotic gene transcription.

The genomes of eukaryotes are assembled into the highly condensed structure of chromatin. Chromatin is composed of repeating units of nucleosomes that are comprised of DNA coiled around an octameric particle consisting of two molecules of each core histone, H2A, H2B, H3, and H4 (1, 2). The acetylation state of these histone protein tails has for some time been known to be correlated with gene expression, where transcriptionally competent loci are hyperacetylated and silenced loci hypoacetylated (3–5). Histone acetyltransferases (HATs)1 compose a superfamily of enzymes broken into subfamilies based on sequence similarities, active site size, and the presence or absence of other protein domains (6). In each HAT protein there exists a structurally homologous region that contains the active site and includes four universally present motifs, designated A–D, that form the scaffold of the catalytic core (6). Recently published three-dimensional structures of various acetyltransferases (ATs) have allowed for a comprehensive view of the roles played by each motif as well as the roles of many amino acids structurally conserved within (6–8). HATs and histone deacetylases (HDACs) act competitively within large multiprotein complexes that recruit them to their nucleosomal substrates on DNA and give them the ability to contribute to the activation or repression of gene expression, respectively (9).

The covalent addition of the monosaccharide, N-acetylgalactosamine (GlcNAc) to serine or threonine residues of proteins is catalyzed by the enzyme, O-GlcNAc transferase (OGT) that is encoded by a single gene (10). Recently, we reported that the coenzyme, mSin3A, known to recruit HDAC (9, 11), also recruits OGT via its TPR domains to specific genes (12). OGT can thereby contribute along with HDAC to the repression of gene expression through addition of O-GlcNAc modifications on transcriptional activators, inhibiting their activity. Such inhibitory effects have been witnessed on the transactivation domain of Sp1 (13), the C-terminal tail of RNA polymerase II (14) and the TAF1130 recruitment domain of CREB (15) among others. It has also been documented that the repression of genes is associated with the hyperglycosylation of the proteins bound to their promoters (12).

Many genes, including those responsive to variable hormone levels, can be activated or repressed to maintain homeostasis. For the repressed gene state to be reversible, the complex responsible for the repressed state must be exchanged with a complex that allows gene activation (16, 17). As part of the activation process, the action of the enzymes residing in the repression complex would need to be removed, including the inhibitory modification of many transcriptional activators by O-GlcNAc. The enzyme O-GlcNAcase, which is the only enzyme capable of catalyzing the removal of these regulatory O-GlcNAc modifications on proteins in the nucleus and cytosol, must necessarily then play a role in cooperation with HATs in the activation of genes, just as OGT plays its role along with HDAC in repression (12). Of interest in this regard, it has recently been reported that the C terminus of O-GlcNAcase contains a domain with similar composition to eleven different AT active sites, as predicted by SMART computer analysis (18). While it has been argued that the O-GlcNAc may have evolved from an AT (19), this protein could potentially play a dual role in the reversibility of corepression by removing O-GlcNAc modification from activators while also adding acetyl groups to histones, allowing a target gene to be expressed. Here

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1 The abbreviations used are: HAT, histone acetyltransferase; AT, acetyltransferase; HDAC, histone deacetylase; OGT, O-GlcNAc transferase; CBP, CREB-binding protein; CREB, cAMP-responsive element-binding protein; NCOAT, nuclear cytoplasmic O-GlcNAcase and acetyltransferase; bNCOAT, bacterial NCOAT; mNCOAT, mammalian NCOAT; GST, glutathione S-transferase; TPR, tetrastricopeptide; STZ, streptozotocin.
we report that O-GlcNacase does in fact possess acetyltransferase activity in vitro for a synthetic histone substrate tail as well as for free core histones and reconstituted oligonucleosome substrates. However, the HAT activity is regulated and can only be observed when the enzyme is expressed in mammalian cells. The active site for this domain lies in the C terminus of the protein, where it resembles other acetyltransferases both structurally and in catalytic mechanism and has complete functional distinction from the N-terminal O-GlcNacase domain. Because the enzyme is bifunctional with two important enzymatic domains, we have renamed it nuclear cytoplasmic Q-GlcNacase and acetyl transferase (NCOAT).

