We examined the phosphorylation and acetylation of histone H3 in ovarian granulosa cells stimulated to differentiate by follicle-stimulating hormone (FSH). We found that protein kinase A (PKA) mediates H3 phosphorylation on serine 10, based on inhibition exclusively by PKA inhibitors. FSH-stimulated H3 phosphorylation in granulosa cells is not downstream of mitogen-activated protein kinase/extracellular signal-regulated kinase, ribosomal S6 kinase-2, mitogen- and stress-activated protein kinase-1, p38 MAPK, phosphatidylinositol-3 kinase, or protein kinase C. Transcriptional activation-associated H3 phosphorylation on serine 10 and acetylation of lysine 14 leads to activation of serum glucocorticoid kinase, inhibit α, and c-fos genes. We propose that phosphorylation of histone H3 on serine 10 by PKA in coordination with acetylation of H3 on lysine 14 results in reorganization of the promoters of select FSH responsive genes into a more accessible configuration for activation. The unique role of PKA as the physiological histone H3 kinase is consistent with the central role of PKA in initiating granulosa cell differentiation.

Maturation of ovarian follicles to a preovulatory stage requires follicle-stimulating hormone (FSH) production by the pituitary gland. The FSH receptor is a member of the G protein-coupled seven-transmembrane receptor family and is coupled to adenyl cyclase (1). It is expressed exclusively on ovarian granulosa cells in female mammals (2). Most of the actions of FSH are mediated by cAMP formation and activation of protein kinase A (PKA), based on the ability of cell-permeable cAMP analogs to mimic the known differentiation responses to FSH in granulosa cells (2) and on the ability of the PKA inhibitors H89 (3) and KT5720 (4) to inhibit granulosa cell differentiation. The downstream consequences of FSH are well established and include, for example, the induction of receptors for luteinizing hormone (LH) and prolactin, induction of enzymes associated with the increased steroidogenic capacity of granulosa cells including P450 aromatase and cholesterol side chain cleavage, induction of proteins associated with PKA signaling including RIβ (2, 4) and AKAP80 (5), and expression of the hormone inhibin (6). However, gene and/or protein induction for these responses to FSH is generally delayed by at least 24 h (2–4, 7). The more immediate responses to FSH, which lead to the induction of immediate early genes such as c-fos and serum glucocorticoid kinase (SGK) (3, 8), are less well understood. Further elucidation of the FSH signaling pathways that lead to the induction of immediate early genes would be useful to understand how FSH initiates granulosa cell differentiation. We have previously shown that FSH (via PKA) promotes activation of the p42/p44 mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway (9). FSH also activates the p38 MAPK pathway (10) and downstream phosphorylation of the heat shock protein (HSP) 27, leading to granulosa cell rounding (10). We and others (11) have recently identified phosphatidylinositol-3 kinase (PI-3 kinase) as a downstream target of FSH and cAMP. The transcription factor cAMP-response element-binding protein (CREB) is also phosphorylated in response to FSH in a cAMP-dependent manner (3, 12, 13). We recently reported that histones H1 and H3 are also downstream targets of FSH and PKA in granulosa cells (14).
Under conditions in which FSH stimulates granulosa cell differentiation and not proliferation (2, 15), histone H3 is transiently phosphorylated exclusively on serine 10 (14). The addition of factors that do not promote granulosa cell differentiation (2), such as epidermal growth factor (EGF), does not lead to histone H3 phosphorylation (14). This phosphorylation event appears to represent an early marker of differentiation and is distinct from the well established marker of chromosome condensation during mitosis (16–18) in granulosa cells as these cells undergo differentiation and not proliferation in response to FSH in serum-free cultures.

While histone H3 phosphorylation on serine 10 has traditionally been viewed as a marker for mitosis (16–18), there were also reports in the early 1980s that histone H3 was phosphorylated in response to differentiative stimuli such as nerve growth factor in PC-12 cells and cAMP/proteorin in C6 glioma cells (19, 20). Ten years later, the rapid phosphorylation of a small fraction of histone H3 on serine 10 was reported in quiescent C3H 10T1/2 mouse fibroblasts in response to phorbol esters, okadaic acid, EGF, and protein synthesis inhibitors coincident with the induction of immediate early genes such as c-fos and c-jun (21, 22).

