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PROSPECT

Synergistic Role of E1A-Binding Proteins and Tissue-Specific Transcription Factors in Differentiation

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Abstract In this review, the complex relationship between tissue-specific transcription factors and genes regulating cell cycle is taken into account. Both E1-A binding proteins belonging to the family of the retinoblastoma gene product and the CBP/p300 coactivator of transcription interact physically and functionally with tissue-specific transcription factor. The relationship between these two classes of molecules regulates cell fate in differentiating cells, deciding whether cells continue to replicate, undergo apoptosis or terminally differentiate. We provide here an update on the recent advances in this field and some models of interaction between E1A binding protein and tissue-specific transcription factors. J. Cell. Biochem. 67:423–431, 1997. © 1997 Wiley-Liss, Inc.

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The focus of this review is the role of E1A-associated proteins, p105-107-130 and p300 in differentiation. Elucidation of the pathways leading to cellular differentiation has been going on for many years and has involved several generations of scientists. DNA tumor viruses have served as valuable tools for uncovering the secrets of cellular differentiation [Dulbecco, 1976]. DNA tumor viruses alter the phenotype of the cell by a complex interaction with the host cell's own transcriptional and cell cycle machineries. First to be identified were the transforming oncoproteins of the DNA-tumor viruses, Large T antigene for SV40 and Polyoma viruses, E1A for adenoviruses, and E7 for papillomaviruses. The cellular targets of the oncoproteins then were examined to establish the biochemical basis of the phenotype alteration. Technical advances obtained in positional cloning led to the identification of the locus and to the subsequent isolation of the retinoblastoma tumor suppressor gene cDNA. It was discovered later that the product of the retinoblastoma gene, pRb, and one of the main host cellular proteins, p105, interacting with E1A, Large T, and E7 were the same molecule [Whyte et al., 1988]. This key observation switched the interest of many toward experiments designed to identify and clone cellular proteins interacting with the DNA tumor virus gene products, as well as with the retinoblastoma protein. This led to the identification of the retinoblastoma gene family and to a number of genes which are part of the "core" cell cycle machinery, as well as to an understanding of their respective roles in the cell cycle.

The Rb-family members, p105/pRb, p107, and p130/pRb2, as well as p300 belong to this group of E1A-binding proteins. Their respective roles in the cell cycle are beyond the scope of this review and we refer the reader to other reviews which deal more extensively with these cellular functions [Paggi et al., 1996].

Abbreviations: bHLH, basic domain-helix-loop-helix; CBP, CREB Binding Protein; CDKI, cell cycle dependent kinase inhibitor; CNS, central nervous system; PNS, peripheral nervous system; TS, tissue specific; TF, transcription factor.

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RB Family Members and Differentiation

Does the role of pRB in differentiation depend on its antagonistic activity on apoptosis induced by cell cycle genes, or does pRB directly cooperate in transactivating tissue-specific genes? Within this question lies the dilemma about the role of pRB in differentiation. We present in this review evidence that support each hypothesis, while at the same time describe, some of the basic phenomenology of the mechanism by which pRB acts as a key molecule regulating both apoptosis and development.

Cell Cycle and Apoptosis Regulation by the RB Family in Terminal Differentiation

Terminal differentiation invariably involves two tightly linked phenomena: biochemical differentiation and permanent withdrawal from the cell cycle [Nadal-Ginard, 1978]. When cells terminally differentiate, they enter into a G0 state. Terminal differentiation is accompanied by pRb dephosphorylation. The cell cycle machinery, which propels cells through the G1/S transition, is frozen in this state and as a consequence pRb remains dephosphorylated [Weinberg, 1995]. In addition, the amount of pRb protein, as well as that of p130/pRb2, is greatly increased during terminal differentiation, though, in some systems, the increase in the level of p130/Rb2 is transient [Chen et al., 1989; Coppola et al., 1990; De Luca et al., 1997). A comparison between the relative amount of p105/pRb, p107 and p130/pRb2 shows that p105 easily is the most abundant of the three both in cycling and in differenting cells. Evidence derived from homologous recombination studies in mice clearly demonstrates a dose-dependent effect in the developing embryo phenotype. The absence of p105 is embryonically lethal and animals are dead at day 13 of development from severe hematopoietic abnormalities, in particular erythroid, as well as CNS cell differentiation [Jacks et al., 1992; Lee et al., 1992]. In contrast, the absence of p107 or of p130/pRb2 alone, which are less abundant in cells compared with p105, was not accompanied by any remarkable defect in mice phenotype [Cobrinik et al., 1996; Lee et al., 1996]. Only animals heterozygous for pRb (RB−/+) and null for p107 show defects in retinal development.

