Direct Binding of INHAT to H3 Tails Disrupted by Modifications*  

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The N-terminal tails of histones are central to the regulation of chromatin structure. They form a binding platform for multiple protein complexes, which in turn regulate DNA processes such as transcription. Using peptide mass fingerprinting we identified INHAT (inhibitor of acetyltransferases) as a specific histone H3 N-terminal tail-binding complex. INHAT comprises two essential subunits, SET and pp32. We demonstrate that both SET and pp32 bind directly to the N terminus of H3. The binding is differentially affected by various modifications within the H3 N terminus. In particular, single phosphorylations within the H3 tail abrogate binding of INHAT, as does the simultaneous acetylation of multiple lysine residues. The histone modifications that affect INHAT binding are therefore compatible with its known role in transcriptional repression. We suggest that the charge of the histone tail is a major determinant in allowing INHAT to bind chromatin and coordinate the activity of multiple histone acetyltransferases.

The packaging of a large amount of DNA into a small nuclear volume is a problem that eukaryotic cells must overcome. The packaging is achieved mainly via the highly ordered folding and compaction of the DNA into chromatin structures. The basic unit of chromatin is the nucleosome, which comprises ~1.75 turns of DNA wrapped around a core histone octamer (1). The N-terminal tails of histones protrude from their originating nucleosome and may contact adjacent nucleosomes as well as chromatin-associated proteins. These interactions are responsible, at least in part, for regulating chromatin structure (2). Histone N-terminal tails are subject to numerous post-translational modifications including acetylation, methylation, and phosphorylation (3–5). These modifications have the potential to affect inter-nucleosomal contacts by stabilizing or destabilizing the interactions involving histone N termini. Furthermore, they can provide binding sites for particular chromatin-regulating complexes or prevent such complexes from binding the histone tails (6). For example, the NuRD chromatin remodeling repressor complex binds to unmodified histone H3 tails (7, 8). This binding is prevented by methylation of lysine 4 (Lys-4) in H3, a mark associated with active genes (9). Indeed, the methylation of Lys-4 forms part of the binding determinant for the ISWI (imitation switch) remodeling complex, which can be involved in activating gene transcription (10). Thus methylation of Lys-4 at active genes serves at least two purposes: (i) it prevents the NuRD remodeling repressor complex from binding, and (ii) it promotes binding of the ISWI remodeling complex.

Although the binding of certain chromatin-associated complexes to histones and nucleosomes has been relatively well characterized, there are many complexes for which binding is poorly understood. For instance, components of the INHAT1 complex (inhibitor of acetyltransferases) are known to associate with core histones, although the molecular determinants have not been determined (11, 12). In its simplest form INHAT is composed of two subunits, the myeloid leukemia-associated oncoprotein SET and the tumor suppressor protein pp32. The INHAT complex specifically inhibits histone acetylation and has been linked to transcriptional repression in vivo (11). In addition, SET and pp32 are inhibitors of protein phosphatase 2A, an enzyme linked to the dephosphorylation of histones (13, 14). Thus, INHAT may possess the ability to regulate chromatin structure via at least two mechanisms: through the inhibition of histone acetylation and via the regulation of histone phosphorylation.

We have screened for proteins capable of binding variously modified histone H3 peptides and have identified these using a mass spectrometry approach. Here we report that INHAT binds to the N terminus of histone H3. Both SET and pp32 proteins bind directly to the H3 tail. We have determined the effect of different histone tail modifications on this binding and suggest that the main binding determinants are through electrostatic interaction.

MATERIALS AND METHODS

Peptides—Histone H3 peptides were made by G. Bloomberg (Bristol University, United Kingdom). The C terminus of all peptides contained a cysteine residue. Peptides were immobilized onto SulfoLink gel (Pierce) via the C-terminal cysteine at a concentration of 1 mg/ml.

Affinity Purification from HeLa Nuclear Extract—HeLa nuclear extract (Computer Cell Culture Center, Mons, Belgium) was diluted in IPH-E buffer (50 mM Tris, pH 8, 325 mM NaCl, 0.5% Nonidet P-40 (v/v)) to a final protein concentration of 4 mg/ml and precleared. Affinity purifications were done with 20 μl of Sepharose-linked peptide and 150 μl of diluted HeLa nuclear extract. The purifications were incubated for 90 min on a wheel at 4 °C and washed three times in IPH-E before resolution on a 15% SDS-polyacrylamide gel. Bound proteins were visualized by silver staining as described previously (21).

