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The D-type cyclins and their role in tumorigenesis

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SUMMARY

The D-type cyclins are expressed during the progression from G_0/G_1 to S phase in the mammalian cell cycle. There is considerable evidence that they contribute to the development of specific cancers, both in humans and in mouse models. For example, cyclin D1 can be activated by chromosomal translocation, DNA amplification and retroviral integration. Cyclins D1, D2 and D3 preferentially associate with two closely related members of the cyclin-dependent kinase family, Cdk4 and Cdk6 and the various complexes are each capable of phosphorylating the retinoblastoma gene product (pRb), at least in vitro. This suggests that the growth promoting effects of the D-cyclins may be manifest via their interactions with tumour suppressor genes.

Key words: cell cycle, cyclin-dependent kinase, cyclins D1, D2 and D3, Cdk4 and Cdk6, DNA amplification, chromosome 11q13, chromosome translocation, retroviral integration, breast cancer, centrocytic lymphoma, *PRAD1*, *CCND1*, *BCL1*, retinoblastoma gene, DNA tumour virus

INTRODUCTION

The discovery of cyclin D1, first reported in 1991, is a striking example of convergent research where several groups, working independently on quite different aspects of biology, found themelves studying the same gene. Some were investigating the regulation of the cell cycle (Lew et al., 1991; Xiong et al., 1991), some were looking for genes induced by specific cytokines (Matsushime et al., 1991), while others were trying to identify oncogenes associated with specific cancers (Motokura et al., 1991; Withers et al., 1991; Schuuring et al., 1992). It is hardly surprising therefore that the D-cyclins have generated enormous interest over the last few years but such is the pace of these activities that the recorded information does not always concur. As well as summarizing the general features of the D-cyclins and the evidence linking them to cancer, this short review will try to distil some order from the recent literature and advance some speculative views as to the possible functions of these proteins.

REGULATION OF THE CELL CYCLE BY CYCLIN-DEPENDENT KINASES

In higher eukaryotes, cell division is regulated by a cyclical series of events in which two protein families play leading roles. The first are the cyclins, the classic example of which was discovered by its marked accumulation and destruction in synchrony with the cell division cycle (Evans et al., 1983). The second are the cyclin-dependent kinases (Cdks), for which the prototype is the 34 kDa serine/threonine kinase encoded by the *cdc2* gene in *Schizosaccharomyces pombe* and the related *CDC28* gene in *Saccharomyces cerevisiae* (reviewed by Norbury and Nurse, 1992; Reed, 1992). As the recently adopted Cdk nomenclature implies (see for example Meyerson et al., 1992), the active enzyme complexes are formed by a partnership between a regulatory cyclin and a catalytic kinase subunit.

Historically, much of this information was gleaned from genetic analyses of the cell division cycle in yeast and studies on oocyte maturation in marine invertebrates and amphibia. These pioneering studies led to a model for the regulation of mitosis by the cyclin B/cdc2 complex in which kinase activity is 'switched on' by specific dephosphorylation of the catalytic subunit and 'switched off' by the rapid destruction of the cyclin (Norbury and Nurse, 1992; Reed, 1992; Solomon, 1993). This paradigm appears to be conserved in all eukaryotes and although the players may be different the same principles are probably relevant to other critical phases of the cell cycle. For example, the G_1/S transition in *Saccharomyces cerevisiae* is regulated by a group of G_1 cyclins, termed CLN1, 2 and 3, acting in conjunction with the CDC28 kinase (reviewed by Reed, 1992). Not surprisingly, the picture appears more complex in mammalian cells, where the numbers of potential Cdks and cyclins have expanded dramatically, and the details are just beginning to be unravelled.