Recent reports have identified two related histone H3 kinases as likely mediators in these pathways. Both ribosomal S6 kinase-2 (RSK-2), which is downstream of p42/p44 MAPK/ERK, and mitogen- and stress-activated protein kinase-1 (MSK-1), which is downstream of both MAPK/ERK and p38 MAPK (23), phosphorylate histone H3 in vitro (24–26). EGF-stimulated histone H3 phosphorylation was restored after the addition of the RSK-2 gene to a fibroblast cell line derived from patients with Coffin-Lowry syndrome with mutations in the RSK-2 gene (24). In quiescent C3H 10T1/2 mouse fibroblasts, anisomycin- and phorbol ester-stimulated histone H3 phosphorylation was inhibited by the p38 MAPK inhibitor SB203580 (27) and the MAPK/ERK kinase (MEK) inhibitor PD98059 (28, 29), respectively. Both phorbol ester- and anisomycin-stimulated histone H3 phosphorylation were inhibited by H89, based on the ability of this kinase inhibitor to inhibit MSK-1 (25) rather than its well recognized inhibition of PKA (30, 31). Similarly in JB6 epidermal C1 cells, ultraviolet B-induced histone H3 phosphorylation at serine 10 was inhibited by the MEK inhibitor PD98059 or by the p38 MAPK inhibitor SB202190 (32). These reports thus establish a prominent role for RSK-2 and MSK-1 downstream of the p38 MAPK and/or ERK/MAPK pathways in growth factor- and phorbol ester-stimulated histone H3 phosphorylation.

As FSH stimulates the MAPK/ERK pathway in granulosa cells in a PKA-dependent manner (9), we hypothesized that, in fibroblasts and epidermal cells, histone H3 phosphorylation in granulosa cells might be mediated by either RSK-2 and/or MSK-1 activated by a MAPK pathway downstream of PKA. We additionally sought direct evidence that histone H3 phosphorylation is functionally linked to the induction of characteristic FSH-responsive immediate early genes. Finally, based on abundant evidence that histone acetylation is associated with transcriptional activation (33, 34), we sought to determine whether acetylation of histone H3 on lysine 14 and/or lysine 9 is stimulated by FSH.

EXPERIMENTAL PROCEDURES

Materials—Ovine FSH (eFSH-19) was kindly provided by Dr. A. F. Parlow of the National Hormone and Pituitary Agency of the National Institute of Diabetes and Digestive and Kidney Diseases (Torrence, CA). The following were purchased: H89, AG1478, GF109203X, KT5720, S-(4-chlorophenylthio)-cAMP (CPT-cAMP), phorbol myristic acid (PMA), and okadaic acid from LC Laboratories (San Diego, CA); PD98059, SB203580, myristoylated PKA inhibitor (PKI) 14–22 amide, and wortmannin from Calbiochem; anti-histone H3 acetylated on lysines 9 and/or 14 (anti-H3AcLys-9/Lys-14), anti-histone H3 acetylated on lysine 14 (anti-H3AcLys-14) or lysine 9 (anti-H3AcLys-9), anti-histone H3 phosphorylated on serine 10 (anti-H3PhSer-10), anti-histone H3 both phosphorylated on serine 10 and acetylated on lysine 14 (anti-H3PhSer-10/Lys-14), anti-CREB phosphorylated on serine 133, and anti-CREB antibodies from Upstate Biotechnology (Lake Placid, NY); anti-histone H3, anti-MAPK/ERK phosphorylated on threonine 202 and tyrosine 204, anti-p38 MAPK phosphorylated on threonine 180 and tyrosine 182, anti-MSK-1 phosphorylated on serine 376, and anti-RSK phosphorylated on serine 381 (which detects RSK-1 and RSK-2) antibodies from New England Biolabs/Cell Signaling (Beverly, MA); anti-phospho-PKA antibody from Santa Cruz Biotechnology (Santa Cruz, CA); anti-MAPK/ERK antibody from Zymed Laboratories Inc. (San Francisco, CA); anti-HSP27 antibody from StressGen (Victoria, British Columbia); trichostatin A (TSA) from Wako (Osaka, Japan); fluorescein isothiocyanate (FITC) from Molecular Probes, Inc. (Eugene, OR); and anti-hemagglutinin peptide antibody and H3 purified from calf thymus from Roche Molecular Biochemicals. All other chemicals were from sources described previously (14, 35, 36). Equivalent results for SDS-PAGE and acid urea gels were obtained with anti-H3PhSer-10 and anti-H3PhSer-10/Lys-14 antibodies obtained from Upstate Biotechnology (and licensed from Dr. David Allis) and directly from the Allis laboratory. Recognition by anti-H3PhSer-10 antibody of H3 phosphorylated on serine 10 was not affected by acetylation of lysines 9 and/or 14 (Fig. 1); anti-H3PhSer-10/Lys-14 antibody was specific for H3 phosphorylated on serine 10 and acetylated on lysine 14 (37).

