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Expression of the cyclin-dependent kinase inhibitor p16 during the ongoing cell cycle

Thomas Soucek[a], Oliver Pusch[a], Elke Hengstschläger-Ottnad[b], Edgar Wawra[c], Gerhard Bernaschek[a], Markus Hengstschläger[a,*]

[a]*Obstetrics and Gynecology, University of Vienna, Department of Prenatal Diagnosis and Therapy, Währinger Gürtel 18–20, A-1090 Vienna, Austria*
[b]*Institute of Molecular Genetics, Vienna Biocenter, University of Vienna, Dr. Bohrgasse 9, A-1030 Vienna, Austria*
[c]*Institute of Molecular Biology, Vienna Biocenter, University of Vienna, Dr. Bohrgasse 9, A-1030 Vienna, Austria*

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Abstract **It has been demonstrated that protein expression of p16, the inhibitor of cyclin-dependent kinase 4 and 6, increases 4 fold at the G1/S transition when serum-arrested cells are restimulated to logarithmic growth. We examined the cell cycle regulation of this cyclin-dependent kinase inhibitor in cells separated according to their cell cycle phases by centrifugal elutriation. Neither p16 mRNA nor its protein expression are regulated during the cell cycle of normal phytohemagglutinin-stimulated lymphocytes, retinoblastoma protein-negative cells, papilloma virus-transformed cells, and acute promyelocytic leukemia cells. p16 mRNA is constitutively expressed in cells in which we detected the normal E2F-dependent S-phase specific expression of thymidine kinase mRNA. We further observed a G1-phase specific expression of cyclin D1 mRNA in the same cells separated by centrifugal elutriation.**

Key words: p16; Cell cycle; Centrifugal elutriation; Cyclin D1; pRb

1. Introduction

p16 was originally described as a protein associated with cyclin-dependent kinase 4 (CDK4) in simian virus 40-transformed fibroblasts [1]. Human p16 cDNA was isolated using a yeast two-hybrid protein interaction screen. The cDNA encodes 148 amino acids, respectively a protein of 15.845 molecular weight comprising four ankyrin repeats. This protein has been shown to inhibit the activities of CDK4 and CDK6 in vitro [2]. The gene encoding p16 (known as $p16^{INK4}$, MTS1, CDK4I and CDKN2) is located at chromosome 9p21 and was found to be homozygously deleted or mutated in different tumor cell lines [3,4]. Since then, functional loss of p16 has been reported in a wide variety of primary tumors and tumor derived cell lines (reviewed in [5]). The function of p16 as a tumor suppressor gene was determined by investigating its ability to arrest cell cycle progression. It has been reported that p16 acts as a potent inhibitor of progression through the G1-phase whereas tumor-associated mutants of p16 do not. This ability of wild type p16 is lost in retinoblastoma protein (pRb)-negative cells [6–11].

Although the role of p16 in regulating cell proliferation and in induction of tumorigenesis has intensively been investigated, little is known about the regulation of p16 expression. It has been shown that p16 expression is low in logarithmically growing primary cells of different origin, whereas several solid tumor cells highly express p16 protein. Interestingly, intracellular p16 protein expression was inducible by transfection with tumor antigens of specific DNA-tumor viruses, such as papilloma virus, adenovirus, and simian virus 40 [12,13]. Restimulation experiments after serum deprivation demonstrated that p16 protein expression increases about 4-fold during the transition from Go to S-phase [12]. Considering these data one could speculate that p16 expression is S-phase-regulated via the transcription factor E2F. This transcription factor is activated by release from pRb prior to the onset of replication. In normal cells this release is caused by the phosphorylation of pRb by the G1-cyclin-dependent kinases, CDK2, CDK4, and CDK6. In addition, it is known that polyoma virus large T, the large T antigen of simian virus 40, the E1A-protein of adenovirus, and the E7 protein of papilloma virus share the capacity to bind pRb and thereby to activate E2F (reviewed in [14]). Accordingly, the observation that p16 is overexpressed in DNA tumor virus-transformed cells and in pRb-negative cells and the fact that p16 protein is regulated during growth indicate the putative role of E2F in the regulation of p16 expression (reviewed in [15,16]). The nucleotide upstream sequence, which has been shown to promote p16 mRNA expression, has been screened for an E2F consensus binding site. Although no apparent E2F site was found so far, the question whether p16 contains a binding site for this factor is still under investigation. Cotransfection of a plasmid containing the p16 promoter cloned upstream of a promoterless luciferase gene, and of expressable pRb demonstrated that pRb represses mRNA expression driven by the p16 promoter [17].

