Increased Ser-10 Phosphorylation of Histone H3 in Mitogen-stimulated and Oncogene-transformed Mouse Fibroblasts*

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When the Ras mitogen-activated protein kinase (MAPK) signaling pathway of quiescent cells is stimulated with growth factors or phorbol esters, the early response genes c-fos and c-myc are rapidly induced, and concurrently there is a rapid phosphorylation of histone H3. Using an antibody specific for phosphorylated Ser-10 of H3, we show that Ser-10 of H3 is phosphorylated, and we provide direct evidence that phosphorylated H3 is associated with c-fos and c-myc genes in stimulated cells. H3 phosphorylation may contribute to proto-oncogene induction by modulating chromatin structure and releasing blocks in elongation. Previously we reported that persistent stimulation of the Ras-MAPK signaling pathway in oncogene-transformed cells resulted in increased amounts of phosphorylated histone H1. Here we show that phosphorylated H3 is elevated in the oncogene-transformed mouse fibroblasts. Further we show that induction of ras expression results in a rapid increase in H3 phosphorylation. H3 phosphatase, identified as PP1, activities in PP1, and c-myc genes in stimulated cells. H3 phosphorylation may contribute to proto-oncogene induction by modulating chromatin structure and releasing blocks in elongation. Previously we reported that persistent stimulation of the Ras-MAPK signaling pathway in oncogene-transformed cells resulted in increased amounts of phosphorylated histone H1. Here we show that phosphorylated H3 is elevated in the oncogene-transformed mouse fibroblasts. Further we show that induction of ras expression results in a rapid increase in H3 phosphorylation. H3 phosphatase, identified as PP1, activities in ras-transformed and parental fibroblast cells were similar, suggesting that elevated H3 kinase activity was responsible for the increased level of phosphorylated H3 in the oncogene-transformed cells. Elevated levels of phosphorylated H1 and H3 may be responsible for the less condensed chromatin structure and aberrant gene expression observed in the oncogene-transformed cells.

Histone H1 and the N-terminal tail of H3 have key roles in the folding and inter-association of the chromatin fiber (1–5). Modification of the N- and C-terminal tails of H1 by phosphorylation or the N-terminal tail of H3 by acetylation and/or phosphorylation could destabilize higher order chromatin structure (6, 7). Myc- and ras-transformed and Rb-deficient fibroblasts have a more decondensed chromatin structure than parental cells (8–10). A general feature of these oncogene-transformed and Rb-deficient cells is increased H1 phosphorylation. H1 phosphorylation may relax chromatin by interfering with its action in chromatin folding and intermolecular fiber-fiber interactions (3).

Continuous stimulation of the Ras mitogen-activated protein kinase (MAPK)1 signaling pathway in mouse fibroblasts transformed with oncogenes ras, fos, mos, and c-myc elevates the level of phosphorylated H1 (9, 11). Activation of the Ras-MAPK signaling pathway of quiescent fibroblasts treated with growth factors or phorbol esters results in the phosphorylation of H3 (12–14). Thus, persistent activation of the Ras-MAPK pathway in oncogene-transformed cells may also increase the level of phosphorylated H3, contributing to the destabilization of the higher order compaction of chromatin.

The N-terminal domain of H3 can be phosphorylated on Ser-10 and/or Ser-28 (15, 16); however, the site of mitogen-induced phosphorylation remains unknown. Phosphorylation of H3 happens concurrently with the transcriptional activation of the immediate early response genes, e.g. c-fos (12, 13). However, inhibition of transcription does not prevent mitogen-activated H3 phosphorylation (12). It was proposed that phosphorylated H3 (pH3) is associated with the immediate early genes and may be a prerequisite to the expression of these genes (13). Unlike the extensive mitosis-specific phosphorylation, which occurs on all H3 molecules (17), this mitogen-stimulated phosphorylation is targeted to a small, hyperacetylation-sensitive nucleosomal fraction (18).

To study H3 phosphorylation in growth factor- or phorbol ester-stimulated quiescent and oncogene-transformed mouse fibroblasts, we used an antibody that specifically recognizes phospho-Ser-10 of H3. We show that mitogenic stimulation, oncogene transformation, or induction of oncogenic ras expression is accompanied with increases in Ser-10 phosphorylation of H3. We provide direct evidence that pH3 is associated with the induced c-fos and c-myc genes in mitogen-stimulated fibroblasts. The elevated levels of phosphorylated H3 in ras-transformed cells were not a consequence of a decrease in the activity of PP1, which was identified as the H3 phosphatase.