Production of TAT-PKI Fusion Protein—An expression vector containing PKI fused to the cell entry leader sequence TAT was constructed by PCR. Oligonucleotides 5′-CGCGTTACCTCCCTGCTATGTAGATTTG and 5′-CCCGTCTGAGCTTGTGATTGATTTG, which add the restriction sites KpnI and XhoI, respectively, were used to amplify PKI using Jurkat cell cDNA as template. The fragment was cut with the above restriction enzymes and subcloned into the pTAT vector (38) generously provided by Dr. Steven Dowdy. TAT-PKI protein was expressed in BL21 DE3 pLysS bacteria and purified by fast protein liquid chromatography. The protein was labeled with FITC and determined to enter cells using a fluorescence-activated cell sorter (Beckman Instruments). Expressed, purified protein was tested for functionality by adding it to an in vitro PKA assay. PKA activity in lysates was stimulated 2.7-fold by the addition of TAT-PKI. This stimulation was inhibited 84% by 250 nm TAT-PKI, which is equivalent to the 83% inhibition obtained using the PKI inhibitor peptide.

Granulosa Cell Culture, Western Blotting, and Immunofluorescence—Granulosa cells were isolated from the ovaries of 26-day-old Sprague-Dawley rats that had been primed with subcutaneous injections of 1.5 mg of estradiol-17β on days 23–25 to promote the growth of preantral follicles (5, 14). Cells were either plated on 33-mm plastic dishes (Falcon) at a density of ~3 × 10⁶ cells/dish (14) or on coverslips (for immunofluorescence) and then treated with the indicated additions 20 h after plating. For subsequent Western blotting, treatments were terminated by aspirating medium, rinsing cells once with phosphate-buffered saline, and collecting total cell extracts in 0.5 ml of SDS sample buffer containing 6 M urea, 5% acetic acid, and triton x-100 0.5% (for immunofluorescence) and then treated with the indicated additions 20 h after plating. For subsequent Western blotting, treatments were terminated by aspirating medium, rinsing cells once with phosphate-buffered saline, and collecting total cell extracts in 0.5 ml of SDS sample buffer containing 1% Triton X-100 in phosphate-buffered saline, and incubated for 2 h at 37 °C with anti-H3PhSer-10 antibody (1:50 dilution) in phosphate-buffered saline containing 5% normal goat serum. Coverslips were washed and incubated for 1 h at 37 °C with FITC-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA). Cells on coverslips were washed and mounted on slides in dianobiphyllate(2,2,2)octane antifading medium (41).
RESULTS

Immunolocalization of Phospho-histone H3 in FSH-treated Granulosa Cells—Phospho-histone H3 is undetectable in vehicle-treated granulosa cells (Fig. 1C). Treatment of cells with FSH for 1 h results in the detection of phospho-histone H3 in small foci in the nuclei of most but not all granulosa cells (compare Fig. 1, B and D). Overexposure results in complete nuclear staining, as seen in the cell nucleus detected in Fig. 1D, lower portion.

