Regulation of Mammalian G_1 Cyclin Dependent Kinase Complexes by Transforming Growth Factor-β

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Academic Dissertation

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ABBREVIATIONS

ARF	alternative reading frame
5-BrdUrd	5-bromo-2’-deoxyuridine
CAK	cyclin activating kinase
Cdk	cyclin dependent kinase
Cdki	Cdk inhibitor
CHX	cycloheximide
GST	gluthathione-S-transferase
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
HA	hemagglutinin
HGF	hepatocyte growth factor
INK4	Inhibitor of Cdk4
IFN-α	interferon-α
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MTS1	multiple tumor suppressor-1
pAb	polyclonal antibody
pRb	retinoblastoma tumor suppressor protein
SMAD	Sma/Mad related mothers against dpp
SV40	simian virus 40
TGF-β	transforming growth factor-β

ORIGINAL PUBLICATIONS

This work is based on the following original publications, referred to by their roman numerals in the text:

I Tsubari, M., Tiihonen E. and Laiho M. Cloning and characterization of p10, an alternative spliced form of p15 CDK-inhibitor. *Cancer Res.*, 57: 2966-2973, 1997.

II Tsubari, M., Taipale, J., Tiihonen, E., Keski-Oja, J. and Laiho M. Hepatocyte growth factor releases mink epithelial cells from transforming growth factor–β1 induced growth arrest by restoring Cdk6 expression and cyclin E-associated Cdk2-activity. *Mol. Cell. Biol.*, 19: 3654-3663, 1999.

III Taipale, M., Tiihonen, E., Heiskanen, A. and Laiho, M. Accumulation of a $p27^{Kip1}$ form not associated with Cdk-cyclin-complexes in transforming growth factor-β arrested Mv1Lu cells. *Submitted.*

IV Kivinen, L., Tsubari, M., Haapajärvi, T., Datto, M.B., Wang, X.-F., and Laiho, M. Ras induces $p21^{Cip1/Waf1}$ cyclin kinase inhibitor transcriptionally through Sp1-binding sites. *Oncogene, in press*.

ABSTRACT

Transforming growth factor-β (TGF-β) is a potent growth inhibitor of mammalian cells, particularly those of epithelial or endothelial origin. The growth inhibitory activity of TGF-β is mediated by retinoblastoma tumor suppressor protein (pRb) functioning in its underphosphorylated form to arrest the cell cycle in G_1-phase. TGF-β regulates the phosphorylation status of pRb by inhibiting the activity of cyclin dependent kinase (Cdk) -complexes that phosphorylate pRb.

In the present study we found novel mechanisms of regulation of Cdk inhibitors (Cdkis) p15 and p27 by TGF-β. In addition, overexpression of Ras oncoprotein was found to induce p21 Cdk inhibitor by transcriptional and posttranscriptional mechanisms. The Cdk inhibitor p15 was found to have an alternative spliced mRNA form termed as p10. p10 mRNA is ubiquitously expressed in normal and tumor cell lines, and its expression is induced by TGF-β1 similarly to p15. Unlike p15, p10 did not associate with G_1-specific Cdks 4 or 6. Further, TGF-β was found to induce accumulation of a subpopulation of p27 Cdk inhibitor in Mv1Lu cells with an exclusively nuclear localization. In contrast to the rest of the p27 pool, it lacks association with Cdks and cyclins. These results suggest further complexity of TGF-β growth regulation in Mv1Lu cells.

TGF-β growth regulation was also studied by using hepatocyte growth factor (HGF) as an antagonist of TGF-β. HGF counteracts the TGF-β mediated growth inhibition and induces Mv1Lu cell proliferation. We found that HGF prevents TGF-β growth arrest mainly by preventing the downregulation of Cdk6 levels by TGF-β suggesting that the regulation of Cdk6 is important for TGF-β growth arrest. The increased Cdk6 levels associate with part of p27 and prevent p27 from binding to and inhibiting the activity of Cdk2-cyclin E-complexes leading to rescue of Cdk2-asociated kinase activity. In addition, cyclin D-associated kinase activity is partially restored, although p15 Cdk inhibitor is still associated at elevated levels with Cdk6. As a consequence pRb is phosphorylated and cell cycle can progress from G_1 to S-phase.

As a conclusion, the study suggests further complexity of G_1 and G_1/S-phase specific Cdk-complex regulation by TGF-β.

A. INTRODUCTION

A 1. Mammalian cell cycle

The purpose of the cell cycle is to multiply cells and to ensure that events of DNA replication and cell division occur in good order with correct timing. The mammalian cell cycle can be divided into four different phases (Fig. 1). The G_1 (gap-1) -phase involves cellular growth and active protein synthesis. In S (synthesis) -phase DNA is replicated and in G_2 (gap-2) -phase the nucleus prepares for cell division. In mitosis (M) the replicated chromosomes are divided into two daughter nuclei followed by the actual cytokinesis, where the cell divides into two daughter cells. Terminally differentiated or mitogen-deprived cells are withdrawn from cell cycle into a so-called G_0-phase, from which they can re-enter in some instances into the mitotic cycle through G_1-phase. Mammalian cells receive various signals from extracellular growth factors, some of which stimulate growth, while others inhibit mitogenesis, induce differentiation or provide positional information. Based on these different signals the cells have to make a decision of whether proliferate or not. The cells are sensitive to extracellular stimuli until they reach a restriction point in late G_1-phase, after which they can complete the division cycle without extracellular signals (Pardee, 1989).

Cell cycle progression of eukaryotic cells is controlled by the sequential activation of cyclin–cyclin dependent kinase (Cdk) complexes (Fig. 1). Formation of the complexes and regulation of

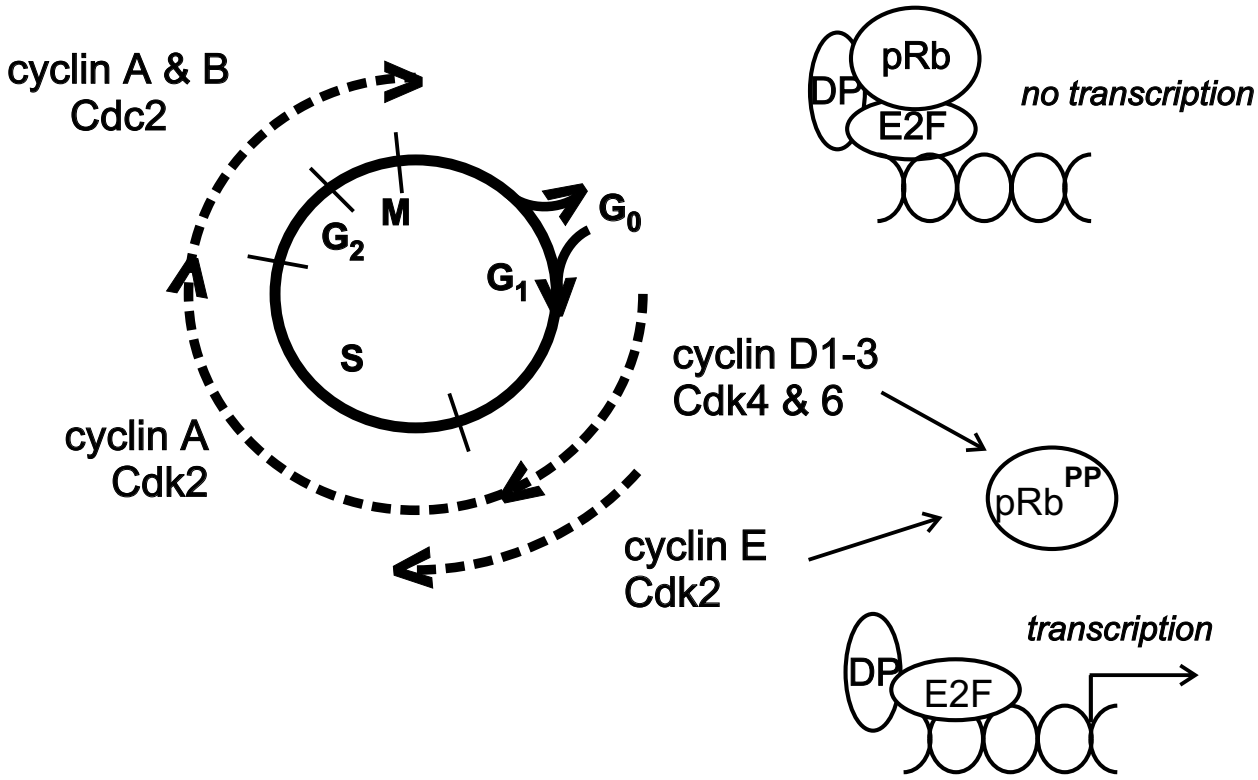

Fig. 1 The mammalian cell cycle is driven by the activity of several Cdk-cyclin complexes. The activities of Cdk4/6 in complex with D-type cyclins together with Cdk2 in complex with cyclin E are required for the G_1-S transition. The best studied target of these Cdk complexes is pRb. Underphosphorylated pRb forms a repressor complex with E2F family of transcription factors and prevents transcription of E2F target genes. When phosphorylated pRb dissociates from E2F, and transcription of genes required for S-phase transition can begin (see text for details).

their activity is highly specific, and distinct complexes drive cell cycle progression in G_1, G_1/S and S-phases, and in mitosis (reviewed in Morgan, 1995; Pines, 1995; Sherr, 1996). The G_1 progression is regulated by D-type cyclins (D1-3) that form complexes with Cdk4 or Cdk6 and later in G_1 by cyclin E in complex with Cdk2 (reviewed by Sherr, 1994). Additionally, a Cdk3-associated kinase activity is found to be induced during G_1-S transition (Meyerson *et al*., 1992; van den Heuvel and Harlow, 1993).

Retinoblastoma/E2F-pathway as a controller of G_1-progression

Retinoblastoma tumor suppressor protein (pRb) is thought to be an important regulator of the G_1 restriction point in mammalian cells, and its activity is vital for orderly cell cycle progression. pRb is the most studied substrate of the G_1 and G_1/S specific cyclin-Cdk-complexes. The *Rb* gene is mutated or deleted in a variety of tumors including retinoblastoma, osteosarcoma, small-cell lung carcinoma, and bladder cancer (reviewed by Weinberg 1991; 1995). pRb is a nuclear phosphoprotein whose activity is regulated by multiple phosphorylations. Different lines of evidence indicate that hypo- and/or unphosphorylated form of pRb restricts cellular growth. First, DNA tumor virus oncoproteins, such as SV40 large T antigen, adenoviral E1A and papillomaviral E7, specifically bind and harvest unphosphorylated, but not phosphorylated, forms of pRb (Whyte *et al*., 1988; DeCaprio *et al*., 1988; Dyson *et al*., 1989a, b; Munger *et al*., 1989; Egan *et al*., 1989). Second, the hypophosphorylated form of pRb binds several cellular proteins such as the E2F family of transcription factors (Chellappan *et al*., 1991), MyoD and c-Abl (reviewed by Wang *et al*., 1994).

Binding to pRb prevents E2F from activating the transcription of its target genes, which are required for the initiation of the S-phase. When phosphorylated, pRb can no longer associate with E2F (Fig. 1; Chellappan *et al*., 1991). The E2F transcription factor is actually a heterodimer composed of two partners, E2F and DP. At least five distinct E2F subunits are known. They all recognize a target sequence present in the promoters of several genes important for cell growth control and S-phase entry, such as *c-myc*, *B-myb*, *Cdc2*, *thymidine kinase* and *E2F-1* itself (reviewed by LaThangue 1994; Weinberg, 1995; Cobrinik, 1996). Of the five E2F family members, pRb binds preferentially to E2Fs 1-3, and also E2F-4 in some cell types, whereas pRb family members p107 and p130 associate with E2Fs 4-5 (reviewed by Weinberg, 1996; Ezhevsky *et al*., 1997). Rather than blocking E2F transcriptional activity, studies suggest that pRb-E2F complex bound to a promoter region is an active transcriptional repressor (reviewed by Brehm and Kouzarides, 1999). Competition of pRb-E2F transcriptional repressor complex by stable overexpression of a mutant E2F containing only the DNA binding domain, but lacking pRb binding and transcriptional transactivator sites, can prevent G_1 arrest suggesting that active repressor complexes formed by pRb and E2F are required for the G_1 arrest (Zhang *et al*., 1999).

In G_1 and G_1/S-phases pRb is phosphorylated by cyclin D- and E-associated Cdks at several specific sites (reviewed by Taya, 1997). Cdk4-cyclin D-complexes have a different phosphorylation consensus site than Cdk2-cyclinE/A-complexes (Kitagawa *et al*., 1996), and they phosphorylate pRb on distinct sites (Zarkowska and Mittnacht, 1997). Furthermore, the phosphorylation of the specific sites is required to inhibit the binding of SV40 large T antigen, c-Abl or E2F to pRb, suggesting that the phosphorylations regulate different protein-protein interactions (Knudsen and Wang, 1996). Although earlier studies suggested that overexpressed D- or E-cyclins can overcame pRb-mediated suppression of proliferation (Hinds et al., 1992; Dowdy et al., 1993; Ewen et al., 1993a), more recent

studies suggest that neither endogenous cyclin D- or E-associated activity alone is enough for Rb inactivation. Several studies indicate that Cdk2-cyclin E-complexes are unable to phosphorylate endogenous pRb unless it has been first phosphorylated by cyclin D-complexes (Resnitzky and Reed, 1995; Leone *et al*., 1997; Lundberg and Weinberg, 1998). Similarly, ectopically expressed human pRb is fully phosphorylated in yeast only in the presence of both cyclin D and E (or their yeast homologues) (Hatakeyama *et al*., 1994). Endogenous cyclin D-complexes have been shown to partially, but not fully phosphorylate pRb in G_1 giving rise to a hypophosphorylated form of pRb (Ezhevsky *et al*., 1997). This study also suggests that the hypophosphorylated pRb can still associate with E2F and is thus active. Taken together these results indicate that a sequential activity of D-type and E-type associated kinases is needed for inactivation of pRb in G_1-S transition and cell cycle progression.

Control of cell cycle progression by G_1 and G_1/S specific Cdk-cyclin complexes

Inadequate activation of G_1 and G_1/S specific Cdk-cyclin complexes can lead to premature entry into S-phase independently of signals that restrict cellular growth such as growth factors or DNA damage. Mouse embryo fibroblasts lacking *Rb* have shortened G_1 phase and elevated cyclin E expression (Herrera et al., 1996a). Similarly, overexpression of the G_1 and G_1/S specific D- and E-type cyclins in fibroblasts shortens the G_1-phase, decreases cell size and decreases the dependency of the cell of mitogens, suggesting that they are rate limiting for G_1 progression (Ohtsubo and Roberts, 1993; Quelle *et al*., 1993; Resnitzky *et al*., 1994).

D-type cyclins

The three different D-type cyclins (D1 to D3) have distinct, although overlapping, expression patterns in tissues (Matsushime *et al*., 1991; Inaba *et al*., 1992; Kiyokawa *et al*., 1992; Motokura *et al*., 1992; Ajchenbaum *et al*., 1993, Sicinsky *et al*., 1995, 1996; Lukas *et al*., 1995a). The expression of the D-type cyclins is induced in G_1 by mitogenic growth factors, and they are synthesized as long as the stimulation persists. Their levels oscillate moderately during the cell cycle being highest in G_1-phase (Kiyokawa *et al*., 1992; Motokura *et al*., 1992; Won *et al*., 1992; Ajchenbaum *et al*., 1993). D-type cyclins are highly unstable proteins ($T_{1/2}$ < 30 min; Matsushime *et al*., 1992) and like many other cell cycle regulatory proteins cyclin D1 has been shown to be degradated via the proteasome pathway (Diehl *et al*., 1997; reviewed by Pagano, 1997). Mitogen withdrawal in G_1-phase leads to rapid degradation of the D-type cyclins and to failure of the cells to enter S-phase. Later in cell cycle degradation of D-type cyclins does not have an effect on cell cycle progression (Matsushime *et al*., 1991). Cyclin D-associated kinase activity is first detected in mid-G_1 and increases towards G_1/S boundary (Matsushime *et al*., 1994; Meyerson and Harlow 1994). Microinjection of cyclin D1 or D2 antibodies into normal fibroblasts or lymphocytes, respectively, in early G_1 prevents their entry into S-phase but fails to do so near the G_1/S transition, indicating that the cyclin D-associated activity is needed in middle to late G_1 (Baldin *et al*., 1993; Quelle *et al*., 1993; Lukas *et al*., 1995a, 1995b).

Homozygous deletion of *Cyclin D1* or *D2* genes does not compromise development of viable animals. Mice lacking cyclin D1 or D2 have focal developmental anomalies such as impaired development of all layers of the retina and the mammary gland during pregnancy in the cyclin D1-deficient animals (Fantl *et al.*, 1995; Sicinski *et al*., 1995). Cyclin D2-deficient female mice are steril due to inability of the granulosa cells to proliferate (Sicinski *et al*., 1996).

The activity of Cdk4 or 6-cyclin D complexes is required for pRb phosphorylation in G_1

The main partners of D -type cyclins are Cdk4 and Cdk6 (Meyerson *et al*., 1991; Matsushime *et al*., 1992, 1994; Meyerson and Harlow 1994), although in some cell types cyclins D2 and D3 have been shown to associate with and activate also Cdk2 (Ewen *et al*., 1993a; Kato and Sherr, 1993; Sweeney *et al*., 1997). Cdk4 and Cdk6 are two close homologues that have overlapping expression patterns in different tissues (Meyerson *et al*., 1991, Matsushime *et al*., 1992, 1994; Meyerson and Harlow 1994). Cdk4 and Cdk6 can be found both in nucleus and cytoplasm, and association with cyclin D is needed for their nuclear transport (Diehl and Sherr, 1997). Only the nuclear complex contains kinase activity, as shown with Cdk6-cyclin D3-complex (Mahony *et al*., 1998).