An obvious effect on development was apparent only in mice rendered null for both p107 and p130/pRb2. The developing embryos had defects in endochondral bone development, shortened limbs and in utero mortality, with death occurring at day 11.5 [Cobrinik et al., 1996]. These results suggest that p130 and p107 together exert a role in the differentiation of specific tissues which is not fulfilled by p105 alone. Table 1 summarizes the data from mouse genetic approaches.

The cell loss that occurs in RB−/− mouse embryos, at least in some tissues, was shown to be due to p53-dependent apoptosis. In fact, the lenses of developing pRb−/− mice show abnormal apoptotic events. When embryos with a genotype RB−/−; p53−/− are generated, lens apoptosis is much reduced. Therefore, it seems that the apoptosis within the lens tissue of RB−/− mice is p53-dependent [Morgenbesser et al., 1994]. Similarly, apoptosis in the CNS of RB−/− mice is p53-dependent. RB−/− CNS cells show an increased level of cyclin E and E2F concomitant with an increased expression level of p53, in good correlation with an increased number of apoptotic events [Macleod et al., 1995]. The same correlation, however, does not hold true for the PNS, where dying cells in RB−/− embryos do not display any increase in p53 expression. Interestingly, PNS neurons expressed p21-WAF protein in the absence of p53

TABLE I. An Overview of the Developmental Effects in Mice Embryos of Homologous Recombination of p105, p107, pRb2/p130, and p53 Genes*

p105	p107	pRb2/p130	p53	Developmental effects (EMBR)	Tissue affected
−/−	+/+	+/+	+/+	+(death at D13)	CNS/Hematop
−/+	+/+	+/+	+/+	−	−
+/+	+/+	+/+	−/−	−	−
+/+	−/−	+/+	+/+	−	−
+/+	+/+	−/−	+/+	−	−
+/−	−/−	+/+	+/+	+	Retina
+/+	−/−	−/−	+/+	+(death at D11)	Bone

*EMBR, embryonal; CNS, central nervous system; Hematop, hematopoietic development; D, day.

[Macleod et al., 1995]. The mechanism of p21 transactivation in differentiation will be discussed later in this review. In addition, the apoptotic effects of the pRb-interacting DNA tumor viruses oncoproteins E1A and E7, respectively, can be blocked, or at least significantly reduced, by the contemporary expression of E1B or E6, which directly (E6) [Scheffner et al., 1990; Werness et al., 1990] or indirectly (E1B, which blocks the effects of the apoptosis-inducing protein bax, transactivated by p53 [Han et al., 1996]) hampers the pro-apoptotic effects of p53. These data confirm that p53 and pRB act synergistically in regulating cell growth and differentiation.

The concept that p105 plays a role in differentiation has been challenged by the evidence that RB−/− mice can be rescued by an in vivo complementation with RB+/+ mice. Most of the hematopoietic and neuronal cells in the resulting chimeric animal are RB−/−, thus indicating that p105 may be dispensable for full differentiation [Williams et al., 1994]. Some believe the absence of terminal differentiation in RB−/− mice is because of defects in extracellular signals, possibly derived from a pathologic environment, not from intrinsic defects of the differentiating cell. More recent evidence from transgenic mice, however, points toward a role for p105 in activating an intrinsic differentiation program. For example, when an overexpression of the p105 antagonist E2F1 is specifically targeted in megacaryocytes of transgenic mice, terminal differentiation of this cell type is blocked [Guy et al., 1996]. At the same time, the number of apoptotic events in differentiating megacaryocytes is increased. In another example, overexpression of cyclin D 1, another antagonist of p105 Rb, in mammary glands, generates abnormal differentiation. After a certain time-lag, such mice develop tumors [Wang et al., 1994].