The identification of new members of the cyclin family was largely stimulated by the search for mammalian equivalents of the yeast CLN genes in complementation assays (Koff et al., 1991; Lew et al., 1991; Xiong et al., 1991). Cyclins C, D1 and E were all identified in this way but in hindsight it seems that the assay may have been scoring the presence of a so-called 'cyclin box', a region of sequence homology that is conserved in all cyclins (Fig. 1) and is probably critical in the interaction between cyclins and their respective kinase partners (Kobayashi et al., 1992; Lees and Harlow, 1993). The other notable characteristic of cyclins is their rapid turnover. In cyclins A and B, this is mediated by a motif near the amino terminus that appears

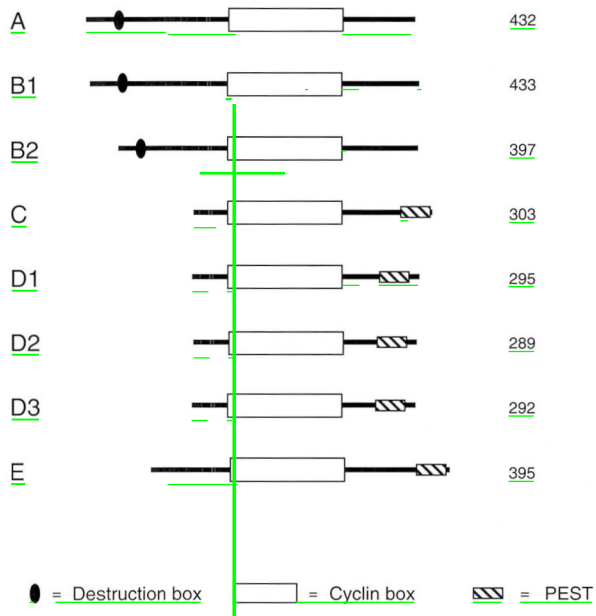

Fig. 1. The mammalian cyclin family. The figure depicts 8 of the known cyclin-related polypeptides identified in human cells, designated A through E as indicated. The number of amino acid residues in each protein is shown on the right. The open box locates the region of homology termed the cyclin box. Other symbols show the presence of protein destabilization elements.

to target the protein for destruction via the ubiquitin pathway (Glotzer et al., 1991) whereas cyclins C, D and E have so-called PEST sequences near their carboxy termini (Fig. 1). Although such concentrations of proline, glutamic acid, serine and threonine residues are thought to contribute to protein instability (Rogers et al., 1986), their significance to cyclin turnover has yet to be confirmed experimentally.

Protein stability is clearly important for controlling the levels of the different cyclins throughout the cell cycle but only cyclins A, B and E show the classical periodic fluctuations that gave the family its name. Significantly, the levels of these proteins and their associated kinase activities peak in a distinct temporal order as illustrated in Fig. 2. A relatively cohesive model can thus be formulated in which specific cell cycle transitions are regulated by the consecutive action of these cyclin/kinase complexes (Fig. 2). In this scheme, the cyclin E/Cdk2 complex regulates events at the G_1/S transition (Dulic et al., 1992; Koff et al., 1992; Ohtsubo and Roberts, 1993; Tsai et al., 1993), cyclinA/Cdk2 operates in S and G_2 (Girard et al., 1991; Walker and Maller, 1991; Pagano et al., 1992; Tsai et al., 1993), and cyclin B/cdc2 orchestrates mitosis (Norbury and Nurse, 1992; Reed, 1992). However, this is clearly an oversimplification since the partnerships between cyclins and Cdks are not monogamous. For example cyclin A can form active complexes with either Cdk1 (i.e. cdc2) or Cdk2 and, as discussed in more detail below, the latter can be found associated with cyclins A, E or D.

