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Synergistic Coupling of Histone H3 Phosphorylation and Acetylation in Response to Epidermal Growth Factor Stimulation

Peter Cheung,* Kirk G. Tanner,† Wang L. Cheung,* Paolo Sassone-Corsi,‡ John M. Denu,† and C. David Allis*§

*Department of Biochemistry and Molecular Genetics
University of Virginia
Charlottesville, Virginia 22908
†Department of Biochemistry and Molecular Biology
Oregon Health Sciences University
Portland, Oregon 97201
‡Institut de Génétique et de Biologie
Moléculaire et Cellulaire
CNRS, INSERM, ULP, B.P. 163
67404 Illkirch-Strasbourg
France

Summary

Histone acetylation and phosphorylation have separately been suggested to affect chromatin structure and gene expression. Here we report that these two modifications are synergistic. Stimulation of mammalian cells by epidermal growth factor (EGF) results in rapid and sequential phosphorylation and acetylation of H3, and these dimodified H3 molecules are preferentially associated with the EGF-activated *c-fos* promoter in a MAP kinase-dependent manner. In addition, the prototypical histone acetyltransferase Gcn5 displays an up to 10-fold preference for phosphorylated (Ser-10) H3 over nonphosphorylated H3 as substrate in vitro, suggesting that H3 phosphorylation can affect the efficiency of subsequent acetylation reactions. Together, these results illustrate how the addition of multiple histone modifications may be coupled during the process of gene expression.

Introduction

In eukaryotic cells, genomic DNA is packaged into nucleosomes, the fundamental organizational units of chromatin. Although not yet fully defined, the regulated folding of nucleosomal filaments into higher order structures and the establishment of distinct functional domains in chromatin can impact on a wide range of nuclear processes including transcription and replication (Wolffe, 1998; Kornberg and Lorch, 1999). While numerous studies have demonstrated the repressive nature of chromatin on gene expression (reviewed in Workman and Kingston, 1998; Tyler and Kadonaga, 1999), there is also evidence that nucleosomal folding of the cellular genome may facilitate transcription of some genes (Schild et al., 1993; Wyrick et al., 1999). Regardless of its positive or negative effect, chromatin structure clearly plays a critical regulatory role in gene expression, and one question central to this process is how RNA polymerase and associated proteins gain access to chromatin templates. To that end, increasing evidence indicates that both chromatin remodeling factors and covalent histone modifications can facilitate access of DNA binding factors to chromatin and, thereby, regulate expression of a wide range of genes (reviewed in Wu et al., 1998; Spencer and Davie, 1999).

A number of posttranslational modifications are known to occur on histones (reviewed in van Holde, 1988; Wolffe and Hayes, 1999; Strahl and Allis, 2000), and acetylation is currently the best studied of these modifications. The levels of histone acetylation have long been correlated with the transcription status of many genes: transcriptionally active euchromatic regions of the genome are often associated with hyperacetylated histones, whereas transcriptionally silent regions such as heterochromatin and the inactive X chromosome are associated with hypoacetylated histones (reviewed in Grunstein, 1997). The steady-state levels of histone acetylation in vivo are maintained by the balance of opposing histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities, and these enzymes are associated with activation and repression of gene expression respectively (Struhl, 1998; Kornberg and Lorch, 1999). Since the discovery that the yeast transcription coactivator Gcn5 possesses HAT activity (Brownell et al., 1996), many more transcription-associated HATs have now been identified (reviewed in Berger, 1999). In general, these HATs preferentially target histones H3 and H4, and acetylate specific lysine residues of their N-terminal tails. Moreover, the Gcn5 family of HATs (yeast Gcn5 and human PCAF) display a strong preference for lysine 14 (Lys-14) of H3 in vitro (Kuo et al., 1996; Schiltz et al., 1999), suggesting that acetylation at this particular site may correlate with transcriptional activation.

Besides acetylation, the H3 N-terminal tail is also phosphorylated in association with different cellular processes. A large fraction, if not all, of the H3 molecules are phosphorylated at serine 10 (Ser-10) during entry into mitosis (Hendzel et al., 1997; Wei et al., 1998), and mutation of this phosphorylation site impairs chromosome condensation and segregation in vivo (Wei et al., 1999). Ser-10 is also phosphorylated in a small population of H3 in a rapid and transient manner upon mitogen stimulation of quiescent cells (Mahadevan et al., 1991). Insofar as this phosphorylation event correlates closely in time with the transcriptional activation of immediate-early genes (Mahadevan et al., 1991; Thomson et al., 1999b), it has been hypothesized that H3 phosphorylation is involved in the transcriptional activation of mitogen-induced genes. Recently, Rsk-2 kinase and other Rsk-related kinases, such as Msk1, have been identified as kinases that contribute to mitogen-stimulated H3 (Ser-10) phosphorylation (Sassone-Corsi et al., 1999; Thomson et al., 1999a). Indeed, Coffin-Lowry (CLS) cells, which bear mutations in the *Rsk-2* gene, but not in the highly related *Rsk-1* and *Rsk-3* genes, are defective in mitogen-induced phosphorylation of H3 (De Cesare et al., 1998). Moreover, *c-fos* expression upon growth fac-

§To whom correspondence should be addressed (e-mail: allis@virginia.edu).

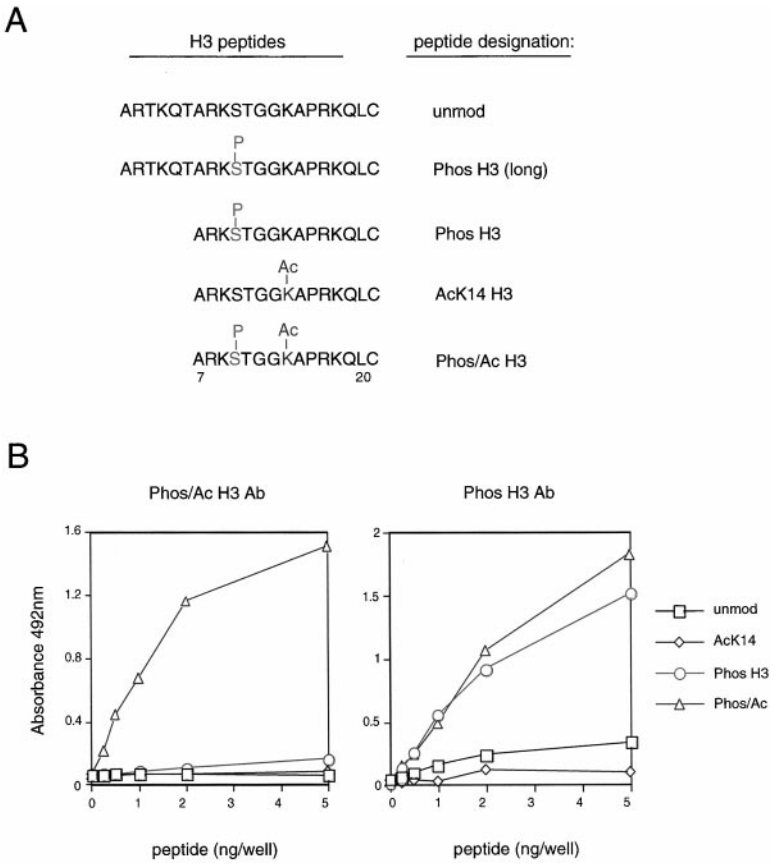

Figure 1. The Phos/Ac H3 Antibody Specifically Recognizes Phosphorylated and Acetylated H3 Peptide

(A) Amino acid sequences of the different H3 peptides used and their designations.
(B) ELISA analyses of the Phos/Ac H3 and Phos H3 antibodies for their recognition of a panel of H3 peptides.

tor stimulation is severely impaired in these cells. Together, these observations support the model that H3 phosphorylation is a requisite step in the activation of immediate-early gene transcription.

