

Is acetylation the key to opening locked gates?

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Like certain protein kinases, some protein acetyltransferases such as p300 may use an inhibitory loop that can be regulated to limit the accessibility of substrates to its active site. The finding that autoacetylation of this loop activates the acetyltransferase provides the first evidence for an acetylation cascade analogous to protein kinase cascades.

Acetyltransferases catalyze the transfer of acetyl groups from acetyl-coenzyme A (acetyl-CoA) to the ϵ -amino group of specific lysine side chains on proteins. In certain cases, the resulting charge neutralization eliminates electrostatic interactions with DNA. Acetylated lysines are also recognized by protein modules called bromodomains, which are present in several transcriptional regulatory proteins. Thus, acetylation increases the accessibility of genes and provides docking sites for the transcription machinery and its regulators, thereby exerting significant control over gene expression. The importance of acetylation is manifested by cancers, developmental defects and death when protein acetyltransferases such as p300 are disrupted¹. How acetyltransferases are regulated is not known.

In this issue of *Nature Structural & Molecular Biology*, Thompson *et al.*² suggest that p300 is regulated by a pseudo substrate inhibitory loop that blocks access of target substrates to its acetyltransferase active site. It is well documented that certain protein kinases, like the insulin receptor kinase domain (InRK), do this³. These kinases are activated by phosphorylation of the loop, presumably causing the loop to swing out of the way. Thompson *et al.*² demonstrate that acetylation of p300's putative inhibitory loop activates its acetyltransferase activity, providing the first evidence of an acetylation-gated acetyltransferase. Thus, there may be a common, although not pervasive, underlying theme in

which the active sites of protein-modifying enzymes are gated, with the gate being opened by the same type of modification. These positive feedback loops and linear cascades reinforce decisions by creating highly sensitive molecular switches that control cell fate.

How protein kinases are gated

InRK provides a good example of enzyme active site gating⁴ and a framework for interpreting the findings of Thompson *et al.*² (Fig. 1a). Like most protein kinases, InRK's architecture includes a cleft for the binding of the target peptide and an adjacent active site where ATP binding and phosphoryl transfer occur^{3,5}. Part of InRK's polypeptide chain traverses the cleft, thereby blocking target

protein entry. When this gate is opened by phosphorylation, the substrate protein is free to diffuse into the active site (Fig. 1b).

Control of the kinase through this loop can be exerted in two ways. Either the inhibitory loop slips into the kinase active site, resulting in a first-order intramolecular phosphorylation event, or a second kinase comes along and phosphorylates it. The implication for each mechanism is significant. An intramolecular reaction suggests a timing device. In this case, the ease with which the unphosphorylated inhibitory loop enters the active site and becomes phosphorylated could dictate how fast the kinase becomes activated. Intermolecular phosphorylation suggests either a positive feedback loop (autophos-

