Pergamon

Oral Oncol, Eur J Cancer, Vol. 31B, No. 5, pp. 291–298, 1995
Elsevier Science Ltd
Printed in Great Britain
0964–1955/95 $29.00 + 0.00

0964–1955(95)00028–3

Review

Cyclin Kinase Inhibitors Add a New Dimension to Cell Cycle Control

W.A. Yeudall and J. Jakus

INTRODUCTION

NEGATIVE REGULATORS of cellular proliferation play important roles in the suppression of the neoplastic phenotype. Several tumour suppressor molecules have been identified to date, some of which appear to be closely involved in the regulation of progression through the eukaryotic cell cycle. For instance, the product of the retinoblastoma susceptibility gene, pRb [1], undergoes cell cycle-specific phosphorylation events [2] which determine its ability to complex with the E2F family of transcription factors, thereby regulating expression of E2F-responsive genes [3]. Furthermore, in response to DNA damage, the p53 tumour suppressor protein accumulates in the nucleus, eliciting cell cycle arrest in G_1 and thereby allowing DNA repair to take place prior to cell division [4]. Thus, cell cycle control is an important feature of oncosuppression, and dysregulation by means of mutation of cell cycle inhibitory molecules, overexpression of positive cell cycle regulators, or disruption of associated intracellular signalling mechanisms is likely to play a critical role in the development of malignancy.

CELL CYCLE PROGRESSION

Progression of cells through the cell cycle is dependent on the formation of specific protein kinase complexes which form in a cyclical fashion at particular times during the cell cycle, enabling the cell to pass through several regulatory transition points [5]. These complexes consist of a cyclin-dependent kinase (cdk; the catalytic subunit), a regulatory cyclin molecule, and proliferating cell nuclear antigen (PCNA) [6], and are regulated intrinsically by a series of phosphorylation and dephosphorylation events. Activation of specific cdks through complex formation with their cyclin partners serves to drive cell cycle progression. Indeed, it is well-documented that overexpression of cyclins may deregulate cell cycle control during oncogenesis in a number of human malignancies, including oral squamous cell carcinoma [7–10], resulting in unchecked cell proliferation. Gene amplification and/or overexpression of cdks may also contribute to increased progression through the cell cycle [11]. In addition, extracellular signals may also elicit some degree of cell cycle control. For example, transforming growth factor β (TGF-β) is growth-inhibitory for epithelial cells and halts cell cycle progression from G_1 into S-phase in a number of epithelial cell types [12–14], whereas epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) positively regulate cell proliferation [15, 16]. Recently, much work has focused on the identification and characterisation of several novel proteins which contribute to cell cycle regulation by binding to, and inhibiting the activity of, cyclin–cdk complexes. These cyclin kinase inhibitors provide an additional level of complexity for cell cycle control and represent a new motif for negative regulation of cell proliferation and differentiation. Furthermore, their dysfunction may play a fundamental role in tumour progression.

p21 (WAF1/Cip1/Sdi1/CDKN1/Pic1/mda1/Cap20)

The p21 cyclin kinase inhibitor was identified independently by several groups. p21 was detected as a component in cdk immunoprecipitates [17], while the use of a yeast two-hybrid interactive screen identified p21 as a cdk-associated protein with the ability to bind and inhibit the activity of cdk2 (*c*dk-*i*nteracting *p*rotein, Cip1) [18]. A cDNA encoding the same protein was isolated from a subtraction library during a search for genes upregulated by wild-type p53 (*w*ild-type p53-*a*ctivated *f*ragment, WAF1) [19], thus establishing p21 as a potential mediator of at least one of the tumour suppressor functions of p53, namely cell cycle arrest in G_1. Yet another group cloned the same gene from senescent fibroblasts (hence *s*enescent cell *d*erived *i*nhibitor, sdi1) [20]. p21, which is encoded by a gene on human chromosome 6p21.2 [19], has been shown to act as a universal inhibitor of cyclin dependent kinases, as it has the ability to form complexes with cdk4–cyclin D, cdk2–cyclin E, cdk2–cyclin A and cdc2–cyclin B1 (Fig. 1) [17–19, 21]. In normal cells, p21 levels have been demonstrated to vary in a cell cycle-dependent fashion, associating with functional, but not inactive, cyclin–cdk complexes, thereby regulating their enzymatic activity [21].

The identification of *p21* as a wild-type p53 responsive gene has, as expected, aroused a great deal of interest. It has now been demonstrated that *p21* mRNA and protein is induced in a wild-type p53-dependent manner during p53-mediated G_1 cell cycle arrest, in response to physical or chemical stimuli such as ultraviolet or γ-irradiation, or treatment with the

Correspondence to W.A. Yeudall.
Both authors are at the Molecular Carcinogenesis Group, Laboratory of Cellular Development and Oncology, National Institute of Dental Research, Bethesda, Maryland 20892, U.S.A.
Received 15 May 1995; accepted 16 May 1995.