FSH Stimulates Histone H3, MAPK/ERK, and RSK 2 Phosphorylation in a Time-dependent Manner—Recent evidence in fibroblasts that has identified both RSK-2, downstream of p42/44 MAPK/ERK, and MSK-1, downstream of both p42/44 MAPK/ERK and p38 MAPK, as histone H3 kinases (24, 25). We first compared the rates of phosphorylation/activation of the p42/44 MAPK/ERK pathway, histone H3 phosphorylation on serine 10, and CREB phosphorylation on serine 133. Increased phosphorylation of CREB, an established PKA target (46), is first detected by Western blot at 1 min and increases further at 10 min after FSH addition; increased phosphorylation of histone H3 and MAPK/ERK is detected by 10 min, and increased RSK-2 phosphorylation is detected at 20 min post-FSH (Fig. 2). Histone H3 phosphorylation peaks at 1 h. By 2 h, MAPK/ERK and RSK-2 phosphorylations have returned to basal levels; CREB and histone H3 phosphorylations returned to basal levels by 4 h post-FSH. MSK-1 phosphorylation is also detected in vehicle-treated cells but is not affected by FSH (not shown). These results show that FSH promotes both MAPK/ERK and RSK-2 phosphorylation, consistent with our previous evidence that FSH stimulates RSK phosphorylation in granulosa cells incubated with [32P]dCTP (9).

**FSH-stimulated Histone H3 Phosphorylation Is Inhibited by PKA Inhibitors but Is Not Blocked by MEK, p38 MAPK, EGF Receptor Tyrosine Kinase, PI-3 Kinase, Protein Kinase C, or RSK-2 Inhibitors—**Based on evidence in fibroblasts and epithelial cells that histone H3 phosphorylation is blocked by inhibitors of the p38 MAPK and/or the MAPK/ERK pathways, we tested inhibitors of these and other pathways on FSH-stimulated histone H3 phosphorylation. Cells were pretreated with inhibitors for 30, 60, or 90 min, as indicated in the legend for Fig. 3, and then treated for 1 h with FSH, the adenyl cyclase activator forskolin, the protein kinase C activator PMA, or the cell-permeable cAMP analog CPT-cAMP. Although a 1-h treatment with FSH is optimal for H3 phosphorylation, MAPK/ERK phosphorylation is declining or has declined to basal levels by this time (Fig. 2). Neither the MEK inhibitor PD98059 (28, 29) nor the p38 MAPK inhibitor SB203580 (27), separately or together, inhibits histone H3 phosphorylation (Fig. 3A). Similarly histone H3 phosphorylation is not altered by the RSK-2/protein kinase C inhibitor GF109203X (47), although this agent does inhibit the modest stimulation by FSH of histone H3 phosphorylation (Fig. 3B, lanes 5 and 21). PD98059 inhibits all detectable MAPK/ERK phosphorylation stimulated by FSH and forskolin as well as FSH- and forskolin-stimulated RSK-2 phosphorylation (Fig. 3A). The p38 MAPK inhibitor SB203580, which has previously been shown to inhibit FSH-stimulated p38 signaling to HSP27 (10), also abolishes RSK-2 phosphorylation (Fig. 3A), suggesting that in granulosa cells, as in some other cells (48–50), RSK-2 is downstream not only of MAPK/ERK but also of p38 MAPK. Histone H3 phosphorylation is also
unaffected by the EGF receptor tyrosine kinase inhibitor AG1478 (51), which consistently abolishes MAPK/ERK and RSK-2 phosphorylation (Fig. 3B). Likewise, H3 phosphorylation is unaffected by the PI-3 kinase inhibitor wortmannin (52) (Fig. 3B), which effectively reduces PI-3 kinase-dependent protein kinase B (AKT) phosphorylation. The increased MAPK/ERK phosphorylation seen in wortmannin-treated cells could reflect inhibition by wortmannin of the inhibitory PI-3 kinase-dependent Raf phosphorylation (53).

Only the PKA inhibitors H89 (30, 31) (Fig. 3B) and KT5720
histone H3 phosphorylation is reduced (55) and then treated for 1 h with vehicle or FSH. Results (Fig. 3D) show that histone H3 phosphorylation is reduced ~4-fold in cells treated with TAT-PKI. Similarly, in cells pretreated with a myristoylated PKI peptide, FSH-stimulated H3 phosphorylation is ablated (Fig. 3E). These results indicate that histone H3 phosphorylation in granulosa cells in response to FSH treatment is not downstream of MAPK/ERK, RSK-2, MSK-1, p38 MAPK, PI-3 kinase, or protein kinase C. Rather, histone H3 phosphorylation appears to be mediated directly by PKA. In vitro phosphorylation experiments confirm the ability of recombinant PKA catalytic subunit to directly phosphorylate purified histone H3 on serine 10 (Fig. 3F).