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EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—The cell line Ciras-3 was derived from 10T½ cells by transfection with the T-24 Ha-ras oncogene (19). The NIH-3T3 mouse fibroblast cell lines transformed with human c-myc, v-mos, and v-fes were NIH/myc1, Mos 1, Fes 1, and NIH/9IV#5, respectively (20). The 2H1 cell line was derived from 10T½ cells by transfection with the T-24 Ha-ras oncogene under the control of a metallothionein promoter (21). Cell lines were grown in plastic tissue culture plates in a humidified atmosphere containing 7% CO2 in medium supplemented with penicillin G (100 units/ml) and streptomycin sulfate (100 μg/ml). Cell lines were grown in a-minimal essential medium plus 10% fetal bovine serum (Intergen, Purchase, NY). Cells were plated in 15 ml of the above medium at 5 x 10⁶ cells per 150-mm diameter plastic tissue culture dish and grown for 72 h. The proportion of cells in the different cell cycle phases was determined by flow cytometry (9).

Manipulation of Cell Lines—10T½ cells were grown as described above and then serum starved in medium containing 0.5% fetal bovine serum for 24 h. The cells were then either untreated or treated with 50 ng/ml epidermal growth factor (EGF) or 100 nM TPA for 5 or 30 min. 2H1 cells (21) were grown as described above, and then the medium was changed to fresh medium for the control cells or medium containing 100 μM ZnSO₄ (to induce the ras oncogene) for 24 h.

After treatment, the medium was removed and the monolayer was washed twice with phosphate-buffered saline, pH 7.3, and trypsinized. The cells were collected by centrifugation and stored at −80 °C.

Isolation of Histones—For the isolation of histones, approximately 4 x 10⁸ 10T½ cells were homogenized in 5 ml of nuclear preparation buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1.5 mM MgCl₂, 0.65% Nonidet P-40, and 1 mM PMSF) in the presence of protein phosphatase inhibitors (10 mM NaF, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate). For some experiments, the protein phosphatase inhibitors were omitted; we did not observe changes in the relative levels of H3 in samples isolated with and without these inhibitors. Nuclei were recovered by centrifugation at 1500 x g for 10 min. All preparations were carried out at 4 °C. Nuclei were resuspended in 3 ml of RSB buffer (10 mM Tris-HCl, pH 7.6, 3 mM MgCl₂, 10 mM NaCl, 1 mM PMSF, and protein phosphatase inhibitors). Nuclei were extracted with 0.4 N H₂SO₄ to isolate total histones. The samples were precipitated with trichloroacetic acid and then resuspended in double distilled H₂O.

Fractionation of Chromatin—Chromatin was fractionated by a procedure described previously (22). Ciras-3 mouse fibroblasts (1.35 x 10⁶ cells) resuspended in RSB buffer were homogenized several times and then passed through a syringe with a 22-gauge needle. Nuclei were collected by centrifugation. Nuclei were resuspended in 5 ml of RSB buffer to 10 A₂₆₀ units/ml. CaCl₂ and micrococcal nuclease (Worthington Biochemical Corporation) were added to 1 μM and 15 A₂₆₀ units/ml, respectively. The reaction at 25 °C was for 7.5 min and then stopped by the addition of EDTA to 10 mM. The nuclei were resuspended in 3 ml of RSB buffer (10 mM Tris-HCl, pH 7.6, 3 mM MgCl₂, 10 mM NaCl, 1 mM PMSF, and protein phosphatase inhibitors). Nuclei were extracted with 0.4 N H₂SO₄ to isolate total histones. The samples were precipitated with trichloroacetic acid and then resuspended in double distilled H₂O.

Electrophoresis and Western Blotting—Acell pellet containing 4 x 10⁶ 10T½ or Ciras-3 cells were lysed in Nonidet P-40 buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1.5 mM MgCl₂, 0.65% Nonidet-P-40, and 1 mM PMSF). The cellular extract was centrifuged at 10,000 x g for 10 min and the pellet discarded. Okadaic acid was added (at a final concentration of either 2 μM or 100 μM) to 20 μg of cellular-extracted protein in a 50-μl volume of Nonidet P-40 buffer. Five μg of total histone (isolated by sulfuric acid extraction of 10T½ cells that were treated with 0.06 μg/ml colcemid in media for 16 h) was added. The reaction was allowed to proceed at 37 °C for 30 or 60 min. The reaction was stopped by the addition of SDS loading buffer. For the zero min time point, the reaction was immediately quenched by the addition of sample buffer. The levels of protein phosphatase activity from 10T½ and Ciras-3 cell extracts, the extracts were incubated with phosphorylated H3 for 15, 30, 45, and 60 min at 37 °C. The proteins were separated by SDS-polyacrylamide gel electrophoresis, and Western blotting with the anti–phospho H3 antibody was performed. To determine the level of phosphorylated H3 in each sample, densitometric analysis of the Western blots was performed. The assays were typically done in triplicate.
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RESULTS

