THE P130 POCKET PROTEIN: KEEPING ORDER AT CELL CYCLE EXIT/RE-ENTRANCE TRANSITIONS

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1. ABSTRACT

Pocket proteins, including the retinoblastoma susceptibility gene product (pRB) and the related proteins p107 and p130, function at cell cycle regulatory steps that link cyclin/CDK-integrated positive and negative growth signals with E2F transcription factor activity on genes required for cell cycle progression. Protein complex formation between pocket proteins and members of the E2F family of transcription factors determines whether E2F complexes act as transcriptional activators or repressors. Experimental work over the last few years indicates that individual pocket proteins interact with specific E2F members to regulate the transcription of certain genes under diverse cell growth conditions. Among these protein associations, p130-containing E2F complexes seem to be of particular importance in controlling gene transcription in quiescent and differentiating cells by repressing the transcription of a set of E2F-responsive genes. Once the cells are progressing through the G1 phase of the cell cycle, pocket protein-mediated regulation of E2F activity is assumed by pRB and p107. p130-mediated transcriptional regulation thus seems to prevent a gene expression program characteristic of dividing cells at the cell cycle exit and re-entrance transitions and in quiescent cells.

2. INTRODUCTION

The E2F family of transcription factors includes five related proteins, namely E2F-1, 2, 3, 4, and 5, that heterodimerize with 3 more distantly related proteins, DP-1, 2, and 3 (figure 1), which are required for the DNA-binding capacity of E2F (1, 2). Most of the E2F-responsive genes so far identified are required for the G1 transition to the S phase of the cell cycle, being transcriptionally activated at a period of the G1 phase coincident with passage through the restriction point. Some genes that have been demonstrated or proposed to be under E2F control encode for either cell cycle regulatory proteins such as cyclins E (3-5) and A (6, 7), the cell cycle kinase CDC2 (8, 9), the CDC25C phosphatase (10), the protooncogenes B-Myb (11, 12), c-myc (13, 14), and N-Myc (2), the pocket protein p107 (15), and E2F-1 (16-19) and E2F-2 (17, 20) themselves, and some enzymes involved in DNA metabolism such as thymidylate synthase (TS) (21), DHFR (22, 23), DNA polymerase alpha (24), TK (25), RRM2 (26) and HsOrc1 (27). E2F/DP heterodimers function as transcriptional activators of E2F-responsive promoters placed upstream of reporter genes, whereas pocket protein complex formation turns E2F either into transcriptional repressors of some of these promoters or prevents E2F transcriptional activation in other cases (figure 2). Association of pocket proteins with E2F is controlled, at least in part, by the temporal activity of cyclin/CDK holoenzymes during the cell cycle, so that the E2F-binding capacity of pocket proteins is abrogated by cyclin/CDK phosphorylation. Other mechanisms that contribute to regulation of E2F activity include modulation of E2F and pocket protein levels (reviewed by 28), cyclin/CDK-mediated phosphorylation of E2F/DP heterodimers (28, 29), and subcellular localization of E2F complexes (30-33). Moreover, while p130 and p107 seem to have binding capacity only for E2F-4 and E2F-5, pRB associates *in vivo* with either E2F-1, E2F-2, E2F-3 or E2F-4 (figure 1). Furthermore, another putative function of p130 and p107 is to act directly as CDK inhibitors, which provides an additional independent growth suppressor function for these two proteins (34-37) and suggests that novel feedback loop pathways participate in the control of certain cell cycle transitions.

As detailed below, in this review, many cell cycle genes are transcriptionally silent in G0. Analysis of a number of promoters in such genes indicates that these genes are actively repressed through E2F-like sites. Since most of the E2F proteins in quiescent cells are complexed with p130-E2F-4 being the predominant E2F family member in these

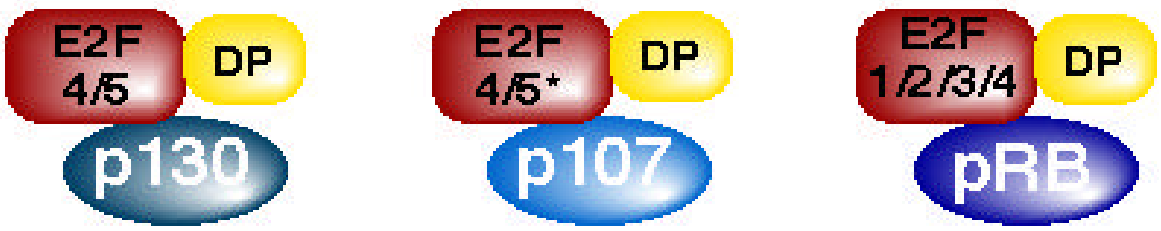

Figure 1. Pocket proteins interact with E2F/DP heterodimers. E2F family members seem to be able to heterodimerize with any DP protein. p130 associates with both E2F-4/DP and E2F-5/DP heterodimers. p107 associates primarily with E2F-4, *and has been found associated with E2F-5 in transfection assays. pRB associates with E2F-1 to 4/DP heterodimers.