The mechanisms by which H3 phosphorylation or acetylation facilitate transcription are not known. Current evidence suggests that these modifications can affect histone–DNA contacts: for example, phosphorylated H3 was less efficiently cross-linked to DNA upon UV exposure compared to unmodified H3, and acetylated histones inhibited higher order folding of reconstituted nucleosomal arrays (Tse et al., 1998; Sauve et al., 1999). Given that addition of either of these modifications leads to a reduction in the overall positive charge of histone N-terminal tails, one long-standing hypothesis is that they attenuate electrostatic interactions between the histone tails and DNA. Such an effect could in turn facilitate binding of nuclear factors to the modified nucleosomal DNA as observed in several studies (Lee et al., 1993; Vettese-Dadey et al., 1996). Alternatively, as illustrated by studies showing that factors such as Tup 1 and CTF-1, a TGF-β-responsive transcription activator, can directly interact with H3 (Alevizopoulos et al., 1995; Edmondson et al., 1996), the modified histones may provide a platform for nuclear factors to bind and target their activities to selected regions of the genome (discussed in Strahl and Allis, 2000).

The fact that multiple activities target the N-terminal tails of histones implies that several modifications may be present simultaneously on the same histone. For example, an earlier study showed that phosphorylated H3 was also acetylated in butyrate-pretreated mammalian cells following EGF stimulation (Barratt et al., 1994). However, little information is available regarding whether one modification affects the subsequent addition or function of other modifications on the same histone molecule. In this study, we examine the possible links between phosphorylation and acetylation of H3 using an antibody that specifically recognizes H3 phosphorylated at Ser-10 and acetylated at Lys-14. Our data show that establishment of both covalent modifications on the same histone molecules is tightly coupled in epidermal growth factor (EGF)-stimulated cells, and kinetic studies suggest that phosphorylation precedes acetylation in the formation of dimodified (phosphorylated and acetylated) H3. This sequential order of histone modifications is further suggested by in vitro studies that demonstrate that the prototypical HAT, Gcn5, displays an up to 10-fold greater preference for Ser-10-phosphorylated H3 peptide as a substrate compared to the unmodified peptide. Moreover, this preference was ablated by substitution of a single amino acid in Gcn5 predicted to contact the phosphoserine 10 of H3. Finally, using chromatin immunoprecipitation assays, we demonstrate that phosphorylated and acetylated H3 are preferentially associated with the *c-fos* promoter in EGF-stimulated cells, and that this association is dependent on the integrity of the MAP kinase signaling pathway. Together, these data show that H3 phosphorylation and acetylation are synergistic and suggest that these combined

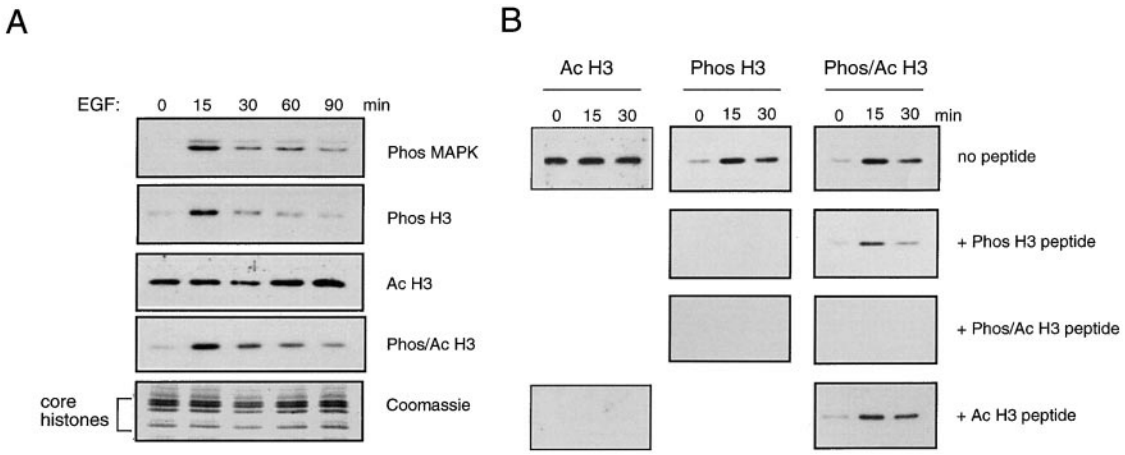

Figure 2. EGF Induces the Accumulation of Phosphorylated and Acetylated H3

(A) Serum-starved 10 T1/2 cells were stimulated with EGF for the indicated lengths of time. Total cellular proteins were harvested in SDS buffer, resolved on 12% polyacrylamide gels, and analyzed by Western blot. Top four panels: Western blots showing the relative protein levels of phosphorylated MAP kinases (ERKs 1 and 2), phosphorylated H3, acetylated H3, and phosphorylated and acetylated H3 in the EGF-stimulated cells. Bottom panel: core histones of the same samples stained by Coomassie blue.

(B) Ac H3, Phos H3, and Phos/Ac H3 antibodies were mock- or preincubated with 3 μg/ml of the indicated peptides and then used for Western blot analyses of the EGF-stimulated cell lysates.

histone modifications may be involved in the expression of mitogen-induced immediate-early genes.

Results

Generation of an Antibody that Specifically Recognizes H3 that Are Both Phosphorylated and Acetylated

Previous studies have suggested that phosphorylation and acetylation may coexist in the same population of H3 molecules as a result of mitogenic stimulation in butyrate-treated mammalian cells (Barratt et al., 1994). To directly study this combination of H3 modifications, we generated an antibody that specifically recognizes dimodified (phosphorylated at Ser-10 and acetylated at Lys-14) H3. A synthetic peptide containing both modifications (Figure 1A, Phos/Ac peptide) was synthesized and used to immunize rabbits after coupling to KLH. The specificity of the affinity-purified antibody (hereafter designated as Phos/Ac H3 Ab; see Experimental Procedures for details) was assayed by ELISA (Figure 1B) and verified by peptide competition experiments (Figure 2B).