The best studied substrate of G_1 specific Cdks is pRb. The D-type cyclins, unlike cyclin E, bind directly to pRb (Dowdy *et al*., 1993; Ewen *et al*., 1993a) and target it to Cdk4 (Kato *et al*., 1993). Unlike Cyclin D2-Cdk2 complexes, cyclin D-Cdk4 or -Cdk6 complexes do not phosphorylate histone H1 (Matsushime *et al*., 1992, 1994; Meyerson and Harlow 1994; Sweeney *et al*., 1997). It is suggested that the substrate specificity can be modulated by both the Cdk and the cyclin subunit. For instance, cyclin D1-Cdk4 complexes phosphorylated 38 and 24 kDa proteins while cyclin D3-Cdk4 specifically phosphorylated proteins of 105, 102, 42 and 24 kDa in T-47D breast cancer cell line lysates *in vitro*. Cyclin D3-Cdk6 complex, instead, did not phosphorylate the 24 kDa protein (Sarcevic *et al*., 1997). These data also suggest that the functions of the different Cdk4/6-cyclin D complexes are not redundant.

Several studies indicate that cyclin D-associated Cdk4 or Cdk6 kinase activity is dispensable in cells lacking functional pRb suggesting that pRb is a key substrate for cyclin D-associated kinase activity (Tam *et al*., 1994a; Lukas *et al*., 1994; 1995b; Medema *et al*., 1995; Parry *et al*., 1995). However, cyclin D-associated kinases can target additional substrates such as pRb-related p107 and p130 proteins (Beijersbergen *et al*., 1995; Mayol *et al*., 1995), and DMP1 transcription factor (Hirai and Sherr, 1996). Additionally, nucleolin was found to be phosphorylated by Cyclin D3-Cdk4/6 complexes in T-47D cell lysates (Sarcevic *et al*., 1997).

E-type cyclins

The expression of cyclin E is periodic, peaking in G_1/S transition, where it assembles with Cdk2 (Dulic *et al*., 1992; Koff *et al*., 1991, 1992). Similarly to the cyclin D family, the half-life of cyclin E is short (approx. 30 min) and it is degradated via proteasome pathway after phosphorylation by Cdk2 (Won and Reed, 1996). Like cyclin D antibodies, microinjection of cyclin E antibodies in G_1-phase, but not close to the G_1/S transition, prevents entry of cells into S-phase (Ohtsubo *et al*., 1995). Similarly to cyclin E, a recently cloned cyclin E2 associates with Cdk2 (Lauper *et al*., 1998; Zariwala *et al*., 1999). The two E-type cyclins have somewhat different expression patterns in different tissues (Zariwala *et al*., 1999). Cyclin E expression is regulated by pRb via E2F transcription factors (Duronio and O'Farrell, 1995; Ohtani *et al*., 1995; Botz *et al*., 1996; Geng *et al*., 1996). Also expression of Cyclin E2 seems to be negatively regulated by pRb, since expression of papilloma virus oncoprotein E7 that inactivates pRb, increases the steady state levels of both E-type cyclins. However, E6, which associates only with p53, upregulates only cyclin E2 suggesting that p53 may be involved in cyclin E2 regulation (Zariwala *et al*., 1999).

Cdk2-cyclin E-complexes in G_1/S-phase transition

Cdk2 is a more closely related to mitotic Cdc2 than to Cdk4 or 6 (Meyerson *et al*., 1991). Cdk2 associates with Cyclin E in G_1/S-phase transition, and later in the S-phase with cyclin A. The Cdk2-

cyclin E-complexes can phosphorylate histone H1, Rb, p107 and p130 (Hinds *et al*., 1992; Herrera *et al*., 1996b; Woo *et al*., 1997; Castaño *et al*., 1998). Several lines of evidence suggest that pRb is not the only important target of cyclin E-complexes. First, in contrast to cyclin D, cyclin E is needed for S-phase entry in cells lacking functional pRb (Ohtsubo *et al*., 1995). Second, cyclin E overcomes G_1 arrest induced by mutant pRb lacking Cdk phosphorylation sites and dominant-negative mutant DP-1, suggesting that cyclin E can induce S-phase without activating the pRb/E2F pathway (Lukas *et al*., 1997). Third, cyclin E overexpression bypasses the G_1 block induced by the Cdk4/6-inhibitor p16 (Alevizopoulos *et al*., 1997). In addition, SV40 large T antigen fails to induce S-phase in the presence of dominant-negative Cdk2 suggesting that Cdk2 has other substrates in addition to pRb (Hofmann and Livingston, 1996).

Recent identification of novel Cdk2 substrates suggests that Cdk2 kinase activity regulates onset of S-phase and DNA replication. NPAT (for nuclear protein mapped to Ataxia-telangiectasia locus) was recently found to be a Cdk2-cyclin E substrate (Zhao *et al*., 1998). NPAT, as the name states, is located next to *ATM* (Ataxia-telangiectasia mutated) gene in human chromosome 11. Overexpression of NPAT in U2OS osteosarcoma accelerates S-phase entry by shortening the G_1-phase. However, there is no evidence so far that the phosphorylation of NPAT by Cdk2-cyclin E is needed for S-phase entry (Zhao *et al*., 1998). Cdc6 is another novel substrate for Cdk2-complexes. Cdc6 is an essential regulator of initiation of DNA replication, and its cellular localization is cell-cycle dependent. Phosphorylation of Cdc6 by Cdk2-complexes removes Cdc6 from the nucleus and prevents re-replication of DNA (Jiang *et al*., 1999; Petersen *et al*., 1999). Interestingly, Cdk2-cyclin E-activity was also found to promote centrosome duplication in *Xenopus* egg extracts (Hinchcliffe *et al*., 1999; Lacey *et al*., 1999).

2. Regulation of Cdk activity

The activity of mammalian Cdks is regulated by multiple mechanisms that include complex formation with cyclin, stimulatory and inhibitory phosphorylations, and negative regulation through association with Cdk inhibitory proteins.

Cdk activity requires cyclin binding and activating phosphorylation

The complex formation with cyclin subunit and stimulatory phosphorylation of a conserved threonine residue (thr-160 in human Cdk2) are absolutely needed for the kinase activity of Cdks (Ducommun *et al*., 1991; Gould *et al*., 1991; Desai *et al*., 1992; Solomon *et al*., 1992; Connell-Crowley *et al*., 1993). In addition, cyclin association contributes to substrate specificity (Holmes and Solomon, 1996; Sarcevic *et al*., 1997). A steric model of Cdk2-cyclin A complex indicates that the association of cyclin subunit promotes the activity of the Cdk subunit by relieving blockage for substrate binding and moving the ATP-binding site into the catalytic cleft. In addition, cyclin binding exposes the thr-160 phosphorylation site of Cdk (Jeffrey *et al*., 1995). Phosphorylation of the conserved thr-residue by CAK (cyclin activating kinase) is needed for the completion of the Cdk activation process (Table 1). The thr-phosphorylation promotes further structural changes in the Cdk-subunit leading to stabilization of the complex with cyclin and affecting the putative substrate binding site (Russo *et al*., 1996a).

Cdk7(MO15)-cyclin H complex has been suggested be the *Xenopus* and mammalian CAK, and it was demonstrated to phosphorylate Cdc2, Cdk2 and Cdk4 *in vitro* (Table 1; reviewed by Harper and Elledge, 1998). Cdk7-cyclin H complex and its *S. cerevisiae* homologue are part of the TFIIH transcription complex and are needed for phosphorylation of C-terminal domain of RNA polymerase II (Roy *et al.*, 1994; Akoulitchev *et al.*, 1995; Shiekhattar *et al.*, 1995) raising the question whether Cdk7-cyclin H is a true mammalian CAK. However, Cdk7 homologue in *Drosophila* is necessary for CAK activity and is required for the activation of Cdc2 *in vivo* suggesting that Cdk7 is the CAK in the multicellular organisms (Larochelle *et al.*, 1998). Thr-dephosphorylation of Cdk2 and Cdc2 can be carried out by Kap-1 phosphatase, which removes the phosphate but only in the absence of cyclin (Poon and Hunter, 1995).

Table 1. Regulation of mammalian Cdk activity by phosphorylations. See text for details and references.

	Thr-phosphorylation at thr-160 (Cdk2)	Tyr/thr-phopshorylation at thr-14, tyr-15 (Cdk2)
Effect on Cdk activity	positive	negative
Phosphorylating enzyme	CAK (Cdk7-cyclin H?)	Wee1 and Mik1 homologues
Phosphatase	Kap1	Cdc25 family

Inhibitory tyrosine phosphorylation

Tyrosine phosphorylation is suggested to prevent premature kinase activation and to maintain it within tightly regulated limits. Human Cdc2 and Cdk2 kinase activity is negatively regulated by phosphorylation of thr-14 and tyr-15 residues (Table 1; Gu *et al.*, 1992). Mutation of Cdk2 thr-14 and tyr-15 to alanine residues stimulates the kinase activity (Gu *et al.*, 1992). Cdk4 and Cdk6, instead, have only the tyrosine residue in this region (Matsushime *et al.*, 1992; Meyerson *et al.*, 1992). Thr-14 and tyr-15 phosphorylation of Cdc2 prevents premature entry into mitosis (reviewed by Nurse, 1990; Solomon, 1993). The role of Cdk2 tyr-15 phosphorylation is less clear, and it is found to be maximal in S and G_2-phase cells (Gu *et al.*, 1992; Sexl *et al.*, 1999). In some cell types Cdk4 and/or Cdk6 are tyrosine phosphorylated after UV- (Terada *et al.*, 1995; Jinno *et al.*, 1999) or TGF-β-induced (Iavarone and Massagué, 1997) G_1-arrest.

Wee1 and Mik1 family members of protein kinases co-operate in phosphorylating thr-14 and tyr-15 of Cdc2 and Cdk2 (or their homologues) in yeast, *Xenopus* and human cells (Lundgren *et al.*, 1991; Müller *et al.*, 1995; Watanabe *et al.*, 1995). A Cdc25 phosphatase family removes the threonine and tyrosine phosphorylations. Cdc25A and Cdc25C function in G_1-S and G_2-M transitions of human

cells, respectively, and their activity is positively regulated by Cdk2-cyclin E and Cdc2-Cyclin B mediated phosphorylation (Hoffmann *et al*., 1993, 1994; Jinno *et al*., 1994).

Cyclin dependent kinase inhibitors

Cyclin dependent kinase inhibitors (Cdkis) are small proteins that bind to and inhibit the activity of Cdk-cyclin complexes. Two families of Cdkis are found in mammalian cells, namely the p21- and p16-related proteins (reviewed by Graña and Reddy, 1995; Sherr and Roberts, 1995). Members of the p21 family are more universal Cdk inhibitors forming ternary complexes with G_1, G_1/S and to some extent also with mitotic Cdks and cyclins (reviewed by Hengst and Reed, 1998). p16 family members, in contrast, form binary complexes and inhibit only the activity of G_1 specific Cdks 4 and 6 (reviewed by Carnero and Hannon, 1998).

p21 family

Structural motifs of p21 family

p21 family consists of three members: $p21^{CIP1,WAF1,Sdi1}$ (El-Deiry *et al*., 1993; Gu *et al*., 1993; Xiong *et al*., 1993a; Harper *et al*., 1993; Noda *et al*., 1994), $p27^{KIP1}$ (Polyak *et al*., 1994a, b; Toyoshima and Hunter 1994) and $p57^{KIP2}$ (Matsuoka *et al*., 1995; Lee *et al*., 1995). The p21 family members associate with and inhibit the activity of Cdk-cyclin complexes through their N-terminal domains that are more conserved than the C-terminal domains (Fig. 2; Toyoshima and Hunter, 1994; Chen *et al*., 1995,

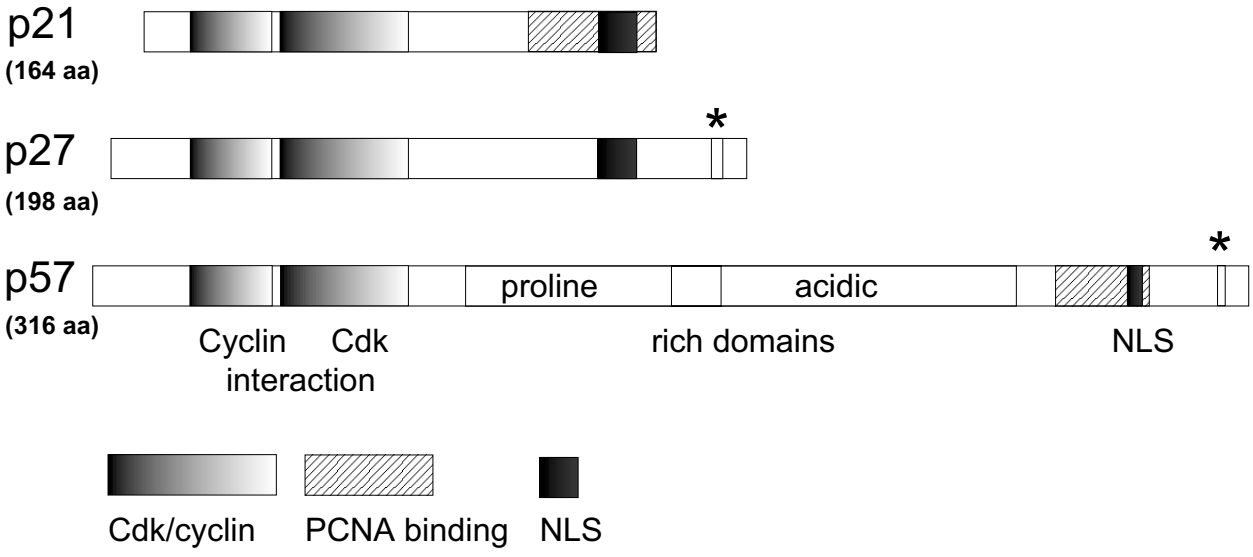

Fig. 2 Schematic representation of the p21 family members. Domains corresponding to Cdk, cyclin and/or PCNA binding, nuclear localization signal (NLS) as well as proline and acidic rich areas of p57 are shown. The asterisk (*) indicates the putative Cdc2 phosphorylation consensus site that in at least p27 is phosphorylated by Cdk2. The total length of each human protein is indicated in amino acids (aa). The figure is adapted from Lee *et al*., 1995. (The Cdk-cyclin interaction domains are according to Russo *et al*., 1996b, the other domains are from Harper *et al*., 1993; Polyak *et al*., 1994b; Lee *et al*., 1995; Matsuoka *et al*., 1995; Watanabe *et al*., 1998).

1996a, b; Goubin and Ducommun 1995; Harper *et al.*, 1995; Lee *et al.*, 1995; Luo *et al.*, 1995; Matsuoka *et al.*, 1995; Nakanishi *et al.*, 1995; Fotedar *et al.*, 1996).

The C-terminal halves of the different p21 family of Cdk inhibitors have different functions. p21 associates with proliferating cell nuclear antigen (PCNA) via the C-terminus (Chen *et al.*, 1995, 1996b; Goubin and Ducummun 1995; Luo *et al.*, 1995). PCNA is a subunit of DNA polymerase-δ, which is the principal replicative DNA polymerase (reviewed by Celis *et al.*, 1987). Binding of p21to PCNA inhibits DNA replication (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994a; Chen *et al.*, 1995), but not PCNA-mediated nucleotide-excision repair (Li *et al.*, 1996). Similar to p21, the C-terminal domain of human p57 contains a short sequence that resembles the p21 PCNA-binding region, and it can inhibit PCNA-dependent DNA synthesis *in vitro* and block the onset of S-phase *in vivo* (Watanabe *et al.*, 1998). Otherwise, the C-terminal domain as well as the N-terminal domain of p57 are more homologous to p27 than p21. Similar to p27, the C-terminal domain of p57 contains a putative Cdk phosphorylation site (Lee *et al.*, 1995; Matsuoka *et al.*, 1995). Also p21 is a phosphoprotein (Zhang *et al.*, 1994; Dulic *et al.*, 1998). In addition, the C-terminal domains of p21 and p27 contain an additional cyclin binding motif (Toyoshima and Hunter, 1994; Chen *et al.*, 1996a), importance of which is unclear. The C-terminal cyclin binding motif of p21 has a lower affinity for cyclins than the N-terminal cyclin interaction domain (Chen *et al.*, 1996a).

Viral oncoproteins negatively affect the function of p21 and p27 by binding to the C-terminal part. Human papilloma virus E7 oncoprotein can block p21-mediated inhibition of Cdk activity and DNA replication by associating with the carboxyl-terminal end on p21 (Funk *et al.*, 1997; Jones *et al.*, 1997). Similarly, E7 inactivates p27 but whether the interaction is direct or depends on a third protein(s) is unclear (Zerfass-Thome *et al.*, 1996). In addition, the adenovirus E1A oncoprotein binds to p27 strongly via the C-terminal domain of p27 and weakly via the N-terminal half (Mal *et al.*, 1996). This interaction overcomes the p27-mediated Cdk2-inhibition, since E1A can dislocate p27 from Cdk2 complexes *in vitro* and *in vivo* (Mal *et al.*, 1996).