THE D-TYPE CYCLINS

The D-cyclins form a distinct subset within the cyclin family based on structural and functional criteria. Although the genes

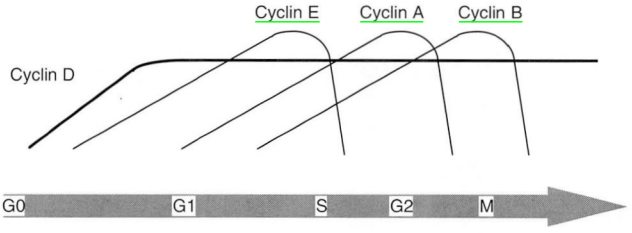

Fig. 2. The mammalian cell cycle. The phases of the cell cycle are shown in a linear form for cells leaving the resting G_0 state and entering the division cycle. DNA synthesis and mitosis occur, respectively, in the S and M phases, separated by two gap phases, G_1 and G_2. The curves above the line are a highly schematized impression of the levels of various cyclin proteins at different phases of the cycle.

map to different chromosomes (Inaba et al., 1992; Xiong et al., 1992a), they encode proteins that are between 57 and 62% identical in pairwise comparisons and absolutely conserved at 140 residues spread throughout the respective molecules (Fig. 3). Similarity to other cyclins is restricted to the cyclin box domain, and the D-cyclins are particularly well conserved in the amino-terminal half of this region. It was sequence relatedness that pointed to the existence of cyclins D2 and D3 by cross-hybridization with cyclin D1 probes (Matsushime et al., 1991), and the other members of the family were not uncovered by the various strategies that led to the discovery of cyclin D1.

As alluded to above, cyclin D1 was among a number of human cDNA clones that were able to complement for CLN activity in yeast (Lew et al., 1991; Xiong et al., 1991). It was also isolated as a 'delayed-early' gene in cytokine-stimulated mouse macrophages by differential screening of cDNA clones (Matsushime et al., 1991). In this system, the expression of cyclin D1 is dependent on the presence of CSF-1 and reaches a maximum late G_1 phase. Subsequent studies have shown that the expression of all three D-cyclins can be regulated by cytokines but the results and their interpretation are rather inconsistent (Matsushime et al., 1991; Cocks et al., 1992; Motokura et al., 1992; Surmacz et al., 1992; Won et al., 1992; Ajchenbaum et al., 1993; Jansen-Dürr et al., 1993; Musgrove et al., 1993; Sewing et al., 1993; Winston and Pledger, 1993). Perhaps the most contentious issue is whether the levels of the RNAs and/or proteins actually cycle, but some of the confusion undoubtedly reflects the way that cells were synchronised and analysed in different labs. Our own findings suggest that while cyclin D1, for example, accumulates significantly in G_1 in serum-stimulated fibroblasts, it does not undergo the dramatic fluctuations that are characteristic of cyclins A and B. Other groups report similar conclusions but suggest that there are more subtle fluctuations and that the protein, which is normally found in the cell nucleus, may become redistributed during S phase (Baldin et al., 1993; Sewing et al., 1993; Lukas et al., 1994).

Clearly, much remains to be learned about the regulation of cyclin D expression and the balance between synthesis and degradation of both transcripts and proteins. The interplay of positive and negative inputs from the signal transduction pathways has yet to be fully explored and there is currently no obvious explanation for the need for three closely related

	Chromosome	RNA	Protein	Molecular weight
CCND1	11q13	4.5 kb	295 aa	33,729 Da
CCND2	12p13	~7 kb	289 aa	33,045 Da
CCND3	6p21	2.2 kb	292 aa	32,482 Da