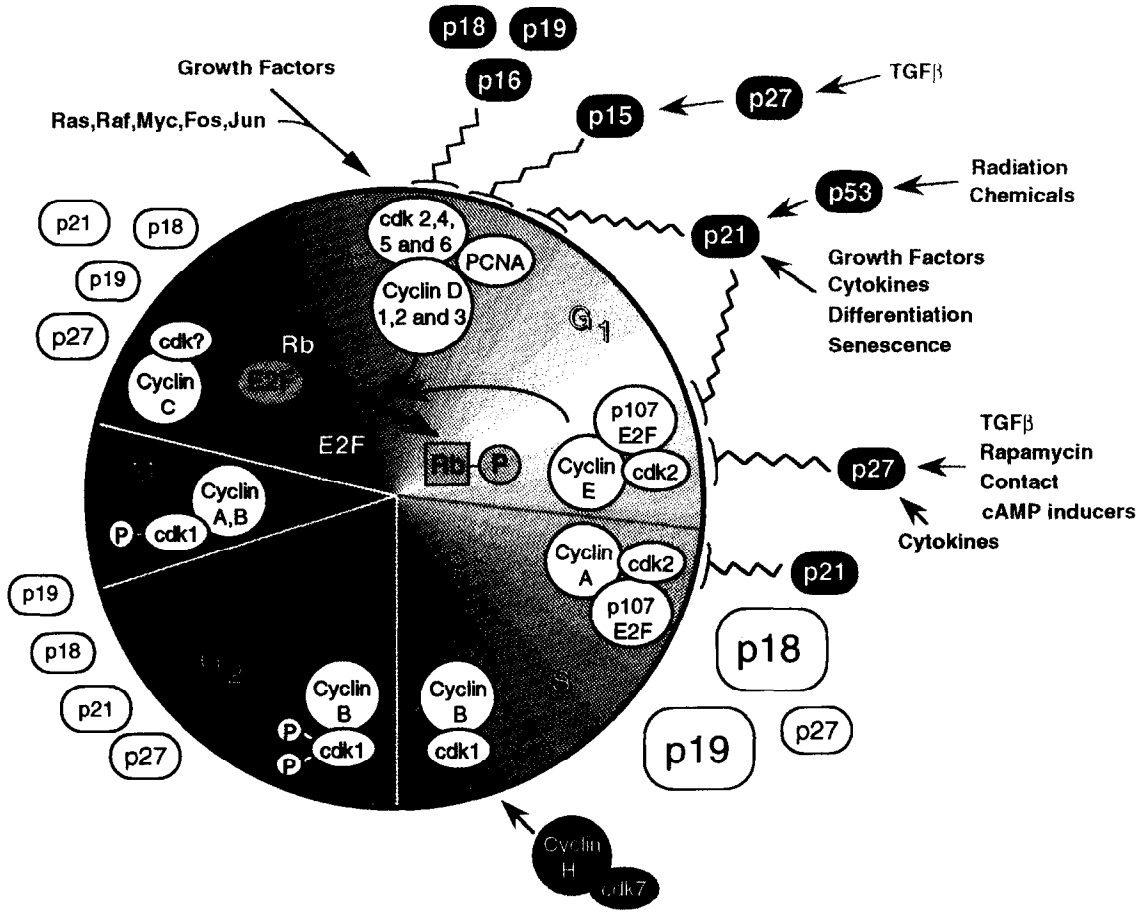

Fig. 1. Interaction of cell cycle inhibitory molecules with extracellular signals and cell cycle events. Cyclin kinase inhibitors integrate signals from cellular and extracellular events to inhibit or activate cyclin–cdk complexes, thus controlling progression through the cell cycle. The abundance of each inhibitor during particular phases of the cell cycle is illustrated by size in the diagram.

chemotherapeutic adriamycin, as cells harbouring mutant p53 molecules failed to induce p21 or arrest in G_1 under these conditions [22]. Furthermore, by using haematopoeitic cells which undergo either G_1 arrest or apoptosis in response to ionizing radiation depending on the presence or absence of IL-3, it has been shown that p21 induction occurs during p53-dependent apoptosis. However, cells undergoing apoptosis by p53-independent mechanisms did not express detectable levels of p21 [22]. In a separate study, the action of p53 and p21 to elicit cell cycle arrest in G_1 was bypassed by constitutive overexpression of the B-*myb* oncogene product [23], a transcription factor which plays a role in controlling the expression of genes crucial for DNA replication and which are normally expressed in late G_1/S-phase [24]. Thus, oncogenic deregulation of cell cycle control by various mechanisms may occur through subversion of p21 function, which further emphasizes its importance for maintenance of normal growth control.

Although mutation of *p53* characterises many malignancies studied to date [25], some tumours have been shown to retain and express a normal p53 gene product. Therefore, if p21 is a crucial mediator of p53 tumour suppression and deregulation of p53 function represents a critical step in oncogenesis, it would seem likely that tumours expressing functional p53 might harbour mutations in *p21*. However, from studies performed thus far, it appears that intragenic mutation of *p21* is a rare occurrence. In a study of over 300 human tumours consisting of a wide variety of tissue types, no *p21* mutations were detected, although specific polymorphisms within the coding region of the gene were identified [26]. This has since been substantiated by subsequent studies of colorectal [27] and other malignancies [28], which failed to demonstrate the presence of somatic *p21* mutations. These data argue that p21 may play a fundamental role in cellular growth control and development, and that inactivation of normal p21 function may be lethal for the cell. One recent study examining Burkitt's lymphomas [29], of which around only one-third have undergone *p53* mutation [30], demonstrated a heterozygous mutation of *p21* in one case, and that the growth-suppressive function of the mutant molecule was compromised compared to wild-type p21. It is notable, though, that both normal and mutant molecules were expressed in the parental cell line, and that some degree of p21-mediated control was likely to be present. It is not yet clear if expression of mutant p21 alone would compromise cell viability.

Although the induction of p21 as a result of the accumulation of wild-type p53 is now considered to be of fundamental importance for cellular growth regulation, control of p21 expression by other cellular factors would be expected, if not mandatory, in view of the fact that many tumours express no functional p53 and that p21 may be vital for cell survival, as discussed above. Thus, it is not surprising that p53-independ-

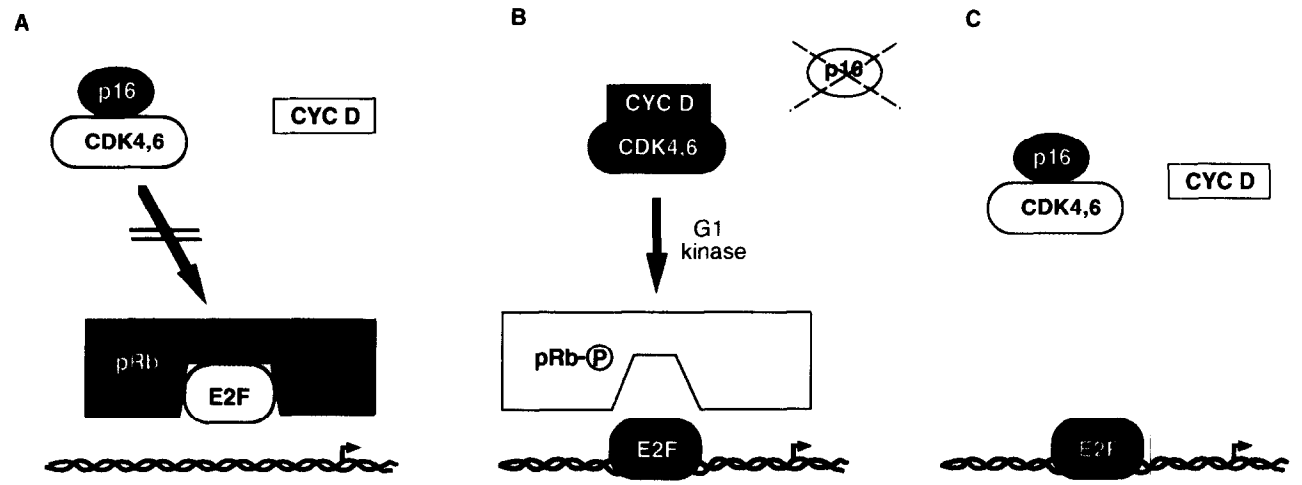

Fig 2. Interplay of p16 and pRb in regulation of cell growth. (A) p16 prevents phosphorylation of pRb by G_1 cyclin dependent kinases, thus pRb remains in a hypophosphorylated, growth suppressive state and sequesters E2F molecules. (B) Absence of functional p16 allows active cyclin–cdk complexes to phosphorylate pRb, resulting in release of E2F which binds to consensus nucleotide sequences in the promoters of responsive genes, activating transcription. (C) The absence of a functional pRb allows constitutive transcriptional activation by E2F, and wild-type p16 is, therefore, unable to modulate the pathway. Active components are shown in red.