The additional functions described for p27 C-terminal part are not related to the ability of p27 to inactivate G_1 and G_1/S specific Cdks. In contrast, the C-terminal domain of p27 has been indicated to activate Cdc2 kinase activity during G_2-M in synchronized culture of NIH 3T3 cells (Üren *et al.*, 1997). During the G_2-M transition a variant of p27 that lacks aminoterminal half is induced. This C-terminal p27 variant, when assayed *in vitro*, stimulates Cdc2 kinase activity (Üren *et al.*, 1997). In addition, the C-terminal half of p27 is able to induce proteolytic cleavage of cyclin A *in vitro* and *in vivo*. The cleavage removes the mitotic destruction box of cyclin A, which leads to accumulation of cyclin A. The truncated cyclin A retains associated kinase activity (Bastians *et al.*, 1998).

The p21 family members are both negative and positive regulators of the Cdk-activity

Based on *in vitro* and overexpression studies p21, p27 and p57 were described initially as efficient inhibitors of at least Cdk4-cyclin D, Cdk2-cyclin E and Cdk2-cyclin A -complexes, and to a lesser extent mitotic Cdc2-cyclin B-complexes (Gu *et al.*, 1993; Harper *et al.*, 1993, 1995; Dulic *et al.*, 1994; Polyak *et al.*, 1994a and b; Toyoshima and Hunter, 1994; Slingerland *et al.*, 1994; Lee *et al.*, 1995; Matsuoka *et al.*, 1995). However, growing evidence suggest that p21 family members are efficient inhibitors of cyclin E- and A-complexes, whereas their association with cyclin D-complexes is required for the normal function of the complexes.

Crystal structure of p27 bound to Cdk2-cyclin A-complex indicates that p27 associates with both Cdk and cyclin subunits. The binding of p27 to Cdk2-cyclin A-complexes seems to be a two-step event, where p27 first binds with cyclin followed by Cdk binding (Russo *et al.*, 1996b). Similarly,

biochemical studies of p27 complexes suggest that p27/p21-cyclin complexes are formed more readily than p27/p21-Cdk-complexes (Hall *et al*., 1995). The association of p27 to Cdk2-cyclin A-complex causes conformational changes in the catalytic cleft of Cdk2 that inactivate the kinase by hindering of ATP binding and blocking of the CAK phosphorylation site (Russo *et al*., 1996b). p27 and p21 have been shown to block the phosphorylation of Cdk4 and Cdk2 (Kato *et al*., 1994; Aprelikova *et al*., 1995), although there is also evidence that p27 is unable to block Cdk4 phosphorylation by CAK (Blain *et al*., 1997). Recent studies also indicate that p27 serves as a substrate to Cdk2-cyclin E, suggesting that p27 exists also in a complex with Cdk2-cyclin E, which have been activated by CAK (Morisaki *et al*., 1997; Müller *et al*., 1997; Sheaff *et al*., 1997; Vlach *et al*., 1997). Alternatively, the phosphorylation of p27 could be accomplished by intercomplex instead of intracomplex kinase activity, so that another p27-free Cdk-cyclin complex can phosphorylate p27 (Montagnoli *et al*., 1999). In accordance to the structural data, studies by Hengst *et al.* (1998) indicate that one molecule of p21 (or p27) bound to Cdk2-cyclin A complex is enough to inhibit the kinase activity.

In contrast to cyclin E- and A-complexes, studies indicate that p21 and p27 are required for the activity of cyclin D-complexes. In exponentially growing cells p27 is found predominantly to associate with Cdk4/6-cyclin D-complexes (Poon *et al*., 1995; Soos *et al*., 1996) and p21 is found as a quaternary complex with Cdk, cyclin and PCNA (Xiong *et al*., 1992; Zhang *et al*., 1993; 1994; Harper *et al*., 1995). In addition, p27 and p21 antibodies immunoprecipitate kinase activity towards pRb that can be attributed to Cdk4 and Cdk6 bound to p27 or p21 (Soos *et al*., 1996; Hauser *et al*., 1997; Blain *et al*., 1997; LaBaer *et al*., 1997; Dong *et al*., 1998). It has also been shown that low concentrations of p21, but not p27 or p57, promote the Cdk4-associated kinase activity, whereas higher p21 concentrations are inhibitory to Cdk4-complexes (LaBaer *et al*., 1997). In fact, it has been shown that p21, p27 and p57 promote the assembly of Cdk4-cyclin D complexes and the proper function Cdk4-cyclin D-complexes requires p21 and p27 (LaBaer *et al*., 1997; Cheng *et al*., 1999; reviewed by Sherr and Roberts, 1999). Primary mouse fibroblasts lacking genes for both p21 and p27 fail to assemble functional Cdk4-cyclin D-complexes, express cyclin D protein at decreased levels, and cannot efficiently direct cyclin D into cell nucleus (Cheng *et al*., 1999). Taken together the data suggest that p21 and p27 are strong inhibitors of Cdk2, whereas the normal activity of Cdk4/6-complexes requires p21 and p27 and is not inactivated by p21 or p27.

The difference of inhibitory activity of p21 family members towards cyclin D- and E/A-complexes is probably due to structural difference of the complex between the Cdk, cyclin and Cdki subunits. Cdk2 and Cdk4 share only a limited sequence homology. The Cdk-inhibitory domain of p21, when not associated with Cdks, lacks fixed secondary or tertiary structure as revealed by spectroscopic studies (Kriwacki *et al*., 1996). However, when bound to Cdk2 the domain has a defined structure. The flexibility of the inhibitory domain that is conserved throughout the p21 family could explain the differency in the ability of p21 family members to inhibit or promote the activity of different Cdks.

p27^{Kip1}

p27 was originally identified as a Cdk2 and Cdc2 inhibitory molecule present in TGF-β and contact arrested mink lung and human mammary epithelial cells (Polyak *et al*., 1994a; Slingerland *et al*., 1994) and in lovastatin arrested HeLa cells (Hengst *et al*., 1994). p27 cDNA was cloned by two different groups by using TGF-β growth arrested Mv1Lu mink lung epithelial cells (Polyak *et*

al., 1994b) and by yeast two- hybrid screen using cyclin D1-Cdk4 as a bait (Toyoshima and Hunter, 1994).

p27 is required in G_1/G_0-cells to prevent Cdk-activity. Overexpression of p27 leads to G_1 arrest (Toyoshima and Hunter, 1994), and antisense expression of p27 prevents mitogen deprived cells from arresting in G_1 (Coats *et al*., 1996; Rivard *et al*., 1996). p27 levels were also found to accumulate in G_1 and decline in S-phase in several human cell lines suggesting a G_1/S-phase periodic regulation (Hengst and Reed, 1996; Millard *et al*., 1997). High levels of p27 protein are detected in quiescent and differentiating cells, whereas its levels are decreased in exponentially growing cells (Nourse *et al*., 1994; Toyoshima and Hunter, 1994; Kato *et al*., 1994; Agrawal *et al*., 1995; Polyak *et al*., 1994a and b; Coats *et al*., 1996; Hengst and Reed, 1996; Millard *et al*., 1997). Similarly, the half-life of p27 is increased in growth arrested cells (Pagano *et al*., 1995; Hengst and Reed, 1996). The regulation of p27 levels is mostly post-transcriptional. p27 mRNA is not regulated in a cell cycle dependent manner or by growth inhibitory treatments (Polyak *et al*., 1994b; Toyoshima and Hunter, 1994). Instead, p27 protein levels are upregulated in G_0-arrested HL-60 human promyelotic cells by increased association of p27 mRNA with polyribosomes (Millard *et al*., 1997). In addition, the p27 protein levels are regulated by ubiquitin-dependent and -independent proteasome degradation pathways (Pagano *et al*., 1995; Montagnoli *et al*., 1999; Nguyen *et al*., 1999; Shirane *et al*., 1999). The degradation of p27 is cell cycle dependent being maximal in S-phase cells, and low in G_1-phase cells. Degradation of p27 via the ubiquitin pathway is regulated by phosphorylation on thr-187, and at least Cdk2-cyclin E/A-complexes phosphorylate p27 on this residue in proliferating cells (Sheaff *et al*., 1997; Vlach *et al*., 1997; Montagnoli *et al*., 1999; Nguyen *et al*., 1999). Coexpression of p27 with Jab1 protein in mouse fibroblasts enhances p27 degradation. Jab1, which was originally identified as a co-activator of Jun, accelerates p27 degradation either by moving p27 from the nucleus into the cytoplasm, or by facilitating p27 phosphorylation at thr-187 (Tomoda *et al*., 1999).

p27 in cellular differentiation and tumorigenesis

Studies of p27 nullizygous mice show that p27 is not required for normal embryonic development, but is necessary for normal growth control. The p27-/- mice develop to term normally, but attain an increased body size within three to six weeks after birth (Fero *et al*., 1996; Kiyokawa *et al*., 1996; Nakayama *et al*., 1996). In addition, the females are infertile due to inability of ovarian follicles to form corpora lutea (Fero et el., 1996; Kiyokawa *et al*., 1996; Nakayama *et al*., 1996). The increased body size is due to increased number of cells in every tissue suggesting that the absence of p27 allows continued cell proliferation (Fero *et al*., 1996; Kiyokawa *et al*., 1996; Nakayama *et al*., 1996). However, terminal differentiation of organs from p27 nullizygous mice is normal indicating that p27 is not essential for differentiation but it is needed for regulation of withdrawal from the mitotic cycle (Fero *et al*., 1996; Kiyokawa *et al*., 1996; Nakayama *et al*., 1996). In concordance, interleukin-2 has been shown to downregulate p27 protein levels leading to Cdk2-activation and T-cell proliferation (Nourse *et al*., 1994), and p27 is required for cell cycle withdrawal in the oligodendrocyte lineage (Casaccia-Bonnefil *et al*., 1997).

The ability of p27 to inhibit cyclin-Cdk-activity and to induce G_1 arrest when overexpressed suggests that it is a tumor suppressor candidate. Abnormally low p27 protein levels in human tumors correlate directly with both histological aggressiveness and patient mortality (Esposito *et al*., 1997; Loda *et al*., 1997; Porter *et al*., 1997; Yang *et al*., 1997). However, no human tumors that have lost the both alleles of the *p27* gene have been found. Therefore p27 is not a tumor suppressor complying Knudson two-hit-theory (Knudson, 1971). Mice nullizygous for p27 exhibit pituitary hyperplasia

and adenomas that showed no sign of invasion or metastasis in mice up to 7 months of age (Fero *et al*., 1996; Kiyokawa *et al*., 1996; Nakayama *et al*., 1996). On the other hand, mice nullizygous or heterozygous for p27 are predisposed to tumors when treated with ionizing radiation or chemical carcinogens suggesting that p27 is indeed a tumor suppressor in mice (Fero *et al*., 1998). Molecular analysis of the remaining allele from tumors of p27 heterozygous mice revealed that the allele was intact suggesting that p27 is a haplo-insufficient tumor suppressor (Fero *et al*., 1998).

p21 ^{CIP1,WAF1,Sdi1} and p57^{Kip2}

p21 was cloned by several groups using different approaches. Initially it was purified from cellular lysates as an inhibitor of Cdk-activity (Gu *et al*., 1993; Xiong *et al*., 1993a). p21 was cloned as a mRNA upregulated by p53 (*Waf1*; El-Deiry *et al*., 1993) and cellular senescence (*Sdi1*; Noda *et al*., 1994), and also identified by yeast two-hybrid screen as a protein binding to Cdk2 and inhibiting its activity (*Cip1*; Harper *et al*., 1993). Finally, a p53-induced Cdk-inhibitor was found in gamma-irradiated cells and identified as p21 (Dulic *et al*., 1994). The approaches by which p21 was identified reflect its function as a Cdk inhibitor that is induced by p53 and cellular senescence.

The role of p21 as a mediator of p53 growth arrest in G_1 after DNA damage is well documented. p53 induces p21 at mRNA level after DNA damage caused by UV- or ionizing radiation or chemicals, such as adriamycin (Dulic *et al*., 1994; reviewed by El-Deiry, 1998). p21-nullizygous fibroblasts are impaired in their ability to arrest in G_1 as response to DNA damage or depletion of nucleotide pools. However, the p53-mediated apoptosis is normal in p21-/- cells suggesting that p21 is needed for p53 mediated G_1 checkpoint but not for apoptosis (Brugarolas *et al*., 1995; Deng *et al*., 1995; Macleod *et al*., 1995). Similarly, a p21-deficient human adenocarcinoma cell line lacks G_1 arrest in response to DNA damage and p53 induction (Waldman *et al*., 1995). On the other hand, p21 has a p53-independent role in development and differentiation of some cell types. p21 expression in various tissues during development and in the adult mouse is p53-independent (Macleod *et al*., 1995), and the expression pattern of mouse p21 correlates with terminal differentiation (Parker *et al*., 1995).

p57 was isolated by using p21 as a probe to screen mouse cDNA library (Lee *et al*., 1995) and also found in a yeast two-hybrid screen using cyclin D1 as a bait (Matsuoka *et al*., 1995). Like other p21 family members, overexpression of p57 leads to G_1 arrest (Lee *et al*., 1995; Matsuoka *et al*., 1995). The mRNA expression pattern of p57 is tissue specific, and most of the cells expressing p57 are terminally differentiated (Lee *et al*., 1995; Matsuoka *et al*., 1995). Interestingly, p57 is subject to imprinting with preferential expression of the maternal allele both in humans and mice (Hatada and Mukai, 1995; Matsuoka *et al*., 1996). The human *p57* gene is located in the 11p15.5 region that is implicated in sporadic cancers and Beckwith-Wiedeman cancer syndrome (Matsuoka *et al*., 1995). However, p57 nullizygous mice do not develop neoplasia (Yan *et al*., 1997), although the phenotype of p57-/- mice resembles that of Beckwith-Wiedeman syndrome patients (Zhang *et al*., 1997a). p57-/- mice have severe defects in cellular proliferation and differentiation and most of them die soon after birth (Zhang *et al*., 1997a; Yan *et al*., 1997).

p16 family

Structural motifs of the p16 family

The p16 family contains of four closely related members, which all inhibit the activity of Cdk4 and Cdk6. The members of p16 family are $p16^{INK4A,MTS1}$ (Serrano *et al*., 1993), $p15^{INK4B,MTS2}$ (Hannon and Beach 1994), $p18^{INK4C}$ and $p19^{INK4D}$ (Guan *et al*., 1994; Hirai *et al*., 1995; Chan *et al*., 1995). p15 and p16 contain four ankyrin repeats, whereas p18 and p19 have five (Serrano *et al*., 1993; Hannon and Beach, 1994; Guan *et al*., 1994; Chan *et al*., 1995; Hirai *et al*., 1995; Russo *et al*., 1998). The ankyrin repeats mediate protein-protein interactions (reviewed by Sedgwick and Smerdon, 1999), and are formed of helix-turn-helix structures (Tevelev *et al*., 1996). p16 family members associate with Cdk4 or 6 at 1:1 stoichiometry and are found both *in vivo* and *in vitro* in Cdk4 or 6 complexes devoid of D-type cyclins resulting in loss of the kinase activity (Serrano *et al*., 1993; Guan *et al*., 1994, 1996; Hall *et al*., 1995; Parry *et al*., 1995; Ragione *et al*., 1996). In addition, p16 can replace cyclin D from Cdk4 complexes *in vitro* (Ragione *et al*., 1996). A peptide of 20 amino acids from the third ankyrin repeat area of p16 has been shown to be able to bind to Cdk4 and 6 *in vitro* (Fåhraeus *et al*., 1996).

In vitro studies and solution structure of p16 suggest that the binding sites for cyclin D1 and p16 in Cdk4 are overlapping and located primarily near the amino-terminus of Cdk4 (Coleman *et al*., 1997; Byeon *et al*., 1998). Structural studies of the p16-Cdk6 and p19-Cdk6 complexes suggest that association with p16 family members prevents ATP binding of Cdk6, and that p16 indirectly prevents Cdk6 from binding to cyclin D (Brotherton *et al*., 1998; Russo *et al*., 1998). The structural studies also suggest that p16 loss-of-function mutations found in cancer cells result in incorrectly folded or insoluble protein (Zhang and Peng, 1996; Luh *et al*., 1997; Brotherton *et al*., 1998; Byeon *et al*., 1998).

$p16^{INK4A,MTS1}$ acts in pRb pathway

p16 was originally cloned in a yeast two-hybrid screen using Cdk4 as a bait, thus the name INK4, inhibitor of Cdk4 (Serrano *et al*., 1993). In addition, p16 was cloned as a gene associated with a chromosome 9p21 locus mutated in melanomas and named MTS1 (multiple tumor suppressor-1; Kamb *et al*., 1994; Nobori *et al*., 1994).

p16 acts on the same pathway as pRb. Overexpression of p16 leads to G_1 arrest in various cell lines and suppresses cellular transformation (Guan *et al*., 1994; Jin *et al*., 1995; Lukas *et al*., 1995c; Medema *et al*., 1995; Serrano *et al*., 1995; Fueyo *et al*., 1996), whereas overexpression of p16 in cells lacking functional pRb did not arrest cells in G_1 (Guan *et al*., 1994; Lukas *et al*., 1995c; Medema *et al*., 1995). Interestingly, p16 levels are elevated in cell lines lacking functional pRb (Serrano *et al*., 1993; Xiong *et al*., 1993b; Otterson *et al*., 1994; Parry *et al*., 1995; Tam *et al*., 1994b). In SV40, adeno- or papillomavirus transformed cells Cdk4 is associated predominantly with p16 (Serrano *et al*., 1993; Xiong, et al., 1993b), whereas in normal human fibroblasts Cdk4 is in quaternary complex with cyclin D, p21 and PCNA (Xiong *et al*., 1992, 1993a; Zhang *et al*., 1993). These results suggest that pRb would function as a sole target of cyclin D-associated kinase in G_1, and in cells lacking pRb cyclin D-associated kinase would not be necessary for cell proliferation. In accordance, prevention of cyclin D expression in cells lacking functional pRb, unlike in their normal counterparts, did not lead in G_1 arrest (Tam *et al*., 1994a; Lukas *et al*., 1995b). In addition, p16 suppresses neoplastic transformation of cultured fibroblasts by oncogenic Ha-Ras and c-Myc but not by Ha-ras and E1A, further verifying that p16 lies in pRb pathway (Serrano *et al*., 1995).