In-gel Digestion and Peptide Mass Fingerprinting—Proteins specifically bound to the unmethylated H3(1–16) peptide were excised from a 15% Coomassie-stained gel. The proteins were carbamidomethylated and cleaved in situ using sequencing grade trypsin (Roche Applied Science) as described previously (22). The digest supernatant (0.5 μl) was applied on a fast evaporation nitrocellulose-cyano-4-hydroxycinnamic acid layer (23) and analyzed by MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry using a
Bruker Reflex mass spectrometer (Bruker Daltonics, Bremen, Germany) in the reflector mode equipped with pulsed ion extraction and a nitrogen laser (337 nm). Spectra were calibrated internally using tryptic autolysis peaks. Data base searches were performed using Mascot (Matrix Science Ltd.) or ProFound (Proteometrics LLC). Mass tolerance was set at ±50 ppm, and we allowed for one missed cleavage.

**Western Blots, Antibodies, and Immunoprecipitations**—Bound proteins were resolved on 15% SDS-polyacrylamide gels, blotted to nitrocellulose, and blocked overnight (4% nonfat milk, 0.5% Tween 20, v/v). Blots were probed with the following antibodies for 1 h at room temperature: anti-SET and anti-pp32 (both purchased from Santa Cruz Biotechnology). Blots were washed in blocking buffer, incubated with horseradish peroxidase-linked secondary antibodies (Abcam), and visualized with ECL (Amersham Biosciences).

**Production of Recombinant Proteins**—The pET30-pp32 and pET26d-SET plasmids were kindly provided by Prof. Judy Lieberman (24). These were transformed into BLR bacteria, and the His-tagged proteins were induced and purified using standard conditions. The purified proteins were either left attached to the Ni₂⁺ affinity resin or eluted from the resin and dialyzed into binding buffer.

**Pull-down Binding Assays**—Histone H3 peptide binding assays were done with 20 μl of Sepharose-linked peptide and ~1 μg of His-tagged SET or pp32 protein. Histone binding assays were performed with 1 μg of His-SET or His-pp32 linked to agarose bead and ~1 μg of the relevant free histone. All assays were incubated for 90 min on a wheel at 4°C and washed three times in IPH-E before resolution on a 15% SDS-polyacrylamide gel. Bound proteins were visualized by Coomassie staining.

**RESULTS AND DISCUSSION**

**Unmodified Histone H3 N Terminus Binds the INHAT Complex**—To identify protein complexes capable of binding specifically to histone N-terminal tails, we applied HeLa nuclear extract to affinity columns displaying either H3 unmodified tails (amino acids 1-16) or the same tails but phosphorylated at threonine 3 (Thr-3). After binding, the columns were washed extensively to remove unbound material. Bound proteins were eluted from the columns, resolved by SDS-PAGE, and visualized by silver staining (Fig. 1A). Two bands appeared to bind specifically to the unmodified H3 tails and not to the phosphorylated tail (Fig. 1A, arrows). The bands were excised and subjected to peptide fingerprinting. This approach identified the two proteins as the INHAT complex subunits SET and pp32 (Fig. 1B). To confirm these findings we used a Western blotting approach with antibodies against SET and pp32. We find that these proteins specifically bind the unmodified H3 tails but not the Thr-3-phosphorylated tails (Fig. 1C). These results suggest that INHAT is capable of binding to histone H3 tails and that the binding can be regulated by post-translational modification of the H3 tail. The N-terminal region of H3 also binds the NuRD remodeling complex (7, 8). However, we performed our H3 peptide binding studies at 325 mM salt, a concentration that prevents NuRD from binding (7). Thus, the binding of SET and pp32 is more resistant to high salt than NuRD binding, suggesting that SET/pp32 bind with higher affinity.

**Both SET and pp32 Bind Directly to the H3 N Terminus**—We next addressed which INHAT subunit(s) has H3 binding activity and whether this is a direct interaction. To this end we expressed both SET and pp32 in *Escherichia coli* and purified them to near homogeneity. We found that both subunits are...
set to H3 tails. Bacterially expressed and purified SET protein (A), pp32 protein (B), or HP1β protein (C) was tested for binding to columns as described for Fig. 1. In C, an additional column containing H3-(1–16) trimethylated at Lys-9 (K9me) was used. Coomassie Blue-stained gels are shown. T3P, Thr-3-phosphorylated; un, unmodified.