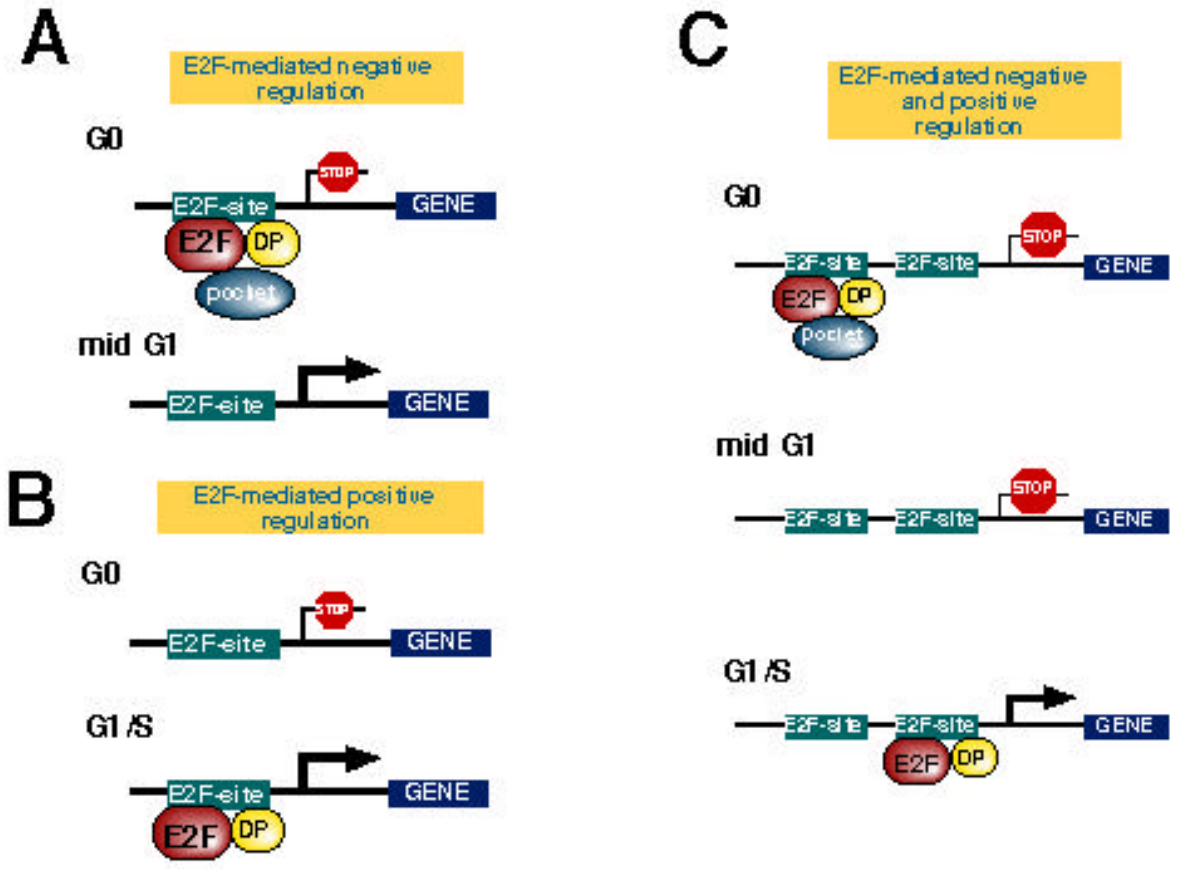

Figure 2. Distinct models of E2F-mediated regulation of E2F-responsive promoters. (A) E2F-mediated negative regulation of E2F-responsive promoters in G0 by binding of pocket protein/E2F/DP complexes. Repression is released in mid- to late G1 by cyclin CDK phosphorylation (see text). (B) E2F-mediated positive regulation of E2F-responsive promoters in mid-to-late G1 and S phases by binding of E2F/DP complexes with transactivation activity. These promoters should be silent in G0 due to the absence of E2F complexes with transactivation activity. (C) E2F-mediated negative and positive regulation of E2F-responsive promoters. This cartoon depicts a dual model that integrates positive and negative regulation of transcription through E2F sites. Genes such as the DHFR gene of Chinese hamster might be regulated in a similar manner in ovarian cells (39). This type of regulation can be envisioned through a single or multiple E2F sites.

cells-, it is assumed that p130 is responsible for this transcriptional silencing (figure 2A). Progression through the G1 phase involves a shift of E2F to pRB- and p107-containing complexes mostly due to complete hyperphosphorylation and downregulation of p130, to expression of pRB-associated E2F members, and to new