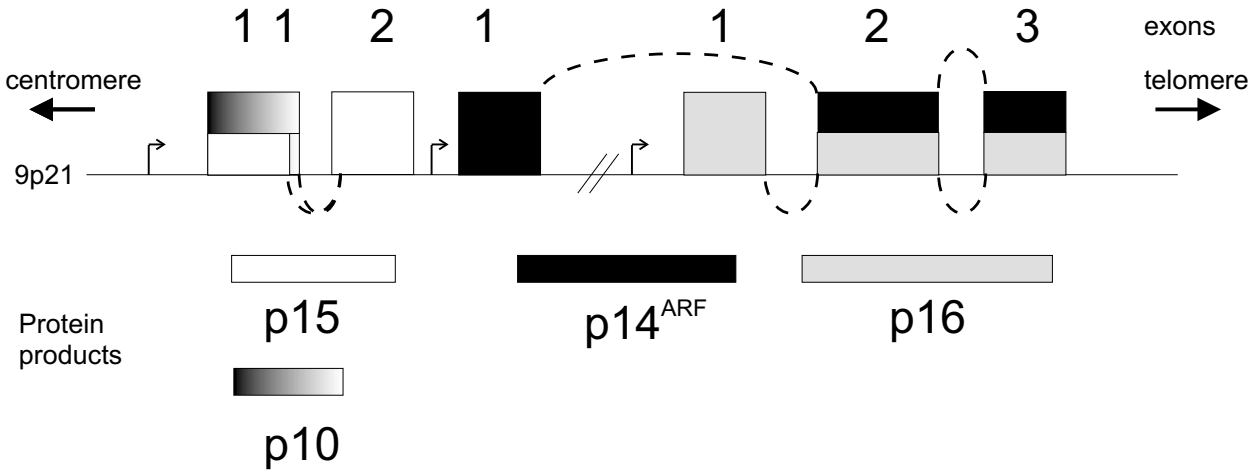

Fig. 3 The genomic organization of p15, p16 and $p14^{ARF}$ in human chromosome 9p21. The exons are marked as boxes, the splicing with dotted lines, and the transcription start sites with arrowheads. The protein products of p15, p16 and $p14^{ARF}$ are shown below. In addition, the alternatively spliced form of p15, p10, together with its *in vitro* translation product is shown (see Results & Discussion for details). The protein products and the locus are not drawn in scale. Studies indicate that the locus spans more than 30 kb, and the exon 1β of p16/$p14^{ARF}$ lies in close proximity (2.3 kb) to exon 2 of p15 and over 20 kb from 1α of p16. The intron 1 area of p15 as well as intron 1 and 2 of $p14^{ARF}$ are approximately 2.5 kb in length (Mao *et al*., 1995; Stone *et al*., 1995b; adapted from Haber, 1997).

The high levels of p16 in cells lacking functional pRb suggested a feedback loop where pRb would regulate the amount of p16 (Serrano *et al*., 1993; Xiong *et al*., 1993b; Otterson *et al*., 1994; Tam *et al*., 1994b; Parry *et al*., 1995). However, the p16 levels are not regulated in a cell cycle specific manner. Instead, p16 expression is tissue specific and p16 accumulates in late-passage, senescent cells (Hara *et al*., 1996; Jen *et al*., 1994; Zindy *et al*., 1997). Similarly, premature senescence of primary human or rodent fibroblasts induced by oncogenic Ras is accompanied by accumulation of p16 and p53 (Serrano *et al*., 1997), whereas immortalization of human cell lines is facilitated by inactivation of p16 and/or p53 tumor suppressor (Reznikoff *et al*., 1996).

$p15^{INK4B, MTS2}$ is regulated by TGF-β

p15 was cloned from a cDNA library of TGF-β treated HaCaT human keratinocytes using p16 as a probe (Hannon and Beach, 1994) and from a HeLa cDNA library (Guan *et al*., 1994). Additionally, Kamb *et al.* (1994) had noted p15 sequence in close proximity of p16 in human chromosome 9 during their effort of cloning a melanoma-linked gene, which turned out to be p16. p16 and p15 are close homologues of each other, especially in the exon two area (97% identity at the amino acid level; Hannon and Beach, 1994). The close linkage and strong homology between p16 and p15 suggest that they arose from a duplication of a single ancestral gene (Fig. 3; Jiang *et al*., 1995).

p15 expression, like that of p16, is low in mouse embryos (Zindy *et al*., 1997). However, in contrast to p16 which has a restricted expression pattern, p15 expression can be detected in several organs of adult mice and humans, such as kidney, lung and testis (Jen *et al*., 1995; Stone *et al*., 1995a; Zindy *et al*.,1997). The mRNA expression of p15 is not regulated in a cell cycle specific manner, neither are p15 mRNA levels elevated in cells lacking functional pRb (Stone *et al*., 1995a). Instead, in TGF-β growth arrested human keratinocytes, mink lung and mammary epithelial cells p15 mRNA and protein are rapidly induced. The increased p15 levels associate with Cdk4 and 6 and inhibit their activity (Hannon and Beach, 1994; Reynisdóttir *et al*., 1995; Sandhu *et al*., 1997). A consensus Sp-

1 transcription factor binding site has been shown to facilitate the TGF-β induction of p15 mRNA (Li *et al*., 1995a). In addition, interferon-α (IFN-α) has been shown to induce p15 mRNA and protein levels in a hematopoietic cell line that is growth arrested by IFN-α (Sangfelt *et al*., 1997).

p16 and p15 loci as tumor suppressors

It is suggested that p16 and 15 are tumor suppressor proteins. They both inactivate the Cdk4/6 kinases (Serrano *et al*., 1993; Hannon and Beach, 1994), and their overexpression prevents cellular growth (Guan *et al*., 1994; Jin *et al*., 1995; Medema *et al*., 1995; Lukas *et al*., 1995c; Serrano *et al*., 1995; Stone *et al*., 1995a; Fueyo *et al*., 1996). They were also cloned from an area linked to putative melanoma tumor suppressor (Kamb *et al*., 1994). p16 was initially named as the multiple tumor suppressor located in human chromosome 9p21 (Kamb *et al*., 1994; Nobori *et al*., 1994). The coding sequence for p15 was not found to be altered in tumor cell lines examined in contrast to p16 sequence, and therefore p15 was first thought to be a pseudogene (Kamb *et al*., 1994). The *p16* gene is altered in high percentage of cells in many human tumors including melanomas, gliomas, lung cancer and leukemias. It is inactivated by several different mechanisms including homozygous deletion, point mutation, and silencing by CpG methylation (reviewed by Kamb, 1998). However, *p16* germline mutations are rare in contrast to common *p16* somatic mutations (Kamb, 1998). Loss of p15 is also common in many transformed cell lines. However, only a couple mutations in p15 have been detected from tumors with normal p16 status. Certain primary non-small cell lung carcinomas were found to have three intragenic mutations of p15 (Okamoto *et al*., 1995) and an esophagus carcinoma one missense mutation (Suzuki *et al*., 1995). In contrast, screens of melanoma-prone kindred with intact p16 did not reveal any p15 mutations (Stone *et al*., 1995a; reviewed by Kamb, 1998). The data suggest that the *p16* locus contains the tumor suppressor, and the loss of the *p16* locus is most probably the primary target, whereas loss of *p15* occurs simply because of its close proximity to p16. However, loss of p15, in addition to loss of p16, could give a growth advantage to cancerous cells.

The phenotype of p16 knock-out mice further indicated a tumor suppressor role for the *INK4A* locus. These mice develop normally suggesting that *p16* is a non-essential gene for viability and proper development (Serrano *et al*., 1996). However, they develop spontaneous tumors at an early age, most of the tumors being soft tissue sarcomas and B-cell lymphomas. The incidence of tumors is accelerated by irradiation or by treatment with chemical carcinogens (Serrano *et al*., 1996). In addition, the p16-null mouse embryo fibroblasts do not undergo replicative senescence in culture. In contrast to normal mouse embryo fibroblasts, they can be transformed oncogenic by Ras without a co-operating oncogene such as myc or adenovirus E1A (Serrano *et al*., 1996).

p16 has an alternatively spliced form called $p14^{ARF}$ in humans or $p19^{ARF}$ in mouse (Alternative Reading Frame) that utilizes the p16 exon 2, but has a novel first exon called 1β . The *p16* and *p14/19^{ARF}*genes are transcribed in different reading frames, and thus the product $p14/19^{ARF}$ has no homology to the p16 Cdki family (Fig. 3; Duro *et al*., 1995; Mao *et al*., 1995; Quelle *et al*., 1995; Stone *et al*., 1995b). Accordingly $p14/19^{ARF}$ does not bind Cdks, but its overexpression arrests cells in G_1 and G_2 (Quelle *et al*., 1995). $p14/19^{ARF}$ has been shown to be involved in regulation of p53 tumor suppressor by binding to Mdm-2 and preventing Mdm-2 induced p53 degradation (reviewed by Sherr, 1998).

The results from p16-/- mice were complicated after the finding that the targeted disruption of *p19^{ARF}*exon 1β generated a similar phenotype as that of the p16-null mice, where the common exons 2 and 3 were disrupted (Kamijo *et al*., 1997). Most importantly, the expression of p16 protein was intact in $p19^{ARF}$-knockout tissues, cultured cells and tumors derived form the mice, suggesting that

p19^{ARF}is the tumor suppressor gene, and the phenotype observed earlier in the p16-/- animals is due to *p19*^{ARF}inactivation and not to that of *p16*. However, the final effect of p16 on the phenotype remains uncertain since currently there are no true p16 knockout mice with normal p19^{ARF} expression.

On the other hand, analysis of the p16 protein structure suggests that p16 is indeed a tumor suppressor. A number of *p16* point mutations found in cancers prevent p16 binding to Cdk4/6, because they encode a protein that is incorrectly folded or insoluble (Koh *et al*., 1995; Zhang and Peng, 1996; Luh *et al*., 1997; Byeon *et al*., 1998; Russo *et al*., 1998). In addition, a Cdk4 mutant found in melanomas has a greatly reduced ability to bind p16 (Wolfel *et al*., 1995). So far no definite germline mutations have been described from melanoma patients that would selectively destroy *p14*^{ARF} exon 1β and leave the common exon 2 intact. In contrast, some mutations are specifically targeted towards exon 1α of *p16* indicating that p16 is a tumor suppressor (FitzGerald *et al*., 1996).

p18^{INK4C} *and p19*^{INK4D}

p18 and *p19* genes were isolated in a yeast two-hybrid screen using Cdk4 and Cdk6 as baits (Hirai *et al*., 1995; Guan *et al*., 1994). p19 was also cloned through its interaction in yeast with Nur 77 orphan steroid receptor that shows no link to cell cycle regulation (Chan *et al*., 1995). Mouse p18 and p19 share approximately 40% identity at protein level (Hirai *et al*., 1995).

Overexpression of p19 causes growth arrest regardless of the pRb status of the cells, whereas p18, similar to p16, can only arrest cells that have functional pRb (Guan *et al*., 1994; Hirai *et al*., 1995). No mutations of p18 or p19 have been found in cell lines or in primary tumors (reviewed by Carnero and Hannon, 1998). The function of p18 is suggested to regulate senescence and terminal differentiation:. p18 levels increase during senescence of mouse embryonic fibroblasts and myogenic differentiation (Franklin and Xiong, 1996; Zindy *et al*., 1997). Interestingly, the phenotype of mice lacking p18 is similar to that of p27-/- animals. The p18-/- mice have increased body size due to organomegaly, and they develop pituitary hyperplasia and adenomas. Mice lacking both p18 and p27 develop pituitary adenomas faster than mice lacking either of the Cdkis, and at a similar rate as mice chimerical for pRb deficiency suggesting functional collaboration of these two Cdk inhibitors in the pRb pathway (Franklin *et al*., 1998).

A 3. Transforming growth factor-β

Transforming growth factor-β (TGF-β) belongs to a large family of growth factors named TGF-β superfamily that regulates cellular growth and differentiation (reviewed by Massagué, 1996, 1998; Taipale *et al*., 1998). TGF-β inhibits the growth of epithelial, endothelial and hematopoietic cells, stimulates extracellular matrix formation and is an immunosuppressor. TGF-β treatment arrests epithelial and endothelial cells in the G_1-phase of the cell cycle. TGF-β is a 25-kDa homodimeric growth factor, and three isoforms are found in mammals, namely TGF-β:s 1 to 3. Also heteromeric active complexes, such as TGF-β1.2 and 2.3 have been found *in vivo* (Cheifetz *et al*., 1987; Ogawa *et al*., 1992). Originally, TGF-β1 was identified as a sarcoma growth factor (SGF) together with TGF-α (transforming growth factor-α) and purified from murine sarcoma virus transformed fibroblasts (DeLarco and Todaro, 1978). SGF stimulated cellular growth and colony formation in soft agar, and was later found to consist of two different growth factors named TGF-α and -β (Anzano *et al*., 1982, 1983).

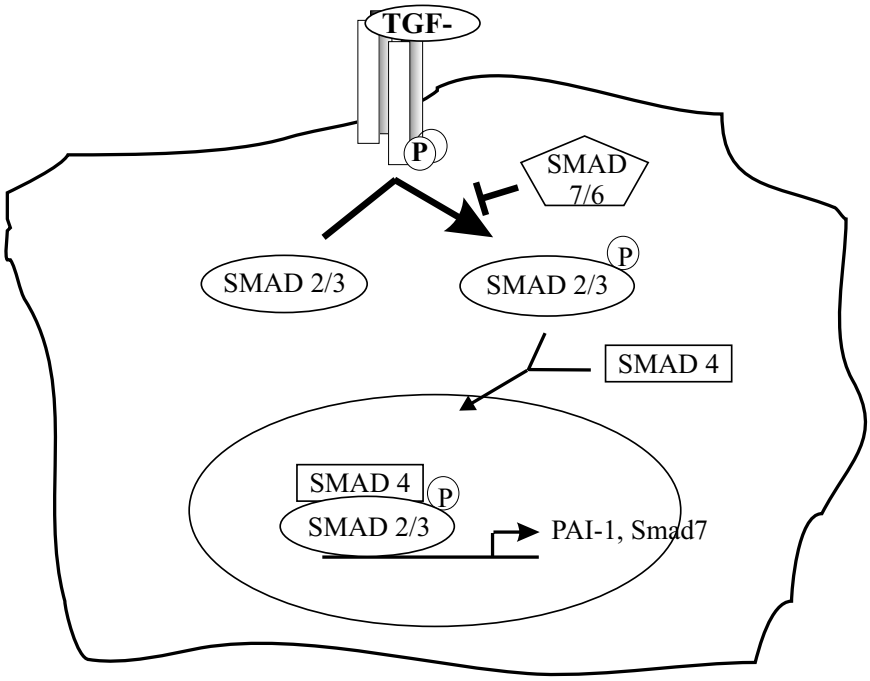

Fig. 4 The TGF-β intracellular signaling pathway. Ligand binding to TGF-β receptor type II induces hetero-oligomerization of the receptor complex and subsequent phosphorylation of receptor type I. The phosphorylated receptor type I then phosphorylates Smad2 and 3 resulting in their association with Smad4 and translocation into the nucleus where they activate transcription of target genes. Smad6 and 7 can inhibit the Smad2 and 3 phosphorylation by preventing their access to receptor type I (modified from Heldin *et al*., 1997; Massagué *et al*., 1998).

TGF-β signal transduction

The TGF-β signaling is initiated by ligand binding to transmembrane heteromeric kinase complex consisting of two serine-threonine kinases. TGF-β and its signaling receptors I and II are expressed in mesenchymal and epithelial cells (reviewed by Taipale *et al*., 1998). Both receptors I and II are needed for signal transduction (Fig. 4; Franzen *et al*., 1993; Ventura *et al*., 1994; Wrana *et al*., 1994). The type II receptor binds to the ligand (Wrana *et al*., 1992), whereas the type I receptor is required for downstream signaling (Carcamo *et al*., 1994; ten Dijke *et al*., 1994). Phosphorylation of the type I receptor by type II receptor, after ligand binding, initiates the downstream signaling (Wrana *et al*., 1994). In addition, TGF-β binds to accessory receptors called betaglycan and endoglin (Gougos and Letarte 1990; Wang *et al*., 1991; Lõpez-Casillas *et al*., 1994; Yamashita *et al*., 1994). They have been suggested to regulate the access of TGF-β to the signaling receptors.