ent regulation of p21 expression occurs [31–34]. Using cultured fibroblasts derived from *p53*-nullizygous mice, it has been demonstrated that p21 expression is induced in response to stimulation of cells with serum, several growth factors, including PDGF and fibroblast growth factor (FGF), phorbol esters and okadaic acid, although no increase in *p21* mRNA was observed upon irradiation of cells (i.e. p53-dependent induction was compromised) [31]. The apparent paradox of induction of a growth-suppressive molecule by mitogenic agents could be explained if p21 were required for the activity of cyclin–cdk complexes, and that inhibition of cdk activity required the level of p21 bound to exceed a certain critical threshold [17].

In addition to its important role as a cyclin kinase inhibitor, p21 has also been shown to function as an inhibitor of DNA replication *in vitro* [35]. Using a cell-free model of eukaryotic DNA replication, Waga *et al.* [36] demonstrated an interaction between p21 and PCNA in the absence of a cyclin–cdk complex. In addition, p21 was able to block PCNA-dependent DNA replication by inhibiting the ability of PCNA to activate DNA polymerase δ. Furthermore, two independent studies subsequently revealed that the interaction of p21 with PCNA inhibited only PCNA-dependent DNA replication, but not PCNA-dependent nucleotide excision-repair functions [37, 38]. Thus, p53-mediated induction of p21 expression in response to DNA damage would have the effect of halting DNA replication, while allowing cells to carry out DNA repair unhindered, a long-recognised attribute of wild-type p53 [39].

p16 (MTS1/CDK4I/INK4A/CDKN2)

The existence of p16 first became apparent from analysis of G_1 cyclin–cdk immunoprecipitates [40], where it was found to be associated principally with cyclin D–cdk4. Complexes of D-type cyclins with cdk4 and cdk6 have the ability to hyperphosphorylate pRb [41], releasing active E2-F molecules which may then upregulate the expression of genes involved in cellular proliferation (Fig. 2) [42]. Therefore, tight regulation of the activity of these cyclin D complexes is a prerequisite for restraining cell growth. p16 represents an additional cyclin kinase inhibitor specific for cdk4 and cdk6 (Fig. 1), and thus is functionally dissimilar to p21, as well as structurally.

Table 1. Relationship between cyclin kinase inhibitors, cyclin dependent kinases, cyclins and cancer

CKI	Potential involvement in carcinogenesis	CDK	Cyclins
p15 (p14)	+	4, 6	D1, 2, 3
p16	+	4, 6	D1, 2, 3
p18	nd	4, 6	D1, 2, 3
p19	nd	4, 6	D1, 2, 3
p21	+	1, 2, 3, 4, 5, 6	A, B, D, E
p27 (p28)	+	1, 2, 3, 4, 5, 6	A, B, D, E

CKI, cyclin dependent kinase inhibitor; nd, not determined.

The gene encoding human p16 has been mapped to the short arm of chromosome 9, at 9p21, the site of a major tumour suppressor locus in several tumour types, as determined by loss of heterozygosity analyses [43–49]. As a negative regulator of the cell cycle machinery, p16 would make a good candidate tumour suppressor encoded by this locus, and it was not surprising when two studies [50, 51] demonstrated that the gene encoding p16 was homozygously deleted in cell lines derived from a wide range of human tumours, including lung, breast, brain, bone, epidermis, ovary and lymphoreticular malignancies. Furthermore, in melanoma cell lines which retained one allele, missense and nonsense point mutations or frameshifting deletions or insertions characterised the majority of cases, strongly suggesting that p16 was, indeed, the tumour suppressor product whose function was ablated by 9p21 chromosomal alterations. However, several subsequent studies using DNAs obtained from primary tumours failed to confirm the high frequency of deletion or mutation previously observed in analyses of cultured cells [52–54], and it was suggested that the high incidence of mutations previously detected in p16 might represent an artefact of *in vitro* propagation [55], although some workers were able to

demonstrate that p16 mutations did occur *in vivo*, both by direct analysis of oesophageal tumours [56] and by utilising cell lines independently derived from individual patients' primary and metastatic oral squamous cell carcinomas to confirm the presence of identical mutations [57]. The reason for these apparently conflicting results is still unresolved, although it may stem, in part, from contamination of tumour tissues by surrounding normal or stromal cells coupled with the use of ultrasensitive detection methods. Alternatively, it may be that tumour cells consist of heterogeneous populations, perhaps as a result of field carcinogenesis, in which only a proportion of cells have undergone mutation in a particular gene (*p16*, for example). Recent reports of a putative senescence gene located at 9p21 [58], together with the demonstration of the loss of 9p21 heterozygosity in immortal but not senescent cells derived from malignant tumours *in vivo* [59], implies that cell lines established from tumours which contained both senescent and immortal variants *in vivo* would comprise only of cells which had inactivated the senescence gene. Mutation of *p16 in vitro* as an explanation for the disparity observed between the mutation frequency in cell lines and tissues is, however, inconsistent with published work [57].