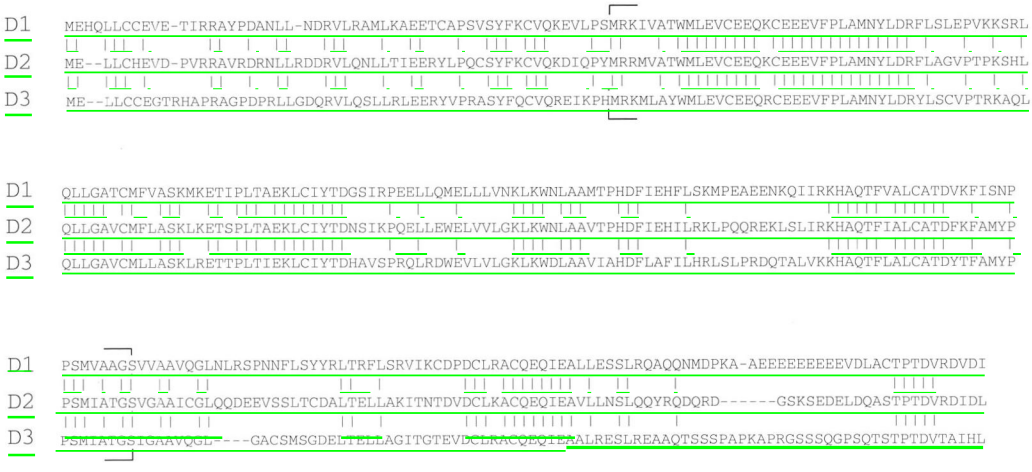

Fig. 3. The human D-type cyclins. The chromosomal locations and transcript sizes for the human D-cyclin genes, *CCND1*, *CCND2* and *CCND3* are indicated along with the characteristics and primary sequences of the encoded proteins, in single letter amino acid code. Vertical lines identify conserved residues and the square brackets delineate the so-called cyclin box.

genes. Many cell types express two and occasionally all three members of the family and few clear patterns have yet become apparent. Perhaps it is significant that, despite sporadic reports to the contrary, we have yet to identify a cell line that does not express at least some levels of cyclin D3 whereas cells lacking either D1 or D2 are much more common (unpublished observations).

CHROMOSOMAL REARRANGEMENTS AFFECTING CYCLIN D1

The other line of research that led to the identification of the D-cyclins was the search for oncogenes associated with specific genetic alterations. Most of the evidence relates to cyclin D1 and although largely circumstantial, it is becoming increasingly persuasive. As indicated in Fig. 4, the gene for cyclin D1 (*CCND1*) maps to the karyotypically defined band q13 on the long arm of human chromosome 11, a region known to be the site of tumour-specific chromosomal abnormalities. For example, some cases of benign parathyroid adenoma show a clonal inversion of chromosome 11 that places the cyclin D1 gene on 11q13 adjacent to regulatory elements of the parathyroid hormone gene on 11p15 (Arnold et al., 1989; Motokura et al., 1991). Although the frequency of this rearrangement in parathyroid adenomas is quite low, its clonality and the resultant increase in cyclin D1 expression are strongly suggestive of a role in the disease (Rosenberg et al., 1991a). It was through this association that the name *PRAD1* was coined, whereas earlier reports referred to the anonymous locus designation D11S287. Both names appear in the recent literature but are essentially synonymous for *CCND1*.

A different and more frequent rearrangement of 11q13 has been observed in B-cell neoplasms, particularly centrocytic lymphoma and multiple myeloma. In this t(11;14)(q13;q32) translocation, a reciprocal exchange occurs between chromosome 11q13 and the Ig heavy chain locus on 14q32 (Erikson et al., 1983; Tsujimoto et al., 1984), exactly analogous to the translocations that activate *MYC* in Burkitt's lymphoma and *BCL2* in follicular lymphoma. When the translocation breakpoint was cloned, and designated *BCL1*, it was naturally assumed that a nearby gene would be activated by juxtaposition to the Ig enhancer. As it turns out, the nearest gene is *CCND1*, which is located some 120 kb distal to the original breakpoint cluster (Withers et al., 1991; Brookes et al., 1992) but it is now clear that the breaks can occur at multiple sites within the intervening DNA (see for example Williams et al., 1992; de Boer et al., 1993). Nevertheless, the original expectations are fulfilled in that the translocation results in increased transcription of the cyclin D1 gene (Rosenberg et al., 1991b; Seto et al., 1992). Although such findings have led to references to the '*BCL1* gene', this is a potential source of confusion since the originally defined *BCL1* probe is quite distant from the *CCND1* gene.