Loss of TGF-β receptors could give a growth advantage to certain preneoplastic cells. Loss of one or more TGF-β signaling receptors occurs in certain tumors such as neuro- and retinoblastomas, prostate carcinoma and in several leukemia cell lines (Kimchi *et al*., 1988; Keller *et al*., 1989; Kim *et al*., 1996a). There is also evidence of specific frameshift or point mutations associated with TGF-β receptors I and II in colon carcinoma and prostate cancers cells (Markowitz *et al*., 1995; Kim *et al*., 1996b).

TGF-β intracellular signaling is at least partly mediated by Smad proteins (Fig. 4; reviewed by Massagué, 1996; Heldin *et al*., 1997). The activated type I receptor phosphorylates Smad2 and Smad3, which form a heteromeric complex with a common mediator Smad4 (Zhang *et al*., 1996, 1997b; Eppert *et al*., 1996; Lagna *et al*., 1996; Macias-Silva *et al*., 1996; Nakao *et al*., 1997a; Wu *et al*., 1997). The complex is then translocated to the nucleus where it regulates transcription of target genes (Chen *et al*., 1996c; Macias-Silva *et al*., 1996; Eppert *et al*., 1996; Liu *et al*., 1997a, b; Nakao *et al*., 1997a; Dennler *et al*., 1998). Smad4 is also called *DPC4* (deleted in pancreatic carcinoma, locus 4), a tumor suppressor gene mutated or deleted in breast, lung, head, neck and ovarian cancers, as well as pancreatic malignancies (Hahn *et al*., 1996; Massagué 1998).

Other Smads, namely Smad6 and 7 may participate in a negative feedback loop to control intracellular signaling. TGF-β rapidly induces expression of Smad7 mRNA (Nakao *et al*., 1997b). Smad6 and 7 associate with the type I receptor but are not phosphorylated. The association blocks the access and activating phosphorylation of Smad2 and 3, and thus prevents signal transduction (Hayashi *et al*., 1997; Imamura *et al*., 1997; Nakao *et al*., 1997b).

TGF-β as a growth inducer

The effects of TGF-β on cell proliferation are dependent on cell type, state of differentiation, and presence of other growth factors (reviewed by Ravitz and Wenner, 1997). Although TGF-β1 was originally identified as part of a growth promoting factor (DeLarco and Todaro, 1978), it is a potent inhibitor of epithelial, endothelial and lymphoid cell growth (Tucker *et al*., 1984; Shipley *et al*., 1986; Silberstein and Daniel 1987; Russell *et al*., 1988). However, it can stimulate the growth of mesenchymal cells, particularly rodent and human fibroblasts (Shipley *et al*., 1985; Soma and Grotendorst, 1989).

The stimulatory effect of TGF-β on mesenchymal cell growth seems to depend on the cell culture conditions, cell density, TGF-β concentration and presence of other growth factors (Battegay *et al*., 1990; Roberts *et al*., 1985; Goodman and Majack, 1989). For instance, TGF-β together with PDGF (platelet derived growth factor) stimulates the colony formation of Fischer rat 3T3 transfected with *c-myc*, whereas it inhibits the growth and antagonizes the effect of epidermal growth factor (EGF) on colony formation of the same cells (Roberts *et al*., 1985). In addition, primary and secondary rodent fibroblasts are growth arrested in G_1 by TGF-β, whereas immortalized cell lines derived from these cells are not (Sorrentino and Bandyopadhyay, 1989). Similarly, TGF-β stimulates growth of Ha-Ras transformed metastatic fibroblasts, whereas it inhibits the growth of the non-transformed parental cell line (Schwarz *et al*., 1988).

TGF-β induced DNA synthesis of mesenchymal cells occurs with slower kinetics than the EGF, PDGF or serum induced synthesis (Shipley *et al*., 1985; Soma and Grotendorst, 1989; Kim *et al*., 1993). The growth stimulatory effect of TGF-β is suggested to be indirect, and mediated by autocrine induction and secretion of PDGF (Mäkelä *et al*., 1987; Soma and Grotendorst, 1989; Battegay *et al*., 1990).

Growth inhibitory effects of TGF-β mediated by cell cycle machinery proteins

pRb as a mediator of TGF-β growth inhibitory signal

The TGF-β induced G_1 growth arrest is reversible, and occurs only if TGF-β is added to cell culture prior to the restriction (R) point in G_1 (Laiho *et al*., 1990; Howe *et al*., 1991). TGF-β treatment of Mv1Lu cells prevents the phosphorylation of pRb, which normally occurs late in G_1 approximately during the time of the restriction point (Laiho *et al*., 1990). When TGF-β is added after the R point, pRb phosphorylation is not prevented, and the cells proceed into S. In addition, expression of SV40 large T antigen in Mv1Lu cells prevents the TGF-β growth arrest without blocking the phosphorylation of pRb (Laiho *et al*., 1990). SV40 large T binds specifically to underphosphorylated pRb and prevents its function (DeCaprio *et al*., 1988). p107 and p130, members of the pRb family, have also been found to be involved in the TGF-β growth arrest pathway. In 32D-123 murine myeloid cells TGF-β treatment prevents p107 phosphorylation, and expression of p107 antisense oligo nucleotides prevents TGF-β growth arrest (Bang *et al*., 1996). In HFK human keratinocytes and HaCaT human

keratinocyte cells TGF-β inhibits the E2F activity by increasing the amount of E2F associated with p130 (Herzinger *et al*., 1995). In addition, infection of Mv1Lu cells with an adenovirus expressing the pRb target E2F-1 prevents TGF-β growth inhibition (Schwarz *et al*., 1995). Taken together, these data suggest that pRb (and its family members) are needed for TGF-β growth arrest and, in addition, that TGF-β has an inhibitory effect on the cyclin dependent kinases that are essential for inactivation of pRb.

Cdk inhibitors as mediators of TGF-β growth arrest

TGF-β has been shown to have inhibitory effects on Cdk2, 4, and 6 kinase activities. The mechanisms of TGF-β growth inhibition have been studied in different cell models, and the mechanisms of Cdk inhibition by TGF-β vary from one cell line to another. The inhibitory mechanisms include downregulation of the amount of Cdk or cyclin subunit, as well as regulation of Cdk phosphorylation status and association with Cdki proteins (Fig. 5 and Table 2).

The Cdk inhibitors p15, p27 and p21 have been found to be involved in TGF-β induced growth arrest, and suggested to be the major mediators of Cdk inhibition by TGF-β. There are cell type specific differences in which Cdkis are induced to accumulate into Cdk-complexes after TGF-β treatment. The Cdkis may have overlapping functions and they may compensate for one another. Alternatively, other mechanisms to inactivate Cdk-cyclin complexes could replace the lack of a certain Cdki in a specific cell type (Table 2). Indeed, TGF-β can still arrest growth of cells lacking either p15 or p27 (Nakayma *et al*., 1996; Iavarone and Massagué, 1997).

Co-operation between p15 and p27 in TGF-β growth arrest

The best known model for TGF-β growth inhibition involves accumulation of p15 and p27 to Cdk4/6- and Cdk2-complexes, respectively. p15 and p27 seem to co-operate at least in HaCaT keratinocytes, Mv1Lu mink lung epithelial and HMEC 184 human mammary epithelial cells to mediate TGF-β growth arrest (Reynisdóttir *et al*., 1995; Reynisdóttir and Massagué, 1997; Sandhu *et al*., 1997). TGF-β induces p15 mRNA. The induction is observed after two hours in HaCaT and Mv1Lu cells (Hannon and Beach, 1994; Reynisdóttir *et al*., 1995) followed by accumulation of p15 to Cdk6 and Cdk4 complexes (Hannon and Beach, 1994; Reynisdóttir *et al*., 1995; Sandhu *et al*., 1997). The accumulation of p15 to Cdk4 and Cdk6 complexes displaces p27 Cdki from these complexes leading to inactive Cdk4 or 6 bound to p15 (Reynisdóttir *et al*., 1995; Reynisdóttir and Massagué, 1997; Sandhu *et al*., 1997). TGF-β treatment does not affect the mRNA or protein levels of p27 (Polyak *et al*., 1994b; Sandhu *et al*., 1997). Instead, p27 is redistributed into Cdk2-cyclin E-

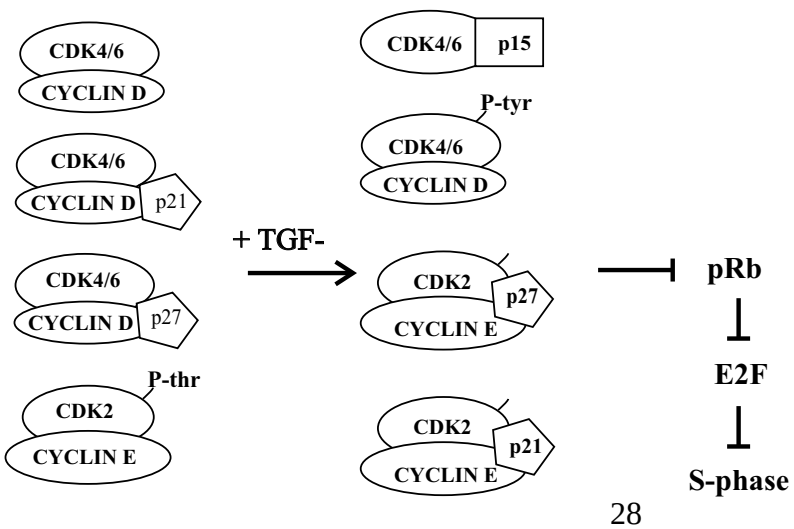

Fig. 5 Inhibitory effects of TGF-β on the activity of Cdk-complexes. TGF-β treatment induces upregulation of p15 and p21 Cdk inhibitors, and their association with Cdk4/6 and Cdk2 complexes, respectively. In addition, upregulation of p15 replaces p27 in Cdk4/6 complexes leading to the association of p27 with Cdk2 complexes and their inactivation. TGF-β prevents also tyr-dephosphorylation of Cdk4/6 by downregulating the Cdc25A phosphatase, and the thr-phosphorylation of Cdk2 (see text for details and references).

complexes, resulting in inhibition of Cdk2 activity (Reynisdóttir*et al.*, 1995; Reynisdóttir and Massagué, 1997; Sandhu *et al.*, 1997).

The different subcellular localizations of p15 and p27 have been suggested to be important for the ability of p15 and p27 to bind to and inhibit different Cdk-cyclin complexes. p15 cannot displace p27 from Cdk4 complexes unless it has access to them prior to p27 *in vitro* (Reynisdóttir and Massagué, 1997). p15 is predominantly cytoplasmic, whereas p27 is nuclear. According to the model by Reynisdóttir and Massagué (1997), the nuclear Cdk4-cyclin D-p27-complexes turn over rapidly since the short half-life of cyclin D (30 min; Matsushime *et al.*, 1991). After dissociation from cyclin D, Cdk4 is returned to cytoplasm where it can bind to p15. Once p15 is associated with Cdk4, Cdk4 can no longer associate with cyclin, and p27 can no longer displace p15 leading to accumulation of free p27. p27 is then able to associate with and inhibit the activity of Cdk2-complexes (Reynisdóttir *et al.*, 1995; Reynisdóttir and Massagué, 1997; Sandhu *et al.*, 1997). In addition, the different binding mechanisms and affinities towards Cdk and cyclin subunits of p15 and p27 support this model. Structural studies indicate that p16 family members bind only to the Cdk-subunit, and that binding of p16 can prevent cyclin D association to Cdk6 (Russo *et al.*, 1998). Structural studies of Cdk2-cyclin A-p27 complex suggest that p27 needs cyclin as a docking site for Cdk binding (Russo *et al.*, 1996b).

p15 mRNA induction by TGF-β is also observed in a derivate of HMEC 184 cell line, 184A1L5 that is not growth arrested by TGF-β (Sandhu *et al.*, 1997), and in several squamous carcinoma cell lines, some of which are growth inhibited by TGF-β while others are not (Table 2; Malliri *et al.*, 1996). One possible mechanism explaining the lack of TGF-β growth arrest in 184A1L5 cells after p15 mRNA induction is a difference in p15 half-life. In the TGF-β sensitive HMEC 184 mammary epithelial cells the half-life of p15 protein is increased by TGF-β treatment, whereas TGF-β treatment had no effect on the half-life of p15 from the 184A1L5 cells (Sandhu *et al.*, 1997). In addition, the authors found that p15 from the TGF-β resistant 184A1L5 cell line was unable to displace cyclin D1 from Cdk4 complexes in contrast to p15 present in the sensitive cell line, suggesting that TGF-β treatment is able to modify either p15 or Cdk4-cyclin D-complexes by some unknown mechanism(s). Subsequently, there was no accumulation of p27 to Cdk2-cyclin E-complexes (Sandhu *et al.*, 1997).

p21 mediates TGF-β growth arrest in some cell types

In addition to inducing accumulation of p27 to Cdk2-complexes, TGF-β can inhibit Cdk2 activity by inducing p21. In contrast to p27, the p21 induction takes place at the mRNA level (Table 2; Elbendary *et al.*, 1994; Datto *et al.*, 1995; Li *et al.*, 1995b). The induction of p21 mRNA is rapid, and it can be observed as early as one hour after TGF-β treatment of asynchronously growing HaCaT cells (Datto *et al.*, 1995), whereas the increased p21 protein levels can be detected after two to four hours (Reynisdóttir *et al.*, 1995). In HaCaT cells three Cdkis seem to co-operate to induce growth arrest as response to TGF-β treatment. p15 inactivates Cdk4/6-complexes, whereas the induced p21 associates with and inactivates Cdk2 together with p27 (Reynisdóttir *et al.*, 1995). TGF-β induces p21 also in several cell lines, such as WM35 melanoma, IEC 4-1 rat intestinal, human squamous carcinoma and prostate cells, some of which lack p15 induction (Table2; Flørens *et al.*, 1996; Malliri *et al.*, 1996; Cipriano and Chen, 1998; Yue *et al.*, 1998). At least in some of these cells where p15 induction is not observed, p21 seems to co-operate with p27 to arrest cells after TGF-β treatment (Flørens *et al.*, 1996; Yue *et al.*, 1998).

Table 2. Effects of TGF- on G_1 and G_1/S specific Cdk-cyclin complexes in different cell lines.

Subunit	Effect/ Mechanism	Cell line	Reference
Cdk4	downregulation of protein synthesis	Mv1Lu HaCaT	Ewen *et al*., 1993 Reynisdóttir *et al*., 1995
	downregulation of protein levels	WM35 melanoma squamous carcinoma cell lines	Flørens *et al*., 1996 Malliri *et al*., 1996
Cdk6	downregulation of protein synthesis	Mv1Lu	Reynisdóttir *et al*., 1995, III
Cdk2/4/6	inhibition of upregulation of mRNA after serum stimulation	HaCaT	Geng and Weinberg, 1993
cyclin D1	downregulation of protein downregulation of mRNA	WM melanoma IEC-6 and RIE-1 rat intestinal epithelial	Flørens *et al*., 1996 Ko *et al*., 1995
Cdk2	downregulation of thr-160 phosphorylated form	Mv1Lu	Koff *et al*., 1993
Cdk4/6	increased tyr-phosphorylation	MCF-10A mammary epithelial	Iavarone and Massague, 1997
p15	upregulation of mRNA and protein	HaCaT Mv1Lu HMEC 184	Hannon and Beach, 1994 Reynisdóttir *et al*., 1995 Sandhu *et al*., 1997
p27	upregulation of protein synthesis	IEC 4-1 rat intestinal epithelial	Yue *et al*., 1998
p21	upregulation of mRNA and protein	HaCaT OVCA 420 ovarian cancer LS1034 and LS513 colon cancer	Datto *et al*., 1995 Elbendary *et al*., 1994 Li *et al*., 1995

Viral oncoproteins prevent TGF-β growth arrest by binding to Cdkis

In addition to direct binding to pRb and its family members (Pietenpol *et al*., 1990; Missero *et al*., 1991), adenovirus E1A oncoprotein can override TGF-β growth arrest by preventing inactivation of Cdk-complexes by Cdkis. E1A oncoprotein can block the transcriptional induction of p15 and p21 by TGF-β in HaCaT cells (Datto *et al*., 1997) explaining how E1A can stimulate pRb phosphorylation in the presence of TGF-β (Wang *et al*., 1991; Datto *et al*., 1997). The effect of E1A on p15 transcription is mediated through the ability of E1A to bind p300 transcriptional co-activator (Datto *et al*., 1997). E1A can rescue TGF-β growth arrest in Mv1Lu cells by binding to and blocking the inhibitory effect of p27 thus restoring the Cdk2-associated kinase activity (Mal *et al*., 1996).

The effects of TGF-β on Cdk and cyclin subunits

Inactivation of Cdk-complexes by Cdkis is not the sole mechanism by which TGF-β prevents kinase activity. However, in the most studied cell models the effects of TGF-β on Cdk levels or phosphorylation status seem to occur in addition to Cdki accumulation. TGF-β treatment has been shown to decrease the amount of Cdk4 and Cdk6 (Table 2; Ewen *et al*., 1993b; Reynisdóttir *et al*., 1995; Flørens *et al*., 1996; Malliri *et al*., 1996). The decrease of the amount of Cdk4/6 protein levels occurs with slower kinetics than upregulation of Cdki proteins to Cdk complexes. At least in Mv1Lu cells the effect of TGF-β on Cdk4/6 levels is post-transcriptional since TGF-β has no effect on Cdk4 mRNA levels (Ewen *et al*., 1993b) but it decreases the amount Cdk4 and Cdk6 synthesis (Ewen *et al*., 1993b; Reynisdóttir *et al*., 1995). However, TGF-β can block growth stimulatory effects of serum stimulation on synchronized HaCaT human keratinocytes by preventing upregulation of Cdk2, 4, cyclin E and A mRNA levels (Geng and Weinberg, 1993).