To date, several independent studies have demonstrated inactivation of p16 in tumours of lung [60–62], oesophagus [60], liver [60], colon [60], pancreas [60], central nervous system [63–65] and head and neck [66]. One study [61] which examined a series of lung tumour cell lines demonstrated a near-perfect correlation between the presence of wild-type pRb and the absence of normal p16 protein. Only 11% of small cell lung cancer cell lines failed to express p16, all of which expressed a functional pRb, whereas pRb was absent or aberrant in those cell lines in which p16 was expressed. In addition, the majority of non-small cell lung cancer lines, which generally express a normal pRb molecule, did not express p16, while p16 expression was maintained in six of seven samples with absent or aberrant pRb. These data present evidence of a common growth suppressive pathway involving p16 and pRb, the inactivation of which may be fundamental for deregulation of cellular growth control during oncogenesis. Further evidence of a working relationship for these two molecules came from a careful comparison of the associations formed between D-type cyclins, cdk4, cdk6 and p16 in cells expressing or lacking a functional pRb [67]. Again, an inverse correlation was found between pRb status and p16 expression. In addition, it was demonstrated that p16 competes with D cyclins for binding with cdk4 and cdk6, as in cells where pRb function had been compromised neither cdk4 nor cdk6 were found to associate with D cyclins, but instead were bound to p16. These data imply that p16 is one component of a feedback mechanism which controls pRb function [67]. Thus, in cells where pRb is inactivated through deletion, mutation, or binding to viral oncoproteins, there is no selective advantage to be gained by inactivating p16. Hence, inactivation of p16 or pRb, or amplification and overexpression of D-type cyclins or their cognitive kinases would be predicted to result in deregulation of the same growth control pathway (summarised in Fig. 2). A further study of p16 protein present in normal cells from different lineages demonstrated differential expression of p16, with minimal p16 detected in ductal epithelium from breast and no expression in T lymphocytes [68]. Although the explanation for this remains unclear, it is likely that T cells, which express cdk6, express another related cdk inhibitor instead of p16 (such as p18, see below). These authors also demonstrated cell cycle dependent expression of p16 in normal cells, with the highest levels occurring during S-phase and implying a requirement to inhibit cdk4 activity at the G_1/S transition.

The familial melanoma susceptibility locus, *MLM*, has recently been mapped to chromosome 9p21 [69], the location of the gene encoding p16. In order to determine whether the *MLM* gene might be p16, two studies have now analysed p16 coding sequences from familial melanoma kindreds for the presence of germline mutations. One group was able to demonstrate mutations in 33 of 36 melanoma cases in 9p21-linked, but not 1p36-linked, melanoma families [70], suggesting that the *p16* gene is a likely candidate for the *MLM* susceptibility gene. However, a second study [71] reported that germline mutations occur with low frequency in familial melanoma, implying that either the *p16* gene is not *MLM*, or that mutations occur outside the regions analysed. It may be that germline deletion of p16 coding sequences may afford an alternative to intragenic mutation as regards susceptibility to melanoma, although the data available to date appear contradictory. However, in a study of pancreatic adenocarcinoma, a tumour type also characterised by 9p allelic loss, somatic alterations in the *p16* gene were demonstrated to occur with high frequency in both primary tumours and xenografts, and included deletions, insertions and point mutations [72], providing strong evidence that *p16* gene mutations play a role in tumour development. It is notable that some melanoma families show a high incidence of pancreatic adenocarcinoma [71], and it may be that mutation of *p16* sequences contributes to carcinogenesis in both these cell types [73].

Although the high frequency of deletions and mutations of *p16* and the correlative data from studies of cells which express either functional or non-functional pRb are strongly suggestive of a role for p16 as a major regulator of normal cell proliferation, more direct evidence of the important contribution of p16 loss to human tumorigenesis has come from gene replacement experiments where exogenous p16 has been reintroduced into $p16^{-/-}$ cells *in vitro*. In one such study, expression of activated H-*ras* in G_0-arrested rat embryo fibroblasts (REFs) stimulated cell proliferation, whereas co-expression of p16 was found to block H-*ras*-induced entry into S-phase [74]. In addition, p16 was shown to suppress cellular transformation of REF cells transformed by activated H-*ras* and c-*myc*, but was incapable of suppressing transformation of cells transfected with H-*ras* plus adenovirus E1a. These results further confirm previous data that p16 and pRb are components of the same growth suppressive pathway, as E1a is known to bind and sequester cellular pRb and other pRb-like molecules [75]. In a separate study, re-introduction of a functional p16 into human glioma cells lacking endogenous p16 resulted in inhibition of cell growth by up to 90% of control values, whereas no significant growth-suppressive effect was seen when cells expressing endogenous wild-type p16 were transfected [76]. Thus, it would appear that p16 does suppress growth of tumour cells, dependent on the mechanism of transformation. However, confirmation of the precise contribution of p16 loss to tumour development awaits targeted disruption of the gene *in vivo*.

p15 (INK4B/MTS2), p18 and p19

Analysis of immunoprecipitates of proteins extracted from cells radiolabelled in culture revealed that, as well as binding to

p16, the G_1 cyclin kinases cdk4 and cdk6 form associations with other small molecules in the 15–20 kDa range [40]. The likelihood existed that these represented additional cdk inhibitors, and this has now been confirmed by cloning of cDNAs encoding three such proteins, p15 [77] (also known as p14 [78]), p18 [78, 79], and p19 [79, 80], and performing functional analyses both *in vitro* and *in vivo* which demonstrate their abilities to bind and inhibit the activity of G_1 cyclin kinases. p15 is encoded by a gene also situated on chromosome 9p21, proximal to that which encodes p16 [50], and, therefore, represents an additional candidate for a tumour suppressor at this locus. Several studies have now presented evidence that p15 function is lost in some human tumours and cell lines, including non-small cell lung cancer [81], leukaemias and lymphomas [82] and glioblastomas [83]. Although a few samples harboured intragenic mutations within the p15 coding region [81, 82], homozygous deletion was found to be more common. Thus, loss of 9p may represent an efficient method through which cells deregulate their growth control mechanisms by deleting both *p16* and *p15* genes in one event. To date, no studies have reported mutations of *p18*, which maps to human chromosome 1p32, a locus which shows structural abnormalities in a range of different malignancies [78]; loss of heterozygosity at this chromosomal region was noted in one lung tumour cell line [81]. p18 has been shown to associate strongly with cyclin D–cdk6 complexes *in vitro* and *in vivo*, and inhibit phosphorylation of pRb [78]. Furthermore, ectopic expression of p18 is able to suppress growth of cells which express a functional pRb, a similar finding to that observed for p16 [74]. p19 also inhibits the G_1 kinases cdk4 and cdk6 [79, 80]. Expression of p19 was shown to vary at different times during the cell cycle, with maximal levels present during S-phase, which led the authors to speculate that it might be involved in limiting the activity of G_1 kinases after cells had exited from G_1 [79]. However, it has also been demonstrated that constitutive expression of p19 results in cell cycle arrest in G_1 [79].

p27 (KIP1)