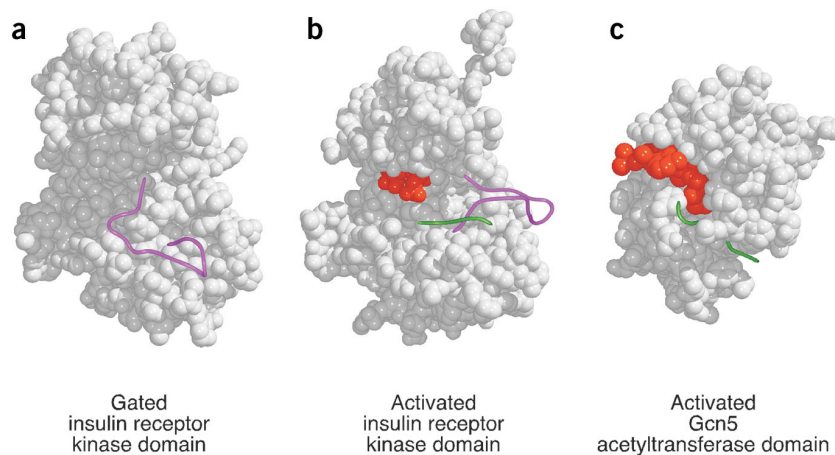


Figure 1 Activation of kinase and acetyltransferase domains through post-translational modification. (a) Space-filling structure of the insulin receptor kinase domain (PDB entry 1IRK) in an unliganded inactive state. The inhibitory loop domain is magenta. (b) Space-filling structure of the liganded insulin receptor kinase domain (PDB entry 1IR3). ATP analog, red. The displaced phosphorylated inhibitory loop is magenta. A green peptide substrate occupies the region vacated by the inhibitory loop. (c) Structure of the Gcn5 HAT domain (PDB entry 1QSN) in which the acetyl-CoA (red) and bound histone H3 peptide (green) are oriented similarly to that in b.

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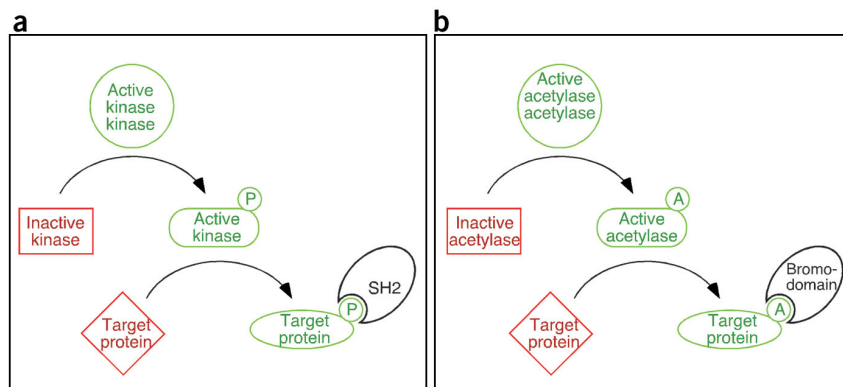


Figure 2 A simplified parallel relationship between well-established kinase cascades and the potential for an acetyltransferase cascade. (a) P, phosphorylation event. (b) A, acetylation event.

phorylation) or a linear regulatory cascade in which one kinase relays its activation to another kinase. InRK autophosphorylation and Cak2 phosphorylation of Cdk2 typify this behavior^{4,6}.

The structure of histone acetyltransferases

The X-ray crystal structures of several histone acetyltransferases (HATs) have been determined⁷. Despite a lack of overall sequence conservation among classes, they are similar in structure. This is remarkable because not all HATs transfer acetyl groups by the same chemical mechanism. All have a cleft that binds the target peptide and an acetyl-CoA-binding site (Fig. 1c). Although the presence of a peptide-binding cleft and an adjacent active site in kinases and acetyltransferases might simply be a design constraint, it does suggest other parallel relationships between the two intracellular signaling strategies. Because many HATs also acetylate other proteins, which in some cases might be their prime targets, HATs are referred to here by the more general term protein acetyltransferase.

An acetylation-gated acetyltransferase

The acetyltransferase p300 acetylates itself, histones and many other proteins⁷. But the functional significance of autoacetylation has remained unclear. Addressing p300 autoacetylation has been problematic for two reasons. First, the catalytic acetyltransferase domain is toxic to recombinant expression systems, probably because of rampant non-specific acetylation of host proteins. Second, the purified enzyme is hyperacetylated at many sites, making it difficult to assess the contribution of individual acetylation events to enzyme activation. Thompson *et al.*² derive an elegant solution to both problems. They bacterially express and purify a truncated

form of the p300 acetyltransferase domain lacking activity, and then synthetically attach the missing piece to restore activity. This system can now address several questions. First, does acetylation regulate its acetyltransferase activity? Second, does the acetyltransferase domain contain any exposed loops that are important for regulating acetyltransferase activity? Third, is any putative loop regulated by acetylation? And fourth, does such acetylation occur *in vivo* and is it associated with gene regulation?