As shown in Figure 1B, the Phos/Ac H3 Ab specifically recognized the dimodified H3 peptide with little or no cross-reactivity to control peptides (H3 peptides that were either unmodified, monophosphorylated at Ser-10, or monoacetylated at Lys-14; see Figure 1A for peptide designations). By comparison, a previously characterized antibody that recognizes Ser-10 phosphorylated H3 (Phos H3; Hendzel et al., 1997) reacted equally well with the singly phosphorylated and the dimodified H3 peptides (Figure 1B), indicating that acetylation at lysine 14 did not interfere with recognition of the phosphorylated H3 epitope by that antibody.

Epidermal Growth Factor Induces the Accumulation of Dimodified H3

To study mitogen-induced H3 modifications in vivo, extracts from EGF-stimulated cells were examined by Western blot analysis using a panel of antibodies. Serum-starved mouse 10 T1/2 cells were stimulated with EGF for various lengths of time, and equal amounts of lysate (shown by the comparable levels of histones stained by Coomassie blue) were immunoblotted with antibodies specific for the indicated epitopes (Figure 2A). As expected (see Thomson et al., 1999b for review and references), MAP kinases and H3 were rapidly and transiently phosphorylated upon EGF stimulation. In contrast, bulk levels of acetylated H3 did not significantly change after EGF treatment, presumably because acetylated H3 is generally associated with active chromatin, whereas mitogen-responsive genes represent only a small subset of the genome (see below).

Peptide competition confirms the specificity of the Phos/Ac H3 antibody on Western blots (Figure 2B). Addition of 3 μg/ml of Phos H3 or acetylated H3 peptide to the primary antibody solution did not prevent detection of the modified H3 by the Phos/Ac H3 antibody but completely abolished detection of H3 substrates by Phos H3 and Ac H3 antibodies, respectively, in parallel control blots. Only addition of the Phos/Ac peptide ablated detection of modified H3 by the Phos/Ac H3 antibody. Together, these peptide competition experiments clearly demonstrate the presence of H3 that are doubly modified by phosphorylation and acetylation (at Ser-10 and Lys-14) in an EGF-dependent manner.

Only a Small Fraction of Total H3 Is Dimodified Following EGF Stimulation

To better resolve the modified forms of H3 generated in response to EGF, acid-extracted histones from mock-treated or EGF-stimulated cells were fractionated in long acid–urea (AU) gels (Figure 3). Protein migration in AU gels is determined by mass and charge (Lennox and Cohen, 1989), and because phosphorylation and acetylation each alter the net charge of histone molecules at the operating pH of these gels, H3 bearing these

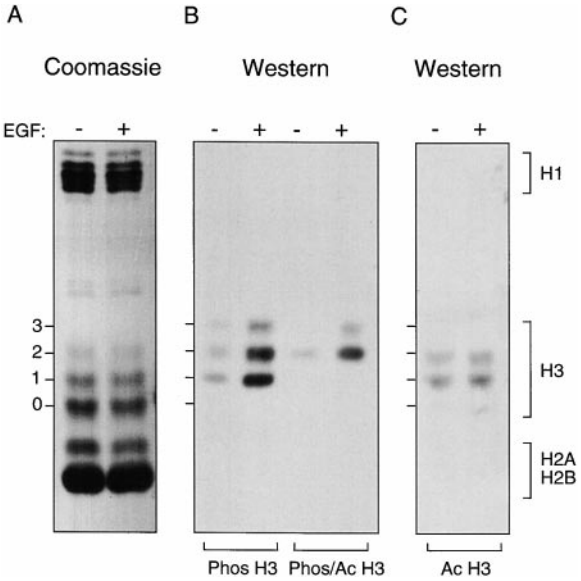

Figure 3. Separation of the Different Forms of Modified H3 on Acid–Urea Gel

(A) Histones from mock- or EGF-stimulated 10 T1/2 cells were extracted in 0.4 N H_2SO_4, resolved on long acid–urea (AU) gels, and stained by Coomassie blue. The migration positions of unmodified, mono-, di-, and trimodified forms of H3 are referred to by 0, 1, 2, and 3.

(B and C) Western blot analyses of the H3 resolved in AU gels using Phos H3, Phos/Ac H3, and Ac H3 antibodies.

modifications can be resolved from the unmodified form. With or without EGF stimulation, Coomassie staining showed that the majority of H3 was not modified (labeled as 0), with progressively lesser amounts of singly and doubly modified H3 present (labeled as 1 and 2, respectively, Figure 3A). In addition, a trimodified form of H3 that was not sufficiently abundant to be stained by Coomassie blue was detected by Western blotting (Figure 3B).

Upon EGF stimulation, the Phos H3 antibody reacted strongly with the three slower migrating forms that represented singly, doubly, and trimodified H3 (Figure 3B). However, only the two more slowly migrating species were detected by the Phos/Ac H3 antibody, indicating that the fastest migrating band recognized by the Phos H3 antibody (labeled as 1) represents monophosphorylated H3. The decreased mobility of monophosphorylated H3 compared to unmodified H3 on AU gels was independently confirmed by analysis of bacterially expressed *Xenopus* H3 treated or mock treated with PKA (data not shown). In agreement with our earlier results (Figure 2A), EGF treatment did not greatly affect bulk levels of acetylated H3 (Figure 3C) but did induce dramatic increases in the levels of phosphorylated and dimodified forms detected by the respective antibodies (Figure 3B). However, this increase was not apparent by Coomassie staining (Figure 3A), nor did EGF stimulation appear to alter the relative proportions of the slower migrating forms (compare the H3 levels in the − and + lanes in Figure 3A). These results are entirely consistent with the report that suggested that EGF-induced H3 phosphorylation only occurs on a small fraction of total H3 (Barratt et al., 1994). Moreover, the predominant accumulation of H3 modified only by phosphorylation upon EGF stimulation suggests that phosphorylation does not preferentially occur on acetylated H3.

Phosphorylation and Acetylation Are Tightly Coupled in Response to EGF Stimulation

The accumulation of phosphorylated and acetylated H3 upon mitogenic stimulation could potentially arise from two scenarios: (1) phosphorylation and acetylation may be completely independent of one another with indiscriminate phosphorylation of unmodified and acetylated H3 leading to accumulation of monophosphorylated and dimodified H3, or (2) these two modifications may be sequentially linked such that phosphorylation occurs preferentially on unmodified H3 and a fraction of this phosphorylated H3 is subsequently acetylated. If such a preference exists, we reasoned that we may detect temporal differences in the accumulation of the different forms of H3.

