Fibroblasts transformed by combinations of *ras*, *myc* and mutant p53 exhibit increased phosphorylation of histone H1 that is independent of metastatic potential

William R. Taylor[a,b,d], Deborah N. Chadee[a,b], C. David Allis[c], Jim A. Wright[a,b], James R. Davie[b,*]

[a]*Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Manitoba, RW3 0V3, Canada*
[b]*Department of Chemistry and Molecular Biology, University of Manitoba, Winnipeg, Manitoba, R3E 0W3, Canada*
[c]*Department of Biology, Syracuse University, Syracuse, NY 13244-1270, USA*
[d]*Department of Molecular Biology, Cleveland Clinic Foundation, Cleveland, OH 44195, USA*

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Abstract H1 histones play an important role in regulating higher order structure of chromatin and are potential regulators of gene expression. H1s are phosphorylated, a modification which alters their interaction with DNA. We measured the abundance of three phosphorylated H1 subtypes in mouse fibroblasts transformed by combinations of *ras, myc* and mutant p53 which differ in metastatic potential. We found that there is an increase in phosphorylation of H1 subtypes in fibroblasts transformed with *ras, myc* and mutant p53. This increase was found to correlate with cellular transformation but not with induction of the metastatic phenotype.

Key words: Chromatin; Transcription; Malignancy

1. Introduction

Malignant progression is accompanied by multiple changes in the expression of cellular genes [1]. *ras* and several other oncogenes can induce the tumorigenic and metastatic phenotypes in permissive cell lines [2–4], and therefore *ras* provides a very useful model of oncogene mediation of these processes. Further, current evidence suggests that *ras* participates in early stages of neoplastic development [5]. Some *ras*-induced changes in gene expression are likely due to regulatory changes in transcription factors [6–8]. However, alterations in chromatin structure may also have roles in aberrant gene expression [9,10].

The H1 histones bind to linker DNA where the DNA enters and exits the nucleosome, and they compact the chromatin fiber into higher order structures [11]. H1 subtypes differing in primary structure include H1a, H1b, H1c, H1d, H1e, H1t and H1° [12–14]. Posttranslational modification adds to heterogeneity in the H1s. Phosphorylation of H1 on serine and threonine in their amino and carboxyl terminal tails occurs in vivo and alters their interaction with DNA [11,15,16]. H1 phosphorylation destabilizes higher order chromatin structure which is thought to allow accessory factors to participate in replication, mitotic condensation, and gene activation [16].

ras-transformed murine NIH3T3 fibroblasts have a more decondensed chromatin structure compared to normal fibroblasts [17]. Recently, we reported that murine 10T½ fibroblasts transformed by one of several oncogenes utilizing the *ras* signal transduction pathway had both a decondensed nucleosomal structure and highly phosphorylated H1 subtypes [18]. In this study we compared the level of histone phosphorylation in cells transformed with combinations of *ras*, *myc* and mutant p53 which differ in malignant potential [19]. We show that H1 is highly phosphorylated in these cell lines and that histone phosphorylation correlates with cellular transformation but appears to be independent of metastatic potential.

2. Materials and methods

2.1. Cell lines and culture conditions

Cell lines were grown in a humidified atmosphere containing 7% CO_2 in α-minimal essential medium (α-MEM) (Gibco, Grand Island, NY) supplemented with penicillin G (100 units/ml) (Sigma, St. Louis, MO), streptomycin sulfate (100 μg/ml) (Sigma, St. Louis, MO) and 10% calf serum (Gibco). The mouse 10T½ fibroblast cell line obtained from American Type Culture Collection (ATCC) (Rockville, MD) was used to isolate cell lines expressing combinations of *ras*, *myc* and mutant p53. The derivation of these cell lines by transfection of 10T½ cells with plasmids expressing *ras*, *myc* and mutant p53 has been previously described [19]. The transfectants used in this study and the oncogenes they express are R-2 (T24-Ha-*ras*), RM-5 (T24-Ha-*ras*, plus c-*myc*), RP-4 and RP-6 (T24-Ha-*ras* plus the proline-193 mutant form of murine p53), and RMP-6 (T24-Ha-*ras*, c-*myc* plus the proline-193 p53 mutant). Cell cycle analysis was performed as described previously [18]. Malignant potential was determined by injecting single cell suspensions into the tail veins of syngeneic mice followed by examination of tumor formation in the lung 21 days later [19].