AMPLIFICATION OF CYCLIN D1

The distinction between *BCL1* and *CCND1* becomes relevant in considering the other chromosomal abnormality that affects cyclin D1, DNA amplification. It has been widely documented that a significant subset of human breast cancers and squamous cell carcinomas show amplification of markers at 11q13, whereas this amplicon is rarely observed in other tumour types (reviewed by Lammie and Peters, 1991; Fantl et al., 1993). The amplification was originally detected using probes for two known oncogenes in the region, *FGF3* and *FGF4*, often in conjunction with the *BCL1* translocation breakpoint probe. The

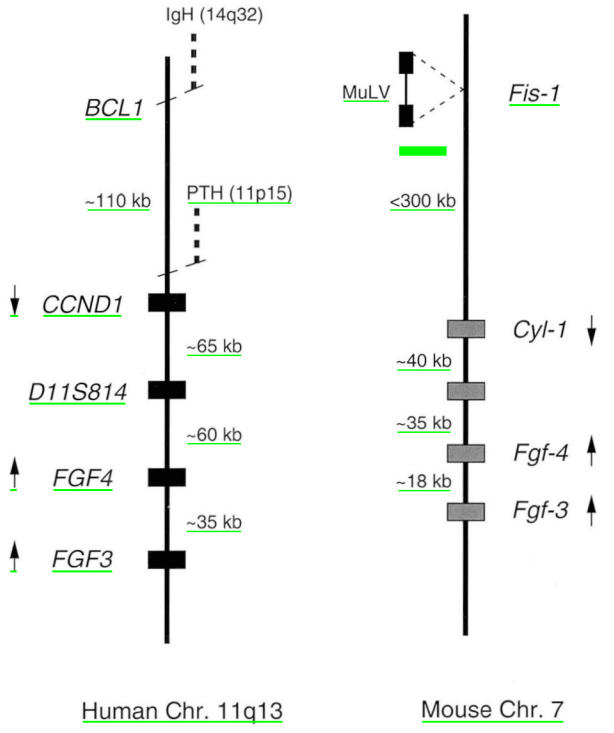

Fig. 4. Chromosomal perturbations that affect cyclin D1. Syntenic regions of human chromosome 11 and mouse chromosome 7 are depicted in which the shaded boxes represent known CpG islands. The distances between these loci were established by a combination of cosmid walking and pulsed field gel electrophoresis. The islands corresponding to the cyclin D1 gene (*CCND1* and *Cyl-1*), and to *FGF3* and *FGF4* are as indicated and the arrows refer to the transcriptional orientation of the gene. The originally defined translocation breakpoints on human chromosome 11q13, in parathyroid adenomas and B-cell lymphomas, and the MuLV integration locus *Fis-1* on mouse chromosome 7 are as indicated.