From these data a model has been suggested to explain the timing of both, the expression and the function of p16 during the ongoing cell cycle [12,15–17]. Expression of D-type cyclins is high in early G1. These cyclins activate CDK4 and CDK6 to phosphorylate pRb. Phosphorylation of pRb leads to activation of a transcription factor, which promotes the expression of p16. Thereupon, p16 inhibits the activity of CDK4 and CDK6 by displacing D-type cyclins, which are thereafter (in late G1) degraded. In accord, one would expect to see a sharp induction of p16 expression in G1 of the ongoing eukaryotic cell cycle. To answer this question we investigated p16 expression after centrifugal elutriation of normal phytohemagglutinin-stimulated human lymphocytes. We compared this normal p16 cell cycle regulation with its regulation in cycling pRb-negative cells and DNA tumor virus-transformed cells. As

*Corresponding author. Fax: (43) (1) 40400-7848.

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a control of a transformed cell line with no obvious changes in the pRb-involving pathway, we also analysed p16 cell cycle regulation in acute promyelocytic leukemia cells.

2. Materials and methods

2.1. Human cells

HeLa cervix carcinoma cells (papilloma virus-transformed; American Type Culture Collection CCL2), Y79 retinoblastoma cells (retinoblastoma protein-negative; ATCC HTB18), and an acute promyelocytic leukemia cell line (in this report indicated as APL) were grown in RPMI 1640 medium supplemented with 10% calf serum and antibiotics (30 mg/liter penicillin, 50 mg/liter streptomycin sulfate). Normal human lymphocytes (indicated as PHA-lymph) were isolated from heparinised blood of a normal male subject using the Ficoll-Hypaque gradient method. These cells were stimulated with 5 μg phytohemagglutinin per ml RPMI medium, composed as described above, for about two cell doubling times. Lymphocytes were analysed after reaching logarithmic growth. All cultures were maintained at 37°C and 5% CO_2, and routinely screened for the absence of mycoplasma.

2.2. Centrifugal elutriation

Separation of cell cycle phases by centrifugal elutriation was performed as described [18]. The elutriation system consisted of a Beckman J2–21 M centrifuge and a JE-6B rotor eqipped with the standard separation chamber. The rotor was kept at a speed of 2000 rpm, temperature was 20°C, and medium flow was controlled with a Cole-Parmer Masterflex pump. Elutriation medium was phosphate buffered saline containing 0.9 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$, and 2% calf serum. Consecutive fractions of 150–300 ml were collected at increasing flow rates. Cytofluorometric analyses of cell cycle distributions were performed on fixed cells using a PAS-II flow cytometer (Partec). DNA was stained with 6 μmol/l 4,6-diamidino-2-phenylindol-dihydrochloride (DAPI).

2.3. Western blot analysis

Protein extracts were prepared as described [19]. After washing with phosphate-buffered saline cell lysis was performed using a buffer consisting of 10 mM Tris-HCl (pH 7.5), 250 mM sucrose, 160 mM KCl, 1.5 mM $MgCl_2$, 3 mM β-mercaptoethanol, 50 mM ε-amino-*n*-capronic acid, and 0.8 mg/ml digitonin. Extracts have been stored at −70°C and protein concentration was determined using the Bio-Rad protein assay reagent with bovine serum albumin as a standard.

Protein was denatured and reduced in a loading buffer containing 200 mM dithiotreithol and 4% SDS for 5 min at 95°C. 50–200 μg protein per lane were run on a 12.5% SDS-PAA gel and transferred to nitrocellulose by electroblotting. Blots were stained with Ponceau-S to confirm the loaded amount of protein. After blocking overnight at 4°C, immunodetection was performed using anti-p16 antibody (PharMingen, Cat.No. 15126E) at a dilution of 1:1000 and anti-pRb antibody (PharMingen, Cat.No. 14001A) at a dilution of 1:2000. Signals were developed using enhanced chemiluminescence (Amersham). Ratio of protein expression was determined by scanning the respective signal-densities. These values were set relative to the amount of loaded protein.