Fig. 3A shows that when recombinantly expressed SET and pp32 are used as affinity columns they efficiently bind H3 purified from mammalian cells (H3) and H3 expressed in and purified from E. coli (rH3), the latter being devoid of post-translational modifications. These results demonstrate that both SET and pp32 directly bind H3. Furthermore, both INHAT subunits are capable of binding to nucleosomes (Fig. 3A, bottom panel). In contrast, SET and pp32 failed to bind histone H1 (data not shown).

The binding specificity of INHAT appears to come from several determinants within the histone tail. Deletion analysis indicates that residues 1–30 allow binding of SET and pp32, but residues 29–43 do not (Fig. 3B). The difference cannot be due to a simple charge distribution because both regions have a similar charge density. Dissection of residues within 1–30 indicates that amino acids 1–16 and 10–25 possess binding determinants for both SET and pp32.

Modifications within the H3 Tail Affect INHAT Binding—As SET and pp32 bind differentially to unmodified and modified H3 tails, we next determined the effect of different H3 modifications on the binding of the two INHAT subunits. Since INHAT inhibits histone acetyltransferases (11), we first considered whether acetylation of histone tails affects INHAT binding. INHAT has been shown previously to inhibit the activity of the CBP/p300 acetyltransferase, and it is known that CBP/p300 preferentially acetylates Lys-18 and Lys-23 of H3 at genes such as the estrogen-responsive pS2 gene (11, 17). We therefore tested whether these modifications could affect SET and/or pp32 binding to H3 tails. Fig. 3C shows that acetylation of Lys-18 and Lys-23 reduced, but did not abolish, the binding of both SET and pp32 to the H3 tail (lanes 11, and 12). However, when a set of multiply acetylated tails was used for testing SET and pp32 binding (combinations of acetylated Lys-9, Lys-14, Lys-18, and Lys-23) binding of both proteins was abolished (Fig. 3C, lanes 14–18). Interestingly, the actual positioning of the acetyl groups does not appear critical, but rather it is the presence of multiple acetylated lysines that leads to the reduction in INHAT binding. This suggests that acetylation of the H3 tails may form part of a feedback loop regulating the INHAT inhibition of histone acetyltransferases.

We next asked whether phosphorylation of residues other than Thr-3 affects the binding of SET and pp32 to histone H3 tails. Fig. 3C shows that phosphorylation of Ser-10 (lane 6) or Thr-11 (lane 8) abolishes the binding of SET and pp32 as effectively as phosphorylation of Thr-3 (lane 2). In contrast, methylation of adjacent lysine residues (Fig. 3C, lane 3, and Lys-9, lane 5) does not abolish binding of either SET or pp32, although a consistent reduction of binding is seen when Lys-4 is methylated. These results demonstrate that phosphorylation at individual Ser/Thr residues within histone H3 abolishes the binding of SET and pp32 to histones. Thus, as in the case of multiple acetylation, this points to a charge distribution as an inhibitory mechanism for the binding of INHAT to histone H3.

It has been pointed out that methylated lysines within histone tails are often adjacent to phosphorylatable Ser or Thr residues that may form part of a binary switch mechanism (18). We therefore examined a series of peptides that was phosphorylated as well as lysine-methylated. Fig. 3C shows that in every instance we examined, T3P/K4me, K9me/S10P, and K9me/T11P (lanes 4, 7, and 9) binding to INHAT components was abolished. These results indicate a dominant effect of H3 tail phosphorylation on the binding of INHAT relative to methylation. This suggests that cross-talk between methylation and phosphorylation does not take place at these sites with respect to INHAT binding.

What are the binding determinants that allow INHAT to bind the H3 N terminus stably? Our results suggest that charge makes a significant contribution to INHAT binding affinity. Neutralizing a single lysine positive charge by acetylation has only a partial inhibitory effect on INHAT binding. However, neutralizing three or four positive charges by acetylation of 3 or 4 lysines, respectively, completely abrogates binding (Fig. 3C). Likewise, adding two negative charges by singularly phosphorylating either serine or threonine residues also has a significant detrimental effect on INHAT binding. Methylation of lysines within the tails (which does not affect charge) has relatively little effect on INHAT binding. It is notable that both SET and pp32 are relatively negatively charged proteins, and therefore it makes sense that reducing the basic nature of the H3 tail reduces the ability of INHAT to bind. This of course is a mechanism that can easily operate in vivo. However, in addition to charge there must be other binding determinants within the H3 tail that dictate binding specificity. For instance, a very similarly charged region of H3 (amino acids 29–43) does not bind either SET or pp32. This indicates that binding requires the appropriate charges within the context of the H3 N-terminal tail.