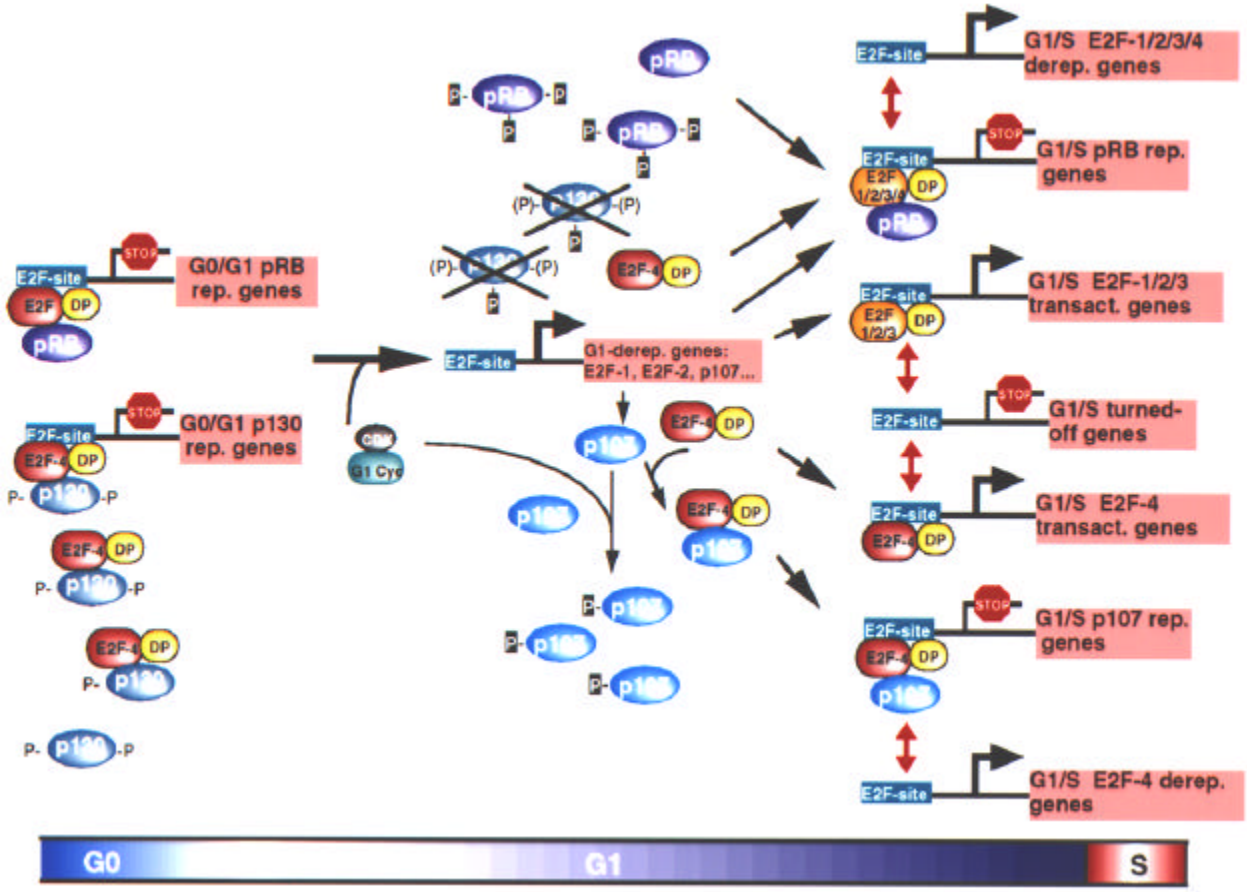

Figure 3. Cell cycle entry: regulation of E2F-dependent transcription by pocket proteins. High levels of phosphorylated p130 forms 1 and 2 are present in quiescent cells (G0 phosphorylation is represented by *P*). E2F-4 is the major E2F species in quiescent cells and it is bound to p130. p130/E2F-4/DP complexes repress the transcription of some genes whose products are required for cell cycle progression (G0/G1-repressed genes: indicated by *G0/G1-rep. genes*). Although low levels of pRB/E2F complexes are detected in quiescent cells, these complexes seem to participate in the repression of some genes (see text). Cell cycle re-entry results in the activation of G1 cyclin/CDK holoenzymes, which phosphorylate p130 to form 3 and abrogate p130/E2F-4 interaction (cyclin/CDK catalyzed phosphorylations are represented by boxed P). In addition, p130 levels abruptly decrease (represented by a crossed out molecule). G0 phosphorylation sites in p130 may not be phosphorylated from mid G1 to the remainder of the cell cycle (represented by *(P)*). Among the genes containing E2F sites which are repressed in G0, there is E2F-1, E2F-2 and several other genes, including the p107 gene itself (see text). De-repression of transcription by disruption of p130/E2F-4 complexes results in the expression of E2F-1 and E2F-2 and allows then for the positive regulation of transcription of other genes containing E2F sites (G1 and S-transactivated genes: indicated by *G1/S transact. genes*). The free E2F-4/DP complexes released from p130 may also transactivate genes in mid to late G1 and S phases. p107 accumulates abruptly in mid G1 and is also phosphorylated by G1 cyclin/CDK holoenzymes. However, low levels of hypophosphorylated p107 associate with free E2F-4/DP complexes to repress the transcription of some genes during late G1 and S phases (G1/S repressed genes: indicated by *G1/S rep. genes*). pRB is hyperphosphorylated in mid G1, and the remaining low levels of hypophosphorylated pRB associate with newly synthesized E2Fs 1 through 4 and the E2F-4 released from p130/E2F-4 complexes by cyclin/CDK-mediated phosphorylation of p130. pRB/E2F complexes might repress genes containing E2F sites during mid-to-late G1 and S phases. In addition, promoters with E2F complexes bound to E2F sites, for instance E2F/DP/pocket or E2F/DP, might be turned on/off respectively (red reversible arrows) by cyclin /CDK phosphorylation of diverse complex components (not shown in this figure).

synthesis of p107. Then, a balance between hypo- and hyper-phosphorylated pRB and p107 is thought to tightly regulate, at least in part, E2F activity during the late G1 and S phases. This scenario is summarized in Figure 3, and more details are given in Section 3 of this review.

The specificity of interaction between individual pocket proteins and E2F family members and the timing of formation of particular pocket protein/E2F complexes during the cell cycle suggests that individual E2F complexes have distinguishable effects on gene transcription. Moreover,

transcriptional regulation of specific genes by individual pocket protein-E2F complexes is suggested by recent experiments employing mouse fibroblasts deprived of individual and/or combinations of pocket proteins (38). In this respect, DNA-sequence specificity may exist for some E2F members (39, 40), and the presence or absence of certain consensus sites in some E2F-regulated promoters correlates with the response to E2F-mediated activation or repression (41). Moreover, the E2F-target gene specificity also seems to be determined to a great extent by the availability of individual E2F complexes. In other words, many E2F-responsive promoters would be activated or repressed depending on the presence of certain E2F complexes in the nucleus. This is the case for genes that are transcriptionally repressed during G0 by p130/E2F complexes, as well as the case for some genes regulated in late G1 and S phases where a balance between hypo- and hyper-phosphorylated forms of pRB and p107 is kept. It is conceivable that the biological significance of the existence of such diverse pocket and E2F protein families is to contribute in a precise manner to transcriptional regulation under a multitude of growth conditions in different cell types. In this respect, p130 has proved to be an example of how certain sets of genes are regulated at transitions involving cell cycle exit to, or cell cycle entrance from, a quiescent state. The regulation of p130 activity mainly with respect to its interaction with E2F complexes and the functional implications of this pathway on the physiology of the cell cycle and of differentiation processes will be discussed in this review.