Serum-starved 10 T1/2 cells were stimulated with EGF, and histones, extracted at different times following stimulation, were resolved on AU gels and analyzed by Western blotting (representative blots shown in Figures 4A and 4B, top panels). The relative levels of the monophosphorylated and phosphorylated/acetylated H3 species detected by the respective antibodies in three independent trials were quantified by densitometry, normalized to the peak values in each experiment, and plotted as a function of time following EGF stimulation (Figures 4A and 4B, bottom panels). The amount of monophosphorylated H3 detected by the Phos H3 antibody was dramatically increased by 5 min post EGF stimulation, peaked at 10 min, and slowly declined thereafter (Figure 4A). In contrast, the levels of dimodified H3 detected by the Phos/Ac H3 antibody accumulated more slowly and consistently peaked at 12.5 min in each experimental trial (Figure 4B). The staggered accumulation profiles of monophosphorylated and dimodified H3 suggest that, at least for a fraction of H3, acetylation occurs on H3 that is already phosphorylated.

A Prototypical HAT Prefers Phosphorylated H3 Peptide as a Substrate In Vitro

The previous results suggest that upon EGF stimulation, H3 phosphorylation and acetylation are tightly coupled and that phosphorylation appears to precede acetylation in the generation of dimodified H3. To further examine the link between these two modifications, we asked whether site-specific phosphorylation of H3 influences the ability of a HAT to recognize and bind to the H3 tail in vitro. Two synthetic H3 peptides, differing only by the presence or absence of a phosphate group at Ser-10 (Figure 1A, unmod and Phos H3 [long] peptides), were used as substrates in HAT assays with the prototypical HAT yeast Gcn5. Two separate assay methods were used (endpoint and continuous; see Experimental Procedures for details), and steady-state kinetic parameters K_{cat} and K_m for these reactions were determined by fitting the data to the Michaelis-Menten equation. A representative data set for wild-type Gcn5 is displayed in Figure 5A, and the collective results are summarized in Table 1.

Strikingly, the K_m value for the phosphorylated H3

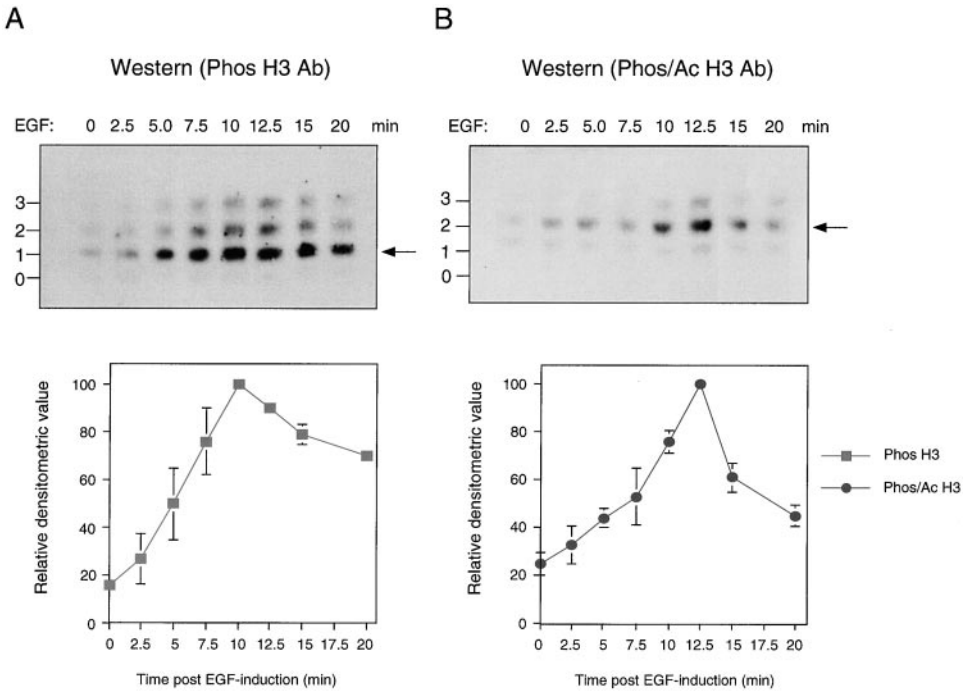

Figure 4. Time Course of Accumulation of Modified H3

Serum-starved 10 T1/2 cells were stimulated with EGF for the indicated lengths of time, and histones extracted from these samples were resolved on AU gels ([A and B], top panels). Representative immunoblots of AU gel-fractionated histones using the indicated antibodies. The relative levels of the monophosphorylated and phosphorylated/acetylated H3 (arrows, top panel) from three independent trials were quantified by densitometry, normalized to the peak densitometric value in each experiment, and plotted as a function of time post EGF treatment (bottom panel). The error bars represent the standard deviation at each time point calculated from the data of the multiple trials.

peptide was up to 10-fold lower than that of the unmodified peptide, whereas only a small apparent change in K_{cat} was observed (Table 1). The significantly lower K_m values for phosphorylated H3 peptide indicate that the enzymatic reaction is occurring much faster with the phosphorylated H3 peptide than with the unmodified peptide, and that Gcn5 binds and reacts with the Ser-10 phosphorylated H3 peptide with higher affinity compared to its unmodified counterpart. These in vitro findings are entirely consistent with our in vivo observation that H3 phosphorylation precedes acetylation in the EGF-induced accumulation of dimodified H3.

To understand the basis of the preference for phosphorylated H3 displayed by the recombinant Gcn5, we examined the recently solved structure of *Tetrahymena* Gcn5 cocrystallized with CoA and H3 peptide (Rojas et al., 1999). By computer modeling, we found that a positively charged arginine residue at position 164 of the yeast Gcn5 could favorably interact with two of the negatively charged oxygen molecules of phospho-Ser-10 (Figure 5C). Such an interaction could potentially stabilize the enzyme–substrate complex and account for the apparent preference of wild-type Gcn5 for phosphorylated H3 (Figure 5A).

To test this prediction, we generated a mutant Gcn5 in which the arginine residue at position 164 was changed to a lysine residue (R164K mutant). The enzyme activity of this recombinant mutant was analyzed using unmodified and phosphorylated H3 peptides as done with wild-type Gcn5. In contrast to wild-type Gcn5, the K_m values and the saturation curves for unmodified and phosphorylated H3 peptides were indistinguishable for the mutant Gcn5, indicating that the Arg-to-Lys substitution completely abolished Gcn5's preference for phosphorylated H3 peptide (Figure 5B and Table 1). Since the K_{cat} values for the R164K mutant were not significantly different from those of wild-type enzyme (Table 1), the loss of the preference for phosphorylated H3 peptide is not likely to be due to gross structural perturbations but, rather, the result of deletion of an important amino acid mediating the interaction between Gcn5 and substrate. Collectively, these data demonstrate that site-specific H3 phosphorylation can enhance the subsequent acetylation reaction by a prototypical HAT in vitro.