**EXPERIMENTAL PROCEDURES**

**Plasmids and Recombinant NCOAT**—A pUC118-pTM hybrid expression vector was designed by removing a 2-κb fragment of pTM containing T7 and GST elements and inserting it into the pUC118 MCS. This construct was used for GST peptide expression. A PCR product containing full-length mouse NCOAT was then cloned into the pUC118-pTM hybrid for N-terminal GST fusions or into pcDNA 3.1 (Invitrogen) for GAL4 fusions. Mouse NCOAT was also inserted into a pGEX vector (Amersham) as described for expression of domains of other recombinant DNA manipulations were performed by standard procedures (20).

**Splice Variant Identification and Cloning**—NCOATs were cloned by reverse transcription-PCR from Goto Kakazaki or Sprague-Dawley rat cDNA to create chimeric NCOAT molecules with the missing exons. These constructs were cloned into the pUC-pTM vector.

**Truncations and Site-directed Mutagenesis**—Full-length pcDNA-NCOAT was digested with EcoRI to excise a fragment that consisted of nucleotides 1–1747 of NCOAT. This digested plasmid was religated onto a T7 promoter pBluescript expression vector for a GAL4 fusion to the NCOAT acetyltransferase domain (NCOAT nucleotides 1748–2771). The AT domain fusion was also placed into the pGEX vector for bacterial expression. For the expression of NCOAT with a deletion of its AT domain, the pcDNA-GAL4-NCOAT vector was transformed into DM-1 particles in both 0.7% agarose gel and 5% polyacrylamide gel as described elsewhere (22). Shifted bands were excised from these gels to ensure only nucleosomal histones were used. These gel fragments were then used in nucleosomal HAT assays. The resulting vector, missing NCOAT nucleotides 2086–2771 was then used for expression of NCOAT acetyltransferase domain (NCOAT nucleotides 1748–2771).

**Characterization of the HAT Domain of NCOAT**—Histone octamers were assembled as described by Dyer et al. (21) using unfraccionated type IIIA calf thymus whole histones (Sigma). Briefly, these histones were dissolved in 4 ml of unfolding buffer (6 μm guanidine HCl) at a concentration of 2 mg/ml. Solubilized histones were then dialyzed and refolded in a 1 M sodium acetate refolding buffer (2 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM dithiothreitol) at 4 °C for 6 h. Precipitated protein was removed, and the histone mixture was concentrated in a YM-10 protein concentrator (Amicon) down to 1 ml. Octamer reconstitution was confirmed by non-denaturing gel electrophoresis. Nucleosomal DNA was prepared using 188-hp HeLa cell α-satellite DNA as described by Tenzer et al. (22). NCOAT cDNA was inserted into pGEX vector for bacterial expression. Bacterial protein expression was achieved by transforming a 500-ml culture of BL-21 (Escherichia coli) induced with 1 mM isopropyl-1-thio-D-galactopyranoside (Amersham Biosciences). Precleared lysates were then incubated with an N-19 GAL4 DNA binding domain antibody (Santa Cruz Biotechnology) for 1 h at 4 °C and then with a 100-μl mixture of 50:50 head bead mixture for an additional 2 h. Immunoprecipitates were collected by centrifugation and washed. An aliquot of each purified enzyme was quantitated by Bio-Rad protein assay to ensure equivalent amounts of protein were used in each assay and were comparable in concentration to the positive controls. Bacterial O-GlcNacase proteins were also run on SDS-PAGE gels with an α-GST according to standard protocols. Resin-bound incubations with mammalian whole cell lysates were carried out for 1 h at 4 °C and then washed.

**Oligonucleosome Reconstitution**—Histone octamers were assembled as described by Dyer et al. (21) using DIG-labeled in vitro transcribed bacterial RNA as a hybrid for N-terminal GST fusions or into pcDNA 3.1 ( Invitrogen) for GAL4 fusions. Mouse NCOAT was also inserted into a pGEX vector (Amersham) as described for expression of domains of other recombinant DNA manipulations were performed by standard procedures (20).