3. DISCUSSION

3.1. Regulation of E2F transcription factor activity by p130 during the G0/G1 transition

Many genes required for cell cycle progression are transcriptionally silent in quiescent cells. *In vivo* footprinting assays using the promoters of some of these genes have shown that consensus E2F-like sites are protected in quiescent cells, and this protection is abrogated in cells progressing through G1 (6, 9, 11). In agreement with a role of E2F in the silencing of these promoters, transcriptional repression is relieved by mutation of the E2F-like sites (6, 7, 9, 11, 12, 15, 17, 27), suggesting that E2F-repressor complexes exist in quiescent cells (figure 2A and Figure 3). A number of G0 silent genes are believed to be regulated in this manner, including CDC2, E2F-1, p107, B-myb, HsOrc1, CDC25C, and cyclin A, among others (see references above). E2F-4 is by far the most abundant E2F protein family member in quiescent cells (42, 43). A number of studies have also reported that most of the E2F activity in G0 cells contains p130 as a pocket protein component, including serum deprived cell lines (17, 44-48), primary human hematopoietic cells (42, 43, 49, 50), and cells differentiated *in vitro* including, muscle cells (51-54), neuronal PC19 cells (53), 3T3-L1 adipocytes (55) and melanocytes (56) (see section 3.3.). These data altogether suggest that most of the E2F-mediated repression of transcription is dependent upon p130 complex formation in quiescent cells. Other silent E2F-dependent genes which are not known to be repressed in non-dividing cells may simply not be expressed because of p130 sequestration of E2F protein and/or because the E2F member that positively regulates their expression is not available in such resting cells (figure 2B).

As summarized in figure 3, upon growth factor stimulation of quiescent cells, p130 is hyperphosphorylated most likely by cyclin/CDK holoenzymes in mid G1 to protein forms unable to bind to E2F (57, 58). Eventually, p130 protein levels drop dramatically to almost undetectable levels which may be due to instability of these p130 hyperphosphorylated forms (see below). Rupture of these complexes correlates with accumulation of free E2F, with the expression of E2F-responsive genes, and is concomitant to the expression of p107 and of additional E2F family members, namely E2F-1 and E2F-2, whose transcription was repressed in G0, most likely through E2F sites. Although less is known about the regulation of E2F-3 protein levels, transcription of E2F-1 and/or E2F-2 in mid G1 and the release of E2F-4 from p130 probably leads to the formation of complexes containing pRB. In a similar manner, new synthesis of p107 from mid G1 also promotes complex formation between p107 and E2F-4. Interestingly, cyclin/CDK phosphorylation also takes place on pRB and p107, but in contrast to p130, pools of hypophosphorylated pRB and p107 able to associate with E2F are detected in late G1 and S phases. Presumably, the existence of different phosphorylated forms of pRB and p107 is responsible of keeping a balance between activator and repressor E2F complexes in these stages, and the resulting overall E2F activity may determine the degree of transcription of E2F-responsive genes. Cyclin/CDK activation in mid G1 thus separates two fundamental functional stages regarding E2F activity: i) total silencing of E2F-negatively-regulated genes in G0 and early G1 mainly by p130-conferred transcriptional repressor activity (see below); and, ii) a shift to pRB- and p107-mediated E2F regulation from mid G1 that coincides with the period of transcription of E2F-responsive genes. As summarized in figure 3, these regulatory steps suggest that p130 is negatively controlling or, at least, participates in the negative control of a gene expression program during G0 and early G1 that involves repression of genes such as E2F-1, E2F-2, p107, and cyclins, among others (see above). As we shall see in this review, p130-mediated gene repression frequently coincides with stages where E2F regulation by pRB and p107 is diminished or non-existent.

Mammalian cells commit to DNA replication and cell division at a point in G1 known as the restriction point (RP). Beyond this point, mammalian cells are independent of growth factor stimulation for cell cycle completion and are not responsive to growth inhibitory factors such as TGF-beta (59). It was postulated that a labile protein might exist that regulates this commitment step. The protein/s regulating the RP transition has/have been a subject of debate, and one possibility is that the coordinate activity of all the G1 components shown in figure 3 determines whether to commit through the cell division cycle. This is likely to happen by a convergence of various early G1 pathways into a critical protein/s or pathway. In support of this hypothesis, various cell cycle regulators are sufficient by themselves to assume commitment or non-commitment decisions *in vivo* when ectopically overexpressed, either blocking cell cycle