2.2. Isolation and analysis of H1

Highly phosphorylated H1 was detected by immunoblotting procedures, using an antiserum raised in rabbits against phosphorylated H1 from *Tetrahymena* [20]. The antibody reacts specifically with phosphorylated murine H1b [18]. Perchloric acid extraction of H1, transfer to polyvinyl difluoride membranes and immunochemical detection were done as previously described [18].

3. Results

3.1. Phosphorylation of H1 in cell lines transformed with combinations of ras, myc and mutant p53

H1 histones undergo two types of phosphorylation. One type causes a reduction in electrophoretic mobility on SDS polyacrylamide gels (c-phosphorylation), while the other does not affect mobility [14,18]. Murine H1 subtypes H1c and H1b undergo c-phosphorylation [18]. H1 subtypes isolated from murine fibroblasts transformed with combinations of *ras*, *myc* and mutant p53 were electrophoretically separated in SDS poly-

*Corresponding author. Fax: (1) (204) 783 0864.

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acrylamide gels. H1 subtypes H1°, H1c, H1d, H1e, and phosphorylated forms c-pH1b, c-pH1c and pH1b were resolved in this gel system (Fig. 1A). H1d and H1e comigrated as a single band [18]. All of the *ras*-transformed cell lines had higher levels of c-pH1b and c-pH1c compared to 10T½ cells. The transformed fibroblasts had 1.6- to 2.7-fold higher levels of c-pH1b and 1.3 to 2.3-fold higher levels of c-pH1c (Table 1). The increase in these two phosphorylated species was found in all *ras*-transformed cell lines, and the presence of either *myc* or mutant p53 did not significantly alter this effect.

3.2. Detection of phosphorylated H1 histones with a specific antibody

Recently an antiserum recognizing phosphorylated H1 but not unphosphorylated H1 has been described [20]. This antiserum was raised against hyperphosphorylated H1 of *Tetrahymena thermophila* and recognizes the phosphorylated form of murine H1b (pH1b) which exhibits the same electrophoretic mobility as its parent band [18]. Since the abundance of this phosphorylated band could not be determined by SDS gel electrophoresis alone, we employed immunoblot analysis with the pH1b antiserum to analyze this phosphorylated species. H1s isolated from cells transfected with *ras*, *myc* and mutant p53 were separated on SDS polyacrylamide gels, and probed with the antiserum by immunoblotting. Sample loading onto the membranes was inspected by staining with india ink (Fig. 1B). The immunoblot shown in Fig. 1C demonstrates that the parental 10T½ cell line had a low level of phosphorylated H1b. However, cells transformed by combinations of *ras*, *myc*, and p53 contained 3.7- to 5.9-fold higher levels of this modified H1 subtype (Table 1).

3.3. Cell cycle analysis

The level of phosphorylation H1s varies considerably during the cell cycle [20,21]. In G1 phase, H1 generally contains approximately 1 phosphorylated amino acid per molecule. However, the level of phosphorylation rises to approximately 6 residues per molecule for some H1 subtypes when cells enter M phase. H1 becomes dephosphorylated as cells exit M phase. A different cell cycle distribution for the transformed cell lines compared to that of the control cells may account for the changes in the levels of phosphorylated H1. Table 1 shows that there were no significant differences in the cell cycle distributions of the transformed cells compared to 10T½ cells. Furthermore, some of the transformed cells (R-2 and RP-6) had fewer G2/M cells than did the 10T½ cells, making it unlikely that the observed increase in H1 phosphorylation was secondary to altered cell cycle distributions.