2.4. Northern blot analysis

mRNA expression was analysed as described previously [20]. 15 μg RNA, prepared by the guanidine isothiocyanate method, were denatured in 1 M deionized glyoxal, 4.8% dimethyl sulfoxide and 10 mM sodium phosphate (pH 6.8) for 1 h at 50°C. After electrophoresis in a 1% agarose gel, RNA was transferred to nylon membranes in 20 × SSC. After UV fixation, filters were hybridized in 1% BSA, 7% SDS, 0.5 M sodium phosphate (pH 6.8) and 1 mM EDTA for at least 10 h at 65°C. After washing for 20 min in 0.5% BSA, 5% SDS, 40 mM sodium phosphate (pH 6.8), 1 mM EDTA, and for 40 min in 1% SDS, 40 mM sodium phosphate (pH 6.8), 1 mM EDTA at 65°C, filters were exposed.

The probes used for hybridization were full length human p16/MTS1 cDNA [2], full length human thymidine kinase cDNA and cyclin D1 cDNA, and a 1300 bp cDNA fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Filters were stripped between hybridizations by incubation in 0.1 × SSC, 0.1% SDS for 25 min at 95°C. Autoradiographs were densitometrically scanned and quantitated.

3. Results

3.1. Separation of cells in different phases of the cell cycle by centrifugal elutriation

Regulation of gene expression which occurs during the stimulation of arrested cells (Go) to logarithmical growth is not really comparable to gene regulation throughout the ongoing cell cycle. Stimulation experiments mainly provide insights into the first round of cell cycle, which occurs after cells have been arrested by withdrawal of growth factors. Studying the ongoing cell cycle means to investigate logarithmically growing cells at specific moments when they pass G1-, S-, G2-, and/or M-phase. Counterflow centrifugal elutriation is the best methodical approach so far to analyse a regulation occurring during ongoing cell cycling. By this method cells are separated on the basis of size, and to a lesser extent by density, under very moderate conditions. This makes centrifugal elutriation ideal for isolating populations of cells in specific phases of the cell cycle with minimal perturbation of metabolic function. We separated normal phytohemagglutinin-stimulated human lymphocytes (PHA-lymph) and three different permanent human cell lines (Y79, HeLa, APL) according to their phases of the cell cycle by centrifugal elutriation. In the presented experiments, fewer and larger fractions were drawn in order to ensure a sufficient number of cells per fraction for the analyses of mRNA and/or protein expression. About half a million cells per separated fraction were stained with 4,6-diamidino-2-phenylindol-dihydrochlorid (DAPI) and cytofluorometrically analysed for DNA distribution. As indicated by the flowcytometric data presented in Figs. 1, 2 and 3, the separation quality of all performed elutriations was comparable. The first fraction contained constantly more than 80% G1 cells (mean 82%). The best S-phase fractions derived from each of the four cell lines also had comparable amounts of cells of different cell cycle positions. The average distribution of the obtained S-phase fractions was 15% G1, 71% S, and 14% G2/M. The amount of G2 cells (also including mitotic cells) in the end fractions varied from 69% (PHA-lymph, Fig. 3) to 84% (HeLa, Fig. 3) (mean of all elutriations 78%). In general, the separation data were very comparable to earlier obtained results with the same or similar cells [18]. Since the quality of separation was that comparable, we concluded that any differences possibly occuring during the cell cycle regulation between the studied cell lines would not be caused by experimental variations.