How does pRb block apoptosis in differentiating cells? The answer probably lies within its antagonistic effect on E2F activity. E2F induces apoptosis when overexpressed in cells with wild type p53 gene, but favors proliferation in p53−/− cells [Qin et al., 1994; Shan and Lee, 1994]. E2F-induced apoptosis follows entry of the cell into S-phase. The E2F death-promoting effect can be blocked by co-expression of p105 [Qin et al., 1994]. Conversely, by gene knock-out studies, it has been possible to demonstrate that E2F is critical for the normal development of diverse cell types. Mice null for the E2F1 gene show defects at a young age in the terminal differentiation of cell types in which apoptosis plays an important role, namely T-cells or epithelial cells of the testis or of other exocrine glands. With increasing age, these animals develop wide-spread tumors. [Field et al., 1996; Yamasaki et al., 1996]. These data indicate that E2F plays a physiological role in normal development, probably by inducing apoptosis in a specific set of developing cells.

Apoptosis is a constant phenomen in differentiating cells [Jacobson et al., 1997]. In developing organisms as well as in many adult tissues like the hematopoietic system, cells replicate and differentiate at the same time. Therefore, many stages of development are needed to reach terminal differentiation. This process occurs only if a specific chain of events proceeds in an orderly fashion. In particular, gene products which stimulate the cell cycle have to be counterbalanced by genes that induce cell cycle arrest. For the E2F family, replicating and differentiating cells need pRb or pRB-family members to counterbalance its apoptotic effect. The more E2F is expressed, the more pRb is needed for the cell to survive. A clear example of this phenomenon has been shown in lymphocytes. Resting primary T lymphocytes express a small amount of pRB as well as E2F. They acquire additional differentiated characteristics when they are challenged to proliferate by external stimuli. The amount and activity of E2F increases when cells enter the S phase in primary T lymphocytes induced to proliferation by IL2 and concanavalin A. pRb protein levels are raised accordingly with the increase in E2F [DeCaprio et al., 1992; Furukawa et al., 1990]. Similarly, in a system of human hematopoietic progenitor cells induced to differentiate toward the erythroid lineage in the presence of appropriate interleukin concentrations, the amount of pRB protein peaked at the level of Burst Forming Units (BFU-E), a stage at which cells "commit" toward the erythroid lineage. Proliferation is also at its maximal level at this time [Condorelli et al., 1995].

How does one explain the paradoxical effect of the absence of pRb (lack of terminal differentiation) or of E2F (defective development and tumors)? The answer may lie in the stochastic nature of development. It is conceivable that out of millions of cells a small percentage of normal, non-tumoral cells may exist in which,

by chance (stochastically), genes which activate the cell cycle are abnormally more active than genes which block cell cycle progression. If so, cells can activate the apoptotic program and self-eliminate from differentiation. E2F and pRB then would be in continuing balance with each other. If one of the two prevails in an untimely fashion or at an abnormal concentration, cells would either stop proliferating or would undergo apoptosis. In either cases, the cells would not reach terminal differentiation. The model represented in Figure 1 could apply to in vitro conditions of cell cycle and terminal differentiation control. By contrast, in knockout mice, there may be a chronic adaptation to the absence of a gene, as in the case of E2F-1, which induces the appearance of a clear-cut phenotype only after several months of age.

Regulation of Tissue-Specific Expression by pRb

Another possibility, which by no mean excludes the first, is that pRb is involved directly in the transactivation of tissue-specific (TS) genes. Many examples have been reported of TS-Transcription Factors (TF) interacting with pRb. We present here some models of pRb-involvement in terminal differentiation that can be drawn, based on published observations.

Myogenesis

The most studied in vitro differentiating system in which the effects of pRb have been followed is probably myogenesis.