Association of Dimodified H3 with the *c-fos* Promoter Is Dependent on MAP Kinase Signaling

Our earlier data show that only a small population of H3 is phosphorylated upon mitogenic stimulation and that an even smaller fraction is subsequently acetylated (Figure 3). Indeed, only a limited number of genes are activated in mammalian cells upon mitogen stimulation, and the Phos H3 antibody has been used in chromatin immunoprecipitation (ChIP) assays to show that phosphorylated (Ser-10) H3 is associated with *c-fos* and *c-myc* genes upon EGF treatment (Chadee et al., 1999). To examine whether dimodified H3 is associated with EGF-activated genes, we used the Phos/Ac H3 antibody in

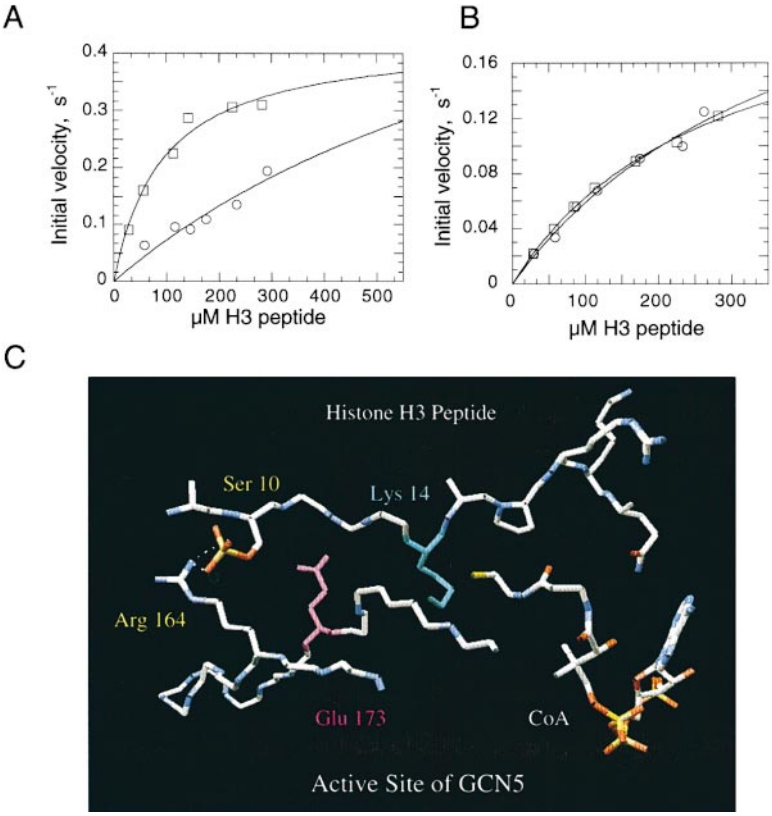

Figure 5. Gcn5 Prefers Phosphorylated H3 as Substrate

(A) Saturation curves for wild-type Gcn5 using unmodified H3 peptide (open circles) and phosphorylated (Ser-10) H3 peptide (open squares) as substrates. The data fitted to the Michaelis-Menten Equation were displayed as solid line graphs. The results from several different experiments are summarized in Table 1.

(B) Saturation curves for the R164K Gcn5 mutant using unmodified H3 peptide (open circles) and phosphorylated (Ser-10) H3 peptide (open squares) as substrates.

(C) Structural model of phosphorylated H3 interacting with Gcn5 HAT. This model was generated using the solved ternary cocrystal structure of *Tetrahymena* Gcn5 with bound CoA and unmodified H3 peptide (Rojas et al., 1999). By modeling a phosphoserine residue into the peptide substrate at position 10 (Guex and Peitsch, 1997), we found that the positively charged arginine residue at position 164 (yeast Gcn5) could favorably interact with two of the negatively charged oxygens of phospho-Ser-10 (hydrogen bonds are represented as dashed lines). The conserved general base Glu173 (Tanner et al., 1999) of Gcn5 is displayed in purple, and the site of acetylation Lys-14 is shown in light blue. Atom coloring scheme: red, oxygen; blue, nitrogen; yellow, sulfur; gold, phosphorus. Backbone carbonyl oxygens have been omitted for clarity.

ChIP assays. In parallel, we also employed an antibody specific for acetylated H3 (acetylated at either lysine 9 or 14) to monitor the association of acetylated H3 with the *c-fos* promoter. Mock-treated and EGF-stimulated cells were cross-linked with formaldehyde, and sonicated chromatin from these cells was immunoprecipitated with the various modification-specific H3 antibodies. The amount of *c-fos* promoter DNA present in immunoprecipitated chromatin fractions was then determined by quantitative PCR. Primers to histone *H4*, a constitutively expressed gene, were included in each PCR reaction to control for DNA input and amplification efficiency.

Immunoprecipitation of equivalent amounts of chromatin from mock-treated and EGF-stimulated cells (compare + and − input lanes in Figure 6A) with the Phos/Ac H3 antibody showed an approximately 2-fold increase in the association of dimodified H3 with the *c-fos* promoter after EGF stimulation (Figures 6A and 6C). Almost identical levels of H4 sequences were immunoprecipitated from cells treated or mock treated with EGF, suggesting that the increased association of dimodified H3 with the EGF-activated *c-fos* gene is a significant finding. A similar enrichment of acetylated H3 at the *c-fos* gene was seen using the acetylated H3-specific antibody (Figures 6A and 6C). Therefore, whereas our earlier immunoblotting results (Figures 2A and 3C) show that EGF treatment does not significantly alter the bulk levels of acetylated H3, our ChIP data demonstrate that the levels of acetylated H3 do increase at this locus

Table 1. Kinetic Analysis of Histone H3 and phospho-H3 Acetylation by GCN5 and R164K Mutant

Enzyme/Assay	$K_{m(H3)}$ mM	$K_{m(phos\text{-}H3)}$ mM	k_{cat} s^{-1}	$k_{m(H3)}/K_{m(phos\text{-}H3)}$ Ratio
GCN5 HAT endpoint	0.28 ± 0.07	—	0.34 ± 0.05	10
GCN5 HAT endpoint	—	0.028 ± 0.011	0.28 ± 0.03	
GCN5 HAT continuous	0.57 ± 0.09	—	0.51 ± 0.34	6.1
GCN5 HAT continuous	—	0.093 ± 0.008	0.42 ± 0.04	
R164K GCN5 continuous	0.28 ± 0.03	—	0.24 ± 0.01	0.7 ± 0.3
R164K GCN5 continuous	—	0.42 ± 0.15	0.30 ± 0.08	

Data were collected and analyzed as described in Experimental Procedures. Conditions: 50 μM acetyl-CoA, 22°C, 100 mM Na Acetate, 50 mM Tris, 50 mM Bis-Tris, and pH 7.5.