**Oligonucleosome-Assay**—Filter binding assays were performed as described elsewhere (23, 24). 60 μl of purified enzyme were added to 20 μg of synthetic histone H4 peptide (Upstate Biotechnology), 100 μl of 5 mg/ml bovine serum albumin, 29 μl of 10X HAT buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium butyrate), and 2 μl of [14C]Acetyl-CoA (4.7 mCi/mmol). Reactions were carried out for 1 h at room temperature and then spotted onto 0.45-μm nitrocellulose membranes (Whatman), dried, and unincorporated [3H]removed with three 15-min washes using 50 mM Na2CO3, pH 9.2. Dried filters were then suspended in 100 μl of 0.1 M acetic acid, washed three times with 0.1 M acetic acid, dried, and then solubilized in 100 μl of 0.1 M acetic acid. Radioactivity was determined using a beta-gamma counter and normalized to the positive controls. Bacterial protein expression was confirmed by mobility shift assay using 15 μg of nucleosomes particles in both 0.7% agarose gel and 5% polyacrylamide gel as described elsewhere (22, 23). Shifted bands were excised from these gels to ensure only nucleosomal histones were used. These gel fragments were then used in nucleosomal HAT assays. The resulting vector, missing NCOAT nucleotides 2086–2771 was then used for expression of NCOAT acetyltransferase domain (NCOAT nucleotides 1748–2771).

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**RESULTS**

O-GlcNAcase Possesses Intrinsic Histone Acetyltransferase Activity in Vitro—To determine whether O-GlcNAcase possessed acetyltransferase activity, recombinant GST-tagged O-GlcNAcase was expressed in both mammalian BSC-40 cells and E. coli BL-21 cells and affinity-purified. The purity of the protein samples were determined by Coomassie-plus staining (Fig. 1A). GST-O-GlcNAcase was visible as a dominant band at 130 kDa. There were other slightly detectable products copurified, most of which were determined by Western blot to be truncated GST-O-GlcNAcase products. Eluted protein was incubated with a synthetic histone H4 tail and [3H]acetyl-CoA for a filter binding assay or with core histones and [14C]acetyl-CoA for resolution on 15% SDS-PAGE and detection by autoradiography. These results were directly compared with those for the global coactivators CBP or p300. Assays were performed in triplicate. The asterisk indicates preincubation with mammalian whole cell lysate. These results were directly compared with those for the global coactivators CBP or p300. The HAT activity, as measured by spectrophotometric absorbance at 405 nm, represent the amount of para-nitrophenol cleavage when 1 mmol of para-nitrophenol-GlcNAc is used in the presence of 1 μmol of enzyme.

**Fig. 1.** Mammalian-expressed O-GlcNAcase has intrinsic HAT activity. A, Coomassie staining confirming expression and purity of O-GlcNAcase samples used in subsequent assays. Lane 2 shows that the same slightly detectable copurified products also coprecipitate with a catalytically dead mutant enzyme. B, filter binding assays were performed on O-GlcNAcase expressed in either mammalian (mOGN) or bacterial (bOGN) cells with a synthetic histone H4 tail and [3H]acetyl-CoA and compared with CBP. Assays were performed in triplicate. The asterisk indicates preincubation with mammalian whole cell lysate. C, autoradiograph using core histones and [14C]acetyl-CoA. Molecular mass markers in kDa are to the left of the gel. p300 was used as a positive control. Reactions in the absence of enzyme were used as a negative control (NC). In these reactions, O-GlcNAcase and bovine serum albumin showed levels of labeling similar to background (data not shown). D, O-GlcNAcase activity associated with mammalian O-GlcNAcase (mOGN), bacterial O-GlcNAcase (bOGN), and bacterial O-GlcNAcase treated with mammalian whole cell lysate (indicated by the asterisk). Units on the y axis, as measured by spectrophotometric absorbance at 405 nm, represent the amount of para-nitrophenol cleavage when 1 mmol of para-nitrophenol-GlcNAc is used in the presence of 1 μmol of enzyme.
greater activity may perhaps be due to the ability of NCOAT to acetylate a greater number of sites on the histones or can be a function of the fact that only the HAT domain of p300 was used in these assays, whereas the full-length p300 may possess greater activity. No HAT activity was measured when using NCOAT expressed in bacteria. The results in either assay, when NCOAT was expressed in bacteria, were similar to those seen when histone substrate was incubated with either GST or in the absence of any enzyme, a finding that may be indicative of the total lack of activity of the bacterially expressed NCOAT is comparable nucleosomal histones. While we cannot conclude at this time how promiscuous NCOAT is as an acetyltransferase, it is likely to have specific targets in the context of nucleosomes, as do all other previously characterized HATs. Further selectivity is likely provided for by the complex(es) in which NCOAT is bound at the promoter.