progression in G1 or allowing S phase entrance in the absence of growth stimuli. In this context, and although possibly not participating directly in the RP transition, p130-mediated repression on positive regulators of the cell cycle may allow post-quiescence, early G1 events to take place in an orderly manner. Thus, the cell cycle regulatory machinery can integrate properly the growth signals to which the cell is exposed (also reviewed in 28). In fact, the effects of overexpression of some E2F proteins in cells might illustrate the biological significance of p130 function during the G0/G1 transition. Overexpression of E2F-1 in serum-deprived cells is sufficient to induce DNA synthesis, yet it is followed by apoptosis due to abnormal S-phase entry (60-65). This indicates that G1 events in addition to E2F-1 activation are required for normal cell cycle progression. Moreover, E2F-1 overexpression, but not E2F-4, can overcome G1 arrest mediated by TGF-beta or p16 (63, 66-68) suggesting that E2F-1 functions in a pathway that directly controls the G1/S transition, and that the events that trigger activation of E2F-1 might indeed represent the RP switch. In conclusion, E2F-1 activation would take place at a mid-to-late G1 stage once G1 events have been integrated to a decision of RP passage. Consistently, p130-conferred negative regulation on E2F-1 transcription during G0 and early G1 (17, 38, 46) is only relieved upon growth factor-induced disruption of p130/E2F-4 complexes, most likely by cyclin/CDKs in mid G1 (46, 58). Therefore, p130 negative control on E2F-1 transcription may impede that a key RP player, such as E2F-1, is activated before growth factor signals have been integrated into a single positive RP passage decision. A close look at the scheme in figure 3 also highlights the presence of feedback regulatory loops in E2F-regulated pathways. Interestingly, while G1 cyclin/CDK activation is responsible of abrogating p130/E2F-4-mediated repression, transcription of cyclins E and A is known to be, at least in part, under E2F control (4, 5, 7). In this regard, transcription of cyclins E and A, but not D-type cyclins, is strongly induced upon E2F-1 overexpression (26, 69), and cyclin E expression is de-regulated in pRB -/- cells (70) and primary pRB -/- mouse fibroblasts (38). Since cyclin promoters seem to be regulated by a number of growth situations, in particular cyclin D1 can be induced by the early response genes encoding the constituents of the AP1 transcription factor (71), it is tempting to speculate that transcription of D-type cyclins is one of the early G1 events that take place before E2F activation in mid G1. This hypothesis is in agreement with the notion that D-type cyclins control the kinases primarily responsible for pocket protein hyperphosphorylation in mid G1 (72, 73). With respect to p130 function in early G1, D-type cyclin-induced disruption of p130/E2F-4 complexes independent of E2F activity would be a plausible mechanism of initiating the regulatory cascade shown in figure 3. On the other hand, although very low levels of pRb/E2F complexes are detected in quiescent cells, we cannot disregard the repressor function of these complexes in quiescent cells (see below). Once E2F complexes have been released from p130, and perhaps pRB control, E2F-dependent expression of cyclins E and A would contribute to regulation of pRB- and p107-E2F interactions. In this regard, cyclin E and A genes have been shown to be de-regulated in pRb -/- and double p107 -/-: p130 -/- quiescent mouse embryonic fibroblasts (MEFs), respectively (38). This suggests that phosphorylation of pRB may result in cyclin E expression while phosphorylation of p130 may result in the expression of cyclin A. This issue, however, is further complicated by additional regulatory loops in this pathway, such as the fact that E2F-dependent cyclin A transcription is activated by the late-G1 cyclin E-associated kinase activity in E2F/p107 complexes (74) or recent experiments suggesting that cyclin E can promote S phase entry in the absence of E2F ctivation (73). Therefore, the possibility that cyclin E is physiologically induced in parallel of the E2F pathway provides and additional point of control of restriction point transition. There is also evidence suggesting that regulation of p107 protein levels during the G1 phase might be regulated by p130. First, p107 protein levels are in the range from very low to undetectable, in quiescent and early-to-mid G1 cells (75), a behavior that can be extended to a number of cell growth and differentiation conditions where hypophosphorylated forms of p130 are present (76). Second, the p107 gene promoter contains E2F sites, and transcription from this promoter can be repressed by pocket protein/E2F co-transfection (15). Third, p107 is expressed in quiescent fibroblasts isolated from p130 -/- mice although changes in p107 mRNA levels are not observed (38, 77). The fact that p107 levels might be directly or indirectly regulated by p130 in G0 and early G1 implicates a functional specificity of p130 as a pocket protein in these stages, since both proteins share partnership with the same E2F members, i.e. E2F-4 and perhaps E2F-5. Moreover, p107 protein and mRNA levels are upregulated in pRB -/- MEFs indicating that pRB might repress p107 transcription in quiescent cells (38). This observation together with the upregulation of cyclin E in these cells indicates that although pRB/complexes are not the major E2F activity in quiescent cells, these complexes are essential for the regulation of at least some E2F-responsive genes.

In addition to the genes mentioned above, the expression of a number of genes thought to be regulated through E2F sites is deregulated in quiescent and G0/G1 p130 -/-; p107 -/- MEFs. The deregulated genes include the B-myb, cdc2, E2F-1, TS, RRM2 and DHFR genes (38, 77). These data together with the analysis of the expression, phosphorylation status and protein-complex availability of both pocket proteins and E2F transcription factors strongly indicate a role of p130 in the repression of a set of genes in G0 and early G1 cells. p107, most likely, assumes the regulation of these genes when p107/E2F complexes are generated. Nevertheless, it is very important to consider that the effects of lack of the expression of a particular pocket protein in these knockout mice have to be understood within the context of their genetic background. In this regard, p130 -/- and p 107 -/- mice have been generated on a different genetic background resulting in severe growth deficit not apparent in the ones reported earlier (M. Rudinicki, personal communication). In this regard, it seems that gene modifiers might exist in particular mice strains thus explaining this phenotype disparity.