Effect of FSH on Histone H3 Acetylation—We next sought to determine whether the acetylation of histone H3 is linked to its phosphorylation. The results shown in Fig. 2 show strong reactivity to anti-histone H3 acetylated on either lysines 9 and/or 14 (anti-H3AcLys-9/Lys-14) antibody in granulosa cells in the absence of FSH treatment (lane 1). This result suggests that a portion of histone H3 is already acetylated in untreated cells. Treatment of granulosa cells with FSH over a 24-h time course does not cause a detectable change in this bulk acetylation of histone H3 (Fig. 2). Similarly, neither forskolin, PMA, CPT-cAMP, nor the addition of protein kinase inhibitors exerts a detectable modulation of histone H3 acetylated on lysines 9/14 (Fig. 3, A–C). To determine whether a selective deacetylation event precedes histone H3 phosphorylation on serine 10, cells were pretreated with vehicle or with the histone deacetylase inhibitor TSA. Results show that whereas pretreatment of cells for 1 h with TSA promotes the expected increase in the bulk acetylation of histone H3 (Fig. 4A, lanes 6–10 versus lanes 1–5), the FSH-stimulated phosphorylation of histone H3 on serine 10 is unaffected. Consistent with the characteristics of the phospho-H3 antibody (37), and in contrast to another phospho-H3 antibody (56), this result shows that increased acetylation of H3 does not appear to impede the ability of the anti-H3PhSer-10 antibody to detect phosphorylated H3. This result also suggests that acetylation does not predispose histone H3 to phosphorylation. We next sought to determine whether the fraction of histone H3 that becomes phosphorylated (on serine 10) also becomes acetylated (on lysine 14) in response to FSH, using an antibody reactive only with histone H3 both phosphorylated on serine 10 and acetylated on lysine 14 (anti-H3PhSer-10/AClys-14) (37). Results show that, as is true of H3 phosphorylation, this dual modification of histone H3 is detected by 10 min (Fig. 4B) and increases with time after FSH treatment of cells (Fig. 4C). Moreover, the increase in the relative signals detected by the two antibodies over time is not significantly different at any time point (Fig. 4C). Similar results were obtained when granulosa cell acid-soluble histones were separated on acid urea gels (Fig. 4D). These results suggest that FSH stimulates the phosphorylation of histone H3 molecules that are already acetylated on lysine 14 or that both modifications of H3 occur concurrently. That FSH most likely stimulates the apparently concurrent phosphorylation and acetylation of histone H3 is suggested by results showing that FSH also produces a modest increase by 10 min in the acetylation of histone H3 on lysine 14 (Fig. 4E) but not on lysine 9 and 14 (Fig. 4F). Consistent with the results shown in Fig. 3, the PKA inhibitor H89 abolishes the signal detected by the anti-H3PhSer-10/AClys-14 antibody (Fig. 4G). Taken together, these results indicate that FSH stimulates the phosphorylation of serine 10 via PKA as well as the acetylation on lysine 14 of a small fraction of histone H3.

FSH Stimulates the Association of Phosphorylated and Acetylated Histone H3 with Promoter Sequences of Serum Glucocorticoid Kinase, Inhibin α, and c-fos, but Not with the Progesterone Receptor—Finally we sought to determine whether the phosphorylation of histone H3 in response to FSH promotes the activation of genes specific for FSH but not those responsive to LH. Granulosa cells were treated with vehicle or FSH for 1 h, chromatin was cross-linked with formaldehyde, and histone H3 that was either phosphorylated on serine 10, acetylated on lysines 9 and/or 14, or phosphorylated on serine 10 and acetylated on lysine 14 was immunoprecipitated and protein-denatured. PCR was conducted on immunoprecipitated DNA using primers specific for promoter sequences of the FSH early response genes SGK and c-fos as well as for the later response gene inhibit a and for the progesterone receptor, which is an LH early response gene. As a control, PCR was also conducted on total genomic DNA. SGK and inhibit a are established downstream targets in granulosa cells of FSH and PKA (3, 7), whereas c-fos is generally thought to be downstream primarily of MAPK/ERK and RSK-2 (57, 58). Results show that there is ~3-fold more amplified PCR product in anti-H3PhSer-10 immunoprecipitates corresponding to SGK and inhibit a promoter sequences and to a lesser extent to c-fos from cells treated with FSH versus vehicle (Fig. 5A, lanes 1–4, lanes 9–12, and lanes 17–20). The response to FSH for the SGK, inhibit a, and c-fos promoters was completely inhibited by pretreating cells with the PKA inhibitor H89 (compare lanes 1 and 2 with lanes 5 and 6, compare lanes 9 and 10 with lanes 13 and 14, and compare lanes 17 and 18 with lanes 21 and 22). Promoter DNA corresponding to the progesterone receptor was not detected in the phosphorylated histone H3-chromatin pool of vehicle- or FSH-treated cells (Fig. 5A, lanes 25 and 26), although it was readily detected in total genomic DNA (lanes 27 and 28). This result is consistent with evidence that FSH does not promote activation of the progesterone receptor gene (4).

