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RB Gene Family: Genome-Wide ChIP Approaches Could Open Undiscovered Roads

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ABSTRACT

Many in vitro and reporter assays have helped to clarify how transcription factors regulate gene transcription. Today, it is important to decode the map of all transcription factor binding sites in the genome context. Chromatin immunoprecipitation followed by genome-wide analyses have tremendously opened new ways to analyze the mechanisms of action of DNA binding factors, cofactors and epigenetic modifications. It is now possible to correlate these regulatory mechanisms with genomic features such as the promoter, enhancer, silencer, intragenic, and intergenic DNA sequences. These approaches help to clarify the complex rules that govern many biological processes. In this review we discuss the genome-wide approaches applied to the retinoblastoma gene family (RBF), the central player of cell cycle control. There are also new, possible directions that are suggested within the review that can be followed to further explore the role of each pRb members in the transcriptional networks of the cell. J. Cell. Biochem. 109: 839–843, 2010.

KEY WORDS: RBF; CELL CYCLE; ChIP GENOME-WIDE

pRb is the key gene in a rare pediatric eye neoplasm (sporadic and hereditary) arising from retinal cells that harbor either a deletion or mutational inactivation of both pRb alleles [Knudson, 1971; Dunn et al., 1988; Paggi and Giordano, 2001; Cobrinik, 2005] pRb is a bona fide tumor suppressor gene, and its mutation or deletion is shared by several malignancies [Paggi et al., 1996]. For these reasons, pRb is considered one of the hallmarks of human malignancies [Hannon et al., 1993; Mayol et al., 1993; Zhu et al., 1993].

The pRb gene is considered as the founder of the RB family since two other genes have been identified, both of which are structurally and functionally related. These genes are named p107 [Ewen et al., 1991; Zhu et al., 1993] and Rb2/p130 [Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993]. Cytogenetically, Rb2/p130 maps to the 16q12.2–13, a genomic region repeatedly altered in human cancers [Goodrich et al., 1991; Hannon et al., 1993; Li et al., 1993]. We have demonstrated that, Rb2/p130 has tumor-suppressor properties in JC virus-induced hamster brain tumor cells [Howard et al., 1998], and the genetic alteration of the Rb2/p130 genes have often been detected in human cancers. Specifically, Rb2/p130 is altered in breast, ovarian, prostate, small-cell lung cancers and many other tumor types [Paggi and Giordano, 2001]. p107 maps to the human chromosome region 20q11.2, a locus not frequently found involved in human neoplasms [Ewen et al., 1991; Ichimura et al., 2000]. It should be noted, however, that p107 suppresses the development of Retinoblastoma in pRb-deficient mice [Robanus-Maandag et al., 1998].

In the recent past, genome-wide approaches have elucidated the mechanism of action of RBF on target genes and yielded some unexpected results. In this review, we summarize the recent findings, give a critical point of view on what has been done up to this point, and finally, highlight the anticipated steps to be taken in the near future.

RBF AND E2F PROTEINS IN CELL CYCLE CONTROL

The first data, identifying pRb in the cell cycle regulation, emerged more than 10 years ago. pRb controls the cell cycle through the interaction with E2F transcription factors [DeGregori et al., 1997; Attwooll et al., 2004; DeGregori and Johnson, 2006]. These interactions are regulated during cell cycle by a phosphorylation

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mechanism. In the early and mid-G1stages, D type cyclins bind CDK4 or CDK6 proteins, and in late G1, cyclin E (or A), along with CDK2 proteins, gradually phosporylate pRb. Hyper-phosphorylated pRb releases E2F transcription factors and allows the expression of genes that mediate S phase entry [Flemington et al., 1993; Helin and Ed, 1993].