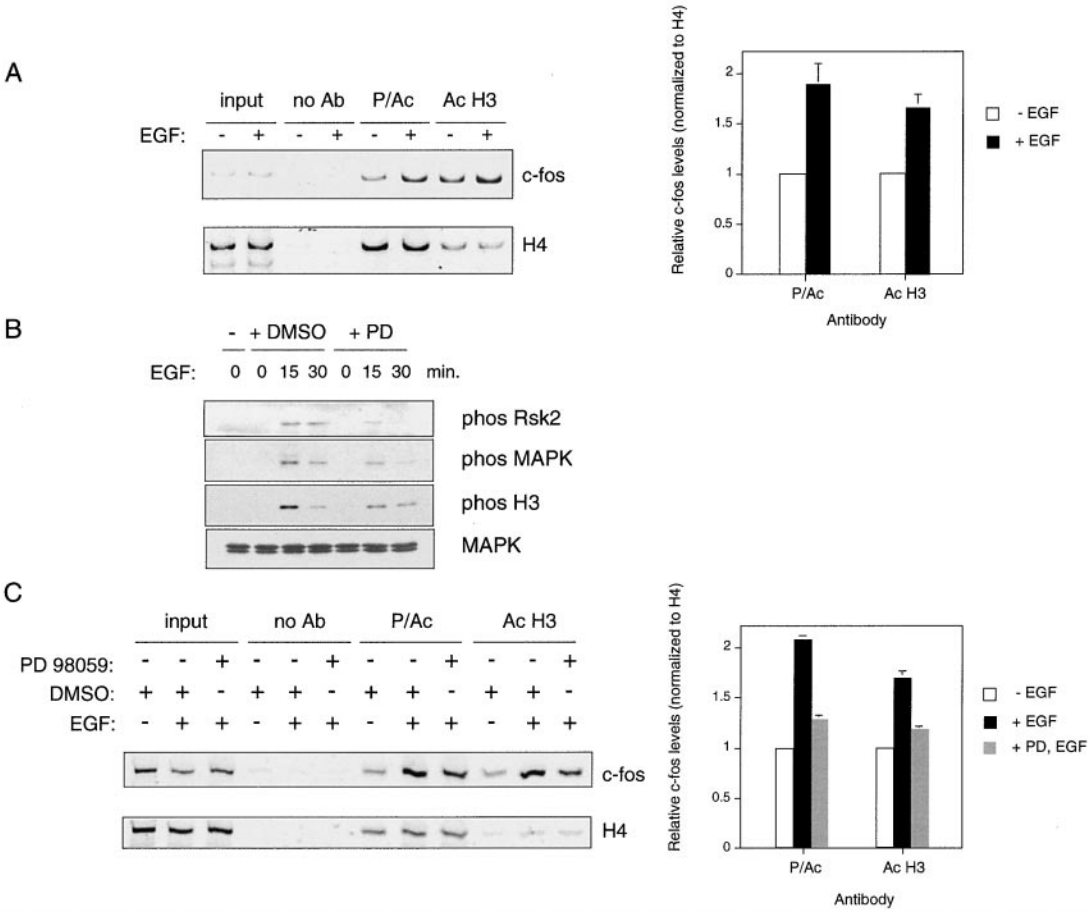

Figure 6. Association of Phosphorylated and Acetylated H3 with the *c-fos* Promoter

(A) Chromatin from serum-starved and mock- or EGF-stimulated cells were harvested and immunoprecipitated with the indicated antibodies. The *c-fos* and *H4* genomic DNA sequences present in the immunoprecipitated samples were amplified by quantitative PCR. The relative levels of *c-fos* DNA were calculated by normalizing to the level of H4 DNA. The average, normalized levels of *c-fos* DNA from two independent trials are presented in graphical form. The error bars indicate standard deviation calculated from duplicate trials.

(B) Serum-starved cells were pretreated with DMSO (mock treatment) or PD98059 for 90 min prior to EGF stimulation. The relative protein levels of phosphorylated Rsk2, phosphorylated MAP kinases (ERKs 1 and 2), and phosphorylated H3 present in the mock- or EGF-treated cells were determined using the indicated antibodies in Western blots.

(C) Chromatin from cells treated as in (B) were analyzed by chromatin immunoprecipitation assays using the Phos/Ac and Ac H3 antibodies. Quantification analyses similar to (A) were performed and presented as graphs. The error bars indicate the standard deviation calculated from duplicate trials.

upon EGF stimulation. Analyses of cells pretreated with PD98059, an inhibitor of the MAP kinase signaling pathway (Alessi et al., 1995), further support the link between acetylation and phosphorylation at the *c-fos* locus. Western blotting showed that, as expected, cells treated with PD98059 prior to EGF stimulation displayed significantly reduced levels of phosphorylated forms of MAP kinases, Rsk-2, and H3, compared to mock-treated (DMSO-treated) cells (Figure 6B). Interestingly, ChIP analysis indicated that treatment with PD98059 reduced the enrichment of dimodified H3, and also acetylated H3, at the *c-fos* gene to similar levels observed without stimulation (Figure 6C). These findings demonstrate that EGF-stimulated acetylation of H3 at the *c-fos* promoter is dependent on the integrity of the MAP signaling pathway and are consistent with our hypothesis that these two modifications are coupled.

Discussion

Synergistic Coupling of H3 Phosphorylation and Acetylation In Vivo

Given the high density of covalent modifications known to occur within the amino-terminal tails of histones, an intriguing question that arises is whether these modifications positively or negatively affect one another (Strahl and Allis, 2000).

Indeed, an earlier study reported that phosphorylated H3 is hyperacetylated in EGF-stimulated cells pretreated with butyrate, suggesting that these two modifications are somehow linked (Barratt et al., 1994). However, the use of butyrate to induce global hyperacetylation of histones may obscure any mitogen-induced acetylation of histones at specific loci. Therefore, we did not use any histone deacetylase inhibitors for this purpose to better

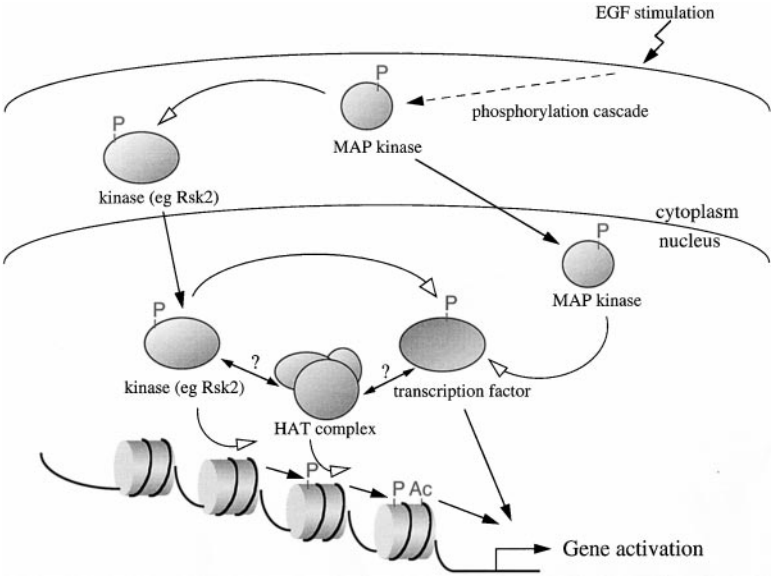

Figure 7. Model of Sequential Modifications of Chromatin in Response to EGF Stimulation

Activation of MAP kinase pathway by EGF stimulation leads to the sequential phosphorylation of MAP kinases and effector kinases such as Rsk2 (Hunter, 2000). These kinases then migrate into the nucleus where the effector kinases can phosphorylate H3 or other transcription factors. We suggest that the phosphorylated H3 is then rapidly acetylated due to its enhanced efficiency as a substrate for HAT complexes, and the potential association of the histone kinase with a HAT complex (question marks). Such localized changes in chromatin structure, in combination with the activated transcription factors, results in expression of selected downstream genes. Straight closed arrows indicate proposed sequential course of events, curved open arrows indicate enzyme reactions, and double-headed arrows represent potential physical interactions between different molecules and complexes.

define the links between histone phosphorylation and acetylation in response to EGF stimulation.