The SV40 Large T antigen has been shown to block cellular differentiation and growth arrest and to reactivate DNA synthesis in terminally differentiated myogenic cells [Cardoso et al., 1993; Gu et al., 1993]. It has been shown in this system that pRB associates with tissue-specific transcription factors, namely, members of the MyoD bHLH transcription factor family [Gu et al., 1993]. It has been suggested that the binding of pRB to MyoD plays a role in inducing the activation of muscle specific genes. In fact, myogenic cells obtained from RB−/− ES cells fail to terminally differentiate. They express muscle-specific markers but undergo DNA synthesis in the presence of fetal growth factors, in contrast to pRb+/+ myogenic cells, in which DNA synthesis cannot be reactivated [Schneider et al., 1994]. It has been demonstrated that pRb is critical for the myogenic cell both to display a fully differentiated phenotype and to progress through the cell cycle restriction points without undergoing apoptosis [Novitch et al., 1996]. In addition, in assays in which MyoD expression converts undifferentiated cells into myogenic cells, the contemporary expression of cyclinD 1, a p105 functional antagonist, blocks differentiation [Skapek et al., 1995]. p21-WAF and p16 CDKI's (cell cycle dependent kinase inhibitor) had an opposite effect on the same assay to that of cyclin D1. On the other hand, it is worth noting that the inhibition of myogenesis by cyclinD 1 might be due partly to other p105 independent effects, because non phosphorylatable pRB only partly restores cyclin D1 inhibition of myogenesis [Skapek et al., 1996].

The important role of pRB in muscle development has been demonstrated in another experimental system. In RB-knock out mice, a transgene which drives pRB expression by low copy number rescues the development of most tissues but not skeletal muscle, thus indicating that a high amount of pRB is required for skeletal muscle terminal differentiation [Zacksenhaus et al., 1996]. What is the role of other Rb-like molecules in this scenario? As mentioned above, it has been shown that the p107 protein can partially substitute for p105 and myogenic lines derived by p105−/− ES cells can differentiate into large myotubes. In addition, it is possible to detect DNA synthesis within the nuclei of these myotubes in the presence of growth factors, in contrast to C2C12 myoblastic p105+/+ cells, which are withdrawn permanently from the cell cycle. Myogenin, another muscle specific bHLH, also binds to p107 in these cells. These results suggest that p107 can substitute for the differentiative

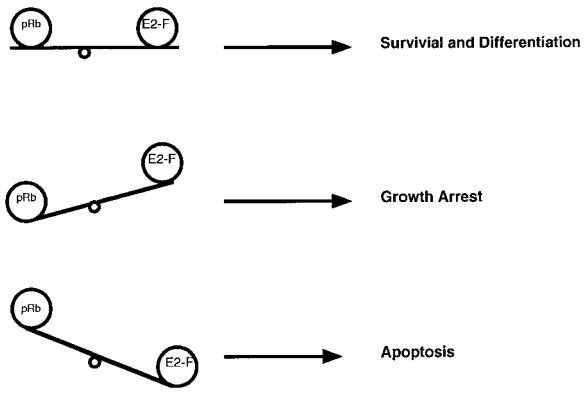

Fig. 1. A simplistic view of the conterbalancing effects of pRb and E2F in differentiation.

role of pRb, but not for its role in determining a permanent cell cycle withdrawal [Schneider et al., 1994]. In fact, while the amount of p107 was increased in myogenic p105−/− cells in comparison with myogenic p105+/+ cells, the level may not have been sufficient to establish a permanent exit from the cell cycle.

In regard to the role of p130/pRb2 in myogenic development, it has been shown that p130/pRb2 levels increase during muscle differentiation. In addition, it has been determined that p130/pRb2 interacts with the cdk2/cyclin complex and blocks its kinase activity, therefore pointing out a role for p130/pRb2 in differentiation [De Luca et al., 1997].