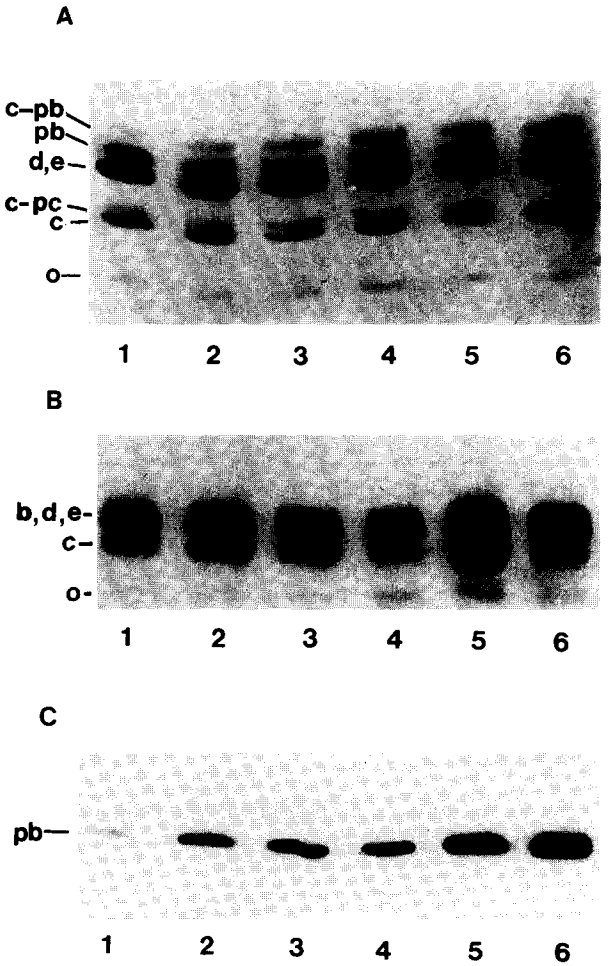

Fig. 1. Electrophoretic analysis of H1 from fibroblasts transfected with *ras*, *myc*, and mutant p53. H1 was extracted and separated by SDS polyacrylamide gel electrophoresis. Two μg of histone were loaded per lane. Gels were either stained with Coomassie blue (A), or transferred to polyvinyl difluoride membranes which were stained with india ink (B), and then probed with the antiserum against hyperphosphorylated H1 by immunoblotting (C) as described in section 2. In this figure, the same membrane used for immunoblotting was stained with india ink. Lanes are: (1) 10T½, (2) R-2, (3) RM-5, (4) RP-4, (5) RP-6, (6) RMP-6. c-pb and c-pc are the c-phosphorylated forms of histone H1b and H1c, respectively. pb is the phosphorylated form of H1b exhibiting the same mobility as the parent H1b band. The remaining H1 subtypes are labeled as follows: H1° (o), H1c (c), H1d (d), H1e (e). H1a is not detectable in this analysis [18].

Table 1
Cell cycle distribution and level of phosphorylated h1 histones of 10T½ murine fibroblasts transformed with *ras*, *myc* and mutant p53

Cell line	Cell cycle phase (% distribution)			Relative level of phosphorylated H1 subtype		
	G1	S	G2/M	c-pH1b	pH1b	c-pH1c
10T½	55	26	19	1.0	1.0	1.0
R-2	61	26	13	1.6	3.7	1.3
RM-5	54	25	21	2.2	4.8	1.6
RP-4	49	31	19	2.6	2.9	1.7
RP-6	50	33	17	2.6	6.7	1.8
RMP-6	45	31	24	2.7	5.9	2.3

H1 isolated from the oncogene-transformed and parental 10T½ cell lines were resolved on SDS 15% polyacrylamide gels, and the level of phosphorylated H1s on Coomassie blue-stained gel patterns or Western blots (shown in Fig. 1) was determined by densitometric analysis.