A p21-related cdk inhibitor, p27, has been described by three independent laboratories [84–86], and the cDNA clone isolated by means of a yeast two-hybrid screen [84] and by a reverse-genetics approach [85]. Sequence analysis revealed 44% identity with p21 in the N-terminal part of the protein [85] (which in p21 contains the motif responsible for inhibition of DNA synthesis [87]), and the gene encoding p27 has now been mapped to human chromosome 12p12.3-pter [88–90]. Functional studies have demonstrated inhibition of cyclin E–cdk2, cyclin A–cdk2, cyclin B1–cdc2 (cdk1), cyclin D1–cdk4 and cyclin D2–cdk4 by exogenous p27 [84–86], and, therefore, the spectrum of activity appears to be similar to that of p21, and broader than the activities of p16 family members. Unlike p21, p27 protein levels do not seem to fluctuate during the cell cycle, remaining relatively constant throughout. Thus, the activity of p27 may be determined by the availability of, and its associations with, other molecules at particular points during the cell cycle. At present, no mutations of p27 have been described in human tumours or cell lines [88–90]. In addition, another cdk inhibitor of similar molecular mass, p28, was observed in lovastatin-arrested HeLa cells [91] which was also able to inhibit a broad spectrum of cyclin–cdk complexes. Whether this is the same protein as p27, or another similar molecule, remains to be determined.

GROWTH FACTOR-MEDIATED REGULATION OF CYCLIN KINASE INHIBITOR ACTIVITY

Sequentially regulated expression or activation of cyclin kinase inhibitors represents an attractive mechanism through which extracellular signals might control cell cycle progression. Of particular interest are the effects of transforming growth factor-β (TGFβ) as a negative regulator of proliferation in many cell types and the loss of cellular response to this ligand that occurs during oncogenesis in some systems. Recent studies from several laboratories have demonstrated cell cycle specific effects of TGFβ, including a block on S-phase entry [12–14, 92], repression of transcription of cyclin A and cyclin E mRNAs [92], and inhibition of cdk4 translation [93, 94]. Some evidence now exists to suggest that part of the growth-suppressive activity of TGFβ may be mediated through cyclin kinase inhibitors, as *p15* mRNA was rapidly induced by several-fold in HaCaT keratinocytes following treatment with TGFβ [77]. Furthermore, p27 was originally isolated from cells arrested following exposure to this ligand [85, 86], and it has been proposed that, upon treatment with TGFβ, the distribution of p27 may alter such that cyclin E–cdk2 complexes are inhibited, resulting in a G_1/S block, and that the redistribution of p27 molecules may occur as a result of increased cellular levels of p15 which preferentially bind cdk4 and cdk6 complexes, displacing p27 [95] (Fig. 1). In murine fibroblasts, for which TGFβ is mitogenic, treatment with TGFβ has been shown to downregulate p27 expression and activate cyclin E–cdk2 kinase [96], providing further evidence of a role for this inhibitor in TGFβ signalling. p21 may also participate in the cellular response to TGFβ, as *p21* mRNA was induced and cyclin E-associated kinase activity inhibited in two TGFβ-sensitive colon cancer-derived cell lines in response to growth factor exposure [97]. Induction of p21 transcription in fibroblasts in response to mitogens such as PDGF and FGF has been discussed above. In addition, A431 human squamous carcinoma cells are known to overexpress the EGF receptor and to proliferate in response to picomolar concentrations of EGF, while nanomolar concentrations of ligand inhibit cell growth [98]. We have recently demonstrated that nanomolar, but not picomolar, concentrations of EGF induce p21 expression rapidly and to a high level (J. Jakus and W. A. Yeudall, manuscript submitted). Furthermore, p21 induced by this mechanism associates with cyclin dependent kinases and inhibits their activity, providing a molecular explanation for the biological responses of these cells to EGF. In quiescent T lymphocytes, p27 binds to cdk2 and inhibits its kinase activity. However, upon mitogenic stimulation with interleukin 2, p27 becomes downregulated and cells enter the cell cycle [99]. Thus, both stimulatory and inhibitory extracellular signals may target cyclin kinase inhibitors. To date, no growth factor modulation of p16 expression or activity has been described.

INVOLVEMENT OF CYCLIN KINASE INHIBITORS IN CELLULAR DEVELOPMENT AND DIFFERENTIATION

In order for terminal differentiation to occur, dividing cells must be removed from the cell cycle into a quiescent state (G_0). Inhibition of cyclin kinase activity through interaction with inhibitory molecules provides a means through which this can be achieved. It is, therefore, not surprising that several groups have been able to demonstrate increased expression of cyclin kinase inhibitors in terminally differentiating cells [100–102].

In skeletal muscle, functional pRb is required for myogenic differentiation to occur. As discussed previously, active cyclin–cdk complexes phosphorylate and inactivate pRb, hence inactivation of cyclin-cdks through the binding of cdk inhibitors allows pRb to suppress growth. Induction of p21 transcription in response to treatment of skeletal muscle cells with MyoD results in terminal cell cycle arrest and correlates with onset of differentiation [100, 101]. Furthermore, differentiation is inhibited by ectopic expression of cyclin D1, while transfection of cells with p21 or p16 expression plasmids enhances muscle-specific gene expression [102], thus providing additional evidence that active cyclin–cdk complexes antagonise differentiation pathways. Overexpression of cyclins or cdks, or mutation or deletion of cdk inhibitors and their partners on the respective growth-suppressive pathways, might, therefore, be expected to contribute to the undifferentiated state of some human tumours.

In summary, while much knowledge of cell cycle control mechanisms has been gained from research in this highly active field, many questions remain unanswered. The function of cell cycle proteins in mediating extracellular signals, the mechanisms of activation of cdk inhibitory proteins and their role in differentiation in different tissue types, the contribution of dysregulation of cyclin kinases and their associated molecules to oncogenesis and, indeed, the potential for use of these molecules as prognostic and therapeutic tools is only beginning to be explored.