Adipogenesis

In the developing mouse, adipogenesis only starts at the very last days of fetal life. Like skeletal muscle, it has been shown that pRb plays an important role in determining the final phenotype in adipocytes. In fact, p105+/+ mouse fibroblasts can be converted into adipocytes by transfecting C-EBP, a zinc finger TF. In contrast, pRb−/− fibroblasts cannot be converted into adipocytes by C-EBP [Chen et al., 1996]. This is similar in a way to the myoD-pRb paradigm of skeletal muscle: cells can be transformed into adipocytes only when pRb is cotransfected into RB null fibroblasts. The two molecules, c-EBP and p105, form a protein complex in the E2F pocket region of pRB. In fact, a point mutation within the E2F-binding pocket region of pRB abolishes both C-EBP binding and adipocite conversion. Thus, pRB seems to play a role in determining the phenotype in this system also. It is of interest that pRb, in its underphosphorylated form, is able to bind both E2F and C-EBP through its pocket region. However, the interplay between the three molecules has not yet been fully elucidated.

Hematopoiesis

The expression of pRb is tightly controlled in differentiating hematopoietic cells [Condorelli et al., 1995]. In fact, pRb expression is controlled in a lineage-specific fashion in this system. An interaction between pRb and TS-TF also has been shown within hematopoietic cells. A member of the c-EBP TF family, NF-IL6, interacts with pRb in differentiating U937 monocytes [Chen et al., 1996] but the IL6-NF/pRb interaction is not stable. It has been suggested that p105 binds to E2F and to NF-IL6 at the same time.

Furthermore, in erythroid cells, pRb is part of a multiprotein complex which comprises the bHLH transcription factors Tal1 and E2A and the LIM-motive TF Lmo2. The proteins Tal1-E2A-Lmo2 form a unique complex in erythroid cells [Condorelli et al., 1995]. The trimer, however, does not possess any transactivating activity. The trimer complex acquires a transactivating capacity only in the presence of pRb. Like the muscle paradigm, the tetramer tal1-E2A-Lmo2-pRb complex is inhibited by cyclin D1 [Condorelli et al., submitted]. A protein interacting with both Lmo2 and p105, the retinoblastoma binding protein 2 (Rbp2), has been cloned which can potentially bridge pRb with Tal1 [Mao et al., 1997]. The Zinc finger protein GATA1 also might be part of this process. Inhibition of the assembly of this erythroid-specific TF complex by the absence of p105 may be partly responsible for the absence of terminally differentiated erythroid cells in developing RB−/− mice. Because the interaction between p105 and the multiprotein complex TF is not very strong, as is also true for p105/NF-IL6, a model has been proposed in which p105 acts as the chaperon to carry the transcription factor to the target sequence and release it immediately following delivery (Fig. 2). The concept of pRb acting as a chaperon is suggested by the fact that pRb is a nuclear matrix protein. It is present in a high amount in the nucleus, and its affinity for the nucleus is regulated by a varying degree of phosphorylation [Weinberg, 1995]. pRb itself possesses little transactivation activity. A major effect of pRb is the inhibition of E2F transactivating properties [Chow et al., 1996; Smith et al., 1996]. This is why pRB historically has been considered an inhibitor instead of an activator of transcription, which recent evidence begin to confirm.

p300/CBP

p300 is an E1A-binding protein with sequence similarities to the CREB Binding Protein CBP/p165 [Arany et al., 1994]. The role of p300 in terminal differentiation had been suggested by experimental findings long before its cloning. In fact, mutants of E1A, which were able to bind p300 but not p105, still were able to block cellular differentiation in different cellular systems including skeletal muscle cells, in-