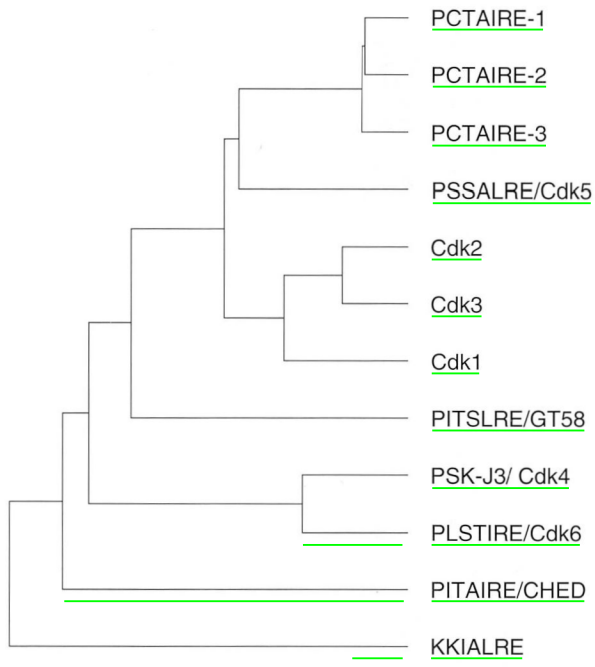

Fig. 5. Evolutionary tree of known CDC-2 related polypeptides. An evolutionary tree was constructed from the published sequences of 12 human proteins that show homology with p34^{cdc2} (Hanks et al., 1988; Bunnell et al., 1990; Lapidot-Lifson et al., 1992; Matsushime et al., 1992; Meyerson et al., 1992; Okuda et al., 1992). The region of each protein that aligns with residues 11 to 230 inclusive of human CDC2, were subjected to pairwise comparisons and the percentage divergence converted into a linear distance using the Intelligenetics GeneWorks software.

now know that *CCND1* (alias D11S287) maps between *BCL1* and *FGF4* (Brookes et al., 1992) and is therefore a consistent component of the amplicon, a fact that was reported before the true nature of the gene became apparent (Lammie et al., 1991). More importantly, *CCND1* is expressed at relatively low levels in normal breast epithelium and its expression is elevated upon DNA amplification (Lammie et al., 1991; Buckley et al., 1993; Gillett et al., 1994). Since this is not true for either *FGF3* or *FGF4*, which remain silent in the adult mammary gland, cyclin D1 is currently the best candidate for the key oncogene on the amplified DNA (Fantl et al., 1993).

The only point of contention is that *CCND1* is not the only expressed gene affected by the amplification (Schuuring et al., 1992, 1993), leading to suggestions that there may be more than one focus for amplification in the 11q13 region (Gaudray et al., 1992). Thus, in some tumours, the *BCL1* probe appears to be more highly amplified than *CCND1*, while in others the converse is true. As we have argued elsewhere (Fantl et al., 1993; Gillett et al., 1994), it is difficult to interpret these observations without a better understanding of the function of cyclin D1, since it may have both positive and negative effects on proliferation depending on the cell type or level of expression achieved (see below). Whatever the resolution of these issues, it is now possible to detect the amplification of the *CCND1* gene at the protein level, by staining tumour sections with cyclin D1 antibodies (Jiang et al., 1993b; Gillett et al., 1994). This should greatly facilitate the analysis of clinical material and there are preliminary indications that the frequency of over expression of cyclin D1 may be much higher than concluded from DNA analyses. Thus, in our own study, approximately one in three breast tumours stained above normal for cyclin D1 (Gillett et al., 1994). As these are almost exclusively tumours that are positive for oestrogen receptor, staining for cyclin D1 holds considerable promise for refining the classification of breast cancers and may well have prognostic significance.

VIRAL ACTIVATION OF CYCLINS D1 AND D2

The final piece of evidence connecting D-cyclins and tumorigenesis is their activation by tumour viruses. For example, both the cyclin D1 and D2 genes are transcriptionally activated by the nearby integration of murine leukaemia virus in mouse T-lymphomas (Lammie et al., 1992; Hanna et al., 1993). For cyclin D1, the insertions occur at the previously defined *Fis-1* locus on mouse chromosome 7 in a region that is a direct parallel of 11q13 (see Fig. 4). Although the exact distance between *Fis-1* and the cyclin D1 gene has not been established, it seems reasonable to conclude that proviral insertions at *Fis-1* are functionally analogous to translocations at *BCL1*

(Lammie et al., 1992). With cyclin D2 on the other hand, the insertions occur adjacent to the gene and the genomic DNA was cloned independently as a common site of viral integration, termed *Vin-1* (Hanna et al., 1993). To date, there are no obvious parallels for *Vin-1* disruption in human tumours. However, we have noted one potential link between cyclin D2 and lymphomagenesis in that the immortalisation of primary B-lymphocytes by Epstein-Barr virus is accompanied by transcriptional activation of cyclin D2 (Palmero et al., 1993; Sinclair et al., 1994). We are currently exploring the possiblity that this is a direct effect of viral gene expression and a key step in the immortalisation process.