1. Hinds PW, Weinberg RA. Tumor suppressor genes. *Curr Opin Genet Devel* 1994, **4**, 135–141.
2. Buchkovich K, Duffy LA, Harlow E. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. *Cell* 1989, **58**, 1097–1105.
3. Nevins J. Cell cycle targets of the DNA tumor viruses. *Curr Opin Genet Devel* 1994, **4**, 130–134.
4. Kastan MB, Zhan Q, El-Deiry WS, *et al.* A mammalian cell cycle checkpoint pathway utilizing *p53* and *GADD45* is defective in ataxia-telangiectasia. *Cell* 1992, **71**, 587–597.
5. Nurse P. Ordering S phase and M phase in the cell cycle. *Cell* 1994, **79**, 547–550.
6. Sherr CJ. Mammalian G1 cyclins. *Cell* 1993, **73**, 1059–1065.
7. Schauer IE, Siriwardana S, Langan TA, Sclafani RA. Cyclin D1 overexpression vs retinoblastoma inactivation: implications for growth control evasion in non-small cell and small cell lung cancer. *Proc Natl Acad Sci USA* 1994, **91**, 7827–7831.
8. Keyomarsi K, Pardee AB. Redundant cyclin overexpression and gene amplification in breast cancer cells. *Proc Natl Acad Sci USA* 1993, **90**, 1112–1116.
9. Gillett C, Fantl V, Smith R, *et al.* Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. *Cancer Res* 1994, **54**, 1812–1817.
10. Lammie GA, Fantl V, Smith R, *et al.* D11S287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to BCL-1. *Oncogene* 1991, **6**, 439–444.
11. Tam SW, Theodoras AM, Shay JW, Draetta GF, Pagano M. Differential expression and regulation of cyclin D1 protein in normal and tumor human cells: association with cdk4 is required for cyclin D1 function in G1 progression. *Oncogene* 1994, **9**, 2663–2674.
12. Pietenpol JA, Stein RW, Moran E, *et al.* TGFβ1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. *Cell* 1990, **61**, 777–785.
13. Laiho M, DeCaprio JA, Ludlow JW, Livingston DM, Massague J. Growth inhibition by TGFβ linked to suppression of retinoblastoma protein phosphorylation. *Cell* 1990, **62**, 175–185.
14. Howe PH, Draetta G, Leof EB. Transforming growth factor β1 inhibition of p34cdc2 phosphorylation and histone H1 kinase activity is associated with G1/S phase growth arrest. *Mol Cell Biol* 1991, **11**, 1185–1194.
15. Carpenter G, Cohen S. Epidermal growth factor. *Annu Rev Biochem* 1979, **48**, 193–216.
16. Heldin C-H, Westermark B. Signal transduction by the receptors for platelet-derived growth factor. *J Cell Sci* 1990, **96**, 193–196.
17. Xiong Y, Zhang H, Beach D. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell* 1992, **71**, 505–514.
18. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 1993, **75**, 805–816.
19. El-Deiry WS, Tokino T, Velculescu VE, *et al.* WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993, **75**, 817–825.
20. Noda A, Ning Y, Venable SF, Pereira-Smith OM, Smith JR. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp Cell Res* 1994, **211**, 90–98.
21. Li, Jenkins CW, Nichols MA, Xiong Y. Cell cycle expression and p53 regulation of the cyclin-dependent kinase inhibitor p21. *Oncogene* 1994, **9**, 2261–2268.
22. El-Deiry WS, Harper JW, O'Conner PM, *et al.* WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res* 1994, **54**, 1169–1174.
23. Lin D, Fiscella M, O'Conner PM, *et al.* Constitutive expression of B-*myb* can bypass p53-induced Waf1/Cip1-mediated G1 arrest. *Proc Natl Acad Sci USA* 1994, **91**, 10079–10083.
24. Travali S, Reiss K, Ferber A, *et al.* Constitutively activated c-*myb* abrogates the requirement for insulin-like growth factor 1 in 3T3 fibroblasts. *Mol Cell Biol* 1991, **11**, 731–736.
25. Harris CC. p53: at the crossroads of molecular carcinogenesis and risk assessment. *Science* 1993, **262**, 1980.
26. Shiohara M, El-Deiry WS, Wada M, *et al.* Absence of WAF1 mutations in a variety of human malignancies. *Blood* 1994, **11**, 3781–3784.
27. Li Y-J, Laurent-Puig P, Salmon RJ, Thomas G, Hamelin R. Polymorphisms and probable lack of mutation in the WAF1–CIP1 gene in colorectal cancer. *Oncogene* 1995, **10**, 599–601.
28. Chedid M, Michieli P, Lengel C, Huppi K, Givol D. A single nucleotide substitution at codon 31 (Ser–Arg) defines a polymorphism in a highly conserved region of the p53-inducible gene WAF1/CIP1. *Oncogene* 1994, **9**, 3021–3024.
29. Bhatia K, Fan S, Spangler G, *et al.* A mutant p21 cyclin-dependent kinase inhibitor isolated from a Burkitt's lymphoma. *Cancer Res* 1995, **55**, 1431–1435.
30. Gaidano G, Ballerini P, Gong J, *et al.* p53 mutations in human lymphoid malignancies: association with Burkitt's lymphoma and chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 1991, **88**, 5413–5417.
31. Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE, Givol D. Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res* 1994, **54**, 3391–3395.
32. Zhang W, Grasso L, McClain CD, *et al.* p53-independent induction of WAF1/CIP1 in human leukemia cells is correlated with growth arrest accompanying monocyte/macrophage differentiation. *Cancer Res* 1995, **55**, 668–674.
33. Sheikh MS, Li XS, Chen JC, Shao ZM, Ordonez JV, Fontana JA. Mechanisms of regulation of WAF1/Cip1 gene expression in human breast carcinoma: role of p53-dependent and independent signal transduction pathways. *Oncogene* 1994, **9**, 3407–3415.
34. Steinman RA, Hoffman B, Iro A, Guillouf C, Leibermann DA, El-Houseini ME. Induction of p21 (WAF1/CIP1) during differentiation. *Oncogene* 1994, **9**, 3389–3396.
35. Strausfeld UP, Howell M, Rempel R, Maller JL, Hunt T, Blow JJ. Cip1 blocks the initiation of DNA replication in *Xenopus* extracts by inhibition of cyclin-dependent kinases. *Curr Biol* 1994, **4**, 876–883.
36. Waga S, Hannon GJ, Beach D, Stillman B. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* 1994, **369**, 574–578.
37. Li R, Waga S, Hannon GJ, Beach D, Stillman B. Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature* 1994, **371**, 534–537.
38. Shivji MKK, Grey SJ, Strausfeld UP, Wood RD, Blow JJ. Cip1