The interaction between RBF members and E2F proteins brings about a repressive function that is mediated by two different mechanisms. The first general mechanism relies on the finding that the E2F transactivation domain and the pRb binding domain physically overlap at the E2F-C terminal [Flemington et al., 1993; Helin and Ed, 1993]. This interaction suggests a competitive model between pRb and the promoters of E2F target genes for activate transcription. The effects of this simple mechanism are not enough to explain why pRb, alone, can reduce E2F luciferase reporter activity in the absence of E2F [Weintraub et al., 1992] or why artificially pRb fused to E2F binding domain could act as a repressor on a basic promoter [Sellers et al., 1995]. The second general mechanism is based on the interaction between pRb and different chromatin modifier enzymes. pRb is able to interact with HDAC1,2,3 histone deacetylases, SUV39H methylases, and Brg1 and Brm chromatin-remodeling enzymes on the promoters of target genes [Cobrinik, 2005]. Further evidence of pRb's repressive function can be derived from site-direct mutagenesis of E2F binding elements on B-Myb, Cdc2, cyclin E and E2F1 target genes, which result in increased gene expression in quiescent and G1 cells. Genomic footprinting also supports these results because E2F complexes are bound to the B-Myb, cyclin A, and Cdc2 genes in quiescent cells and during early G1 when these genes are repressed [Liu et al., 1996]. Collectively, these data support the hypothesis that the RBF/E2F complex can bind the promoters of target genes and repress their expression.

RBF UNIQUE AND OVERLAPPING FUNCTIONS

Due to structural similarities, pRb, Rb2/p130, and p107 have many overlapping functions. All three proteins can repress gene transcription, cause an arrest of the cell cycle in the G1 phase, interact with viral oncoproteins, and share many protein partners [Mulligan and Jacks, 1998; Morris and Dyson, 2001]. Although RBF members possess many sequence similarities, they have additionally unique functions. Examples of differences include their expression pattern, E2F family member interaction (p107 and Rb2/p130 interact with E2F4/5 (repressing E2Fs) and pRb interacts with E2F1-3 (activating E2Fs)), cyclin/cdk complexes [Nevins, 1998; Classon and Dyson, 2001; Classon and Harlow, 2002; Cobrinik, 2005] and sets of target genes. Rb2/p130 is highly expressed in quiescent and differentiated cells while p107 is most often expressed in proliferating cells. pRb is ubiquitously expressed and can be detected in proliferating, quiescent and differentiated cells [Cobrinik, 2005].

An important distinction among the pocket proteins is observed during development. pRb nullizygous mice die during mid-gestation with defects in the nervous system, hematopoietic system and lens. In contrast, p107 and Rb2/p130 nullizygous mice having the same genetic background develop normally. Mice nullizygous for both Rb2/p130;p107 die at birth with abnormalities in endochondral bone formation and epidermal development. Embryos nullizygous for p107 or Rb2/p130, together with pRb loss, die approximately 2 days earlier than pRb null embryos and show more severe defects in the nervous and hematopoietic systems [Wikenheiser-Brokamp, 2006]. The RBF confirmed overlapping functions as well in development. Rb2/p130 is able to compensate for pRb deficiency in cardiac muscle development [MacLellan et al., 2005] and p107 can compensate for the loss of pRb function in the epidermal tissue [Ruiz et al., 2004]. These results support the observation that E2F transcription factors that normally bind pRb (i.e., E2F1, E2F2, E2F3) may bind p107 in pRb-deficient cells [Lee et al., 2002].

Pocket proteins have unique and overlapping functions in tumorigenesis as well in development. pRb heterozygous mice are prone to developing tumors of the pituitary, thyroid, and adrenal glands. p107 and Rb2/p130 ablation, alone or in combination, does not predispose to tumor formation. However, p107 and Rb2/p130 can function to suppress tumorigenesis in the context of pRb deficiency. Mice nullizygous for pRb do not develop retinoblastoma as is seen in humans. However, loss of p107 or Rb2/p130 in combination with pRb results in retinoblastoma [Wikenheiser-Brokamp, 2006]. Additionally, pRb ablation in astrocytes [Marino et al., 2000], mammary [Robinson et al., 2001] and prostate epithelial cells results in no phenotypic abnormalities, whereas loss of total pocket protein function by expression of a truncated form of SV40 large T antigen leads to tumor formation [Xiao et al., 2002; Simin et al., 2004]. Furthermore, chimeric pRb;p107 and pRb;Rb2/p130 null mice develop tumors in addition to those seen with pRb ablation alone [Dannenberg et al., 2004]. The tumor spectra in pRb, pRb;p107 and pRb;Rb2/p130 deficient mice do not totally overlap, providing evidence that the pocket proteins have unique as well as overlapping functions in tumor suppression.