Regulation of the activator thr-phosphorylation seems to be another mechanism of Cdk2 inhibition by TGF-β. TGF-β prevents the accumulation of active Cdk2-cyclin E complexes without affecting the protein levels of Cdk2 or cyclin E by decreasing the amount of thr-phosphorylated form of Cdk2 (Koff *et al*., 1993). The decrease of the amount of thr-phosphorylated Cdk2 is probably due to increased association of Cdk2 with p27 that blocks the CAK phosphorylation site of Cdk2 (Russo *et al*., 1996b). Increased tyrosine-phosphorylation of Cdk4 and 6 is suggested to be an alternative mechanism of Cdk inhibition when p15 induction is lacking. In MCF-10A mammary epithelial cells TGF-β has been shown to downregulate Cdc25A phosphatase mRNA and protein levels leading to accumulation of tyrosine phosphorylated, inactive forms of Cdk4 and 6 (Iavarone and Massagué, 1997). In these cells Cdk2 was similarly phosphorylated in thr-and tyr-residues both in control and TGF-β treated cells. In HaCaT cells, Cdc25A levels decrease slowly after TGF-β treatment suggesting that Cdc25A downregulation is a secondary event in the arrest of the cells in a quiescent state. In HaCaT cells the decrease in Cdc25A levels is mediated by inhibitory complexes of E2F-4, p130 and histone deacetylase (Iavarone and Massagué, 1999).

Downregulation of cyclin subunit seems not to be the major mechanism of Cdk inactivation by TGF-β. TGF-β has been reported to decrease the cyclin D1 mRNA and protein levels (Table 2; Ko *et al*., 1998; Flørens *et al*., 1996). In addition, TGF-β was found to decrease the amount of cyclin A (Flørens *et al*., 1996). Since TGF-β arrests cells in late G1, prior the cell cycle point where cyclin A increase normally occurs, the effect on cyclin A levels by TGF-β is probably secondary to the loss of cyclin E-associated kinase activity needed for cyclin A mRNA synthesis (Zerfass-Thome *et al*., 1997).

A 4. Hepatocyte growth factor

Hepatocyte growth factor (HGF)/ Scatter factor induces growth, invasion, mobility and scattering of epithelial cells (Nakamura *et al*., 1986, 1989; Stoker *et al*., 1987; Gherardi *et al*., 1989; Weidner *et al*., 1990). Thus, the effects of HGF on epithelial cell growth are largely opposite to those of TGF-β.

HGF is expressed predominantly in mesenchymal cells, whereas HGF receptor, the c-met proto-oncogene, is found in epithelial and endothelial cells (Naldini *et al*., 1991a; Bussolino *et al*., 1992; Birchmeier and Birchmeier, 1993; Sonnenberg *et al*., 1993). c-Met is a transmembrane receptor tyrosine kinase (Naldini *et al*., 1991 b,c).

HGF mediates mesenchymal-epithelial signals during mouse development (Sonnenberg *et al*., 1993; Schimdt *et al*., 1995; Uehara *et al*., 1995). HGF has been shown to stimulate several different intracellular signaling pathways including phosphatidylinositol-3-kinase, Ras family members, STAT and Smad leading to different kind of cellular responses. Activation of phosphatidylinositol-3-kinase, Ras and Rac after HGF signal stimulates scattering and motility (Ridley *et al*., 1995; Royal and Park, 1995), and Ras-pathway is stimulated during HGF induced cellular growth (Ponzetto *et al*., 1996). During HGF induced epithelial tubulogenesis STAT pathway is induced (Boccaccio *et al*., 1998). In addition, HGF has been shown to induce phosphorylation of Smad2 and its nuclear translocalization (de Caestecker *et al*., 1998). However, the magnitude of the effects on Smad2 induced by HGF were less prominent than those by TGF-β, and the induction of Smad2 phosphorylation by HGF was transient (de Caestecker *et al*., 1998).

A 5. Ras oncogene as a cellular growth regulator

Ras is an important regulator of eukaryotic cell growth. It is an intracellular membrane linked G-protein, which can be activated in response to peptide growth factors, cytokines and hormones. One of the best known intracellular Ras-signalling pathways involved in cellular proliferation is the Ras/ Raf/ERKs (extracellular signal-regulated kinases) cascade. Activated Ras stimulates the kinase activity of Raf, which in turn activates the ERK kinases that are part of of the mitogen-activated protein kinase (MAPK) pathway. Activation of this pathway leads to stimulation of various transcription factors, such as Jun (reviewed by Hunter, 1997; Kerkhoff and Rapp, 1998; Vojtek and Der, 1998).

Ras activity can lead to either induction or arrest of cellular proliferation. Mitogenic Ras activity is required throughout the G_1-phase, and it is essential for S-phase progression of fibroblasts (Dobrowolski *et al*., 1994). It is suggested that Ras signal is mediated through pRb. Ras expression induces activation of the Raf/MAPK pathway (Lavoie *et al*., 1996), upregulation of cyclin D mRNA levels and it shortens G_1-phase (Filmus *et al*., 1994; Liu *et al*., 1995; Winston *et al*., 1996). The induced cyclin D levels lead to accumulation of active Cdk4/6-complexes and inactivation of pRb. In addition, murine or rat fibroblasts treated with neutralizing antibodies against Ras, or expressing a dominant-negative form of Ras fail to arrest when functional pRb is lacking (Leone *et al*., 1997; Mittnacht *et al*., 1997; Peeper *et al*., 1997).

Another cell-cycle machinery protein linked to Ras/Raf pathway growth stimulation is p27 Cdki. Overexpression of Ras and c-Myc in rat embryo fibroblasts leads to downregulation of p27 in a

posttranscriptional manner, while expression of either Myc and Ras alone has no effect on p27 (Leone *et al.*, 1997). In addition, downregulation of p27 levels by serum can be blocked by overexpression of dominant negative Ras mutant (Aktas *et al.*, 1997). These data suggest that Ras/Raf pathway participitates in p27 regulation.

In addition to growth stimulatory effect, Ras can arrest the proliferation of primary rat Schwann cells, pheochromocytoma cells, and rodent fibroblasts (Bar-Sagi and Feramisco, 1985; Franza *et al.*, 1986; Ridley *et al.*, 1988; Benito *et al.*, 1991), and induce cellular senescence of human diploid fibroblasts and primary mouse fibroblasts (Serrano *et al.*, 1997). It has been suggested that constitutively active versions of Ras or Raf are growth inhibitory, whereas cellular proliferation requires activation of Ras or Raf and an additional oncogene, or inactivation of a tumor suppressor. Expression of activated Ras in primary mouse fibroblasts causes growth arrest and premature cellular senescence that is associated with elevated levels of p53 and p16 (Serrano *et al.*, 1997). In the absence of p53 or p16 Ras expression allows transformation of the cells. Similarly, in the absence of Cdki p21 (Missero *et al.*, 1996; Michieli *et al.*, 1996) or in the presence of co-operative oncogenes such as Myc (Land *et al.*, 1983) Ras causes transformation. In addition, the strength and duration of Ras/Raf signal seem to determine whether cells proliferate or differentiate. Pheochromocytoma PC12 proliferate as response to transient Ras/Raf signal, whereas the sustained activation causes these cells to differentiate and eventually cease to proliferate (Qui and Greene, 1992). The differentiation of PC12 was accompanied with elevated p21 Cdki levels (Yan and Ziff, 1995). Similarly, high levels of Raf induce cell cycle arrest and p21, whereas in p21 deficient fibroblasts high Raf levels do not arrest cellular growth (Sewing *et al.*, 1997; Woods *et al.*, 1997).

B. AIMS OF THE PRESENT STUDY

This study was aimed at elucidating the regulation of cell cycle machinery proteins associated with the TGF-β induced G_1 arrest of epithelial cells. Although the TGF-β signal transduction pathway has been well studied, very little was known of how simultaneous inhibitory and stimulatory signals are integrated. The finding that HGF releases Mv1Lu cells from TGF-β induced growth arrest was used as a model to study the effects of TGF-β on endogenous cell cycle proteins. The detailed aims of the study were:

1) to study the role of p15 Cdki in TGF-β growth control,
2) to understand the mechanisms by which HGF allows cells to escape from TGF-β1 growth arrest,
3) to study the effects of TGF-β and Ras oncoprotein on p27 and p21 Cdkis.

C. MATERIALS AND METHODS

The cell lines, cDNA libraries, plasmid constructs, growth factors, antibodies and methods used in this study are listed in the tables below.

Cell lines

Cell line	*Description*	*Rerence or source*	*Used in*
COS-7	African green monkey kidney epithelial	ATCC	I, IV
DU-145	prostate carcinoma	ATCC	I
HaCaT	human keratinocyte	ATCC	I
HeLa	cervical carcinoma	ATCC	I
J-82	bladder carcinoma	ATCC	I
Mv1Lu	mink lung epithelial	ATCC	I-III
NIH 3T3	mouse fibroblast-like, immortalized	ATCC	I, III-IV
SaOS-2	osteosarcoma	ATCC	I
SW-480	colon adenocarcinoma	ATCC	I
T-24	bladder carcinoma	ATCC	I
WI-38	human lung fibroblast	ATCC	I
NIH 3T3 Ras clones	NIH 3T3 stable transfected with pSVlacOras and pHβINLneo	Kivinen *et al.*, 1996	IV

Libraries and plasmids

Library/ plasmid	*Description*	*Reference or source*	*Used in*
Human placental cDNA library	oligo d(T) primed in λgt11 vector	Clontech	I
pcDNA3	mammalian expression vector with CMV promoter	Invitrogen	I
pSG5	mammalian expression vector with SV40 promoter	Stratagene	I, III
pP10HA	HA-tagged p10 cDNA in pcDNA3	cDNA from placental cDNA library	I
pP10	p10 cDNA in pSG5	placental cDNA library	I
pP15	p15 cDNA in pSG5	cDNA from HaCaT cells	I
pP16	p16 cDNA in pSG5	cDNA from HeLa cells	I
pP27	p27 cDNA in pSG5	cDNA from HaCaT cells	III
pCdk2	Cdk2 cDNA in pRc/CMV	Dr. Tomi Mäkelä, University of Helsinki	III

pCdk4	Cdk4 cDNA in pRc/CMV	Dr. Tomi Mäkelä	III
pCdk6	Cdk6 cDNA in pCMV	Dr. J. LaBaer, Massachusetts General Hospital	III
pCyclin E	cyclinE cDNA in pRc/CMV	Dr. Tomi Mäkelä	III
pCyclin D1	cyclinD1 cDNA in pRc/CMV	Dr. Tomi Mäkelä	III
pCyclin D2	cyclinD2 cDNA in pRc/CMV	Dr. Tomi Mäkelä	III
pSVlacOras	Ha-ras under SV40/ lactose inducible promoter	Liu *et al.*, 1992	IV
pHβlacINLSneo	lacI lactose repressor under β-actin promoter	Liu *et al*., 1992	IV
pGEJ6.6	genomic Ha-ras in pGEM7Zf(+)	Mäkelä *et al*., 1992	IV
p21 promoter constructs		Datto *et al*., 1995b; Kivinen *et al*., 1999	IV

Growth Factors, Antibodies and Chemicals

Growth Factor/ Antibody/ Chemical	*Reference or source*	*Used in*
TGF-β1	human platelets	I-III
HGF	R&D Systems	II
α-p15, rabbit or goat polyclonal (C-20)	Santa Cruz	I-II
α-p21, rabbit polyclonal (13436E)	Pharmingen	IV
α-p27, mouse monoclonal	Transduction Laboratories	II-IV
α-p27, rabbit polyclonal (C-19)	Santa Cruz	II-III
α-p27, rabbit polyclonal	Dr. J. Massagué, Sloan-Kettering Institute	II-III
α-Cdk2, rabbit or goat polyclonal (M2)	Santa Cruz	II-IV
α-Cdk4, rabbit polyclonal (C-22)	Santa Cruz	I-IV
α-Cdk6, rabbit polyclonal (C-21)	Santa Cruz	II-IV
α-Cdk6, mouse monoclonal (DCS-83)	Dr. Jiri Bartek, Danish Cancer Society	II-III
α-Cyclin E, rabbit polyclonal (M-20)	Santa Cruz	II
α-Cyclin E, rabbit polyclonal (06-459)	Upstate Biotechnology	II-III
α-Cyclin D1, rabbit polyclonal (H-295)	Santa Cruz	II-IV

α-Cyclin D1, mouse monoclonal (DCS-11)	Dr. Jiri Bartek, Danish Cancer Society,	II-III
α-Cyclin D2, rabbit polyclonal (C-17)	Santa Cruz	II-III
α-pRb, rabbit polyclonal (C-15)	Santa Cruz	II
α-p53, rabbit polyclonal (Pab240)	Pharmingen	IV
α-Ras, mouse monoclonal (Ab-1)	Oncogene Science	IV
α-Sp1, mouse monoclonal (1C6)	Santa Cruz	IV
α-Sp3, rabbit polyclonal (D-20)	Santa Cruz	IV
α-HA, mouse monoclonal	BabCo	I
α-BrdUrd, mouse monoclonal	Amersham Life Sciences	II-III
α-BrdUrd, rat monoclonal	Accurate Antibodies	I
TRITC-conjugated α-mouse, rabbit polyclonal	DAKO	I-III
Cy3-conjugated α-mouse, donkey polyclonal	Jackson Immunochemicals	I
Cy3-conjugated α-rabbit, donkey polyclonal	Jackson Immunochemicals	I
FITC-conjugated α-rabbit, swine polyclonal	DAKO	I
FITC-conjugated α-rat, donkey polyclonal	Jackson Immunochemicals	I
Biotin-conjugated α-mouse, swine polyclonal	DAKO	II-III
Biotin-conjugated α-rabbit, swine polyclonal	DAKO	II-III
Peroxidase-conjugated α-mouse, rabbit polyclonal	DAKO	IV
Peroxidase-conjugated α-rabbit, swine polyclonal	DAKO	IV
PD 098059	Calbiochem	IV
Calyculin A	Calbiochem	IV

Methods

Technique	*Used and described in*
cDNA cloning and sequence analysis	I
Screening of bacteriophage λ library	I
RT-PCR	I
Northern blot analysis	I-II, IV
Southern blot analysis	I
Western blot analysis	II-IV

Immunoprecipitation	I-IV
Immunohistochemistry	I-III
5-BrdUrd analysis	I-III
Flow cytometry	II
Cell transfection by calcium phosphate precipitation	I, III-IV
Colony assay	I
Metabolic labeling	I-IV
Pulse-chase analysis	II-IV
Kinase assays	II-III
Phosphatase treatment	III
Luciferase assay	IV
EMSA	IV

D. RESULTS AND DISCUSSION

D 1. Cloning and characterization of p10, an alternative spliced form of p15 Cdk inhibitor (I)

Ubiquitous expression and TGF-β regulation of p10 mRNA

After the cloning of p15 Cdki and the finding that it is regulated by TGF-β in HaCaT keratinocytes (Hannon and Beach, 1994), we wanted to use the p15 cDNA in our studies concerning TGF-β growth inhibitory mechanisms in Mv1Lu cells. In the process of cloning of p15 cDNA from human placenta cDNA library using p16 exon 2 PCR product as a probe, a 2.3 kb cDNA was isolated. It contained a novel 123 bp cDNA sequence flanked by exons 1 and 2 of p15. The 123 bp cDNA sequence, named exon 1β, was identical to 5' end of p15 intron 1 (Fig. 3; **I**: Fig. 1; Guan *et al.*, 1994). In the 2.3 kb cDNA exon 1 was not spliced to exon 2. Instead the cDNA contained exon 1 of p15 followed by exon 1β that was spliced to exon 2 by a new splice site downstream of that used in the splicing of exon 1 to exon 2 in p15. A stop codon at nucleotide position 554 in the exon 1β terminated the translation, giving rise to a predicted 78 amino acid protein homologous to the p15 on its aminoterminal half but lacking the p15 carboxyterminal half encoded by exon 2 sequence. Instead the protein, designated as p10 based on the migration of an *in vitro* translation product in SDS-PAGE, contains a 25 amino acid novel, basic carboxyterminal half, which lacks significant homology to known protein products.

p10 mRNA was found to be expressed in several normal and tumor cell lines including human keratinocytes, fibroblasts, osteosarcomas, colon adenocarcinoma and bladder carcinomas when exon 1β was used as a probe in Northern analysis (**I**: Fig. 2). Similarly to p15 (Hannon and Beach, 1994; Reynisdóttir *et al.*, 1995), the p10 specific 5.2 and 7.4 kb mRNAs were upregulated in TGF-β treated HaCaT cells (**I**: Fig. 3). The ubiquitous presence of p10 splice products suggests that at least in cultured cell lines and in placenta the splicing takes place constitutively. In addition, the results suggest that the p10 mRNAs share the same TGF-β1 regulatory promoter region as p15.