Similar to results following immunoprecipitation with anti-H3PhSer-10 antibody, immunoprecipitation with anti-H3AcLys-9/Lys-14 antibody evokes ~2-fold increased amplification of SGK and inhibit a promoter DNA (Fig. 5B, lanes 1–4 and lanes 9–12) but not of the progesterone receptor promoter (lanes 13–16). However, in contrast to phospho-histone H3-associated chromatin, the progesterone receptor promoter is readily detected in the chromatin pool associated with histone H3 acetylated on lysines 9 and/or 14 (lanes 13–16). Only a minor increase in c-fos promoter amplification is detected in response to FSH in the anti-H3AcLys-9/Lys-14 immunoprecipitates (lanes 5–8).

We additionally assessed whether there is a direct link between both phosphorylation on serine 10 and acetylation on lysine 14 in histone H3 and promoter activation. Immunoprecipitation with anti-H3PhSer-10/AClys-14 antibody results in ~4-fold more amplified PCR product corresponding to SGK, inhibit a, and c-fos promoter sequences in FSH versus vehicle-treated cells (Fig. 5C).

These results demonstrate that although FSH treatment of granulosa cells does not promote a detectable increase by Western blotting in bulk levels of acetylated histone H3 (on lysines 9 and/or 14) (Figs. 2 and 3), in the ChIP assay, more promoter DNA characteristic of FSH response genes is associated with

4 O. K. Parke-Sarge, personal communication.
the acetylated histone H3 after FSH treatment of granulosa cells. These results therefore support our evidence (presented in Fig. 4E) showing that FSH stimulated a modest increase in H3 acetylation on lysine 14, and these results are consistent with the idea that only a small fraction of total cellular histone H3 is acetylated at specific loci in response to FSH treatment of granulosa cells. In contrast to bulk histone H3 acetylation levels, increased levels of phosphorylated histone H3 as well as of histone H3 both phosphorylated on serine 10 and acetylated on lysine 14 are readily detected by Western blotting in FSH-treated granulosa cells and are associated with increased promoter amounts of FSH-responsive genes. These results thus...
provide direct evidence that both phosphorylation (on serine 10) and acetylation (on lysine 14) in histone H3 are associated with FSH-responsive promoters.

DISCUSSION

FSH stimulates the differentiation of granulosa cells to a preovulatory phenotype (2). In the intact ovary, FSH also stimulates granulosa cell proliferation (59, 60), although this response to FSH is lost in serum-free granulosa cell cultures (2, 15), presumably because of the loss of the contribution of growth factors by adjacent thecal cells (61). It is generally believed that most of the cellular pathways downstream of the FSH receptor that lead to both granulosa cell proliferation and differentiation are mediated, directly or indirectly, by PKA (2, 4, 62). It is interesting that granulosa cells appear to express the same signaling pathways that in other cells are activated by growth factors or phorbol esters and are often inhibited by PKA (63, 64), yet in granulosa cells, these pathways are activated by PKA. A prime example is the MAPK/ERK pathway, which in granulosa cells is activated downstream of PKA (9, 10, 65). Thus, granulosa cells must have evolved unique routes for PKA to modulate these conserved signaling pathways.

