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PROHIBITIN EXPRESSION DURING CELLULAR SENESCENCE OF HUMAN DIPLOID FIBROBLASTS

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SUMMARY: Prohibitin is an evolutionarily conserved gene postulated to possess tumor suppressor activity and to contribute to the limited lifespan of human diploid fibroblast-like cells. Prohibitin mRNA and protein expression and its ability to become post-translationally modified were determined in human diploid fibroblast-like cells of different in vitro ages. The expression of prohibitin mRNA and protein changes little with increasing in vitro age; however, its protein product is post-synthetically modified in younger but not older cells. These results suggest that prohibitin is similar to the retinoblastoma gene product whose anti-proliferative activity remains active in older cells because it is not post-synthetically modified.

INTRODUCTION: HDF lose the ability to replicate their DNA and divide when they are maintained in culture for extended periods of time [1]. This loss of proliferative potential has been equated to the process of senescence on a cellular level [2]. Pereira-Smith and Smith [3] have presented evidence that this limited in vitro lifespan is the dominant phenotype and cellular immortalization associated with tumorogenesis is a recessive trait. Additional studies suggested that inhibitors of DNA synthesis, possibly tumor suppressors, are responsible for the limited in vitro lifespan exhibited by HDF; and unlimited growth and immortalization results when the functional activity of these inhibitors is lost [4-9].

The inhibitors of DNA synthesis responsible for limited proliferation were hypothesized to be expressed not only in senescent cell populations but also in normal tissues which exhibit little proliferative activity [10]. Using a microinjection assay system, mRNA coding for

Abbreviations: HDF, human diploid fibroblast-like cells; RB, retinoblastoma; PDL, population doubling level; kD, kilodaltons; kb, kilobases.

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inhibitors of DNA synthesis has been detected not only in senescent HDF [11] but also in human and rat liver [12,13] and human T lymphocytes [14]. The activity from rat liver inhibits the cell cycle traverse of HDF and is considered to be a model for inhibitors of DNA synthesis involved in senescence and immortalization [13,15]. A cDNA coding for this inhibitory activity was isolated and named prohibitin [16,17]. The prohibitin cDNA codes for two mRNA species of 1.2 and 1.9 kb; and both mRNAs code for a novel 272 amino acid peptide with a molecular weight of 30,000 daltons. Other studies have shown that the gene coding for prohibitin is highly conserved with homologs found not only in rat but also in yeast, fruit fly, and man [18-20]. Although the physiological function of prohibitin has yet to be defined, it is required for the normal development of *Drosophila* [19]; and it plays a role in determining the replicative lifespan of *Saccaromyces cerevisieae* [18].

The present experiments were undertaken to determine if there is a relationship between age related alterations in prohibitin expression or potential regulatory mechanisms and cellular senescence in HDF. Because prohibitin inhibits the cell cycle traverse of HDF and has been implicated in the control of the replicative lifespan of yeast [16,18], prohibitin expression and post-synthetic modification were analyzed in HDF of different in vitro ages. The results indicate that there are limited cell cycle and age related variations in prohibitin expression at both the mRNA and protein levels. They also indicate, however, that prohibitin is post-synthetically modified in low but not high PDL cells. These results suggest that, similar to RB, prohibitin activity in senescent cells may be altered by changes in a secondary mechanism of regulation [4].

MATERIALS AND METHODS

Cell Culture

The cells used in these studies were derived from newborn foreskin and designated CF-3. These cells have a characteristic in vitro lifespan of 65±10 population doublings. Cells with a PDL of 18 to 31 were considered low PDL and those of 54 to 71 high PDL. Cells were maintained as previously described [21] and were determined to be free of mycoplasma contamination by the method of Barile [22]. Cells at various stages of the cell cycle were obtained by stimulating serum restricted, preconflent populations to re-enter the cell cycle [23,24].

Northern Analysis

Total RNA was isolated from the cells by extraction with guanidine thiocyanate as described by Kingston [25]. Northern analysis was performed as previously described using a 500 bp fragment of the prohibitin cDNA clone pPRO1 as the probe [17]. Autoradiographs from the Northern blots were analyzed using a JAVA video analysis system (Jandel) and normalized as percent of total intensity. RNA loading was monitored by glyceraldegyde 3-phosphate dehydrogenase, pHcGAP, expression.