Assessment of the kinetic parameters associated with the hypo- and hyperacetylated forms of the p300 acetyltransferase domain reveals that the catalytic efficiency of peptide acetylation increases by more than ten-fold when in the hyperacetylated state. This large difference in activity suggests that the p300 acetyltransferase can be regulated by acetylation. Although not discussed by Thompson *et al.*², it is assumed that the hypoacetylated form is not significantly autoacetylated during the acetylation assays. If this were correct, it would imply either that intramolecular autoacetylation was inefficient, or that any intermolecular regulatory acetylation occurred infrequently at the low enzyme concentrations.

Thompson *et al.*² reveal that the p300 acetyltransferase domain has many acetylated lysines. The three-dimensional structure of this domain is not known, nor is its primary sequence conserved with other acetyltransferases whose structure has been determined; therefore, it is not yet possible to obtain a structural view of the relationship between the location of the acetylated lysines and the binding sites for acetyl-CoA and peptide substrate. Nevertheless, Thompson *et al.*² provide an initial glimpse by mapping protease-sensitive domains. They find a single region of trypsin sensitivity, possibly reflect-

ing an exposed loop on the protein. This region also contains many lysines that can be acetylated, consistent with its location in an accessible loop.

Mutations of the putative loop region showed that certain lysines are important for acetyltransferase activation, and that an acidic stretch within the loop is inhibitory. These observations are consistent with the speculation that the proposed loop is a pseudo substrate that blocks access of target peptides to the active site. Acetylation of this loop might then displace it from the binding cleft. Although the molecular basis for activation remains to be determined, it is plausible that loss of positive charges on specific lysines through acetylation might reduce intraloop ionic interactions with the inhibitory acidic patch, thereby releasing the loop from the vicinity of the peptide-binding site. Much of this will remain speculative until a high-resolution three-dimensional reconstruction of the p300 acetyltransferase domain is achieved.

Several pieces of evidence suggest that acetylation of p300 is physiologically important. First, Thompson *et al.*² demonstrate that one of the critical regulatory lysines in the loop is acetylated *in vivo*. Second, a known p300 target protein, p73, shows increased acetylation when the loop is deleted, presumably creating a constitutively active acetyltransferase. Third, increased transcriptional activation of a target gene is observed with the loop-deleted version as compared with wild type. These types of experiments are difficult to interpret because indirect effects cannot be excluded. Additionally, the magnitude of the effects is not large, perhaps owing to the possibility that transfected wild-type p300, used as a control comparison, is constitutively active in the assayed cell lines. In this regard, it would have been informative to assay arginine-substitution mutants in the loop, which retain the positive charge but are not acetylated. These mutants are expected to be substantially less active. Nonetheless, the data are consistent with the possibility that acetylation regulates p300 acetyltransferase activity.

Remaining uncertainties

Several new questions arise from the idea that p300 acetyltransferase activity is regulated by a pseudo substrate. First, is autoacetylation of the loop domain intramolecular, suggesting that it might serve as a timing device? Alternatively, if acetylation is intermolecular, then is the physiological acetyltransferase p300 itself or another acetyltransferase? Is the loop domain part of a regulated acetylation switch akin to phosphorylation switches in

kinase loop domains? Or is p300 constitutively acetylated and therefore regulated by other mechanisms? With a conceptual framework now laid out, answers to many of these questions are within reach.

The apparent parallel relationship between phosphorylation and acetylation regulatory switches has been discussed by Kouzarides⁸, but the question of an acetylation cascade has remained open. Kinases phosphorylate other kinases, which phosphorylate other proteins (Fig. 2a). Phosphorylation sites are bound by specific recognition motifs such as SH2 domains. So protein phosphorylation mini-

mally serves two essential purposes, regulation of enzyme activity and creation of protein docking sites. Does a similar regulatory cascade exist for acetylation (Fig. 2b)? Several pieces of this puzzle are now in place. We know that an acetyltransferase can be regulated by acetylation, and have known that acetylation provides docking sites for bromodomain proteins. An important missing piece in this puzzle is whether another acetyltransferase activates p300 via acetylation of the proposed looped domain, or whether autoacetylation simply reinforces the activation decision.