Induction of H3 Phosphorylation in Serum-starved 10T½ Cells in Response to Growth Factors and Phorbol Esterst-To determine whether Ser-10 of H3 is phosphorylated in response to mitogen stimulation, we used an antibody that was generated against Ser-10 pH3; henceforth called anti-pH3 antibody. Serum-starved 10T½ cells were treated with either EGF or TPA for 5 or 30 min, and the level of pH3 was analyzed in immunoblotting experiments. We observed a 7.1–10.3-fold increase in the level of pH3 in the EGF- or TPA-treated cells in comparison with the untreated 10T½ cells (Fig. 1). These results show that Ser-10 of H3 is phosphorylated in response to EGF or TPA stimulation.

Immunolocalization of pH3 in 10T½ Cells Treated with TPA—In murine cell lines, AT-rich centromeric heterochromatin can be seen as regions of intense DAPI staining. These regions colocalize with the domains of intense H3 phosphorylation observed in G2/M phase cells, reflecting G2 phosphorylation of centromeric heterochromatin (17). In TPA-induced 10T½ cells, a second pattern is seen. The pH3 was located in numerous small foci scattered throughout all interphase nuclei (Fig. 2, panel A). The pH3 foci shown in Fig. 2 were found not to colocalize with centromeric heterochromatin in three-dimension reconstructions. In the vast majority of the cells, all of the foci were located outside of condensed regions of chromatin (Fig. 2, panel B). This observation is consistent with the hypothesis that pH3 of mitogen-stimulated cells is associated with less condensed chromatin.

Stimulated c-fos Gene Is Associated with pH3—The CHIP (chromatin immunoprecipitation) assay was used to test directly if pH3 was associated with immediate early genes, the expression of which is stimulated in mitogen-stimulated cells (12, 29). Fig. 3 shows that pH3 of TPA-stimulated cells was associated with c-fos and c-myc DNA sequences (coding regions). In three separate experiments, the c-fos DNA probe reproducibly gave a stronger signal than did the c-myc DNA probe. Note, however, that both probes generated a comparable signal when hybridized to the input DNA (from TPA-treated cells). In contrast to expressed genes, transcriptionally inactive signal when hybridized to the input DNA (from TPA-treated probe. Note, however, that both probes generated a comparable signal). In three separate experiments, the c-fos regions colocalize with the domains of intense H3 phosphorylation (see arrows in Fig. 5A), in agreement with previous results (17). These observations suggest that pH3 of most ras-transformed mouse fibroblasts is associated with relaxed chromatin regions.

Phosphorylated H3 Is Associated with Micrococcal Nucleasesensitive Chromatin in Ciras-3 Cells—Nuclei isolated from Ciras-3 cells were digested with micrococcal nuclease, and the chromatin fragments were sequentially solubilized with buffers of increasing ionic strength. As the ionic strength of the extracting buffer increased so did the lengths of the chromatin fragments, in agreement with our previous results (22) (Fig. 6A). Fractions SS 0.05, SS 0.10, and SS 0.20 accounted for approximately 30% of the chromatin, with 70% of the chromatin being present in fractions SS 0.40 and the residual nuclear pellet (P). Histones isolated from these chromatin fractions were electrophoretically resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membranes, which were stained with India ink (Fig. 6B). Fig. 6C shows that pH3 levels were higher in the chromatin fractions SS 0.05, SS 0.10, and SS 0.20 than in fractions SS 0.40 and P. This result provides evidence that the pH3 observed outside of condensed chromatin regions (Fig. 5) is associated with less condensed regions of chromatin in Ciras-3 cells.

Ras-transformed and Parental Mouse Fibroblasts Have Similar H3 Phosphatase Activities—Increased phosphorylation of H3 in the ras-transformed cells may be a consequence of increased H3 kinase activity and/or decreased H3 phosphatase activity. Histones incubated with cellular extracts isolated from 10T½ and Ciras-3 cells were electrophoretically resolved on SDS gels and then transferred to nitrocellulose. Western blot analysis with anti-pH3 antibodies determined the amount of pH3 remaining following incubation with the cellular extracts, providing a measure of the H3 phosphatase activity. Fig. 7 shows that the H3 phosphatase activities were similar in the cellular extracts.