Our results indicate that INHAT has a preference for binding to unphosphorylated and hypo-acetylated H3 tails. This type of modification status is typically associated with repressed genes. Lysine methylation such as at Lys-9 H3 may well be present at these genes, but this modification has little effect on
INHAT binding. Thus, the types of histone modifications that are found to affect INHAT binding are perfectly compatible with its known role in transcriptional repression (11, 12).

Why is INHAT binding sensitive to the overall degree of charge rather than dependent on an absolute sequence requirement? This may be explained by the fact that in vitro multiple histone-modifying enzymes act together to effect changes in chromatin structure. Indeed, if one focuses on histone acetyltransferases, these enzymes often work combinatorially to stimulate gene transcription. For example, hGCN5, P/CAF, and CBP/p300 operate together to regulate the interferon-β promoter (19). Different HATs acetylate overlapping but distinct sites within histone tails (20). If INHAT binding to H3 tails is sensitive to the overall change in charge, then it could “monitor” the level of acetylation and hence the level of activity of a gene. Coupled with the fact that INHAT inhibits multiple HATs, its H3 tail binding ability could be part of a feedback mechanism allowing the fine-tuning of HAT activity.

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REFERENCES

1. Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997) Nature 389, 251–260

2. Khorasanizadeh, S. (2004) Cell 116, 259–272

3. Fischle, W., Wang, Y., and Allis, C. D. (2003) Curr. Opin. Cell Biol. 15, 689–697

4. Strahl, B. D., and Allis, C. D. (2002) Nature 413, 49–54

5. Turner, B. M. (1999) Cell 99, 5–9

6. Hampsey, M., and Reinberg, D. (2003) Cell 113, 429–432

7. Zegerman, P., Canas, B., Pappin, D., and Kouzarides, T. (2001) J. Biol. Chem. 276, 11621–11628

8. Nishioka, K., Chuikov, S., Sarma, K., Erdjument-Bromage, H., Allis, C. D., Tempst, P., and Reinberg, D. (2002) Genes Dev. 16, 479–489

9. Santos-Rosa, H., Schneider, R., Bannister, A. J., Sherriff, J., Bernstein, B. E., Emure, N. C., Schreiber, S. L., Mellor, J., and Kouzarides, T. (2002) Nature 419, 407–411

10. Santos-Rosa, H., Schneider, R., Bernstein, B. E., Karabetsou, N., Morillon, A., Weise, C., Schreiber, S. L., Mellor, J., and Kouzarides, T. (2003) Mol. Cell. 12, 1325–1332

11. Seo, S. B., McNamara, P., Hong, R., and Kouzarides, T. (2001) Cell 104, 119–130

12. Seo, S. B., Macfarlan, T., McNamara, P., Hong, R., and Kouzarides, T. (2002) J. Biol. Chem. 277, 14005–14010

13. Li, M., Makkinje, A., and Damuni, Z. (1996) Biochemistry 35, 6998–7002

14. Li, M., Makkinje, A., and Damuni, Z. (1996) J. Biol. Chem. 271, 11059–11062

15. Bannister, A. J., Zegerman, P., Partridge, J. A., Miska, E. A., Thomas, J. O., Allshire, R. C., and Kouzarides, T. (2001) Nature 410, 120–124

16. Lachner, M., O’Carroll, D., Rea, S., Mechtler K., and Jenuwein, T. (2001) Nature 410, 116–120

17. Daujat, S., Bauer, U. M., Shah, V., Turner, B., Berger, S., and Kouzarides, T. (2002) Curr. Biol. 12, 2090–2097

18. Fischle, W., Wang, Y., and Allis, C. D. (2003) Nature 425, 475–479

19. Agalioti, T., Lomvardas, S., Parekh, B., Yie, J., Maniatis, T., and Thanos, D. (2000) Cell 103, 667–678

20. Roth, S. Y., Denu, J. M, and Allis, C. D. (2001) Annu. Rev. Biochem. 70, 81–120

21. Morrissey, J. H. (1981) Anal. Biochem. 117, 307–310

22. Shlevchenko, A., Wilm, M., and Mann, M. (1996) Anal. Chem. 68, 850–854

23. Vorm, O., Roepstorff, P., and Mann, M. (1994) Anal. Chem. 66, 3281–3287

24. Fan, Z., Beresford, P. J., Oh, D. Y., Zhang, D., and Lieberman, J. (2003) Cell 112, 659–672