Table 1
Properties of logarithmically growing human cells

	PHA-lymph (%)	Y79 (%)	HeLa (%)	APL (%)
pRb[a]	100	0	82	91
p16	4	3	100	6
G1[b]	61	44	46	44
S	25	37	34	41
G2/M	14	19	20	15

[a]Retinoblastoma protein (pRb) and p16 protein expression were determined by Western blot analyses. The blots were densitometrically quantitated and the highest value was set 100%.
[b]DNA distributions were determined by Flow Activated Cell Analyses. The values are means of three independent determinations. PHA-lymph, phytohemagglutinin-stimulated normal lymphocytes; Y79, pRb-negative cells; HeLa, papilloma virus-transformed cervic carcinoma cells; APL, acute promyelocytic leukemia cells.

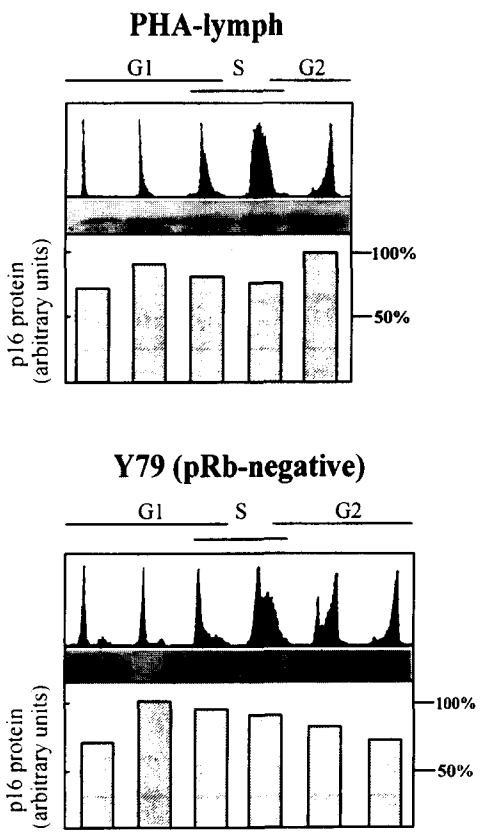

Fig. 1. Regulation of p16 protein expression throughout the ongoing cell cycle. Logarithmically growing phytohemagglutinin-stimulated lymphocytes (PHA-lymph) and Y79 cells were separated into fractions of different cell cycle phases by centrifugal elutriation. The fractions were cytofluorometrically analysed for DNA distribution (upper panel) and for p16 protein expression by Western blot analysis (middle panel). The Western blot signals were densitometrically quantified and related to each other by setting the highest value to 100% (lower panel).

3.2. *p16 protein expression throughout the ongoing cell cycle*

It has been observed that p16 protein expression is elevated in pRb-negative cells and in DNA tumor virus-transformed cells [6,12,13,17]. We compared the levels of protein expression of pRb and p16 in four different human cell lines. Western blot analyses using an anti-pRb antibody as well as an anti-p16 antibody revealed that the papilloma virus-transformed human cell line HeLa expressed 25-fold higher levels of p16 than normal phytohemagglutinin-stimulated lymphocytes (Table 1). In addition, p16 protein expression was about 16-fold higher in logarithmically growing HeLa cells than in cycling acute promyelocytic leukemia cells. In contrast to data described so far, the pRb-negative cell line Y79 did express p16 protein at very low levels, comparable to normal lymphocytes. Since it has been demonstrated that protein expression of this CDK-inhibitor is growth-regulated [12], one could speculate that its expression is also S-phase-regulated during the ongoing cell cycle. This correlation stands true for some growth regulated genes (e.g. thymidine kinase), but certainly not for all (e.g. c-*myc*). Accordingly, we correlated the amount of S-phase cells to the p16 protein expression in logarithmically growing cells (Table 1). Normal cycling lymphocytes contained about 17% more G1 cells and about 14% less S-phase cells than the pRb-negative Y79 cells and the acute promyelocytic leukemia cells (APL). Nevertheless, the amount of p16 protein expression was almost identical in all three lines. Furthermore, although logarithmically growing HeLa cells, Y79 cells, and APL cells presented a comparable distribution of G1, S, and G2/M cells, the level of p16 expression is much higher in HeLa cells than in the other two lines. From these data we can conclude that there is no correlation between p16 protein expression and S-phase content of these logarithmically growing cells. We also want to speculate that the capacity of papilloma virus to increase p16 expression in cycling cells is distinct from the capacity of this virus to induce cell entry into S-phase and thereby fast cell cycling of growing cells.