3.4. Correlation of H1 phosphorylation with cellular transformation but not induction of the malignant phenotype

We have previously shown that *ras*, *myc* and mutant p53 cooperate in the induction of the metastatic phenotype in 10T½ cells [19]. This furnished us with an opportunity to test if increased phosphorylation of histone H1 might play a role in regulating the metastatic phenotype. It is evident that there is no correlation of any of these three phosphorylated histone subtypes with metastatic potential (Tables 1 and 2). In contrast all transfectants exhibited an increase. For example, the highly

metastatic RMP-6 cell line formed an average of 85.1 lung nodules when injected intravenously, whereas RP-6 cells did not metastasize in this assay, yet both cell lines had similar levels of all three phosphorylated H1 histone subtypes. In contrast, all of the *ras*-transfected cell lines described in this study form tumors when injected subcutaneously (Table 2). Therefore, increased levels of c-pH1b, pH1b and c-pH1c correlated well with cellular transformation but not with induction of the metastatic phenotype.

4. Discussion

The H1 histones are potential regulators of gene transcription [22]. Phosphorylation of H1 alters its interaction with chromatin and may be important in the transcriptional state of genes to which it is bound [15,16]. We observed in cells transformed by combinations of *ras*, *myc* and mutant p53 that there is an increased amount of the phosphorylated H1 subtypes c-pH1b, pH1b and c-pH1c. This was observed in Coomassie Blue-stained gel patterns of histones resolved on SDS polyacrylamide gels and using an antibody specific for phosphorylated H1b. The increase is unlikely to be a secondary effect of altered cell cyle distribution as some transformed cell lines, containing high levels of phosphorylated H1, contain fewer G2/M cells than the parental cell line.

We have recently shown that cells transformed with oncogenes utilizing the *ras* signal transduction pathway have elevated levels of the phosphorylated H1 subtypes, and that these increases persist in the transformed cells blocked at the G1/S boundary with hydroxyurea [18]. This is consistent with *ras* activation leading to increased H1 phosphorylation and may explain the increase in chromatin decondensation previously observed in *ras*-transformed cells [17,18]. Furthermore, increased H1 phosphorylation in G1/S might potentially be involved in regulating gene transcription.

ras, *myc* and mutant p53 cooperate in the induction of the metastatic phenotype in murine fibroblasts [19]. This gave us an opportunity to test if altered histone phosphorylation might be important in the many changes in gene expression which occur during induction of the metastatic phenotype [1]. We found that there was no correlation between the level of c-pH1b, pH1b and c-pH1c and the metastatic potential of the transformed cell lines analyzed. In contrast, increased H1 phosphorylation was observed in all cell lines expressing the *ras* oncogene. This is consistent with our recent observations that stimulation of the *ras* pathway leads to increased H1 histone phosphorylation. In 10T½ fibroblasts, which are rendered tumorigenic by *ras* overexpression, we found that H1 phosphorylation is associated with this phenotype. Our studies show that acquisition of high levels of phosphorylated H1 is an early event in neoplastic development in this in vitro system. Thus, H1 phosphorylation may affect the expression of genes involved in the loss of contact inhibition, growth factor dependence and growth control [1–4].

Table 2
Tumorigenic and malignant properties of 10T½ mouse fibroblasts transformed by combinations of *ras*, *myc* and mutant p53

Cell line	Gene	Tumor latency* (days)	Metastatic potential[b] (number of lung tumors)
10T½	-	NA	0
R-2	*ras*	15	0
RM-5	*ras*, *myc*	11	0
RP-4	*ras*, p53	12.8	11
RP-6	*ras*, p53	10.2	0
RMP-6	*ras*, *myc*, p53	7.4	85.1

The properties of these cell lines have been previously described [19].
*Tumor latency was determined after subcutaneous injection of 1×10^5 cells into immunocompetent C3H/HeN syngenic mice as previously described [19]. 10T½ cells are nontumorigenic [19,23].
[b]Metastatic potential was determined after intravenous injection of 1×10^5 cells into the tail veins of immunocompetent C3H/HeN syngeneic mice (five mice/clone) as described previously [19,23].

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