INTERACTION OF D-CYCLINS AND CDKS

Apart from one isolated case of hepatitis-B virus integration into the cyclin A gene (Wang et al., 1990), none of the other cyclins have yet been implicated in tumorigenesis. Does this mean that there is something fundamentally different about the D-cyclins? An obvious question is whether they interact with catalytic subunits in the same way as cyclins A, B and E, and several groups set out to determine the kinase partners for cyclins D1, D2 and D3. The strategies used were dictated by the availability of cDNA clones and specific antisera to potential candidates but the most significant outcome was the demonstration that a 33 kDa protein kinase designated PSK-J3 (now renamed Cdk4) is a major partner for the D-cyclins (Matsushime et al., 1992; Xiong et al., 1992b). PSK-J3 had been isolated previously in a general screen for new kinases and was recognised as a distant cousin of cdc2 (Hanks et al., 1988). However, its close association with the D-cyclins not only solved some of the mysteries surrounding this kinase but provided encouraging evidence that the cyclin/kinase paradigm might be extended to new members of the respective families. For example, the growing list of cdc2-related sequences in the literature included a 38 kDa protein, originally referred to as PLSTIRE, that is very closely related to Cdk4 (see Fig. 5). We and others have recently shown that this protein, now designated Cdk6, also associates with the D-cyclins (Bates et al., 1994a; Meyerson and Harlow, 1994). Thus, just as the D-cyclins form a distinct subset of the cyclin family, so Cdk4 and Cdk6 form a distinct subset of the Cdk family, and appear to interact exclusively with the D-cyclins. All six possible pairings can be detected by immunoprecipitation of cell lysates (Bates et al., 1994b).

These are not the only interactions observed for the D-cyclins; Xiong et al. (1992b) reported that in primary human fibroblasts, cyclin D1 can be associated with Cdk2 and yet another member of the family designated Cdk5. The significance of the latter remains uncertain but the association with Cdk2 clearly demands some explanation, since this is also a partner for cyclins A and E. A possible clue may be that it is the hypophosphorylated and hence inactive form of Cdk2 that is found associated with cyclin D1 (Dulic et al., 1993; Bates et al., 1994a). A further clue may be that this complex accumulates as primary cells undergo senescence (Dulic et al., 1993; Lucibello et al., 1993). It is therefore conceivable that cyclin D1 performs two contrasting functions, as a positive regulator of Cdk4 and Cdk6 and as a negative regulator that sequesters Cdk2 in an inactive form. Our inability to detect the cyclin D1/Cdk2 complex in tumour cell lines (Bates et al., 1994a) and its absence in transformed cells (Xiong et al., 1993) would therefore tie in with an escape from senescence. If the recently described p21 protein is also part of this complex (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993; Noda et al., 1994), this would provide an additional arm to p53-mediated cell cycle arrest.

The notion that the D-cyclins may have double lives could of course explain why some functional experiments have produced paradoxical results. For example, transfection of cells with vectors expressing D-cyclins from constitutive or inducible promoters has been shown to accelerate the G_1/S transition, exactly as one might expect for over-expression of a G_1 cyclin (Ando et al., 1993; Jiang et al., 1993a; Quelle et al., 1993; Resnitzky et al., 1994). However, at least one published and several anecdotal reports testify to the toxicity of the D-cyclins in transfection assays (Quelle et al., 1993). This would make some sense if cyclin D is having contrasting influences depending on its Cdk partner. It might also explain why early attempts to demonstrate the oncogenic potential of cyclin D1 by DNA transfection were unrewarding, yet if the appropriate levels are achieved then it can cooperate with *RAS* in transforming primary rodent cells (Hinds et al., 1994; Lovec et al., 1994). Finally, such considerations might explain why the over-expression of cyclin D1 as a result of DNA amplification is not always as dramatic as one might expect and why in some tumours there appear to be rearrangements that down-regulate cyclin D1 expression from the amplified DNA (Gillett et al., 1994).