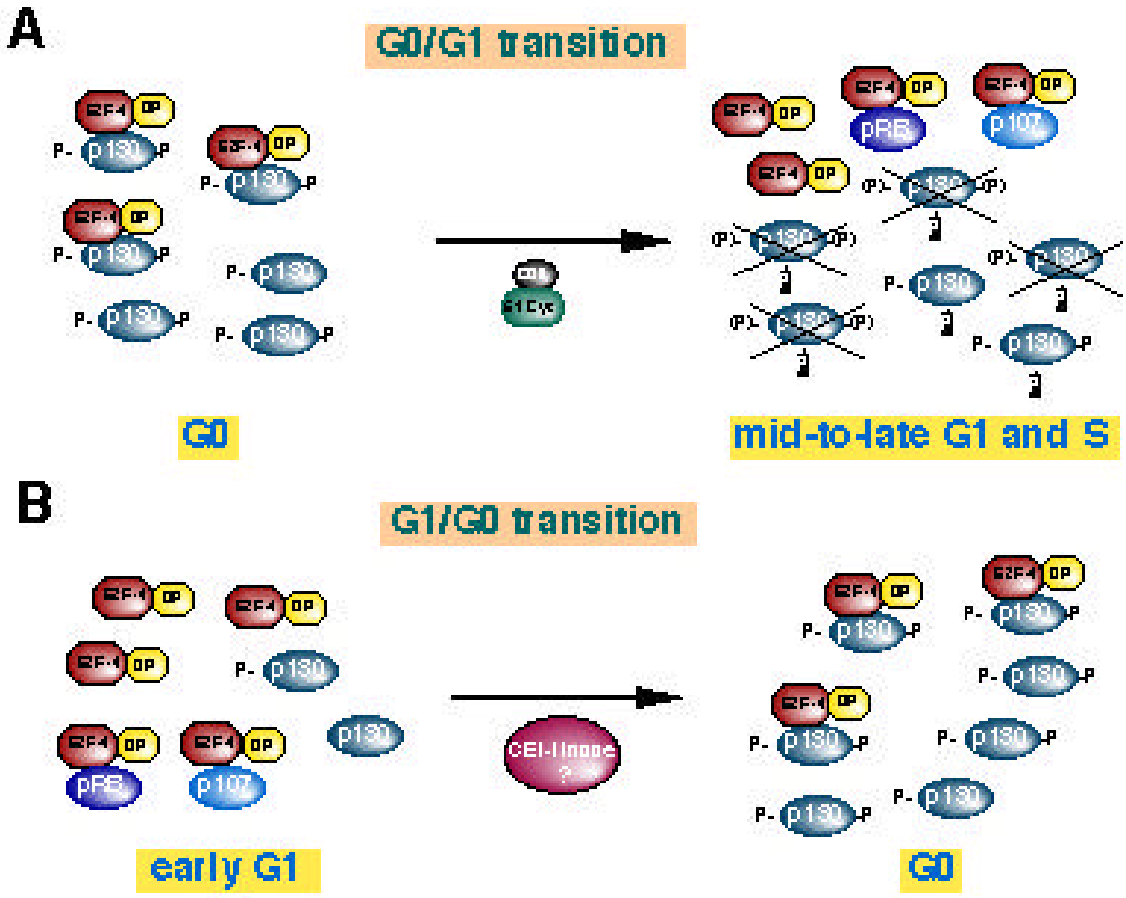

Figure 4. Regulation of p130 by phosphorylation. (A) The G0/G1 transition. In G0, E2F-4 is the major E2F activity and it is bound to p130. p130 is mainly found as a phosphorylated protein that we have named p130 form 2 (represented by a p130 molecule with 2 P). p130 form 1 is also detected in G0 at variable levels depending on the cell line or cell type. In mid G1, p130 is phosphorylated to p130 form 3, most likely by G1 cyclin/CDKs resulting in the disruption of E2F-4/DP/p130 complexes. In addition, p130 levels drop dramatically (represented by a crossed out molecule). G1 cyclin/CDK-mediated phosphorylation is represented by boxed P. G0 phosphorylation sites in p130 may not be phosphorylated from mid G1 to the remainder of the cell cycle (represented by *(P)*). Free and newly synthesized E2F-4 form now complexes with pRB and p107. (B) Regulation of p130 at the cell cycle exit. Cells in G1 prior to the restriction point exit the cell cycle into G0 in response to growth inhibitory and/or differentiation signals. However, when cells have passed the restriction point, they are committed to divide and re-enter G1, prior to exit the cell cycle into G0. In post-mitotic G1, low levels of unphosphorylated p130 and p130 form 1 are detected. E2F-4/p130 complexes are scarce, thus E2F-4 might be free or complexed to pRB and p107. At the G1/G0 transition, phosphorylation of p130 to specific forms -mainly form 2- coincides with its accumulation and leads to the formation of p130/E2F-4 complexes, which in turn are thought to repress the transcription of several genes required for cell cycle progression (see text and Figure 2 and 3). A Cell-cycle Exit Induced (CEI)-kinase might target underphosphorylated p130 to generate p130 forms with higher stability, thus, triggering the formation of E2F/p130 repressor complexes. This putative kinase may play a major role in regulating this transition.

3.2. Cell cycle exit-induced modulation of the phosphorylation status of p130 is unique among pocket proteins

Pocket proteins are known to be regulated by phosphorylation during the cell cycle. Cyclin/CDK activities from mid G1 to mitosis result in hyperphosphorylated pocket protein forms unable to bind to E2F transcription factors as well as some other associated proteins (figure 3 and figure 4). Moreover, the analysis of p130 phosphorylation during different cell growth conditions has revealed that additional kinase activities targeting the p130 protein are likely to exist (figure 4).

Unphosphorylated or hypophosphorylated pocket protein forms shift to electrophoretically-slower migrating forms by cyclin/CDK-induced hyperphosphorylation

(reviewed in 78, see also 57, 75). Three phosphorylated forms of p130 can easily and reproducibly be resolved by SDS/PAGE (57, 58, 76). Conditions involving either cell cycle exit by growth factor deprivation, intercellular contact inhibition, TGF-beta-induced G1 arrest, or terminal differentiation, all lead to a pattern of discrete p130 phosphorylated forms that is not observed with pRB or p107 (57, 58, 76). One of the p130 phosphorylated forms, p130 form 2, has been well characterized during these processes, and its pattern of appearance upon serum removal in cultured cells suggests that a Cell cycle Exit-Induced kinase (CEI kinase) specifically phosphorylates p130 (figure 4B) (58, reviewed in 28). Although such a putative p130 kinase has yet to be identified, the possibility that p130 form 2 arises from post-transcriptional or post-translational mechanisms other than phosphorylation -production of a larger protein or dephosphorylation- seems unlikely. First, *in vitro* treatment of p130 form 2 with a phosphatase reconstitutes the electrophoretic mobility of unphosphorylated, full length p130, thus indicating that form 2 is a phosphorylated form of p130 but not a p130 polypeptide of different size. Second, forcing synchronized cells in early G1 to exit the cell cycle results in accumulation of p130 form 2 at a time where hyperphosphorylated forms of p130, which could act as substrates of a putative cell cycle exit-induced phosphatase, are not detected. Finally, cell cycle exit-induced accumulation of p130 form 2 occurs only in early G1 cells prior to restriction point passage. At this time, aside from not detecting hyperphosphorylated p130 (see above), there are no G1, S and G2/M cyclin/CDK activities in the cell that could provide a hypothetical cell cycle exit-induced phosphatase with a hyperphosphorylated p130 substrate.