Western Analysis and Two Dimensional Electrophoresis

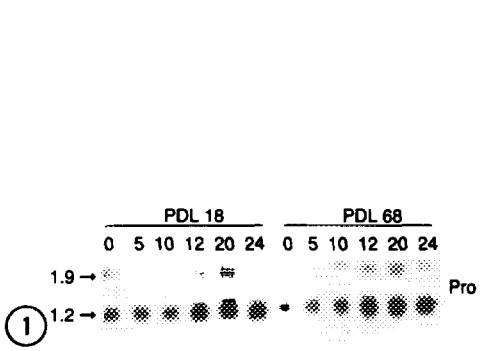
For Western analysis, cells were harvested directly into SDS sample buffer, protein was determined by the method of Bensadoun and Weinstein [26], and the electrophoresis, blotting,

detection and analysis were as previously described [27]. In some experiments, protein samples were subjected to two dimensional electrophoresis according to the method of O'Farrell [28] as modified by Dell'Orco and Whittle [29] prior to Western analysis.

RESULTS

Since prohibitin mRNA inhibits DNA synthesis and cell cycle progression when microinjected into HDF [17], normal prohibitin mRNA levels were determined in HDF during the G0 to S phase transition. Figure 1 shows a Northern blot of RNA from both low and high PDL cells stimulated to enter the cell cycle following serum restriction. Densitometric analysis showed prohibitin mRNA levels for both the 1.2 and 1.9 kb transcripts increased 2 to 3 fold as low and high PDL cells moved through the cell cycle. Although prohibitin mRNA levels were slightly lower in the high PDL populations, especially at G0 and early G1, the same profile of expression was observed in both populations. Prohibitin mRNA levels were lowest in G0 (0 time), increased throughout G1 (5 and 10 hrs), peaked in late G1/early S (12 and 20 hrs), and began to be fall as the cells proceeded through S phase (24 hrs). The data show that prohibitin mRNA is constitutively expressed throughout the cell cycle of both low and high PDL cells but that the level of its expression is partially regulated in a cell cycle manner.

Western analysis was performed during the G0 to S phase transition of low and high PDL HDF to determine if the mRNA changes observed in Figure 1 were translated into similar changes in prohibitin protein levels. Figure 2 shows a representative Western blot reacted with



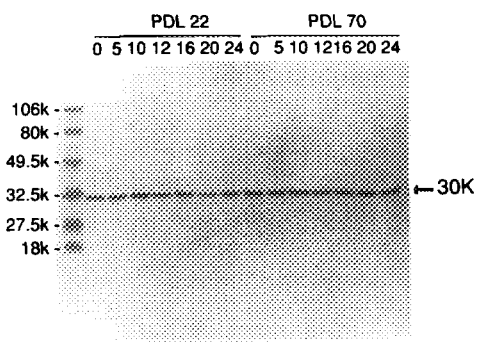


Figure 1. Prohibitin mRNA levels detected by Northern analysis of low (18) and high (68) PDL CF-3 cells during G0 to S phase transition. Synchronized cells were stimulated to enter the cell cycle and were subjected to Northern analysis at various times post-stimulation as described in Materials and Methods. The number at the top of each lane is the time in hours after the cells were stimulated to re-enter the cell cycle. The numbers on the left are the sizes of the two prohibitin (Pro) mRNA transcripts in kilobases.

Figure 2. Prohibitin protein levels detected by Western analysis of low (22) and high (70) PDL CF-3 cells during G0 to S phase transition. Synchronized cells were stimulated to enter the cell cycle and were subjected to Western analysis at various times post-stimulation as described in Materials and Methods. The number above each lane is the time in hours after the cells were stimulated to re-enter the cell cycle. The far left lane contains stained molecular weight markers with their molecular weights noted in kilodaltons (k). The number on the right is the molecular weight of prohibitin given in kilodaltons.

antibodies for the detection of prohibitin protein. In contrast to what was observed in the Northern analyses, these data show little difference in prohibitin protein levels as both the low and high PDL cells are stimulated to move through the cell cycle. Additionally, in high PDL cells, prohibitin protein levels were the same or slightly higher than those detected in low PDL cells. These data suggest that if prohibitin is a factor in cellular senescence its functional activity is altered by an age related change in a post-translational regulatory mechanism.