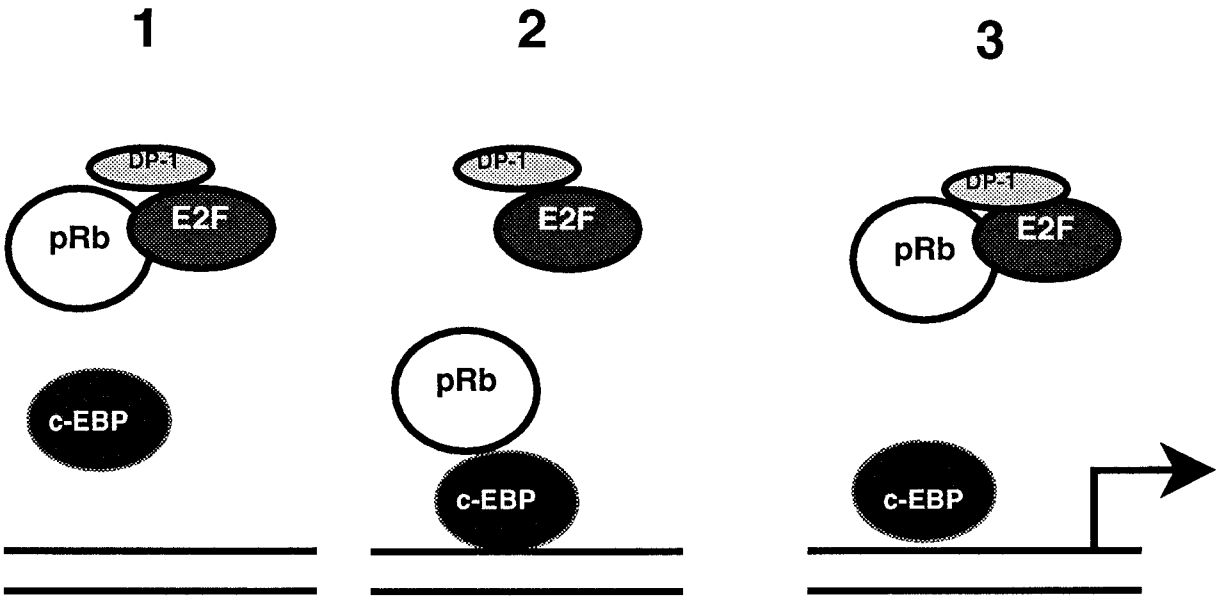

Fig. 2. A model of pRb-cEBP interaction during adipogenesis.

directly pointing out the role of the E1A-associated protein in differentiation [Caruso et al., 1993; Mymryk et al., 1992; Webster et al., 1988]. When p300 was cloned, functional experiments demonstrated that p300 had the properties of a transcriptional adaptor. In fact, p300 is a coactivator of universal as well as TS-TF [Arany et al., 1994]. It has the ability to bring the transcription factors close to the basal transcription machinery. p300/CBP is able to interact with members of the universal transcriptional initiator complex TFIID and TBP [Lundblad et al., 1995; Yuan et al., 1996] and with TS-TF, at the same time. To date, the list of TS-TF interacting with and co-activated by p300 continues to grow. It has been shown that p300 interacts with the muscle-specific bHLH TF MyoD as well as with the universal bHLH E protein in skeletal muscle cells [Eckner et al., 1996; Puri et al., 1997; Yuan et al., 1996]. p300/CBP is also able to cooperate in transactivation with MEF2, another class of skeletal muscle TF [Eckner et al., 1996; Sartorelli et al., 1997]. In addition, p300 plays a critical role in mediating cell cycle arrest during terminal differentiation. In fact it was shown that the expression of p21 during differentiation is independent of p53 but is dependent on TS-TF. In p53−/− mice, the expression of p21 is normal and terminal differentiation takes place regularly [Parker et al., 1995]. However, p21 expression in skeletal muscle is dependent on bHLH proteins [Guo et al., 1995; Halevy et al., 1995]. In addition, it has been shown in other cellular systems such as the keratinocytes, skeletal muscle cells, hematopoietic and neuronal cells, that the expression of p21 as well as terminal differentiation is blocked if cells are transfected with an E1A mutant that retains the ability to bind to p300 but is not blocked by mutants that bind exclusively to p105 and not to p300 [Billon et al., 1996; Datto et al., 1997; Missero et al., 1995; Steinman et al., 1994]. It can therefore be stated with a certain degree of confidence that TF-TF and p300 cooperate in order to transactivate the p21 CDKI universal inhibitor of cell cycle during development (Fig. 3).