The subcellular localizations of transiently overexpressed p15 and HA-epitope-tagged p10 were similar. In Mv1Lu cells both were found in cytoplasm and nucleus, whereas in NIH 3T3 cells they localized predominantly to the cytoplasm (**I**: Fig. 4). Reynisóttir and Massagué (1997) have also observed cytoplasmic and nuclear localization of exogenously expressed p15.

p10 does not associate with Cdks or cyclins

Since p15 is a Cdk inhibitor and involved in TGF-β induced G_1 arrest (Hannon and Beach, 1994; Reynisdóttir *et al.*, 1995), we tested whether p10 is able to restrict cellular growth, and if so, is it due to its association with Cdks or cyclins. However, stable overexpression of p10 in NIH 3T3 or Mv1Lu cells had no effect on cellular growth as compared to colony formation of cells transfected with an empty vector. As a control, p15 and p16 decreased the number of cell colonies by over 30% in NIH 3T3 cells (**I**: Table 2). Interestingly, stable expression of p15 in Mv1Lu cells did not decrease the number of cell colonies, whereas p16 efficiently suppressed the growth (**I**: Table 2).

Unlike p15, p10 did not associated with Cdks. Immunoprecipitation analysis of overexpressed p15, p10HA and Cdk4 in COS-7 cells indicated that anti-Cdk4 and -Cdk6 antibodies co-precipitated p15 but not p10 (**I**: Fig. 5). In addition, expression of p10 in the cells did not prevent co-precipitation

of p15 with either Cdk4 or Cdk6 antibodies suggesting that p10 does not compete with p15 for interaction with these Cdks (**I**: Fig. 5). The exon 2 area that is not translated in p10, is almost totally conserved (94% identity) in p15 and p16, and functionally relevant for binding to Cdk4/6 (Wick *et al*., 1995; Fåhraeus *et al*., 1998; Luh *et al*., 1997; Russo *et al*., 1998). Thus our results, which suggest that p10 is devoid of Cdk4 and Cdk6 interaction and, additionally, that it does not compete with p15 for interaction with the Cdks, were not unexpected.

We were unable to verify the existence of endogenous p10 protein by using p10 specific antibodies for immunoprecipitations and western blotting analysis suggesting that either p10 specific mRNAs are not translated or that the protein levels of p10 are extremely low even in TGF-β treated cells. p15 is not the only Cdki with multiple transcripts. p18 has at least four different transcripts which are differentially expressed in various tissues (Guan *et al*., 1994; Hirai *et al*., 1995). The regulation of the distinct mRNA forms is different in response to starvation or G_1-S phase transition (Hirai *et al*., 1995). p57 has two different sized mRNAs (Lee *et al*., 1995), and three different cDNAs were isolated during p57 cloning (Lee *et al*., 1995; Matsuoka *et al*., 1995). The shorter cDNA forms lack 13 amino acids from the N-terminus including the first translation initiator codon, and they are started from a potential initiator codon downstream (Lee *et al*., 1995; Matsuoka *et al*., 1995). However, there is no evidence that the shorter forms are translated (Lee *et al*., 1995). The alternatively spliced form of p16, $p14/19^{ARF}$, raises from usage of an alternative first exon with a different promoter than that used for p16. In addition, $p14/19^{ARF}$ is translated in different reading frame coding a non-Cdki protein (Duro *et al*., 1995; Mao *et al*., 1995; Quelle *et al*., 1995; Stone *et al*., 1995b). Instead, $p14/19^{ARF}$ is involved in the p53 regulation (reviewed by Sherr, 1998).

D 2. HGF releases Mv1Lu cells from TGF-β induced G_1 arrest by restoring Cdk6 expression and Cdk2 activity (II)

The finding that HGF releases Mv1Lu cells from TGF-β growth inhibition (Taipale and Keski-Oja, 1996) gave us a good model system to study the separate and simultaneous effects of a growth inhibitory growth factor (TGF-β) and a stimulatory growth factor (HGF) on endogenous cell cycle machinery proteins.

HGF decreased the number of Mv1Lu cells arrested in G_1 phase by TGF-β as indicated by flow cytometry and 5-BrdUrd incorporation analyses (**II**: Fig. 1). Most of pRb was found in phosphorylated forms after treatment of the cells with both growth factors, which is in line with the four-fold increase in DNA synthesis in cells treated with both TGF-β and HGF as compared to TGF-β treated cells (**II**; Fig. 1 and 2). As TGF-β is a very potent growth inhibitor of Mv1Lu cells (ED_{50} = 5 pM), the rescue from TGF-β growth arrest by HGF was significant, albeit not complete.

HGF counteracts the effects of TGF-β on Cdk6 protein level and complex formation with cyclin D2 and p27, but not on p15 induction

pRb is phosphorylated in G_1 by cyclin D complexes (Kato *et al*., 1993; Connell-Crowley *et al*., 1997; Zarkowska and Mittnacht, 1997; Lundberg and Weinberg, 1998). To study how HGF signaling interacts with TGF-β signaling to prevent the shift of pRb to its underphosphorylated form, we analyzed the effects of TGF-β and HGF on the levels on G_1 Cdks and their complexes. Interestingly, TGF-β decreased the expression of Cdk6 and its association with cyclin D2, whereas HGF prevented these effects (**II**: Fig. 3A and 4A). Additional studies suggested that TGF–β had no

effect on the Cdk6 mRNA levels (**II**: Fig. 3C). Instead TGF–β decreased the synthesis and increased the turnover of Cdk6, whereas HGF opposed both of these effects (**II**: Fig. 3B and D).

Despite that HGF antagonized the TGF–β induced growth arrest, it did not prevent the TGF–β-mediated induction of p15 or its complex formation with Cdk6 (**II**: Fig. 3A and 4A). Upon TGF–β treatment the association of Cdk6 with cyclin D2 was decreased, as well as the binding of p27 to Cdk6. The results reflect the presence of lesser amounts of Cdk6 in the TGF–β-treated cells as well as displacement of p27 from Cdk6-complexes by p15 as suggested earlier (Reynisdóttir *et al*., 1995). HGF restored the association of Cdk6 with cyclin D2 and Cdk6 binding to p27 in cells treated with both growth factors (**II**: Fig. 4A).

The restoration of Cdk6 expression in cells treated with both growth factors correlated with the partial rescue of the cyclin D2-associated kinase activity towards GST-Rb (**II**: Fig. 4B). This suggests that though p15 induction is unperturbed in cells treated with both growth factors and it forms avidly complexes with Cdk6, its levels are not high enough to fully prevent the activity of Cdk6-cyclin D2 complexes. In addition, the levels of p15 may not be sufficient to sequester all Cdk6 present in the cells. This in turn leads to the presence of active Cdk6-cyclin D2-complexes that can harvest p27 from binding to Cdk2. Thus, the decrease in Cdk6 expression may contribute an important part to the induction, maintenance and adaptation of the cells to the TGF–β-mediated growth arrest. However, since HGF could only partly rescue the cyclin D2-associated kinase activity, HGF may also override the TGF–β block by mechanisms other than solely regulation of the G_1 cyclin-Cdk complexes.

HGF counteracts TGF–β regulation of Cdk2-cyclin E activity

Cdk2-cyclin E-complexes phosphorylate pRb in the late G_1-phase (Akiyama *et al*., 1992; Lundberg and Weinberg, 1998). In accordance with the earlier studies, we found that TGF–β treatment enhanced the association of p27 to Cdk2-cyclin E-complexes (**II**: Fig.5B; Polyak *et al*., 1994a, b; Reynisdóttir *et al*., 1995; Reynisdóttir and Massagué, 1997; Sandhu *et al*., 1997). Concomitantly, TGF–β decreased the amount of the faster migrating, active form of Cdk2 (**II**: Fig. 5A; Koff *et al*., 1993; Reynisdóttir *et al*., 1995). Both these events lead to a decrease in Cdk2- and cyclin E-associated kinase activities (**II**: Fig. 6A and B; Koff *et al*., 1993; Polyak *et al*., 1994a, b; Reynisdóttir *et al*., 1995; Reynisdóttir and Massagué 1997). When the cells were treated with both growth factors, the TGF–β-mediated decrease in the faster migrating form of Cdk2 was efficiently prevented by HGF, although HGF could not prevent the induction of p27 association with cyclin E or Cdk2 (**II**: Fig. 5A and B). HGF also restored the TGF–β suppressed Cdk2 and Cyclin E-associated kinase activities towards Histone H1 and GST-Rb, which is in line with the observed phosphorylation status of pRb (**II**: Fig. 6A and B).

The observed Cdk2 and cyclin E-associated kinase activity in cells treated with both growth factors could result from complexes devoid of p27. The possibility was addressed by immunodepletion analyses using polyclonal anti-p27 antibodies. The depletion steps did not significantly affect on the total levels of cyclin E or Cdk2 (**II**: Fig. 7B). However, all cyclin E–bound Cdk2 present in TGF–β-treated cells was removed. Instead, in cells treated with both growth factors, 30% of cyclin E-bound Cdk2 was left after immunodepletion, suggesting that less p27 remains attached with cyclin E/Cdk2 complex. Histone H1-kinase assays, carried out in parallel with the above experiment, indicated that TGF–β decreased the cyclin E-associated kinase activity to close to basal levels in both p27-depleted and non-depleted lysates (**II**: Fig. 7A). Depletion of p27 from cells treated with both

growth factors had no effect on the activity. The p27-depletion assays suggest that though p27 is associated with Cdk2-cyclin E-complexes in cells treated with both TGF–β and HGF and part of these complexes are removed, there appears to be enough active complexes present in the cells sufficient for cellular proliferation.

HGF is able to induce proliferation of cells that have been growth arrested by TGF–β

HGF was found to bypass the TGF–β-mediated growth arrest also under conditions in which the cells were first arrested into G1 by TGF–β followed by addition of HGF without the removal of TGF–β. HGF increased the amount of the DNA synthesis after 12 h of addition of HGF, and significant amount of the cells replicated their DNA after 24 h of HGF addition (**II**, Fig. 6C). In contrast, serum stimulation of TGF–β arrested cells was without effect on DNA replication. Analyses on Cdk6 and Cdk2 indicated that HGF restored expression of Cdk6 and the faster migrating form of Cdk2 with kinetics that paralleled entry of the cells into S-phase. Similarly the Cdk2-associated kinase activity was restored. The results suggest that HGF releases Mv1Lu cells from TGF–β-mediated growth arrest with concomitant upregulation of Cdk6 and increased Cdk2-cyclin E activities.

Our results suggest that HGF modifies the TGF–β response by regulation of Cdk6-complexes and as a consequence affects the activity of Cdk2-complexes, and that both these events serve as means to hinder the growth inhibition induced by TGF–β. In this manner the effect of HGF on TGF–β induced growth arrest is different than that of viral oncoproteins such as SV40, E1A or E7, which sequester and inactivate pRb thus bypassing the requirement of Cyclin D-associated kinase activity (Whyte *et al.*, 1988; DeCaprio *et al.*, 1988; Dyson *et al.*, 1989; Munger *et al.*, 1989; Egan *et al.*, 1989). However, E1A also prevents the induction of p15 and p21 by TGF–β (Datto *et al.*, 1997). Similarly, E1A and E7 bind to and sequester p27 from Cdk2-cyclin E complexes which leads to activation of the complex (Mal *et al.*, 1996; Zerfass-Thome *et al.*, 1996). This suggests that activation of at least Cdk2-complexes is required in addition to pRb inactivation to ensure cell cycle progression.

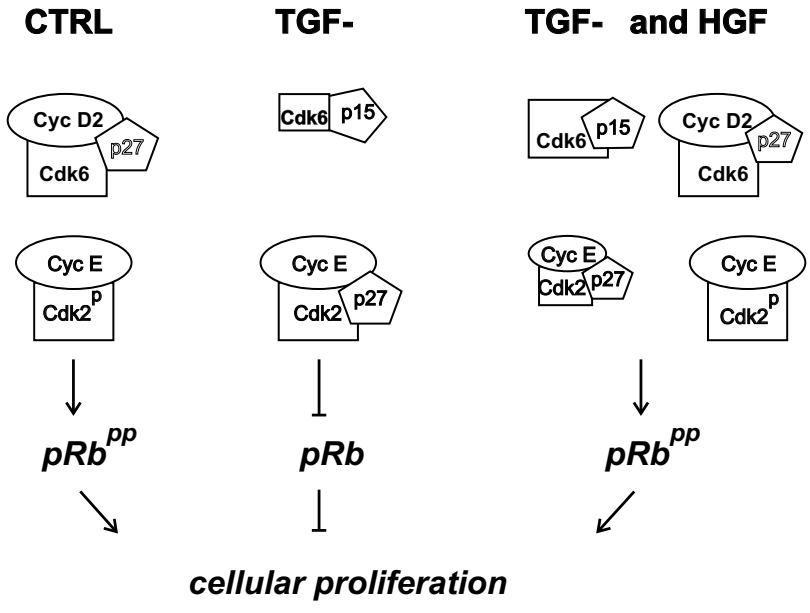

Fig. 6 Schematic model of how HGF prevents TGF-β growth arrest in Mv1Lu cells. HGF prevents the downregulation of Cdk6 by TGF-β leading to increased amounts of Cdk6-cyclin D2-p27 complexes that are active. Since HGF is unable to prevent upregulation of the p15 Cdk inhibitor, there are still inactive Cdk6-complexes present. In addition, less p27 is liberated from Cdk6 complexes than in TGF-β treated cells leading to accumulation of p27-free Cdk2-cyclin E-complexes. This, together with the similar amounts of thr-phosphorylated Cdk2 as in exponentially growing cells, leads to increased Cdk2 kinase activity. Cdk6 kinase activity together with that of Cdk2 allows cell cycle progression.

Based on our results we suggest a model, in which regulation of Cdk6 levels serves as means to regulate both Cdk6- and Cdk2-activities (Fig. 6). The upregulation of p15 and subsequent relocalization of p27 are not enough to prevent cellular proliferation as suggested by the treatment of the cells with both growth factors. The downregulation of Cdk6 levels and presence of an active form of Cdk2 are also required. The release of Cdk2-cyclin E-activity from suppression by p27 occurs as a secondary mechanism through increased binding of Cdk6-complexes to p27. The hypothesis that TGF–β growth arrest involves additional mechanisms besides action of p15 and p27 Cdkis is strengthened by genetic and biochemical evidence, as cells from mice nullizygous for p27 (Nakayama *et al*., 1996) and mammary epithelial cells that lack p15 (Iavarone and Massagué, 1997), are growth inhibited by TGF–β. These models imply that for growth suppression TGF–β must affect multiple cell cycle components. Interestingly, also p16 growth arrest requires the co-operation of p27. Dominant negative Cdk4 that associates with cyclin D forming inactive kinase complexes is unable to arrest growth of U2OS cells when overexpressed (van den Heuvel and Harlow, 1993). In contrast, overexpression of p16 in U2OS cells leads to G_1 arrest due to inactivation of Cdk4 by p16 and Cdk2-complexes by p27 (Jiang *et al*., 1998). p27 is liberated from cyclin D-complexes by p16 suggesting that inactivation of cyclin D-associated kinase activity is not sufficient for G_1 arrest (Jiang *et al*., 1998; McConnell *et al*., 1999).

Furthermore the present data indicates that the positive and negative growth signals are integrated at the level of Cdk-regulation. Little is known of how cells respond to simultaneous inhibitory and stimulatory growth factors, and on what level these signals are integrated. Recent data suggest that they can be integrated by intracellular signaling pathways. Epidermal growth factor and HGF can antagonize the effects of bone morphogenetic protein by inducing phosphorylation of Smad1 via the extracellular signal-regulated kinase mediated pathway. The phosphorylation leads to the inhibition of bone morphogenetic protein signaling (Kretzschmar *et al*., 1997), suggesting that opposing signals are modulated at the level of Smad proteins. Smad1 is not involved in TGF–β signaling (Massagué, 1996), but similar mechanisms utilizing Smad2 and 3 could affect the ability of HGF to alter the growth inhibitory effect by TGF–β. Indeed, recent work shows that HGF can phosphorylate and activate Smad2, although to a lesser extent than TGF–β (de Caestecker *et al*., 1998). In addition, IFN-g was shown to prevent the induction of 3TP-luc reporter by TGF–β. 3TP-luc contains TGF–β responsive elements from the plasminogen-activator inhibitor-1 gene. IFN-γ induces inhibitory Smad7 via the Jak/Stat pathway and prevents TGF–β signal transduction (Ulloa *et al*., 1999). Our data, however, indicates that HGF does not inhibit, nor act synergistically with TGF–β signal transduction, because HGF does not alter the level of induction of p15 or extracellular matrix components such as fibronectin and thrombospondin (Taipale and Keski-Oja, 1996) or PAI-1 (not shown) by TGF–β. Our results are consistent with the hypothesis that HGF does not interfere, in general, with the TGF–β signal transduction pathways, but that the signals involving growth regulation are integrated at the level of Cdk complexes.