Consistent with the hypothesis that FSH uniquely signals predominately via PKA, we have shown in this report that...
histone H3 phosphorylation in granulosa cells appears to be mediated directly by PKA. In other cellular models, histone H3 phosphorylation is downstream of the p38 MAPK and/or MAPK/ERK pathways and mediated by RSK-2 or MSK-1 (24, 25, 32, 66). However in granulosa cells, inhibition of these pathways (by PD98059, SB203580, GF109203X, or AG1478) does not diminish FSH-stimulated histone H3 phosphorylation. Therefore in granulosa cells, histone H3 phosphorylation is not a consequence of the activation of the MAPK pathways. Our results support the hypothesis that the catalytic subunit of PKA directly phosphorylates histone H3, based on stimulation of histone H3 phosphorylation by cell-permeable cAMP analogs as well as by the adenyl cyclase activator forskolin and on inhibition by the ATP competitive inhibitors H89 and KT5720. Although H89 also inhibits MSK-1 (25), the absence of FSH-stimulated MSK-1 phosphorylation coupled with the lack of inhibition of FSH-stimulated histone H3 phosphorylation by inhibitors upstream of MSK-1 indicates that the effect of H89 in granulosa cells is on PKA and not on MSK-1. In support of these data, histone H3 phosphorylation was inhibited on the introduction into granulosa cells of the specific PKA inhibitor protein PKI using the TAT transducing peptide as well as inhibited by myristoylated PKI. We and others have shown that histone H3 is readily phosphorylated in vitro by the catalytic subunit of PKA purified from mammalian sources (14, 67, 68). We show herein that recombinant purified PKA catalytic subunit also directly phosphorylates histone H3 on serine 10, as detected with the anti-H3phSer-10 antibody. Moreover, the catalytic subunit of PKA is known to translocate into the nucleus of granulosa cells in response to FSH treatment to phosphorylate nuclear substrates such as CREB (3, 14). Although we have been unsuccessful in our attempts to cross-link histone H3 and the catalytic subunit of PKA, the inability to demonstrate the physical association of these two proteins most likely reflects the transient nature of this catalytic reaction.

Histones are ubiquitous proteins that organize DNA into nucleosomes and chromatin. Nucleosomes consist of an inner core of two copies each of the four core histones, H2A, H2B, H3, and H4, surrounded by 146 base pairs of double-stranded DNA (69). H1 histones bind to the outer surface of the DNA that surrounds the core histones and to the stretches of linker DNA that connect histones (69). There is compelling evidence that chromatin remodeling involves the modification of core histones in nucleosomes in activated target genes (33, 70–72). These core histone modifications generally consist of the covalent addition of an acetyl or phospho group to lysines or serines, respectively, primarily in the N-terminal tails of the core histones H3 and H4. Acetylation neutralizes the positive charge of the histone, and phosphorylation adds a negative charge to histone, thereby decreasing the affinity of histone for DNA (33). The predicted result of these histone modifications is the destabilization of nucleosomes and chromatin structure, resulting in the access of select promoter regions to transcription factors and co-activators (72, 73). In support of these suppositions, mutational analysis of yeast Gcn5, one of the well known nuclear histone acetyltransferases (HATs), indicates a direct role for histone acetylation in the transcriptional activation of target genes in vivo (74, 75). It has been similarly shown that the substitution in Gcn5 of arginine 164 for alanine, a residue close to serine 10 in the structure of the ternary Gcn5-CoA-histone H3 complex, reduces promoter activity of a set of Gcn5-dependent genes; mutation of serine 10 to alanine in histone H3 impairs transcription of the same set of genes (76).

Recent evidence from yeast and EGF-treated C3H10T1/2 fibroblasts indicates that acetylation on lysine 14 rapidly follows phosphorylation on serine 10 in histone H3 (37, 76). Consistent with these results, in vitro studies show that several HATs, including p300 and p300/CREB-binding protein (CBP)-associated factor (PCAF) and Gcn5, display increased HAT activity toward a histone H3 peptide phosphorylated on serine 10 versus the unphosphorylated or mutated peptide (37, 76). However, these authors did not detect global modifications in histone H3 acetylation (by Western blotting with anti-H3acLys-9/Lys-14 antibody) (37), suggesting that these modifications are restricted to a small portion of growth factor-responsive genes in the genome. While our data show that FSH stimulates both the phosphorylation (on serine 10) and acetylation (on lysine 14) of histone H3 in primary rat granulosa cells and that both modifications occur on at least a pool of the same H3 molecules, phosphorylation and acetylation occurred in a statistically simultaneous manner, although in some experiments, phosphorylation appeared to precede acetylation (Fig. 4C). Additional studies are needed to resolve the precise timing of H3 phosphorylation versus acetylation in response to FSH in granulosa cells. It has been proposed that transcriptional activation requires both the serine 10 phosphorylation and acetylation on lysine 14 of histone H3, whereas mitotic activity requires only histone H3 phosphorylation (73). As granulosa cells exhibit differentiation and proliferation responses to FSH in the absence and presence, respectively (2), of growth factors, this cell offers an ideal model to explore this hypothesis.