Using an antibody selective for Ser-10-phosphorylated and Lys-14-acetylated H3, we demonstrate that EGF induces a rapid and transient accumulation of H3 molecules bearing both modifications in mammalian cells. Furthermore, kinetic studies demonstrate that, following EGF treatment, peak accumulation of the singly phosphorylated H3 preceded that of dimodified (phosphorylated and acetylated) H3. The simplest interpretation of our data is that H3 is first phosphorylated and then rapidly acetylated to generate the dimodified subset of H3. Interestingly, while EGF treatment does not detectably alter the steady-state levels of acetylated H3, there was an increased association of acetylated H3 at the EGF-activated *c-fos* promoter, suggesting that localized H3 acetylation is involved in the mitogen-activated gene expression. Moreover, this locus-specific H3 acetylation is sensitive to PD98059, suggesting that H3 acetylation at this immediate-early gene promoter is dependent on MAP kinase signaling and is likely linked to H3 phosphorylation.

Phosphate-Directed Substrate Recognition by a Prototypical HAT

A clue to the functional link between H3 phosphorylation and acetylation seen in vivo is provided by our in vitro finding that yeast Gcn5 displays a stronger (up to 10-fold) preference for Ser-10 phosphorylated H3 peptide as substrate when compared to an unmodified control peptide. This effect is likely due to phosphate-dependent stabilization of the enzyme–substrate complex since a single amino acid substitution (R164K) of Gcn5 at the predicted site of phospho-Ser-10 interaction completely abolished Gcn5's higher affinity for phosphorylated H3. These data show that the observed differences in enzymatic rates are greatest when substrate concentrations are at or below the K_m value. Since most enzymes operate in this concentration range, we suspect these inherent differences in substrate affinity have significant physiological consequences. At present, the roles of H3 phosphorylation in gene expression in yeast are not well defined. Analyses of the phenotype of yeast strains bearing mutations in Gcn5 at R164 are currently underway to further identify links between H3 phosphorylation and acetylation in yeast (Lo et al., 2000 [this issue of *Molecular Cell*]).

The preference of wild-type Gcn5 for phosphorylated H3 peptide, which we refer to as phosphate-directed substrate recognition, provides evidence that one covalent modification can alter the efficiency of a subsequent enzymatic reaction on the same histone molecule. Several human HATs such as PCAF and p300 also prefer phosphorylated H3 as substrate (Lo et al., 2000); however, whether other families of HATs such as CBP or $TAF_{II}250$ also display such a preference remains to be determined. We note that transcription-associated H3 phosphorylation only occurs in discrete regions of the genome, and therefore acetylation of bulk chromatin at lysine 14 is clearly not dependent on phosphorylation. However, for signaling pathway-activated genes such as *c-fos*, the coupling of histone phosphorylation and acetylation at specific loci may contribute to mechanisms that allow them to be rapidly activated in response to external stimuli. The concept of coupled modifications need not be confined to relationships between histone kinases and HATs. The recent discovery of a nuclear receptor coactivator-interacting protein, CARM1, which possesses arginine-specific H3 methyltransferase activity (HMT), provides evidence that histone methylation also contributes to transcriptional activation (Chen et al., 1999). Interestingly, methylated H3 has been reported to occur in a subset of hyperacetylated H3 molecules in several systems (Annunziato et al., 1995; Strahl et al., 1999); therefore, these two modifications may also be coupled in some functional way. Different combinations of modifications may allow the limited set of histone modifications that are targeted to histone N-terminal tails to regulate a multitude of cellular processes (Strahl and Allis, 2000). Moreover, this general concept can easily be extended to nonhistone substrates such as p53, which is also regulated by phos-

phorylation and acetylation (Gu and Roeder, 1997; Prives and Hall, 1999).

Although the phosphorylated H3 peptide was acetylated more efficiently by Gcn5 in vitro, only a subset of phosphorylated H3 was acetylated in EGF-stimulated cells. The acetylation of phosphorylated H3 in vivo is undoubtedly further determined by the abundance and targeting of the relevant HATs. In response to EGF stimulation, only a small set of genes are activated. Consistent with this, immunofluorescence studies using the Phos H3 and Phos/Ac H3 antibodies show punctate nuclear staining in the EGF-stimulated cells, in contrast to the more diffuse nuclear staining observed with antibodies directed against acetylated H3 (Chadee et al., 1999; data not shown). Therefore, targeting of histone kinases and HATs is likely to play an important role in regulating the mitogen response. It has been shown that pp90Rsk is associated with CBP in mitogen-stimulated cells (Nakajima et al., 1996), and recently we reported that the Rsk-2 isoform is required for EGF-stimulated phosphorylation of H3 at Ser-10 in vivo (Sassone-Corsi et al., 1999). These findings suggest the possibility that kinase and HAT activities in a single complex can be targeted together to selected promoters to activate expression.

Traditionally, nuclear responses to signal transduction pathways are thought to be mediated by activation of transcription factors via a number of well-documented phosphorylation cascades (Treisman, 1996; Cohen, 1997; Hunter, 2000). The discovery that H3 is a physiologically relevant substrate of some of these activated kinases (Sassone-Corsi et al., 1999; Thomson et al., 1999a) suggests that chromatin structure also plays a role in regulating the expression of immediate-early genes. The synergism between H3 phosphorylation and acetylation reported here further suggests that H3 phosphorylation may itself serve as an important integrator for signaling-mediated chromatin remodeling. As illustrated in Figure 7, we postulate that phosphorylation cascades induced upon EGF stimulation lead to targeting of histone kinases (such as Rsk-2) to selected gene loci and rapid phosphorylation of H3 in specific nucleosomes. With the enhanced efficiency of acetylation of phosphorylated H3, and the possible recruitment of a single complex containing both a histone kinase and a HAT, these effects could expedite acetylation of H3 at targeted promoters. Such localized changes in chromatin structure may then facilitate binding of EGF-activated transcription factors to activate expression of the appropriate downstream genes. By linking histone acetylation to the EGF-stimulated phosphorylation cascade, this model provides an attractive explanation of how the chromatin structure of genes, regulated by different factors, can be altered in a coordinate fashion in response to external stimuli.