The activities of the two major protein phosphatases, PP1 and PP2A, in mammalian cells can be distinguished by their sensitivity to okadaic acid (30). PP2A activity is completely inhibited at 1 nM okadaic acid, whereas 50% inhibition of PP1 activity is observed at 10–15 nM okadaic acid. A cellular extract from 10T½ cells was incubated with histones and 2 or 100 nM okadaic acid and incubated for various times. Fig. 8 shows that for control and 2 nM okadaic acid dephosphorylation of pH3, 29

2 M. J. Hendzel, unpublished observations.
and 39%, respectively, of the pH3 remained after 60 min. However, when 100 nM okadaic acid was added to the cellular extract, dephosphorylation of pH3 was not observed following 60 min of incubation. These observations suggest that PP1 is the major H3 phosphatase.

The Level of pH3 Is Increased upon Induction of the ras Oncogene in 2H1 Cells—To test if altered levels of pH3 are an early event in cellular transformation, which occurs upon expression of oncogenic ras, we used the mouse fibroblast cell line, 2H1, which is a 10T1/2 cell line transfected with an inducible -ras oncogene (21). In these cells, ras oncogene expression is controlled by a metallothionein promoter, which can be induced by treating the cells with 100 μM ZnSO4. Fig. 9 shows the levels of Ras and pH3 at different times after the induction of the ras oncogene. One h after the addition of zinc, Ras levels were increased severalfold, diminishing over 18 h. The amount of pH3 increased rapidly for 2 h and then increased at a much slower rate. These observations show the rapidity and sustained phosphorylation of H3 in response to the expression of the oncoprotein Ras. Treatment of parental 10T1/2 cells with 100 μM ZnSO4 for 0, 8, or 24 h did not result in increased levels of phosphorylated H3, and therefore phosphorylation of H3 was not affected by ZnSO4 treatment alone (data not shown).

Phosphorylation of H3 in Cell Lines Transformed with Oncogenes Encoding Protein Kinases—Previously we reported that mouse fibroblasts transformed with oncogenes (e.g. fes, mos, c-myc) whose products stimulate the Ras-MAPK signaling pathway have elevated levels of phosphorylated H3 and therefore phosphorylation of H3 was not affected by ZnSO4 treatment alone (data not shown).
transformed with various different oncogenes have increased levels of pH3.

**DISCUSSION**

In this study, we show that activation of Ras-MAPK pathway in quiescent murine fibroblasts treated with phorbol esters or growth factors results in the phosphorylation of Ser-10 of H3. We demonstrate that pH3 is located with chromatin that is not highly condensed, and we provide direct evidence that pH3 is associated with the induced c-fos and c-myc genes.

The c-fos gene is transcribed in quiescent cells; however, elongation of the gene is blocked approximately 100 nucleotides from the site of initiation (33). Stimulation of the Ras-MAPK pathway results in the release of this block in elongation. It is possible that phosphorylation of H3 associated with the c-fos gene allows the chromatin fiber to be less compact, favoring elongation. Consistent with this hypothesis, the c-fos chromatin becomes more DNase I sensitive following activation of the Ras-MAPK pathway (34). Similarly, phosphorylation of H3 may release the block within the c-myc gene (35). Increased c-myc expression is seen later than that of c-fos (29, 36), concurrent with the induced expression of these genes is the appearance of unfolded nucleosomes along the coding region of the c-fos and c-myc genes (29). Dynamically acetylated H3 is the target of mitogen-stimulated phosphorylation (18). As the H3 tail contributes to the folding and inter-association of chromatin fibers, modification of the H3 tail by acetylation and phosphorylation may destabilize higher order compaction of the chromatin fiber and contribute to maintaining the unfolded structure of the transcribing nucleosome (2, 6, 37–41).

Although the H3 that is the target of mitogen-stimulated phosphorylation is dynamically acetylated, it should be noted that acetylation does not predispose H3 to phosphorylation (18). Further, the anti-pH3 antibody will not bind to hyper-acetylated H3 unless it is phosphorylated (42). In stimulated mouse fibroblasts, most newly phosphorylated H3 is non-, mono-, and diacetylated (18).

**FIG. 5.** Relative organization of pH3 and condensed chromatin in ras-transformed Ciras-3 mouse fibroblasts. Cells were co-stained with anti-pH3 and DAPI. Digital optical sections of 0.3 mm were obtained and false colored red (phosphorylated H3, panel A) and blue (DAPI, panel C), and a composite image was produced using Adobe Photoshop (panel B). The arrows indicate regions of centromeric heterochromatin in a G2 phase cell. The bar represents 10 μm.