INTERACTION BETWEEN THE D-CYCLINS AND pRB

The evidence that D-cyclins can interact with Cdks and can accelerate cell cycle progression raises an obvious question - what are the substrates for the multiple kinase combinations? Perhaps the most attractive candidates and certainly the current favourites are the product of the retinoblastoma gene, pRb and its close relative p107. As discussed elsewhere in this volume, pRb is known to act as a negative regulator of G_1 progression, leading to a relatively robust model in which the inactivation of pRb by phosphorylation is a critical step in permitting entry into S phase. A number of studies have shown that pRb can be phosphorylated by various cyclin/Cdk combinations (Lees et al., 1991; Lin et al., 1991; Hinds et al., 1992; Hu et al., 1992) but the timing of the initial phosphorylation events have encouraged the idea that the D-cyclins may be involved. Certainly, the phosphorylation of pRb can be demonstrated in vitro using mixtures of the D-cyclins and Cdk2, Cdk4 or Cdk6 expressed in insect cells using baculovirus vectors (Matsushime et al., 1992; Ewen et al., 1993; Kato et al., 1994; Meyerson and Harlow, 1994). However, it has proved much more difficult to detect such activities in immunoprecipitates from cycling cells (Matsushime et al., 1994; Meyerson and Harlow, 1994) and there are still some puzzling features about the specificities of the different complexes. It would seem strange that all six complexes involving D-cyclins, Cdk4 and Cdk6 are doing exactly the same thing.

Despite these reservations, it does seem very likely that the function of the D-cyclins is somehow connected to pRb, if for no other reason than the presence of a sequence feature near their amino termini that suggests they may interact directly

with pRb (Dowdy et al., 1993). This is the LxCxE motif that is common to the SV40 T-antigen, adenovirus E1A, and human papilloma virus E7 proteins, each of which is thought to bind to and functionally inactivate pRb. For the respective DNA tumour viruses this would have the advantage of promoting entry into S-phase, a prerequisite for viral DNA replication. For the D-cyclins, it raises the possibility, albeit unlikely, that they can inactivate pRb by direct binding and displacement of associated transcription factors. Supportive evidence for this idea has been reported, based on the reversal of pRb-induced cell cycle arrest in SAOS-2 cells (Hinds et al., 1992), but it remains curious that cyclin D1 can apparently achieve this effect without concomitant phosphorylation of pRb, whereas cyclins D2 and D3 are thought to inactivate pRb by phosphorylation (Dowdy et al., 1993; Ewen et al., 1993). Either way, one can rationalise how the elevated expression of a D-cyclin in a tumour cell could accelerate G_1 progression.

However, there are a number of uncomfortable facets to such ideas, not least of which is the potential for functional redundancy implicit in the findings. Why should elevated expression of a D-cyclin be so critical to a cell that already expresses one of its close relatives? Moreover, tumour cells do not cycle more rapidly than normal cells and it would be much more attractive to postulate that the D-cyclins are in some way regulating exit from G_0 or the cell's ability to return to a G_0 state after completing mitosis. Finally, it seems clear that pRb is not the master regulator of all cell cycles so that it is premature to settle for pRb as the substrate for D-cyclin/kinases. As if matters were not already confusing enough, we have recently noted that, in cells in which pRb has been inactivated, either by DNA tumour virus infection or as a result of naturally occurring mutations, it is very difficult to detect associations between the D-cyclins and any of their kinase partners (Bates et al., 1994b). Taken at face value, the data suggest that the substrate for D-cyclin/Cdk complexes must be present for the active enzyme to be formed.

As in all rapidly advancing fields, it is almost impossible to draw all the published and soon to be published data into a cohesive picture. Models are being formulated, modified and discarded at an alarming rate as each new component appears on the scene. Within the last few months, two more players have entered the arena, the p21 protein that links p53 to the cyclin/Cdk framework (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993; Noda et al., 1994) and p16, a specific inhibitor of Cdk4 (Serrano et al., 1993). Exciting times lie ahead.

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