inhibits DNA replication but not PCNA-dependent mucleotide excision repair. *Curr Biol* 1994, **4**, 1062–1068.
39. Lane DP. p53: Guardian of the genome. *Nature* 1992, **358**, 15–16.
40. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993, **366**, 704–707.
41. Hinds PW, Mittnacht S, Dulic V, Arnold A, Reed Si, Weinberg RA. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* 1992, **70**, 993–1006.
42. Nevins JR. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science* 1992, **258**, 424–429.
43. Nawroz H, Riet P van der, Hruban RH, Koch W, Ruppert JM, Sidransky D. Allelotype of head and neck squamous cell carcinoma. *Cancer Res* 1994, **54**, 1152–1155.
44. Ah-See KW, Cooke TG, Pickford IR, Soutar D, Balmain A. An allelotype of squamous cell carcinoma of the head and neck using microsatellite markers. *Cancer Res* 1994, **54**, 1617–1621.
45. Cairns P, Tokino K, Eby Y, Sidransky D. Homozygous deletions of 9p21 in primary human bladder tumors detected by comparative multiplex polymerase chain reaction. *Cancer Res* 1994, **54**, 1422–1424.
46. Cairns P, Shaw ME, Knowles MA. Initiation of bladder cancer may involve deletion of a tumor suppressor gene on chromosome 9. *Oncogene* 1993, **8**, 1083–1085.
47. Diaz MO, Rubin CM, Harden A, *et al.* Deletions of interferon genes in acute lymphoblastic leukemia. *N Engl J Med* 1990, **332**, 77–82.
48. Fountain JW, Karayiorgou M, Ernstoff MS, *et al.* Homozygous deletions within human chromosome band 9p21 in melanoma. *Proc Natl Acad Sci USA* 1992, **89**, 10557–10561.
49. Olopade OI, Buchhagen DL, Malik K, *et al.* Homozygous loss of the interferon genes defines the critical region on 9p that is deleted in lung cancers. *Cancer Res* 1993, **53**, 2410–2415.
50. Kamb A, Gruis NA, Weaver-Feldhaus J, *et al.* A cell cycle regulator potentially involved in genesis of many tumor types. *Science* 1994, **264**, 436–440.
51. Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* 1994, **368**, 753–756.
52. Cairns P, Mao L, Merlo A, *et al.* Rates of p16 (MTS1) mutations in primary tumors with 9p loss. *Science* 1994, **265**, 415–416.
53. Spruck CH, Gonzalez-Zulueta M, Shibata A, *et al.* p16 gene in uncultured tumors. *Nature* 1994, **370**, 183–184.
54. Kamb A, Liu Q, Harshman K, Tavtigian S, Cordon-Cardo C, Skolnick MH. Rates of p16 (MTS1) mutations in primary tumors with 9p loss. *Science* 1994, **265**, 416–417.
55. Bonetta L. Open questions on p16. *Nature* 1994, **370**, 180.
56. Mori T, Miura K, Aoki T, Nishihira T, Mori S, Nakamura Y. Frequent somatic mutation of the MTS1/CDK4I (Multiple Tumor Suppressor/Cyclin Dependent Kinase 4 Inhibitor) gene in esophageal squamous cell carcinoma. *Cancer Res* 1994, **54**, 3396–3397.
57. Yeudall WA, Crawford RY, Ensley JF, Robbins KC. MTS1/CDK4I is altered in cell lines derived from primary and metastatic oral squamous cell carcinoma. *Carcinogenesis* 1994, **15**, 2683–2686.
58. Porterfield BW, Diaz MO, Rowley JO, Olopade OI. Induction of senescence in two neoplastic cell lines by microcell transfer of human chromosome 9. *Proc Am Assoc Cancer Res* 1992, **33**, 73.
59. Loughran O, Edington KG, Berry IJ, Clark LJ, Parkinson EK. Loss of heterozygosity of chromosome 9p21 is associated with the immortal phenotype of neoplastic human head and neck keratinocytes. *Cancer Res* 1994, **54**, 5045–5049.
60. Okamoto A, Demetrick DJ, Spillare EA, *et al.* Mutations and altered expression of p16INK4 in human cancer. *Proc Natl Acad Sci USA* 1994, **91**, 11045–11049.
61. Otterson GA, Kratzke RA, Coxon A, Kim YW, Kaye FJ. Absence of p16INK4 protein is restricted to the subset of lung cancer lines that retains wildtype RB. *Oncogene* 1994, **9**, 3375–3378.
62. Liu Q, Neuhausen S, McClure M, *et al.* CDKN2 (MTS1) tumor suppressor gene mutations in human tumor cell lines. *Oncogene* 1995, **10**, 1061–1067.
63. Schmidt EE, Ichimura K, Reifenberger G, Collins VP. CDKN2 (p16/MTS1) gene deletion or CDK4 amplification occurs in the majority of glioblastomas. *Cancer Res* 1994, **54**, 6321–6324.
64. He J, Allen JR, Collins VP, *et al.* CDK4 amplification is an alternative mechanism to p16 gene homozygous deletion in glioma cell lines. *Cancer Res* 1994, **54**, 5804–5807.
65. Walker DG, Duan W, Popovic EA, Kaye AH, Tomlinson FH, Lavin M. Homozygous deletions of the multiple tumor suppressor gene 1 in the progression of human astrocytomas. *Cancer Res* 1995, **55**, 20–23.
66. Zhang S-Y, Klein-Szanto AJP, Sauter ER, *et al.* Higher frequency of alterations in the p16/CDKN2 gene in squamous cell carcinoma cell lines than in primary tumors of the head and neck. *Cancer Res* 1994, **54**, 5050–5053.
67. Parry D, Bates S, Mann DJ, Peters G. Lack of cyclin D–Cdk complexes in Rb-negative cells correlates with high levels of p16INK4/MTS1 tumour suppressor gene product. *EMBO J* 1995, 503–511.
68. Tam SW, Shay JW, Pagano M. Differential expression and cell cycle regulation of the cyclin-dependent kinase inhibitor $p16^{Ink4}$. *Cancer Res* 1994, **54**, 5816–5820.
69. Cannon-Albright LA, Goldgar DE, Meyer LJ, *et al.* Assignment of a locus for familial melanoma, *MLM*, to chromosome 9p13-22. *Science* 1992, **258**, 1148–1152.
70. Hussussian CJ, Struewing JP, Goldstein AM, *et al.* Germline p16 mutations in familial melanoma. *Nature Genet* 1994, **8**, 15–21.
71. Kamb A, Shattuck-Eidens D, Eeles R, *et al.* Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. *Nature Genet* 1994, **8**, 21–26.
72. Caldas C, Hahn SA, Costa LT da, *et al.* Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. *Nature Genet* 1994, **8**, 27–32.
73. Ivinson AJ. p16 and familial melanoma. *Nature* 1994, **371**, 180.
74. Serrano M, Gomez-Lahoz E, DePinho RA, Beach D, Bar-Sagi D. Inhibition of ras-induced proliferation and cellular transformation by $p16^{ink4}$. *Science* 1995, **267**, 249–252.
75. Whyte P, Buchkovich KJ, Horowitz JM, *et al.* Association between an oncogene and an anti-oncogene: the adenovirus E1A protein binds to the retinoblastoma gene product. *Nature* 1988, **334**, 124–129.
76. Arap W, Nishikawa R, Furnari FB, Cavenee WK, Huang H-JS. Replacement of the p16/CDKN2 gene suppresses human glioma cell growth. *Cancer Res* 1995, **55**, 1351–1354.
77. Hannon GJ, Beach D. $p15^{INK4B}$ is a potential effector of TGF-β-induced cell cycle arrest. *Nature* 1994, **371**, 257–261.
78. Guan K-L, Jenkins CW, Li Y, *et al.* Growth suppression by p18, a $p16^{INK4}$/MTS1 and $p14^{INK4B}$/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes Devel* 1994, 2939–2952.
79. Hirai H, Roussel MF, Kato J-Y, Ashmun RA, Sherr CJ. Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Mol Cell Biol* 1995, **15**, 2672–2681.
80. Chan FKM, Zhang J, Cheng L, Shapiro DN, Winoto A. Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to $p16^{ink4}$. *Mol Cell Biol* 1995, **15**, 2682–2688.
81. Okamoto A, Hussain SP, Hagiwara K, *et al.* Mutations in the $p16^{INK4}$/MTS1/CDKN2, $p15^{INK4B}$/MTS2, and p18 genes in primary and metastatic lung cancer. *Cancer Res* 1995, **55**, 1448–1451.
82. Otsuki T, Clark HM, Wellmann A, Jaffe ES, Raffeld M. Involvement of CDKN2 ($p16^{INK4A}$/MTS1) and $p15^{INK4B}$/MTS2 in human leukemias and lymphomas. *Cancer Res* 1995, **55**, 1436–1440.
83. Jen J, Harper JW, Bigner SH, *et al.* Deletion of p16 and p15 genes in brain tumors. *Cancer Res* 1994, **54**, 6353–6358.
84. Toyoshima H, Hunter T. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* 1994, **78**, 67–74.
85. Polyak K, Lee M-H, Erdjument-Bromage H, *et al.* Cloning of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitotic signals. *Cell* 1994, **78**, 59–66.
86. Slingerland JM, Hengst L, Pan C-H, Alexander D, Stampfer MR, Reed SI. A novel inhibitor of cyclin-Cdk activity detected in transforming growth factor β-arrested epithelial cells. *Mol Cell Biol* 1994, **14**, 3683–3694.
87. Nakanishi M, Robetorye RS, Adami GR, Pereira-Smith OM,