GENOME-WIDE APPROACHES APPLIED TO RBF PROTEINS

In the last few years, ChIP genome-wide approaches have opened new roads to the analyses of transcription factors and chromatin modifications. These new methodologies are becoming important to identify basic players of different biological processes, such as gene expression, DNA replication and repair. RB family members play a key role in many gene regulatory networks that govern the cellular response to anti-mitogenic signals and whose deregulation constitutes one of the hallmarks of cancer. With the advent of ChIP technology, many important questions can now be addressed. Are the target genes among the RB family shared? Which are the main targets of each member? What are the relations between each RBF members and chromatin modifications on single target genes?

ChIP-on-chip, gene expression microarray and proteomic approaches have allowed different groups to "de-convolute" the specific roles of each pRb members. Many articles have demonstrated that at the genomic level, p107 and Rb2/p130 are the central pocket proteins that bind the E2F responsive promoters during G0 and early G1, and most of them are genes that regulate cell cycle

progression [Cam et al., 2004; Balciunaite et al., 2005; Litovchick et al., 2007; Farnham, 2009]. In particular, ChIP-on-chip in the T98G glioblastoma cell lines have shown Rb2/p130 and E2F4 cooperating to repress a common set of genes under different growth arrest conditions; however p107 or pRb do not function in doing this. The repression involves a set of genes not only involved in the cell cycle but also in mitochondrial biogenesis and metabolism with the NRF1 protein (nuclear respiratory factor-1) as a co-regulator of a number of E2F target genes [Cam et al., 2004]. In early G1 cycling cells, the same investigators reported three new functional categories of target genes uniquely bound to p107 and/or E2F4 (stress response, signal transduction, and immune response) and a distinct set of genes. In addition, specific combination of RBF and E2F4 proteins correspond to a distinct code of histone acetylation and Sin3B corepressor recruitment, highlighting a complex relation between RBF and chromating remodeling [Balciunaite et al., 2005].

In a more recent work, proteomic, ChIP-promoter array, gene expression array and bioinformatics analysis have allowed the discovery of a Rb2/p130-associated protein complex that contributes to repress cell cycle-dependent genes during quiescence [Litovchick et al., 2007]. Combined protein immunoprecipitation with multidimensional protein identification technology (MudPIT), Litovchick et al., identified 12 Rb2/p130 interacting proteins, 9 of which are homologous to Drosophila dREAM complex. In Drosophila this complex was determinated to be essential for the silencing of developmentally regulated genes. Interestingly, the human complex assembles in two different ways during the cell cycle: in G0, Rb2/p130, E2F4/5, and DP1/2 interact with LIN9, LIN37, LIN52, LIN54, and RBBP4 to repress transcription. During S phase LIN9, LIN37, LIN52, LIN54 dissociate from Rb2/p130 and interact with the B-MYB protein. Promoter chip assays revealed that the G0 complex bound and cooperated to specifically repress E2F target genes. Because pRb is not found in this complex, the authors postulated that Rb2/p130, not pRb, serves as the functional ortholog of pRb from fly and worm to human.

The rather surprising result that emerged from ChIP experiments was the difficulty to detect the presence of pRb on the promoters of many well-established E2F target genes. The only exception is the cyclin E gene that is also deregulated in pRb deficient mouse embryonic fibroblasts. It was speculated that pRb forms the repressor complex in the cytoplasm instead of on chromatin [Stevaux and Dyson, 2002; Iaquinta and Lees, 2007]. Another possible explanation was that pRb can bind regulatory regions other than promoters by a direct E2F mediated mechanism (this is improbable because E2F proteins bind preferentially the promoter region of genes) or by a different mechanism involving other factors [Markey et al., 2002]. With the limits of past microarray technology, we could not analyze the regions outside the promoter. We know, from the β-globin locus control region [Misteli, 2007] and genome-wide studies [Farnham, 2009] that, DNA elements apart several kilobases from the gene are able to enhance gene transcription. The mapping of the ERα binding site is one of the best examples [Carroll et al., 2005, 2006; Laganiere et al., 2005]. The group of Myles Brown analyzed the complete non-repetitive sequence of human chromosome 21 and 22 [Carroll et al., 2005]. They established that most of the ERα binding sites mapped outside the promoter in many sequences with enhancer functions, as demonstrated by chromosome capture and luciferase assays. Subsequently, taking advantage of ChIP-on-chip on all the non-repetitive sequence of the human genome, the same group mapped ERα and RNA Pol II proteins binding in MCF7 breast cancer cells. Only 4% of estrogen binding sites mapped within 1 kb promoter. By combining transcriptional profiling arrays and chip-on-chip data, the authors demonstrated a positive correlation between binding sites within 50 kb of transcription start site and gene expression activation [Carroll et al., 2006]. Although the mechanism of action of ERα protein is cell type specific, these results correlate with data obtained on c-Myc, p53 and Sp1 binding on chromosome 21 and 22, suggesting the necessity of genome-wide studies in opposition to promoter analysis [Cawley et al., 2004].