The regulation of differentiation by p300 also can take place through alternative mechanisms. It has been demonstrated that p300 also can bind to the cdk2/cyclinE complex. This binding is inhibited by p21 CDKI. When the binding of the cdk2/Cyclin E complex is blocked, p300 can interact with NF-kB and transactivate another set of genes during cell cycle arrest and DNA damage [Perkins et al., 1997]. In a recent series of experiments, it was demonstrated also that p53 and p300/CBP directly interact in vivo. The interaction between the two induces a block in the transactivation of promoters activated by p300 during cell growth, namely AP1. This block can be reversed by increasing the amount of

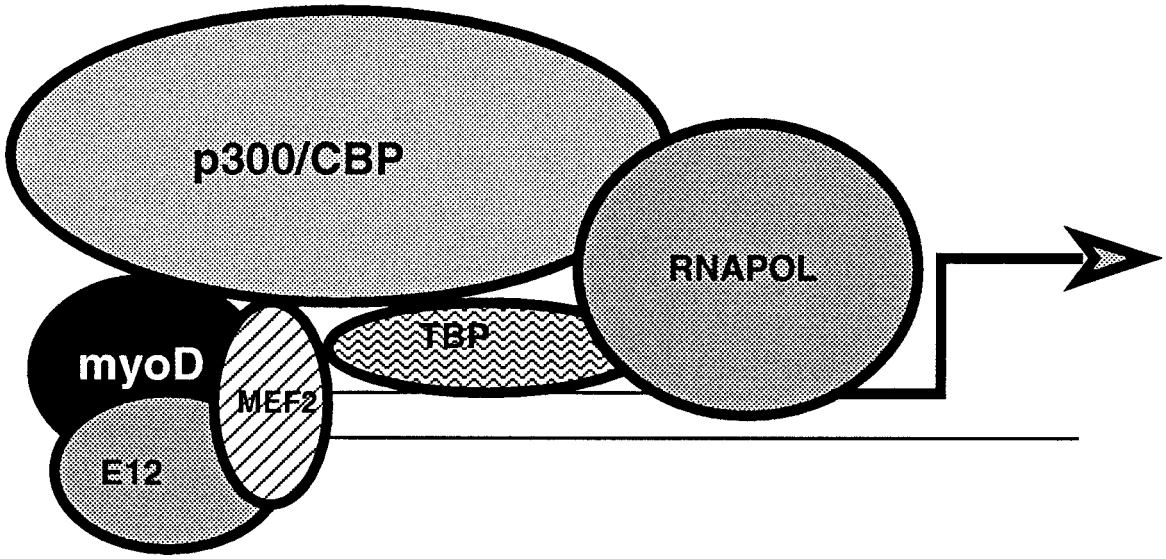

Fig. 3. A model of the cooperative effects of the interaction between p300/CBP and bHLH transcription factor in the transactivation of the p21/WAF promoter during cell cycle arrest in terminal differentiation.

p300/CBP in the assay [Avantaggiati et al., 1997]. At the same time, this cooperation induces an activation of p53-regulated promoters, namely p21, mdm-2 and bax [Avantaggiati et al., 1997; Gu et al., 1997; Lill et al., 1997]. It may be worth noting that p21/WAF expression is not mediated by p53 during differentiation. Only when the cell is hit by DNA damaging agents does p21/WAF expression become p53-dependent. The regulation of promoters during cell growth and differentiation determined by p300/CBP, such as AP1, also may take place through the following mechanism. When p300 binds to the nuclear hormone receptor (NHR) TF, the heterodimers RXR/RAR or RXR/TR can bind to p300/CBP in the presence of the hormone and transactivate their specific promoters [Chakravarti et al., 1996; Kamei et al., 1996]. Binding of p300 to the heterodimers RXR/NHR is incompatible with the interaction of the AP1 complex with CBP/p300 [Kamei et al., 1996]. Competition for the CBP/p300 binding may therefore represent the molecular explanation for the incompatibility between the growth-stimulatory effects induced by the AP1 complex and the growth inhibitory effects induced by steroid hormones. It appears that the emerging role of p300/CBP is that of an integrator of different types of stimuli at the nuclear level, in contrast to the p105 role as a scaffold protein, which anchors transcription factors to nuclear structures.

We have analyzed the current understanding of the role and function of E1A-binding proteins in development in this review. An endless number of questions remain to be answered. It will be exciting to watch as this field unfolds and to learn how much more these DNA tumor viruses can teach us.

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