D 3. TGF-β induces accumulation of a nuclear p27^{Kip1}-form devoid of Cdk-cyclin-association (III)

TGF-β and growth arresting treatments induce accumulation of a nuclear form of p27

TGF-β has been reported to have no effect on the total p27protein levels in Mv1Lu cells (Polyak *et al*., 1994b). However, in the course of the study II we found out that in TGF-β treated Mv1Lu cells a specific form of p27 accumulated. The accumulated p27 form was found by immunoprecipitations using a specific monoclonal antibody (p27 mAb from Transduction Laboratories) (**III**: Fig. 1A). In addition to TGF-β treated cells, the accumulated p27 form was detected in Mv1Lu cells growth arrested by low serum or contact inhibition suggesting that the accumulation is not only due to TGF-β treatment, but associates with G_0/G_1 arrest (**III**: Fig. 1A). No regulation of p27 levels was detected when using a polyclonal p27 antibody (p27 pAb) for immunoprecipitation. Similarly, total p27 levels were not affected by TGF-β or low serum treatments, which is in accordance with earlier studies (**III**: Fig. 1B; Reynisdóttir *et al*., 1995; Sandhu *et al*., 1997). The levels of p27 immunoprecipitated from TGF-β treated cells with p27 mAb antibody were found to be induced also in metabolically labeled cells (**III**: Fig. 1) suggesting that the induction, at least partly, requires protein synthesis. However, since there is no change in the total p27 protein or mRNA levels in the TGF-β-treated Mv1Lu cells (**III**: Fig. 1B; Polyak *et al*., 1994b; data not shown), the observed increase in the metabolic labeled lysates could reflect change in p27 conformation or complexes. Furthermore, the amount of p27 precipitated by the p27 mAb in TGF-β-treated cells was consistently lower than the amount of p27 precipitated by the p27 pAb (**III**: Fig. 1A, compare lanes 2 and 4), suggesting that the TGF-β-inducible p27 represents a fraction of total p27 present in the cells.

p27 localizes predominantly to nucleus (Lee *et al*., 1996; Reynisdóttir and Massagué, 1997; Orend *et al*., 1998; Singh *et al*., 1998; Tomoda *et al*., 1999). The accumulated p27 form recognized by the p27 mAb was found only in nuclei of growth arrested Mv1Lu and NIH 3T3 cells, whereas NIH 3T3 cells that are growth stimulated by TGF-β (Pitkänen *et al*., 1993) lacked completely p27 staining with the p27 mAb (**III**: Fig. 2A and B). In contrast, the p27 pAb detected p27 similarly in both control and growth arrested Mv1Lu and NIH 3T3 cells (**III**: Fig. 2A and B). In the TGF-β treated NIH 3T3 cells the amount of p27 positive nuclei, as detected by the p27 pAb, was somewhat decreased. The results suggest that TGF-β and treatments arresting the cells in G_0/G_1 induce a form of p27 that can be detected in individual cells at high levels with exclusive nuclear localization. That in NIH 3T3 fibroblasts stimulated to proliferate by TGF-β the accumulated p27 form was excluded from the nucleus further verifies that the inducible p27 is sensitive to the growth status of the cells. It also suggest that the p27 form recognized by the p27 mAb is either not present or is in different kind of complexes in growth stimulated than in growth arrested cells.

The TGF-β inducible form of p27 is a subpopulation of the total p27 pool, but does not associate with Cdk-cyclin complexes

The results using either monoclonal or polyclonal p27 antibodies in the above assays suggested that the monoclonal antibody could recognize a subpopulation of the total p27 pool recognized by the polyclonal antibody. The possibility was verified by immunodepletion assay showing that immunodepletion with the p27 pAb removed all detectable p27 from control and TGF-β treated cells (**III**: Fig. 3A). The p27 mAb removed some of p27 from TGF-β treated lysates but none of the protein from the control lysates (**III**: Fig. 3A). When the lysates were denaturated with 1% SDS and boiling

prior to the immunoprecipitations, the p27 mAb immunoprecipitated similar amounts of p27 from control and TGF-β treated cells (**III**: Fig. 3B). The result suggests that upon denaturation the p27 mAb recognizes the total p27 protein pool as shown also by the western blotting analyses of total cell lysates. We also found that phosphorylation of p27 had no effect on the antibody recognition (**III**: Fig. 3C). The results suggest that the p27 mAb recognizes a native TGF-β inducible form of p27, which represents a subpopulation of the total p27 pool present in the cells.

The half-lives of different p27 pools detected by the mAb and pAb antibodies were compared with a pulse-chase experiment. p27 half-life in proliferating human fibroblasts has been reported to be approximately 50 min, and six times longer in quiescent cells (Pagano *et al*., 1995). Similarly, TGF-β increased the half-life of p27 recognized by the p27 pAb from less than two hours to over six hours (**III**: Fig. 4). The half-life of the TGF-β inducible form of p27 was found to be three hours. These findings suggest that the half-life of the TGF-β inducible form of p27 is significantly shorter than that of p27 recognized by the p27 pAb in TGF-β-treated cells. A shorter half-life for the TGF-β-inducible p27 may suggest either its faster degradation or conversion to a form not recognized by the antibody.

p27 binds to cdc2, Cdk2, 4 and 6 complexes (Polyak *et al*., 1994b; Toyoshima and Hunter, 1994). However, we found out that the p27 mAb did not co-immunoprecipitate any of Cdks 2, 4 or 6 or cyclins E or D1-2 or Cdc2 from endogenous lysates (**III**: Fig. 5; data not shown) or from lysates that were overexpressing p27 together with Cdks 2, 4 or 6 and cyclin E, D1 or D2 (**III**: Fig. 6; data not shown). In contrast, the p27 pAb co-immunoprecipitated all the mentioned Cdks and cyclins (**III**: Fig. 5 and 6; data not shown). Furthermore, epitope mapping of the p27 mAb indicated that the p27 mAb recognizes a p27 domain spanning amino acids 1 to 91, which harbors the Cdk and cyclin interaction domains of p27 (Polyak *et al*., 1994b; Toyoshima and Hunter, 1994). The p27 pAb recognizes an epitope mapping to the carboxyterminal amino acids 181-198. Taken together, these findings suggest that the TGF-β inducible form of p27 is not associated with Cdks 2,4 or 6 or with cyclins D1-2 or E.

In accordance with the above results, the p27 mAb did not co-immunoprecipitate any kinase activity towards GST-Rb or histone H1 (**III**: Fig. 5). Instead, the p27 pAb co-immunoprecipitated kinase activity both from control and TGF-β treated cells (**III**: Fig. 5). The GST-Rb kinase activity can, at least partly, be attributed to co-precipitation of Cdk4/6-cyclin D complexes, as p27 has been found not to block the activity of this complex (Poon *et al*., 1995; Soos *et al*., 1996; Hauser *et al*., 1997; Blain *et al*., 1997, Dong *et al*., 1998). The presence of histone H1 kinase activity may result from the presence of active Cdk2 complexes in p27 immunoprecipitates, since Cdk4 or 6 do not utilize histone H1 as a substrate (Matsushime *et al*., 1994). Cdk2 has been shown to associate with cyclins D2 and 3 in Sf9 insect cells and to phosphorylate histone H1 (Matsushime *et al*., 1994). However, there is no evidence that Cdk2 can form complexes with D-type cyclins in Mv1Lu cells, or that these complexes could phosphorylate histone H1.

TGF-β modulates cellular growth by regulating the availability and association of Cdkis with the Cdk-cyclin complexes. Our data indicated that part of the p27 pool is not associated with Cdk-cyclin complexes after TGF-β and other growth arresting treatments. The lack of p27 staining with the p27 mAb in TGF-β growth stimulated NIH 3T3 cells suggest that in exponentially growing cells all p27 is associate with Cdk or possible other cellular complexes. The induction of p27 in metabolically labeled samples, at least in part, could be due to increased synthesis of the protein. However, a major part, if not all, could represent p27 released from Cdk4/6-complexes by the action of p15 (Reynisdóttir and Massagué, 1997; Sandhu *et al*., 1997) and by the downregulation of Cdk6 levels (**II**; Fig. 3 and 4) induced by TGF-β. Together with the finding that in TGF-β treated cells Cdk2-

cyclin E complexes are saturated with p27 (**II**; Fig. 7B), the data suggest that TGF-β releases more p27 from cyclin D-complexes that can be absorbed to Cdk2-cyclin E-complexes. In this manner the TGF-β-inducible p27 may serve as a reservoir, the binding of which to Cdk2-cyclin E/A facilitates the establishment of cell cycle arrest.

D 4. Ras induces the p21 Cdk inhibitor both transcriptionally and posttranscriptionally (IV)

The p21 Cdk inhibitor has been shown to be induced by Ras and its signal mediator Raf (Lloyd *et al*., 1997; Sewing *et al*., 1997; Woods *et al*., 1997; Olson *et al*., 1998). However, the level of p21 regulation was not known. For this reason, we undertook analysis of p21-regulation by Ras in NIH 3T3 fibroblasts that express c-Ha-Ras under an inducible lactose promoter. The expression of Ras is induced by inactivating the lactose repressor with the lactose analog IPTG.

Conditional Ras expression increases p21 protein levels

As p21 is regulated both transcriptionally and posttranscriptionally (Liu *et al*., 1996; Macleod *et al*., 1995; Schwaller *et al*., 1995; Zeng and El-Deiry, 1996), we analyzed the effects of conditionally expressed Ras on p21 protein and mRNA levels. Three Ras clones (ras 8, 22 and 30) with the highest Ras induction levels were chosen for the studies. Northern blotting experiments revealed a slight increase in basal expression of p21 mRNA in the Ras clones as compared to non-transfected NIH 3T3 cells and neo clones (mean induction 1.6-fold; **IV**: Fig. 5A). Similarly, p21 mRNA was only slightly increased by Ras expression (**IV**: Fig. 5A).

The basal protein levels of p21 were found to be increased in all three Ras clones by western blotting analysis (**IV**: Fig. 1A, middle panel). Moreover, induction of Ras expression with IPTG significantly induced p21 expression in the Ras clones with kinetics that paralleled the induction of Ras (**IV**: Fig. 1B). In addition, increased levels of p21 were found to associate with Cdk2 and 4 complexes after Ras induction (**IV**: Fig. 2C). Also the amounts of the immunoprecipitable cyclin D and Cdk4-associated cyclin D increased after IPTG treatment (**IV**: Fig. 2), which is in agreement with earlier studies (Winston *et al*., 1996). However, although Ras expression increased p21 in Cdk2 and Cdk4 complexes, the level of p27 was unaltered by Ras in the exponentially growing cells, or in the complexes with Cdk2 or 4 (**IV**: Fig. 2A and D). These results are concordant with earlier data that Ras alone is unable to affect p27 levels, but that joint expression of Ras and Myc, or additional growth factor stimulation is needed for this effect (**IV**: Fig. 2; Leone *et al*., 1997; Winston *et al*., 1996). In additon, the levels of p53, the best known regulator of p21, were not affected by Ras induction (**IV**: Fig. 1A).

Since the p21 protein levels were increased after Ras induction, we analyzed the effect of Ras on p21 protein synthesis and half-life by metabolic labeling of ras8-cells. Ras expression increased the amount of newly synthesized p21 protein by 2.5-fold (**IV**: Fig. 3, lanes 2 and 8). However, induction of Ras had no effect on the p21 protein half-life (**IV**: Fig. 3). Taken together, the results suggest that p21 induction by conditional Ras expression occurs mainly via increase in p21 protein synthesis.

Sp1-transcription factors mediate the transcriptional activation of p21 by Ras

To investigate possible transcriptional activation of p21 by Ras, a luciferase activity assay was carried out in COS-7 and NIH 3T3 cells transiently transfected with Ha-Ras together with a 2.4 kb p21-promoter luciferase construct (p21p). As compared to controls transfected with pCDneo, Ras increased the activity of p21 2.4 kb promoter by 6.0- and 7.6-fold in COS-7 and NIH 3T3 cells, respectively, indicating that Ras can induce p21 transcriptionally (**IV**: Fig. 6A-B). However, transfection of the 2.4 kb (p21p) or 93 bp (93-S) p21 promoter constructs to ras8-cells and induction of Ras expression by IPTG had no major effect on the luciferase activity further suggesting that in ras8 clones p21 induction is regulated at the posttranscriptional level (**IV**: Fig. 6C).

For more close mapping of the Ras responsive element we used a series of deletion or mutation constructs based on the 2.4 kb p21-promoter in the luciferase assay (**IV**: Fig. 7 and Datto *et al*., 1995b). Experiments using the deletion constructs suggest that that the Ras responsive region in p21 promoter spans between -110 and -62 bp relative to the transcription start site (**IV**: Fig. 6A-B). This area in human p21 promoter carries three GC-rich Sp1-binding sites (numbered 1-3 in **IV**: Fig. 7), the second of which overlaps with a TGF-β responsive element (TβRE; **IV**: Fig. 7) (Datto *et al*., 1995b). Two additional Sp1-sites are located close to the TATA-box, between -61 to -50 (Sp1-binding sites 4 and 5; **IV**: Fig. 7). Mutations of the Sp1 binding sites or TβRE decreased the overall basal luciferase activity. However, none of the mutations alone could totally abolish the transcriptional induction by Ras, although loss of Sp1 binding site 2 or TβRE decreased the Ras induction (**IV**: Fig. 7). The Ras induction was completely lost when both of the Sp1-binding sites 2 and 4 were mutated simultaneously (**IV**: Fig. 7). The activation was absent also in construct SmaΔ1 containing the Sp1-binding site 4, suggesting that intact Sp1-binding site 2 (or TβRE) is required for the function of the Sp1-binding site 4, and indicating a critical role for interactions between transcription factors acting in different regions for full promoter activity. Similarly, induction of p21 during keratinocyte differentiation or by progesterone is dependent on correct spacing of the promoter elements (Owen *et al*., 1998; Prowse *et al*., 1997). The mutant construct that affects the TβRE but does not eliminate any of the Sp1-binding sites (mut 2.3) (Datto *et al*., 1995b), showed a weaker induction of luciferase activity by Ras similarly to the mutants affecting the Sp1-binding site 2 and TβRE simultaneously (mut 2 and mut 2.2). This could refer to the relevance of TβRE as well as the Sp1-binding site 2 in the Ras effect.

Binding of two closely related transcription factors Sp1 and Sp3 to the Sp1-binding site 2/ TβRE was characterized earlier (Datto *et al*., 1995b). Our results indicated that in NIH 3T3 cells Sp3, but not Sp1 transcription factor is associated with this region (**IV**: Fig. 8C). However, in COS-7 cells antibodies against these transcription factors caused supershift in an EMSA assay (**IV**: Fig. 8B) indicating the presence of both Sp1 and Sp3 proteins. The results thus suggest that depending on cellular context, either both Sp1 and Sp3 or Sp3 alone bind the Sp1-binding site 2/ TβRE region. Sp1 transcription factors have been regarded as house-keeping transcription factors. Recently, however, the Sp1 family has been implicated in the induction of p21 by TGF-β (Datto *et al*., 1995b; Li *et al*., 1998), okadaic acid and phorbol esters (Biggs *et al*., 1996), NGF (Yan and Ziff, 1997), and progesterone (Owen *et al*., 1998), as well as in differentiation of keratinocytes (Prowse *et al*., 1997). The DNA-binding of Sp1 and Sp3 was not affected by Ras, and was unaltered also upon p21 induction by TGF-β or NGF (Datto *et al*., 1995b; Yan and Ziff, 1997) suggesting that the transactivation function of these factors is enhanced by mechanisms other than regulation of Sp1 and

Sp3 protein levels or DNA-binding activity. Alternatively, the Ras effect could (also) be mediated by other transcription factors associating with the same promoter region.

Taken together, the data suggest the presence of both transcriptional and posttranscriptional components in the Ras-regulation of p21 that may be determined by the level and/ or duration of Ras expression. Although there is no marked induction of p21 mRNA levels in NIH 3T3 cells conditionally expressing Ras, there is a clear induction of p21 promoter activity in COS-7 and NIH 3T3 cells transiently transfected with Ras. The transcriptional p21 regulation by additional induction of Ras in constitutively Ras expressing cells with higher basal level of Ras may be attenuated (or absent). Transcriptional induction of p21 by Ras may thus depend on the level or duration of Ras-expression, and after reaching a certain threshold transcriptional regulation may be switched to posttranscriptional.

E. CONCLUDING REMARKS

TGF-β has previously been shown to inhibit G_1 and G_1/S specific Cdk-cyclin-complex activities by several different mechanisms including regulation of the complex formation with different Cdk inhibitors, phosphorylation and downregulation of the Cdk-subunit. This study identifies novel ways of regulation of G1 and G1/S specific Cdk-cyclin complexes. Our work suggest that TGF-β growth arrest in Mv1Lu cells requires downregulation of Cdk6 levels in addition to p15 and p27 Cdki accumulation leading to concomitant loss of Cdk6- and Cdk2-activity (**II**). In addition, our data suggest that upregulation (or stable overexpression) of p15 alone is not enough to prevent growth of Mv1Lu cells (**I-II**). We also found that TGF-β induces accumulation of a p27 form that is not associated with Cdks (**III**), and that p15 Cdk inhibitor has an alternatively spliced form that is induced by TGF-β treatment (**I**). In addition, we found that overexpression of Ras-oncogene induces p21 Cdk inhibitor, but not p27 Cdki (**IV**).

These findings support the idea that TGF-β growth inhibition involves regulation of Cdk-complexes at multiple levels. Initiation of TGF-β growth inhibition in Mv1Lu cells may be due to induction of p15 Cdk inhibitor, which occurs within a couple of hours after TGF-β has been added to cells. The decrease in Cdk6 levels provides an additional mechanism by which a growth inhibitory status is induced and maintained. Together the increase in p15 levels and the decrease in amount of Cdk6 inhibit cyclin D-associated kinase activity. In addition, the TGF-β treatment prevents Cdk2 activity by re-localizing p27 into Cdk2-complexes and by blocking the activating phosphorylation of Cdk2. The accumulation of p27 found in study **III** could form a buffer to ensure that all Cdk2 (and Cdc2)-associated kinase activity is kept inactive.

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