**Characterization of the HAT Domain of NCOAT**

**NCOAT Can Acetylate Oligonucleosomal Substrates**—HAT proteins are classically described as being either type A, those that acetylate histones within chromatin, or type B, those that acetylate free histones within the cytoplasm. For NCOAT to act as theorized on gene transcription directly, it would need to act as a type A HAT. As described above, NCOAT, like other type B HATs, can acetylate free core histones. We next tested its ability to act upon nucleosomal histones. For this, histones were reconstituted into oligonucleosome arrays for use as substrates in the HAT assays. Successful formation of these substrates was determined by gel shift assay (Fig. 2A). The shifted oligonucleosomes were purified from these gels and incubated with p300 or NCOAT in the HAT assay and run on 15% SDS-PAGE. NCOAT acetylated all four core histones even when bound by double-stranded DNA in the context of reconstituted nucleosomes (Fig. 2B). Again, mNCOAT and bNCOAT incubated with mammalian whole cell extract were active, while bNCOAT not preincubated with extract was not active. These HAT assays were also repeated using 10 μM unlabeled acetyl-CoA. Western blots of the subsequent gels were then probed with antibodies for specific acetylated lysine residues to determine some of the modified residues. Lysine 14 on histone 3 was efficiently acetylated by NCOAT in these assays (Fig. 2C). Lysine 8 on histone 4 was also acetylated by NCOAT under the same conditions (Fig. 2D). In both cases, mNCOAT was active, while bNCOAT required exposure to mammalian whole cell extract. When these reactions were probed with an antibody to lysine 16 on histone 4, we were unable to detect any acetylation of this particular residue by NCOAT (data not shown). While other modified lysine residues have not been mapped, these data suggest that NCOAT is a type A HAT capable of modifying the histones within chromatin. While we cannot conclude at this time how promiscuous NCOAT is as an acetyltransferase, it is likely to have specific targets in the context of nucleosomes, as do all other previously characterized HATs. Further selectivity is likely provided for by the complex(es) in which NCOAT is bound at the promoter.

**The Active Site for Acetyltransferase Activity Lies in the C Terminus of the Enzyme**—To determine the region of NCOAT containing HAT activity, we constructed an O-GlcNAcase with a deletion of the C-terminal third of the enzyme, thus removing the region identified by SMART analysis to have AT-like composition. This enzyme was expressed as a GAL4 fusion protein and placed in both HAT assays. When the C-terminal 221
amino acids were removed from NCOAT the enzyme no longer exhibited HAT activity in either assay (Fig. 3), suggesting that this portion of the enzyme either comprises the active site or contains a feature requisite for the ability of NCOAT to function as an acetyltransferase. To address this question a GAL4 fusion protein was made using only the C-terminal third of NCOAT to determine whether, like many other AT domains, the AT domain of NCOAT alone can confer HAT activity to an otherwise non-HAT peptide, the GAL4 DNA binding domain. As shown in Fig. 3, the C-terminal 334 amino acids of NCOAT were sufficient to introduce HAT activity to the GAL4-DNA binding domain when the peptide was expressed in mammalian cells. GAL4 alone had no significant activity in either assay. Furthermore, this domain of NCOAT alone, when expressed as a GAL4 fusion, exhibited a full retention of activity when compared with that of the full-length NCOAT or to the positive control for this assay, p300. The same 334-amino acid peptide, when expressed in bacteria as a GST fusion protein failed to show HAT activity, just as seen when the full-length enzyme was expressed in bacteria. However, once again full enzymatic activity was observed after incubation of this peptide with a mammalian whole cell lysate (Fig. 3A, lane 8). These results indicate that the AT domain of NCOAT lies in the C-terminal third of the enzyme and that this domain can act autonomously when expressed in a mammalian cell system.