ACKNOWLEDGMENTS

I thank J. Adams for advice and S. Tan for generating the structures in Figure 1. Research support comes from the US National Institutes of Health (GM059055).

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RISCy business

Two classes of small RNAs influence a wide variety of biological processes by silencing gene expression. MicroRNAs (miRNAs) are produced from stem-loop RNA precursors encoded in the genome and are incorporated into miRNA-induced silencing complexes (miRISCs). These complexes appear to silence gene expression by blocking mRNA translation and play many roles in growth and development. In contrast, small interfering RNAs (siRNAs) are generated from longer double-stranded RNA precursors. siRNAs are incorporated into and guide siRNA-induced silencing complexes (siRISCs) to target messenger RNAs for degradation.

The extent of base-pairing between the small RNA and the mRNA appears to determine the outcome of silencing. Specifically, translation inhibition occurs if the target mRNA is partially complementary to the silencing trigger (miRNA or siRNA). In contrast, direct cleavage of the message requires perfect complementarity. It is unclear whether a single silencing complex can cleave mRNA and block its translation, or whether the silencing triggers assemble into distinct RISCs each with its own activity.

Both mi- and siRNAs are generated by an RNase III enzyme called Dicer. *Drosophila melanogaster* contains two Dicer proteins, Dicer-1 and Dicer-2, which might work together to carry out silencing. In two papers published in a recent issue of *Cell*, the Carthew and Sontheimer laboratories show that in *Drosophila*, Dicer-1 and Dicer-2 have distinct but overlapping roles in the two silencing pathways. Furthermore, they show that Dicer-2 and siRNAs assemble into a RISC intermediate that subsequently forms the functional RISC involved in mRNA cleavage.

Lee *et al.* (*Cell*, in the press, 2004) developed a sensitive way to screen for mutations that affect siRNA-mediated silencing in the eyes of *Drosophila*. The normal red color of the eye (upper left panel) was changed to light orange (upper right panel) by silencing of a pigmentation gene. Mutations in Dicer-1 and Dicer-2 were discovered and these altered the eye color back toward normal (lower panels). Lee *et al.* show that flies with

mutant *dicer-1* have darker orange eyes (lower left). Furthermore, the size and morphology of the eyes were different from that seen with wild-type Dicer-1 (compare upper right and lower left panels). These developmental differences suggest that the miRNA pathway is somehow affected in *dicer-1* mutants. Further analysis showed that siRNA levels were comparable to those in the wild-type flies but no mature miRNAs were detected. These and other data suggest that Dicer-1 is critical for miRNA production, but the mechanism by which this protein functions remains unclear.

In contrast, Dicer-2 is dispensable for miRNA production. Furthermore, flies with a mutated Dicer-2 protein (lower right panel) have very low levels of siRNA suggesting that Dicer-2 is important for siRNA processing. Mutations of single residues in the active site of Dicer-2 confirmed that

RNase III activity is required to generate siRNA. Further analyses showed that both Dicer-1 and Dicer-2 are required for the formation of functional RISCs.

Pham *et al.* (*Cell*, in the press, 2004) used biochemical techniques to show that Dicer-2 facilitates multiple steps in siRISC assembly. The data are consistent with a model in which these complexes form sequentially in an ordered assembly pathway. Dicer-2 interacts with siRNAs in an ATP-independent complex that initiates siRISC assembly, and remains associated with the siRNA in the functional siRISC. They also identify a form of active siRISC that is much larger (~80S) than those characterized previously. These results indicate that Dicer-2 not only processes double-stranded RNA and transfers siRNAs to a distinct complex, but also assembles into a very large siRISC along with the siRNAs.

Is the miRISC assembled using a similar process? Dicer-1 co-immunoprecipitates with miRNA and could initiate miRISC assembly in much the same way that Dicer-2 initiates siRISC assembly. The genetic data from Lee *et al.* showing that Dicer-1 and Dicer-2 play distinct roles in miRNA and siRNA processing lend support to such a model.

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