Smith JR. Identification of the active region of the DNA synthesis inhibitory gene $p21^{Sdi1/Cip1/WAF1}$. *EMBO J* 1995, 555–563.
88. Bullrich F, MacLachlan TK, Sang N, *et al.* Chromosomal mapping of members of the cdc2 family of protein kinases, cdk3, cdk6, PISSLRE, and PITALRE, and a cdk inhibitor, $p27^{Kip1}$, to regions involved in human cancer. *Cancer Res* 1995, **55**, 1199–1205.
89. Pietenpol JA, Bohlander SK, Sato Y, *et al.* Assignment of the $p27^{Kip1}$ gene to 12p13 and its analysis in leukemias. *Cancer Res* 1995, **55**, 1206–1210.
90. Ponce-Castaneda MV, Lee M-H, Latres E, *et al.* $p27^{Kip1}$:chromosomal mapping to 12p12 and absence of mutations in human tumors. *Cancer Res* 1995, **55**, 1211–1214.
91. Hengst L, Dulic V, Slingerland JM, Lees E, Reed SI. A cell cycle-regulated inhibitor of cyclin-dependent kinases. *Proc Natl Acad Sci USA* 1994, **91**, 5291–5295.
92. Geng Y, Weinberg RA. Transforming growth factor β effects on expression of G1 cyclins and cyclin-dependent protein kinases. *Proc Natl Acad Sci USA* 1993, **90**, 10315–10319.
93. Ewen ME, Sluss HK, Whitehouse LL, Livingston DM. TGFβ inhibition of cdk4 synthesis is linked to cell cycle arrest. *Cell* 1993, **74**, 1009–1020.
94. Ewen ME, Oliver CJ, Sluss HK, Miller SJ, Peeper DS. p53-dependent repression of CDK4 translation in TGF-β-induced G_1 cell-cycle arrest. *Genes Devel* 1995, **9**, 204–217.
95. Peters G. Stifled by inhibitions. *Nature* 1994, **371**, 204–205.
96. Ravitz MJ, Yan S, Herr KD, Wenner CE. Transforming growth factor β-induced activation of cyclin E–cdk2 kinase and down-regulation of $p27^{Kip1}$ in C3H 10T1/2 mouse fibroblasts. *Cancer Res* 1995, **55**, 1413–1416.
97. Li C-Y, Suardet L, Little JB. Potential role of WAF1/Cip1/p21 as a mediator of TGF-β cytoinhibitory effect. *J Biol Chem* 1995, **270**, 4971–4974.
98. Gill GN, Lazar CS. Increased phosphotyrosine content and inhibition of proliferation in EGF-treated A431 cells. *Nature* 1981, **293**, 305–307.
99. Firpo EJ, Koff A, Solomon MJ, Roberts JM. Inactivation of a Cdk2 inhibitor during interleukin 2-induced proliferation of human T lymphocytes. *Mol Cell Biol* 1994, **14**, 4889–4901.
100. Parker SB, Eichele G, Zhang P, *et al.* p53-independent expression of $p21^{Cip1}$ in muscle and other terminally differentiating cells. *Science* 1995, **267**, 1024–1027.
101. Halevy O, Novitch BG, Spicer DB, *et al.* Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* 1995, **267**, 1018–1021.
102. Skapek SX, Rhee J, Spicer DB, Lassar AB. Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. *Science* 1995, **267**, 1022–1024.

Acknowledgements—We are grateful to Keith Robbins, Silvio Gutkind and Edith Wolff for comments on the manuscript, and to Dr Robbins for continued support and encouragement.