The phosphorylation event leading to p130 form 2 may have important implications in the regulation of p130 function during cell cycle exit transitions. Experiments measuring *in vivo* p130 synthesis, p130 phosphorylation rates, and p130 steady state protein levels in quiescent versus proliferating cells indicate that cell cycle exit modulation of p130 phosphorylation -that is, conversion to form 2- results in stabilization of the p130 protein (figure 4B) (54, 58, reviewed in 28). This increased stability is probably responsible for the fact that p130 protein levels increase dramatically during cell cycle exit. Indeed, p130 protein accumulation during cell cycle exit allows the formation of the typical p130/E2F-4 complexes observed during G0 (58), whereas these complexes are accordingly scarce in cell growth situations where hypophosphorylated p130 is present but p130 form 2 does not accumulate, i.e. post-mitotic G1 progression (figure 4B, see below). Moreover, association with p130 protects E2F-4 from protein degradation (79), a mechanism that may further contribute to the accumulation of these complexes in the cell. In conclusion, cell cycle exit specific phosphorylation of p130 is responsible, at least in part, for the formation of the high levels of p130/E2F-4 complexes in G0 cells. As explained above, the existence of p130/E2F-4 transcriptional repression in quiescent cells may be determinant regarding the order of events that take place during re-entrance and progression through the G1 phase, hence the importance of regulatory mechanisms which modulate p130 protein interactions, for instance the CEI kinase activity. In this respect, identification of the biochemical activity that results in accumulation of p130 form 2 will allow us to ascertain to what extent this activity is required for the normal control of the cell cycle. In addition to the control of E2F activity, we can also consider another aspect of the p130/E2F-4 association during cell cycle exit. As a consequence of the high levels of p130/E2F-4 complexes in G0, a pool of E2F-4 protein is maintained in these cells such that a certain amount of E2F-4 will be ready to be used by the cell cycle machinery as soon as cyclin/CDKs are activated during the G1 phase (figure 4A). This feature would provide the cells with a minimum threshold of free E2F activity upon disruption of p130/E2F-4 complexes with which they could rapidly initiate a period of E2F-dependent positive transcriptional control. Moreover, it is also interesting to note that G1 progression in continuously dividing cells -that is post-mitotic G1- does not involve the accumulation of p130/E2F-4 complexes seen during cell cycle exit (17, 58), thus indicating that E2F activity in these cells is mainly regulated by pRB and/or p107 (figure 4B). Since the cellular restriction point function is retained in post-mitotic cells, this is an additional support for the involvement of E2F interactions with pRB and perhaps p107 as a mechanism of restriction point control. On the other hand, p130/E2F complex repressors would act at earlier, upstream pathways during the G0/G1 transition.

3.3. p130 and cell differentiation

The patterns of p130 protein accumulation and its association with E2F-4 seen during serum starvation of cultured cells suggest that p130 is a critical regulator of E2F activity during the cell cycle exit transition. This feature is not only characteristic of growth factor deprivation but can also be extended to cell differentiation since p130/E2F-4 complexes not existing in proliferating cells are predominantly formed during diverse situations of *in vitro* and *in vivo* differentiation of mammalian cells (17, 51, 53-56, 80). Consistently, accumulation of p130 phosphorylated forms typical of G0 cells (i.e. p130 form 2) is maintained in differentiated cells and adult mouse tissues (76). Obviously, the physiological inference of this occurrence is that, during the differentiation process, p130 mediates the transcriptional shutting off of some cell cycle genes that are silent in non-proliferating cells. In addition, p107 protein levels drop dramatically during *in vitro* differentiation of several cell types concomitantly with the induction of the p130 phosphorylated forms characteristic of cell cycle exit (44, 55, 76), which agrees with the notion that the p107 gene is among the genes repressed in non-dividing cells. In this regard, E2F-containing p107 and pRB complexes are abundant in cells susceptible to differentiation under conditions of exponential proliferation, but they typically disappear -with a few exceptions (44, 53)- and always give rise to E2F-containing p130 complexes during the differentiation process (see references above). This situation is compatible with the hypothesis that p130 primarily represses a genetic expression program that results in preventing E2F regulation by the other pocket proteins, pRB and p107. Control of this regulatory shift would be partially accomplished by down-regulating p107 protein levels and by depriving pRB of at least two of its

normal E2F partners: E2F-1 and E2F-2. On the other hand, in contrast to the situation during G1 progression where cyclin/CDKs trigger a shift in the control of E2F activity from p130 complexes to pRB and p107 complexes, cyclin/CDKs are not active when p130 assumes E2F regulation by complex formation during differentiation, which agrees with the fact that pocket proteins become hypophosphorylated a this time (76). The cause of p130/E2F-4 complex formation rather seems to be the activity of a putative Cell cycle Exit-Induced kinase (CEI-kinase) on p130, probably increasing its stability (figure 4B, see above). At this point, it is interesting to consider that p107 has been implicated in down-regulating cyclin/CDK activity on pocket proteins as well as modulating substrate specificity of cyclin A/CDK2 complexes by direct binding to cyclins E and A (34, 35, 81). From these data, it follows that before the fall of p107 protein levels during cell differentiation, p107 may be one of the negative modulators of cyclin/CDKs. In this context, we may speculate that the differentiation signals are recognized by the restriction point machinery by inactivating cyclin/CDKs, possibly by induction of CDK inhibitors (82, 83) or by p107-induced direct inhibition of complexed cyclin/CDK holoenzymes. Accordingly, both pRB and p107 would be responsible for stopping cell cycle progression by inactivating E2F and cyclin/CDK activities before the restriction point. Concomitantly, a putative CEI kinase is induced and p130 would relieve the other pocket proteins of performing E2F regulation by assuming the silencing of the expression of certain cell cycle genes. p130 has also been found to inhibit CDK activity (34-36), however, participation of p130 as a CDK inhibitor during cell cycle exit is unlikely since p130 levels are very low in proliferating cells (17, 58) and only accumulate upon cell cycle exit from the post-mitotic G1 previous to the restriction point, a period where these CDKs are already inactive. Nevertheless, this CDK inhibitory activity of p130 might be crucial in other cellular situations.