**FIG. 6.** Fractionation of Ciras-3 chromatin. Ciras-3 chromatin was fractionated as described under “Materials and Methods,” yielding chromatin fractions SS 0.05 (15%), SS 0.10 (7%), SS 0.20 (9%), SS0.40 (22%), and residual nuclear material P (47%). The values given in the parentheses indicate the percentage of chromatin in that fraction. DNA fragments (5 μg) isolated from these fractions were electrophoretically resolved in 1% agarose gels, which were stained with ethidium bromide (panel A). Lane M contains HindIII-digested lambda DNA fragments. Histones (5 μg) isolated from the chromatin fractions were resolved on 15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes, which were stained with India ink (panel B). The membrane was immunocohemically stained with anti-pH3 (panel C).

**FIG. 7.** Comparison of H3 dephosphorylation in lysates from parental and ras-transformed cells. Twenty μg of cellular-extracted protein from 10T1/2 (2.9 × 10⁴ cell equivalents) or Ciras-3 (3.5 × 10⁴ cell equivalents) were incubated with 5 μg of total histone substrate for 0, 15, 30, 45, and 60 min at 37 °C. The reaction was stopped, and the proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and immunocohemically stained with anti-pH3. The percent of pH3 remaining after each time period was determined by densitometric analysis of the Western blots as described under “Experimental Procedures.” Inspection of the India ink-stained immunoblot showed that the protein loads in each lane were equivalent and that protein degradation was not occurring (not shown).
Total histone was extracted from 2H1 cells that were treated with 100 μm okadaic acid in 10T1/2 cell extracts as described under "Experimental Procedures." The percent of pH3 remaining after each time period was determined by densitometric analysis of the Western blots stained with anti-pH3.

Polyacrylamide gel electrophoresis (PAGE) was performed on 10% SDS-PAGE gels, transferred to membranes, and immunochemically stained with anti-pH3. The reaction was stopped, and the proteins were separated on an SDS-polyacrylamide gel, transferred to membranes, and immunologically stained with anti-pH3. The percent of pH3 remaining after each time period was determined by densitometric analysis of the Western blots as described under "Experimental Procedures."

**FIG. 8.** Inhibition of H3 dephosphorylation by okadaic acid in 10T1/2 cell extracts. Cell extracts were incubated in the presence of 0, 2, or 100 nM okadaic acid and 5 μg of total histone as substrate. The reaction mixture was incubated for 0, 30, or 60 min at 37 °C. The reaction was stopped, and the proteins were separated on an SDS-polyacrylamide gel, transferred to membranes, and immunologically stained with anti-pH3. The percent of pH3 remaining after each time period was determined by densitometric analysis of the Western blots as described under "Experimental Procedures."

**FIG. 9.** Effect of induction of the ras-oncogene on pH3 levels. Total histone was extracted from 2H1 cells that were treated with 100 μM ZnSO₄ for 0, 1, 2, 4, 8, or 16 h. The relative levels of pH3 and Ras from each sample were determined by densitometric analysis of Western blots with anti-pH3 as described under "Experimental Procedures."

**FIG. 10.** Phosphorylated H3 of parental and oncogene-transformed mouse fibroblasts. Total histone was isolated from parental NIH-3T3 (lane 1) and NIH-3T3 cells transformed with v-fes (lane 2), v-mos (lane 3), and c-myc (lane 4). The total histone sample (10 μg) was electrophoretically resolved on a 12.5% SDS-polyacrylamide gel, transferred to membranes, and immunologically stained with anti-phospho-H3. Panel A shows a Coomassie Blue-stained gel. Panel B shows the immunologically stained membrane.

(11) Further, we found that the MAPK (ERK-1 and -2) activity was higher in cellular extracts from the ras-, fes-, mos-, and myc-oncogene-transformed cells than in the parental cellular extracts (data not shown). By persistent activation of the Ras-MAPK signaling pathway, the phosphorylated isofoms of H3 would remain elevated, resulting in the destabilization of chromatin observed in oncogene-transformed cells (8, 9, 44). Consistent with this idea is the observation that pH3 of ras-transformed cells is associated with less condensed chromatin regions. The increased steady state levels of pH3 may result in deregulation at transcription at the level of transcriptional elongation, resulting in aberrant gene expression observed in cancer cells (45–47).

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