At least two different articles suggest that the problems encountering in ChIP experiments carried out on the pRb protein could be ascribed to the antibodies [Takahashi et al., 2000; Stengel et al., 2009]. In a pioneering experiment, Takahashi et al., analyzed all three members of pRb and found that the repression of each promoter in T98G glioblastoma quiescent cells is associated with recruitment of E2F-4 and Rb2/p130. The authors tested eight different pRb antibodies without obtaining enrichment on background of E2F target genes [Takahashi et al., 2000]. After this report, the Farnham laboratory analyzed the in vivo binding sites of pRb in Raji cells utilizing chip-on-chip CpG array. Different pRb binding sites were detected in G0/G1 and during S phase. Surprisingly the number of hits was low compared to the other pRb family members [Wells et al., 2003; Balciunaite et al., 2005]. The differences in results found in literature could be ascribed to the cell lines utilized; however, very recently, an independent group reported that a number of pRb antibodies are not able to immunoprecipitate the crosslinked chromatin in SOS-2 cells. To overcome these problems, the authors prepared a GFP-pRb fusion protein as well Rb2/p130 or p107 and ChIP analyses were carried out with anti-GFP antibody. Positive results were obtained on *plk-1* and *dhfr* E2F target promoters. The binding of pRb, Rb2/p130, and p107 on chromatin were also confirmed by Fluorescence Recovery After Photobleaching analyses [Stengel et al., 2009].

These results were supported by another laboratory where it was confirmed that pRb could be detected and associated with chromatin on *cdc6* and *dhfr* E2F target promoters only when special chromatin fixation protocols (dimethyl adipimidate followed by 1% formaldehyde) were applied [Vandromme et al., 2008]. These data strongly suggest that ChiP-grade antibodies are necessary to analyze the pRb-binding site on chromatin to discriminate which unique and overlapping functions have the pRb family of proteins.

CONCLUSIONS AND FUTURE DIRECTIONS

One key point of the post-genomic era is to clarify how the cell machinery utilizes genomic information in normal and anomalous cells. Transcription factors, cofactors, histone modifications, and histone variants participate at different levels to regulate gene transcription in diverse processes including cell growth, proliferation, differentiation, and death [Kouzarides, 2007]. The pRb pathway

is central to regulating cell proliferation, one of the first steps in tumorigenesis. A lot of studies have shown that many chromatin modifier enzymes work in concert with pRb. It is time to investigate through the use of new approaches (e.g., RNA interference) how the RBF members can influence DNA and chromatin modifications and integrate these data with others ''omics'' approaches.

Until now, gene expression profiling has been the principal topic compared with various issues such as disease recurrence, invasive potential, treatment response, and molecular subtype. But at an upper level, integrating expression with protein-protein and protein/DNA interaction can help us better understand the pathways relevant to human pathological diseases such as cancer. Now, there are many integrative analysis platforms that can help discern between important functional connections and to identify relationships among transcriptional programs, protein complexes, disease subpopulations and drug treatments. Several tools (DAVID, Gene set Enrichment analysis, System biology, L2L, Connectivity MAP, etc.) are able to interrogate data from public repositories and display all the information in a network data system (Cytoscape, Osprey, PIANA, GenMAPP, GRAPHVIZ, etc.). Most of the current tools analyze single target signatures across a set of reference signatures. More sophisticated programs are necessary to integrate different types of data, which yields the emergence of all-versus-all, comparing approaches, such as ''Molecular concept map'' in Oncomine.

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