Identification of Two Splice Variants Containing HAT Activity but Not Hexosaminidase Activity—Over the course of our study, the rat NCOAT cDNA was cloned by reverse transcription-PCR from RNA derived from the brains of Sprague-Dawley and Goto-Kakazaki rats. We noted two subtle size variants. One cDNA corresponded in size to the full-length mouse NCOAT, while the two variants were smaller. These reverse transcription-PCR products were sequenced. Whereas the full-length rat NCOAT (GenBank™ accession number NM_131904) was nearly identical to its mouse counterpart (GenBank™ accession number AF132214), the shorter variants had missing sequences corresponding to exon 8 in the Goto Kakazaki rat and exons 8 and 9 in the Sprague-Dawley rat (Fig. 3C). Because the missing regions in these variants correspond exactly to exon boundaries, we believe they may result from alternative splicing of the gene. These variants were expressed as proteins of 90 kDa for the Goto Kakazaki variant due to the deletion of amino acids 250–345 and 84 kDa for the Sprague-Dawley variant due to the deletion of amino acids 250–398. Each of these variants was tested for hexosaminidase activity and was found to be catalytically inactive (data not shown). Both variant enzymes, however, were able to completely catalyze the addition of acetyl groups to the histones when compared with the full-length enzyme (Fig. 3A). This finding lends to the validity that the HAT active site resides in the C terminus of the enzyme, since the resultant proteins are able to retain full activity. These findings also raise the possibility that organisms may utilize alternative splicing to regulate the levels of the enzymatic activities of this bifunctional enzyme within a particular cell type or cell compartment. Interestingly, a splice variant resembling our C-terminal truncation missing the AT domain, has been discovered as well and retains considerable activity in hyaluronidase assays (31), further indicating the significance of splicing to favor one cellular activity over the other.

In addition to splicing, O-GlcNAcase has also been shown recently to be a competent substrate for caspase 3 cleavage, and that the processed product retains O-GlcNAcase activity (27). It has been reasoned that cleavage of the protein is an
Either CBP (streptozotocin) is unaffected by treatment with streptozotocin. We and others have previously shown that the hexosaminidase activity of the enzyme can be inhibited by STZ, which is sufficient to abolish hexosaminidase (25, 32), had a negligible effect on the acetyltransferase activity of NCOAT (Fig. 3A). The slight decrease in activity observed by scintillation counting appears solely the consequence of the incubation conditions or to a trace amount of nonspecific involvement, since CBP and p300 also show a marginal loss of HAT activity when pretreated with STZ. The inhibitory effects of STZ on NCOAT were concluded therefore to be specific only for the hexosaminidase activity of the enzyme and not the result of a nonspecific, global effect such as oxidative damage to the enzyme. It seems likely that the GlcNAc analog streptozotocin is acting directly on the O-GlcNAcase active site and that the C-terminal acetyltransferase domain is, in the active enzyme, completely distinct in function from the N-terminal O-GlcNAcase domain. Such functional independence is consistent with our observations using splice variants, caspase, or the HAT domain alone and also support splice variation and caspase cleavage as plausible methods of cellular control of these activities.

Identification of Residues Essential for HAT Activity—The C-terminal acetyltransferase domain of NCOAT was further characterized. Eight of the ten acetyltransferases that SMART computer analysis identified as having similar domain composition to NCOAT have been crystallized, and a detailed map of their active site secondary structures is known. A comparison of the active sites of these enzymes shows that, while containing only low levels of sequence identity (15%) and similarity (50%), they share a highly conserved structural fold (8). Such low levels of sequence homology make identification of new acetyltransferases by sequence alone difficult. A more defining feature of acetyltransferases is their overall active site core architecture. This core structure includes four universally present motifs, designated motifs C, D, A, and B (Fig. 5), whose lengths and sequences are variable. The intervening loops are also variable, nevertheless, the overall structure of these ATs is superimposable in each case (6–8, 35, 36). All ATs contain the same arrangement of α-helices and β-sheets as indicated in Fig. 4A. Using secondary structure prediction programs (19, 28, 29), the C terminus of NCOATs from five different species can be predicted to contain this exact motif pattern (Fig. 5), providing further evidence that the AT active site of NCOAT lies in the C-terminal third of the enzyme as indicated by measurements of activity. Within this stretch, there are seven invariant residues among the secondary structure alignments. Three of these are glycines, there is one invariant leucine, two aspartic acids (at positions 853 and 884 for mouse NCOAT), and one tyrosine residue (at position 891), which is exchanged with an adjacent phenylalanine in the Caenorhabditis elegans NCOAT. The amino acid conservation at these sites suggests their functional importance to the activity of the enzyme.