Experimental Procedures

Generation of an Antibody Specific for Dimodified Histone H3

Peptide synthesis, conjugation of peptide to keyhole limpet hemocyanin, and immunization of rabbits were done as described previously (Hendzel et al., 1997). In this study, the H3 peptide used for antibody production (ARK*S*TGG*K*APRKQLC), contained a phosphorylated serine residue and an acetylated lysine residue, corresponding to amino acids 10 and 14 of H3 amino-terminal tail respectively (in italic type in the peptide sequence above). Rabbit serum was precipitated with 50% ammonium sulfate and then affinity purified using a Sulfolink (Pierce) column conjugated with the phosphorylated and acetylated H3 peptide.

Enzyme-Linked Immunosorbent Assay

Four separate H3 peptides were used in enzyme-linked immunosorbent assay (ELISA) assays to determine the specificity of the immunized rabbit serum and the affinity-purified antibody (see Figure 1A). The unmodified H3 peptide (ARTKQTARKSTGGKAPRKQL) corresponds to the first 20 amino acids of the H3 amino terminus. The singly phosphorylated, singly acetylated, and phosphorylated/acetylated peptides all correspond to amino acids 7–20 of the H3 amino terminus (ARK*S*TGG*K*APRKQLC; see Figure 1A) but differ by containing either a phosphorylated serine, an acetylated lysine, or both. Increasing amounts of peptides were coated onto ELISA plate wells by incubation in 0.05 M H_2CO_3. Subsequent washes, blocking with 1% BSA, incubation with primary and secondary antibodies were done in PBS containing 0.5% Tween 20. The bound horseradish peroxidase-linked secondary antibody was detected using OPD substrate (Sigma), and the reactions were quantified by measuring the absorbance at 495 nm.

Cell Culture, EGF Stimulation, and Harvesting of Protein Samples

Mouse C3H 10 T1/2 cells were obtained from ATCC and cultured in Basal Medium Eagle (GIBCO-BRL) supplemented with 10% FBS. Cells were grown in medium without serum for 24 hr prior to EGF stimulation. When used, PD98059 was added to cells at a final concentration of 50 μM for 90 min prior to stimulation by EGF. The PD98059 compound was dissolved in DMSO, and equivalent amounts of DMSO was added to cells in mock-treated control samples. EGF (50 ng/ml) was added to cell cultures for various lengths of time, and total cellular proteins were harvested with boiling SDS buffer (10 mM Tris, 10 mM EDTA, 2% SDS, 20% glycerol, 0.05% bromophenol blue), and analyzed by SDS-PAGE and Western blotting. Alternatively, cells were lysed in 100 mM Tris (pH 7.6), 150 mM NaCl, 1.5 mM $MgCl_2$, 0.65% NP-40, 1 mM PMSF, 1 μM Microcystin LR, and 1 μg/ml each of aprotinin, pepstatin, and leupeptin. Nuclei pelleted from the lysis buffer were extracted with 0.4 N H_2SO_4, and acid-soluble histones were precipitated with 20% Trichloroacetic acid (TCA). Histones were resolved either by SDS-PAGE or on AU gels.

Electrophoresis and Western Blotting

SDS-PAGE was done as described by Laemmli (1970). Acid-urea electrophoresis was modified from the protocol described by Lennox and Cohen (1989). Briefly, sample buffer containing 6 M urea, 5% acetic acid, 12.5 mg/ml protamine sulfate, and 0.02% pyronin Y was electrophoresed through the 1.5 cm stacking gel (6% acrylamide, 6 M urea, 5% acetic acid) and 35 cm separating gel (15% acrylamide, 6 M urea, 5% acetic acid) for 16 hr. Histone samples were dissolved in 10 μl sample buffer and electrophoresed through the pre-run gel for 42 hr at 400V constant voltage with 5% acetic acid as the running buffer. Gels were either stained with Coomassie blue for visual inspection or histones were transferred to PVDF membrane for Western blot analysis. The antibody to Ser-10 phosphorylated H3 was described in Hendzel et al. (1997), and the antibody specific for acetylated H3 was raised against peptides acetylated at lysine 9 and 14 (Boggs et al., 1996). Western blot detection was by chemiluminescence using an ECL + kit (Amersham) as per manufacturer's instructions. For peptide competition experiments, 3 μg/ml of peptide was added to the diluted primary antibody prior to incubation of the blot.

In Vitro H3 Acetylation Assays with Recombinant Yeast Gcn5 and R164K Mutant

Expression and purification of the catalytic domain of yeast Gcn5 were performed as previously described (Tanner et al., 1999; Kim et al., 2000). The R164K mutant was generated by site-directed mutagenesis using the Bio-Rad Muta-gene method and the oligonucleotide, 5′-ATCGAAAGGTTTATATGTTAT-3′, harboring the R164K

mutation. The mutation was verified by DNA sequencing. Concentrations of purified enzymes were determined by the Bradford method.

For HAT assays, two separate peptides, ARTKQTARKSTGG KAPRKQL and the identical peptide with phosphoserine at position 10, corresponding to amino acid residues 1–20 of histone H3, were employed. The steady-state initial velocities were determined using increasing concentrations of each peptide (up to 1.5 mM) at a fixed concentration of AcCoA (50 μM), in pH 7.5 buffer at 22°C. Two distinct HAT assays were utilized to fully elucidate any observed changes: the commonly used filter binding endpoint assay (Tanner et al., 1999) and a newly developed continuous assay (Kim et al., 2000). To obtain the steady-state kinetic constants K_{cat} and K_m, the individual data sets were fitted to the Michaelis-Menten equation: velocity $= K_{cat} \times [S]/(K_m + [S])$.

Chromatin Immunoprecipitation

Mock-treated or EGF-stimulated cells were formaldehyde cross-linked, harvested, and disrupted by sonication as previously described (Chadee et al., 1999). Cell lysates were immunoprecipitated using the Phos/Ac H3 or Ac H3 (recognizing H3 acetylated at either Lys-9 or -14) antibodies. Immunoprecipitation and washing of the protein A bound–chromatin were also done as described by Chadee et al. (1999); however, the immunoprecipitated chromatin was then eluted from protein A by incubation in 2% SDS, 0.1 M $NaHCO_3$, and 10 mM DTT. The eluted samples were incubated at 65°C for 6 hr to reverse DNA–protein cross-links, and the DNA was purified by phenol-chloroform extraction and recovered by ethanol precipitation. Immunoprecipitated DNA samples were resuspended in dH_20, a fraction of which was used in PCR analysis. The primer pair CACGGCCGGTCCCTGTTGTTC and GTCGCGGTTGGAGTAGTAG GCG was used to PCR amplify *c-fos* promoter sequences present in the immunoprecipitated DNA. The primers, GACACCGCATGCA AAGAATAGCTG and CTTTCCCAAGGCCTTTACCACC, were also added to all PCR reactions to amplify *H4* DNA sequences as an internal control. PCR products were phenol chloroform extracted once and resolved on 15% polyacrylamide gels.

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