Stein, et al. [4] showed that the gene product of one tumor suppressor gene, RB, exhibits a altered pattern of post-synthetic modification in older HDF populations, marking a change in the regulation of its functional activity. In order to determine if prohibitin protein is post-synthetically modified, protein from low and high PDL cells was subjected to two dimensional electrophoresis followed by Western analysis. The results from one such analysis is presented in Figure 3. Two isoforms of prohibitin are seen on the blot from low PDL cells (Figure 3A). The major form of the protein has a molecular mass of 30 kD and a pI of between 5.0 and 5.5; the minor form of the protein has the same molecular mass but a slightly more acidic pI. The minor component accounts for approximately 10% of the total detected signal. In high PDL cells, the minor form of prohibitin is no longer present (Figure 3B). These data show that a portion of the prohibitin protein is post-synthetically modified in low PDL populations but not high PDL cells. The results suggested that prohibitin was modified by a small molecule because the molecular mass was not altered by the modification. Since the phosphorylation of prohibitin

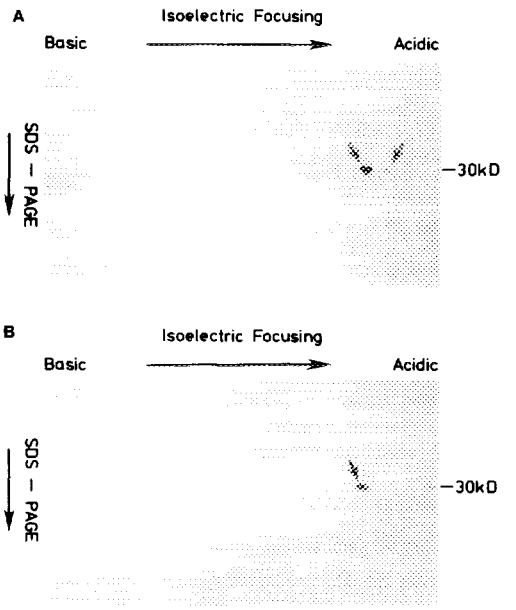


Figure 3. Prohibitin protein isoforms detected by Western analysis following the two dimensional electrophoresis of (A) low (16) and (B) high (66) PDL CF-3 cell protein. Cells were harvested 24 hours after being stimulated to enter the cell cycle. Arrows denote the prohibitin isoforms and their molecular weight is shown on the right.

could account for the minor component in low PDL cells, experiments to illustrate prohibitin phosphorylation were performed but yielded negative results (data not shown).

DISCUSSION: This study sought to determine if prohibitin was a senescence inhibitor which participates in the mechanisms that result in the loss of proliferative activity in high PDL HDF. When prohibitin mRNA levels were analyzed at various stages of the cell cycle in different aged cells, the data presented in Figure 1 were obtained. They showed that prohibitin mRNA levels exhibited some cell cycle and age related differences; however, these were limited, indicating that transcriptional regulation of prohibitin expression was basically unaltered in older populations. Prohibitin protein levels were also essentially unchanged during the cell cycle in both low and high PDL cells with no significant age related differences detected (Figures 2). These results suggest that if prohibitin is involved in the inhibition of cell proliferation in high PDL cells it involves a post-translational mechanism. The data presented in Figure 3 indicate that this may indeed be how prohibitin is altered during cellular senescence. These results indicate that prohibitin is post-synthetically modified in low PDL cells and that this modification is absent in high PDL populations. The moderation of the functional activity of proteins with tumor suppressor activity, particularly p53 and RB, by post-synthetic modifications is well documented [30-34]. Since these two gene products have also been implicated in the process of senescence [6,35], age related alterations in the regulation of the functional activity of proteins which affect cellular growth, such as prohibitin, would appear to play key roles in the cellular aging process.

A role for RB in the age related loss of proliferative activity in HDF is better established than that of p53 and may parallel what happens to prohibitin during cellular senescence [4,6,35,36]. RB does not appear to be primarily regulated at the level of transcription since it is constitutively expressed throughout the cell cycle and to the same extent in both low and high PDL populations [36]. This is similar to the expression profiles determined for prohibitin and presented in Figures 1 and 2. Although there are no significant age related alterations in RB expession at either the mRNA or protein levels, Stein, et al. [4] presented evidence that the post-synthetic modification of RB was different in high PDL cells and that this changed its functional activity in these populations. These investigators showed that RB remains active in senescent cells and inhibits cell cycle traverse because it fails to be phosphorylated. Although we were unable to show that prohibitin is phosphorylated, we did show that a modified form of prohibitin was present in low but not high PDL cells (Figure 3). Therefore, like RB, the failure of high PDL cells to post-synthetically modify prohibitin may allow prohibitin to remain active and inhibit cell cycle traverse in these populations. Experiments are currently underway to define prohibitin's physiological function, to identify its post-synthetic modification, and to determine its precise role in the loss of proliferative potential exhibited by HDF in vitro.

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