It is interesting to note that the role of p130 during cell differentiation may not be restricted to regulation of E2F activity. As an E2F activity regulator, pRB must be critical with respect to the control of the G1 restriction point during cell cycle exit, but the fact that it associates with a number of other transcription factors, some of them related to cell differentiation programs (reviewed in 28), suggests a direct role of pRB in the regulation of gene expression during differentiation. Accordingly, p130 has also been found to associate with HBP1 in differentiated cells (84), which is a transcription factor involved in cell cycle exit during differentiation, thus indicating that p130-mediated regulatory processes during differentiation may involve other transcription factors in addition to E2F. In support of this possibility, formation of p130/E2F-4 complexes during cell muscle differentiation is not sufficient to commit the cells to terminal differentiation (80), which suggests that additional events are required e.g., interaction with other transcription factors. In this context, further study of p130 protein interactions during cell differentiation should provide more clues about its precise relevance in this process.

Knockout of pocket protein genes in mice has led to some relevant conclusions about their role in cell differentiation during *in vivo* development. It is clear that lack of pRB only leads to abnormal development of certain tissues (85-89) indicating that the function of pRB during development is only necessary in specific cell types. The case of p107 and p130 is a more complicated issue since initial reports describing no apparent developmental defects in mice carrying either p107-/- or p130-/- mutations (77, 90) have been put on hold by recent experiments where other p107 and p130 knockouts induce severe phenotypes (M. Rudnicki, personal communication, see above). Nevertheless, double knockout of p130 and p107 in the first animal model reported, led to postnatal lethality with defects in chondrocyte cell differentiation (77). Considering that individual knockouts do not have such an effect, this may reflect functional redundancy between these proteins, as discussed in the next section.

3.4. Pocket proteins act coordinately.

The biological significance of the existence of such large E2F and pocket protein families is at present still unclear. The experimental data reviewed above support the reasoning of a coordinate function among pocket proteins rather than simple functional redundancy or specific functions depending on the cell type, among other possibilities. In this respect, it is obvious that individual pocket proteins exert their function sequentially during cell cycle progression and during cell differentiation, particularly when comparing p130 with pRB and p107, and that p130 is to a great extent responsible of keeping an order in this sequence. Moreover, it is also conceivable that individual pocket proteins regulate the transcription of specific sets of genes. This is particularly likely in the case of the gene expression program repressed by p130 during the G0/G1 transition. In support of this latter possibility, alterations in the expression of some E2F-responsive genes are detected specifically in fibroblasts carrying mutant alleles of pRB or p130 and p107 (38; see above). Nevertheless, functional redundancy has been emphasized in the case of p130 and p107. First, these two proteins are structurally more similar to each other than to pRB (91-95). Second, p130 and p107 specifically associate with the same E2F family members: E2F-4, and perhaps E2F-5, the interaction with E2F-4 being the major one *in vivo* (42, 96-99). In contrast, pRB, aside from associating with E2F-4 (43, 44, our unpublished data), also associates with E2F-1, E2F-2, and E2F-3 (100-104). Finally, and most importantly, p107 might substitute for p130 function in murine cells that carry homozygous mutation of the p130 gene as indicated by the fact that the p107 protein is expressed at higher than normal levels in p130 -/- quiescent fibroblasts and is found associated with E2F in these cells (38, 77). On the other hand, p130 and p107 display opposite patterns of protein expression in all the cell growth and differentiation models studied

hereto, which indicates that under normal conditions their functions do not overlap in time (54, 76). The mechanism by which the absence of p130 results in the upregulation of p107 protein levels is uncertain at this time since p107 mRNA levels do not seem to be deregulated in p130 -/- quiescent MEFs (38). These data altogether suggest that p130 and p107 carry out similar biochemical actions but in different cell growth situations, so that functional redundancy between these two proteins would only be manifested under certain conditions such as the knockout in mice. In this regard and with respect to p130 and p107, it is of interest to consider that, with the exception of a point mutation of the p130 gene in a GLC2 small cell lung carcinoma cell line (105), no other alterations in these genes have ever been reported in human cancers. Despite their structural and functional similarities with the tumor suppressor pRB, this lack of mutations in tumors suggests that they may not be involved in tumorigenesis. On the other hand, the functional redundancy manifested by p107 in p130 -/- cells might also occur in cells of human tissues that undergo p130 mutations. In this manner, p130 inactivation as a growth suppressor would not confer any proliferative advantage that could facilitate neoplastic transformation. It would, therefore, be of interest to measure tumor susceptibility in heterozygous p107 -/+, p130 -/- and p107 -/-, p130 -/+ adult mice to ascertain the validity of this possibility. In respect to a putative role for p130 and p107 in cell transformation, it has been shown that the growth advantage conferred by SV40 large T-antigen in MEFs requires inactivation of both p130 and p107, a feature that might contribute to T-antigen-mediated cell transformation (106-109).

4. PERSPECTIVES

The coordinate function of pocket proteins during processes of cell growth and differentiation also implies that biochemical differences must exist between individual pocket protein/E2F complexes. The effects of p130-mediated repression on transcription of certain E2F-responsive genes constitutes one of the first few pieces of evidence in this regard. Thus, p130 function appears to be in charge of repressing the transcription of some genes that should not be expressed or that are not required in situations of cell cycle exit or re-entrance transitions. Our hypothesis is that repression of this genetic program is required for an ordered passage through the regulatory pathways that co-exist in the G1 phase of the cell cycle, including the restriction point transition. Particularly, the order of events that take place to build the restriction point machinery (partially shown in figure 3) suggests that p130: i) participates in keeping this restriction point machinery silent, at least partially, during G0 and early G1; and, ii) participates in the initiation of this process by releasing a pool of free E2F transcription factor, as well as relieving transcriptional repression of some G0-silenced genes. Moreover, additional regulatory pathways involving pocket proteins and other transcriptional regulators in addition to E2F family members should provide more clues about pocket protein function, and this is specially true regarding the biochemical role of p130 during cell cycle exit and differentiation processes. In this respect, identification of the biochemical factors that regulate p130 during cell cycle exit, for instance the hypothetical cell cycle exit-induced kinase activity and differentiation-specific transcription factors putatively targeted by p130, should provide crucial details about the function of p130 during these processes. Therefore, future prospects in the study of p130 function should include the precise dissection of the gene expression programs controlled by p130 and the identification of additional factors that participate in this control.

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