We detected increased representation of c-fos and SGK promoter DNA in the phosphorylated, acetylated, and dual-phosphorylated and acetylated histone H3-chromatin pools in response to FSH treatment of granulosa cells in ChIP assays. Similarly in C3H 10T1/2 fibroblasts, EGF-stimulated phosphoacetylation of H3 is associated with c-fos activation (37, 56). However, the activation of c-fos in granulosa cells most likely reflects signaling to the c-fos gene via PKA and CREB rather than the more commonly used MAPK/ERK pathway (57, 58) as the c-fos promoter contains a functional CAMP response element (77) that can bind phospho-CREB, leading to the recruitment of CBP and perhaps other co-activators to activate the c-fos gene.

Transcriptional activation of the SGK gene in response to FSH/PKA requires an Sp1/Sp3 binding site (78). Sp1 sites are also required both for FSH/PKA induction of the LH receptor gene (79) and the cholesterol side chain cleavage CYP11A gene (80) and for LH/PKA induction of the progesterone receptor gene (81), but the mechanism by which PKA modulates Sp1-directed gene activation is not known. Because we have shown that both phosphorylation and acetylation of histone H3 are linked to the rapid activation of the SGK gene, based on our evidence that FSH increases the amount of SGK promoter DNA in immunoprecipitates of phosphorylated, acetylated, and dual-phosphorylated and acetylated histone H3, we anticipate that co-activators with HAT activity, such as CBP or PCAF, may complex with Sp1 at the SGK promoter. These findings also raise the possibility that some of the transcriptional effects of PKA may reflect H3 phosphorylation and chromatin reorganization rather than, or in addition to, direct phosphorylation of transcription factors.

We also detected increased representation of inhibin a promoter DNA in the phosphorylated, acetylated, and dual-phosphorylated and acetylated histone H3-chromatin pools in response to FSH treatment of granulosa cells. Inhibin a is generally considered to be a relatively late FSH responsive gene (82) as maximal mRNA levels are not reached until 12–24 h after FSH addition. However, inhibin a mRNA is detectable as early as 4 h after FSH (83). Interestingly in our ChIP assays, histone H3, which contains the dual modifications of phosphorylation and acetylation, is already associated with the inhibin a promoter by a 1-h post-FSH treatment. Perhaps the ChIP
PKA-dependent Histone H3 Phosphorylation and Acetylation

assay detects a reorganization of the promoter into a relatively more accessible configuration for the entry of various co-activators prior to the activation of transcription. Activation of the inhibin α gene is regulated by the nuclear transcription factor steroidogenic factor-1 (SF-1) (36). Recent studies have shown that SF-1, in conjunction with PKA, CREB, and CBP, synergize to strongly activate the inhibin α promoter (36). SF-1 and CREB constitutively bind to adjacent sites on the inhibin α promoter and physically interact (36). CBP is known to be recruited by and to bind not only to phospho-CREB (84, 85) but also to SF-1 (86, 87) and to enhance transcription of SF-1-regulated genes (87). Using the ChIP assay, it was shown that PKA and SF-1 increased the histone H4 acetylation associated with the inhibin α promoter (36). The increased HAT activity is believed to derive either from the recruitment of CBP (88, 89) and to physically interact, as demonstrated both in yeast (90) and in mammalian cell lines (91). The role of PKA in the formation of a complex between phospho-CREB and the inhibin α promoter (86, 87) has also been shown to be duplicated in target cells for such hormones as thyroid-stimulating hormone, LH, epinephrine, and vasopressin, for instance. PKA-mediated histone H3 phosphorylation could prove to be a universal mechanism to regulate gene activation.

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