Examination of the residues identified to be necessary for catalysis in the majority of these eight crystallized acetyltransferases reveals that a carboxylate-containing residue lying on the back end of the β-sheet C-terminal to helix A is the critical residue responsible for the initial base step of the enzymatic reaction. In this step, a proton is abstracted from the substrate lysine ε-amino group in the enzyme-lysine-coenzyme A ternary complex (37). Human PCAF and yeast GCN5 are exceptions in this case, with these enzymes having their critical base carboxylate in the middle of the β-sheet N-terminal to helix A (38, 39). Interestingly, it has been reported that, despite this difference...
in sequence position, these critical base residues superimpose identically in the three-dimensional structures of each of these enzymes (35). After the deprotonated lysine directly attacks the acetyl group of acetyl-CoA, a general acid is needed to donate a proton to the CoA sulfur atom, to facilitate the departure of the thiolate leaving group. In all but two cases (8, 40) the catalytic acid is a tyrosine situated near the middle of helix B (8, 39).

Since the invariant aspartic acids and the tyrosine in these enzymes lie at corresponding positions in the predicted secondary structures of NCOATs (Fig. 5), we wished to determine whether these residues indeed were necessary for catalysis, as they are in their crystallized counterparts. Each aspartic acid was independently mutated to an asparagine, and the tyrosine to a phenylalanine, so that the mutant proteins could be tested for acetyltransferase activity. In the filter binding assay, the counts observed for each mutant protein matched that of the background (Fig. 6A). These mutant enzymes displayed no change on hexosaminidase activity (Fig. 6B), demonstrating again the independence of the two domains and suggesting that the lack of HAT activity observed by the mutation of these residues is not the result of a gross alteration of total protein conformation. Each mutant enzyme was also unable to label any of the core histones as visualized by autoradiograph (Fig. 6C). These results demonstrate the dependence of NCOAT on these residues for its ability to catalyze acetyltransfer. Because their respective positions in the predicted secondary structure of NCOATs are comparable with the essential catalytic acid and base in other AT enzymes, it is reasonable to expect that either Asp\textsuperscript{853} or Asp\textsuperscript{884} for mouse NCOAT is the catalytic acid in the reaction and the tyrosine at position 891 is the general base. Currently, however, in the absence of crystal structure, their exact roles cannot yet to be certified.

**DISCUSSION**

Several transcriptional activating cofactors have been recently identified that contain a domain conferring HAT activity in vitro. HAT activity is required to open the chromatin structure as part of the process of gene activation. Recently, we added O-GlcNAc modification of the transcription apparatus by OGT as part of the repression process (12), therefore making the removal of these sugars also necessary for transcriptional activation to occur. Here, we demonstrate that O-GlcNAcase, the only enzyme encoded in the genome that can remove these sugars, is bifunctional; it contains a domain with intrinsic HAT activity. Because the protein has these two enzymatic activities, we have renamed it NCOAT.

Saccharomyces cerevisiae cells have been shown to use HATs and HDACs to regulate gene transcription (41). In these single cell organisms, however, the post-translation modification of proteins with O-GlcNAc has not been observed. Nevertheless, yeast cells use a combination of proteins, including Ssn6-Tup1, in the repression of several genes (42, 43). Interestingly, Ssn6, while having no known enzymatic function, has TPR domains very similar to OGT. More recently, it has been shown that Ssn6, through its TPR domains, associates with corepressors and HDACs (44), further suggesting a similarity of OGT to Ssn6. We speculate that an Ssn6-like TPR protein fused with a glycosyltransferase domain to form OGT during evolution. This fusion may have been necessitated by the need for more stringent repression of gene expression when organisms became...
multicellular with differentiated cell functions. The enzymatic function of OGT would provide an additional covalent modification needed to cooperate with HDAC in corepression complexes. To activate genes repressed by OGT and HDACs, both the enzymatic removal of the sugars and addition of acetyl groups would therefore be required. That both functions reside in one protein, NCOAT, lends further credence to the importance of both modifications in metazoan gene expression.

We further characterized the HAT domain of NCOAT. It has the ability to acetylate all four core histones when either free or bound by DNA in the context of oligonucleosome arrays. Moreover, it does so with a specific activity rivaling that of the global transcriptional coactivators, p300 and CBP. The capacity of this dual function enzyme to catalyze its two activities, not surprisingly, appears to involve tight control, as displayed by the discrepancy between the levels of intrinsic activities of the same enzyme expressed in a mammalian versus bacterial system. The recovery of optimal activity by the bacterially expressed enzyme when incubated with a mammalian whole cell lysate is suggestive of a currently uncharacterized post-translational modification(s) that correlate with the activation of the enzymatic domains so that they function proficiently. We have also discovered two naturally occurring splice variants of NCOAT, which lack hexosaminidase activity but retain HAT activity. The bacterially expressed C-terminal active site is fully functional acetyltransferase. The bacterially expressed C-terminal third is able to fully recover activity when incubated with a mammalian whole cell lysate, indicating that at least one of the proposed activating post-translational modifications lies within this region. While we have not yet determined the nature of these covalent modifications, this apparent dependence on modification emphasizes the tight control exerted on NCOAT by upstream signaling pathways.

Within the C-terminal active site we have identified three residues that are critical for efficient acetyl transfer. For mouse NCOAT these are Asp^853, Asp^884, and Tyr^891. The acetyltransferases found by SMART analysis to resemble NCOAT are known to utilize one of two distinct mechanisms. The principle mechanism involves an ordered Bi-Bi reaction in which an active site carboxylate base deprotonates the substrate lysine e-amino group, then, in a single step, the acetyl group is transferred from the reactive thioester group of acetyl-CoA to the uncharged substrate lysine. An active site tyrosine (with exception) then donates a proton to facilitate CoASH departure (38, 45, 46). The other mechanism involves the acetyl transfer from CoA to an enzyme nucleophile, creating a covalent intermediate, and then to the substrate (40, 47). We were unable to detect evidence for such a self-acetylated intermediate. A mutation to the only free cysteine in the active site, the most likely site to accept an acetyl group, does not impair HAT activity (data not shown). We therefore propose that NCOAT undergoes the single step reaction described for those members of the Gcn5, HAT1, and GNAT family of acetyltransferases. This mechanism, and the placement of conserved residues in positions corresponding to those shown necessary for catalysis in these enzymes, suggest that the tyrosine at position 891 acts as the general acid involved in NCOAT acetyltransfer. Likewise, the aspartic acids at position 853 or 884 are promising candidates as the necessary catalytic base.

Given the mechanism, and the fact that NCOAT can acetylate all four core histones, like p300 and CBP, other substrates for NCOAT acetylation potentially exist as they do for these other enzymes. Much like the diversity of O-GlcNAcylated proteins, which exist both in the nucleus and cytoplasm (48), NCOAT may be critical for an increasing number of cellular events requiring acetyl modification. The diverse nuclear as well as cytoplasmic activities that are modulated by acetylation make this AT domain perhaps as important as the O-GlcNAc-
nase domain, justifying the change of the name of this bifunctional protein to NCOAT.

In summary, NCOAT contains versatile enzymes. Its O-GlcNAcase can activate proteasomes (49), but another compelling idea about the function of NCOAT is in the reversibility of gene repression. The recent finding that OGT is specifically repressed in vitro, we have come closer to this goal by showing that NCOAT specifically modifies some important lysine residues in recombinational protein to NCOAT.

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