We separated phytohemagglutinin-stimulated human lymphocytes according to their phase of the cell cycle by centrifugal elutriation. These cells are neither immortalized nor transformed, and therefore provide an optimal system to study p16 regulation throughout the normal eukaryotic cell cycle. Western blot analysis revealed that p16 protein expression is not cell cycle regulated in these cells (Fig. 1). The maximal fluctuation of p16 protein amount between the different fractions was not more than 1.3-fold. Loss of retinoblastoma protein did not influence the course of cell cycle regulation of this cyclin-dependent kinase inhibitor. pRb-negative Y79 cells did not exhibit any fluctuation of p16 protein during their ongoing cell cycle (Fig. 1). To ask whether any other form of transformation, which does not influence regulations involving pRb, DNA tumor viruses, and/or the transcription factor E2F, leads to alterations of p16 regulation, we studied acute promyelocytic leukemia cells (APL). Not surprisingly, those cells did not exhibit cell cycle-dependent fluctuations of p16 protein expression either (Fig. 2). Studying logarithmically growing cells, we demonstrated that papilloma virus-transformed HeLa cells exhibit highly increased p16 protein levels compared to normal lymphocytes, pRb-negative cells and APL cells. Accordingly, the question arose whether p16 protein expression is upregulated in all phases of the cell cycle of HeLa cells, or if its expression is deregulated in these cells. Centrifugal elutriation experiments revealed that p16 protein is not cell cycle regulated in HeLa cells (Fig. 2). One must conclude that this CDK-inhibitor is higher expressed in all cell cycle phases (G1, S, G2) of HeLa cells compared to normal cells, such as phytohemagglutinin-stimulated lymphocytes. Absolute quantitative comparison of p16 protein levels in the different phases of the cell cycle of HeLa cells and PHA-lymph cells demonstrated that HeLa cells indeed express much more p16 protein in all phases of the cell cycle (data not shown).

3.3. *p16 mRNA expression throughout the ongoing cell cycle*

We further asked at which level the cell cycle independent regulation of p16 protein is determined. It might be that p16 mRNA is expressed at a very defined time point during the eukaryotic cell cycle, but p16 protein is so stable that mRNA fluctuations are not reflected in protein regulation. However, Northern blot analyses after centrifugal elutriation demonstrated that p16 mRNA is constitutively expressed throughout the cell cycle (Fig. 3).

Furthermore, quantitative comparison revealed that p16 mRNA is constantly much higher expressed in HeLa cells than in normal lymphocytes. These data allow to conclude that papilloma virus transformation leads to an upregulation of p16

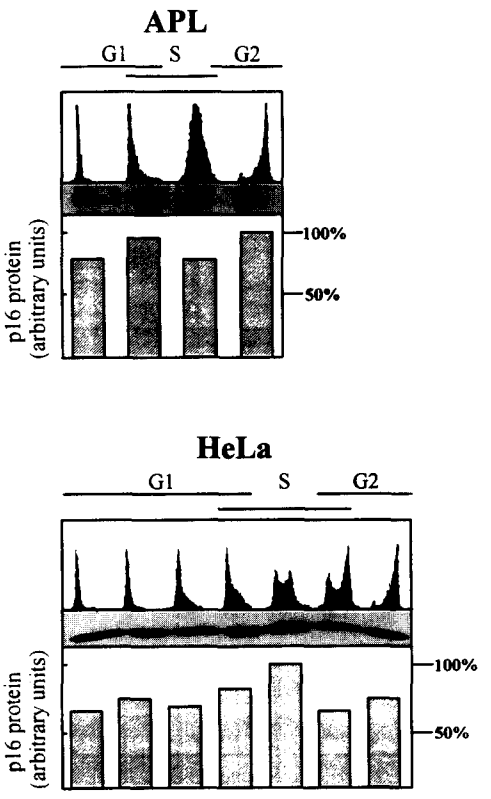

Fig. 2. p16 protein expression throughout the cell cycle of acute promyelocytic leukemia cells (APL) and of papilloma virus transformed HeLa cells. Cells were separated according to their phases of the cell cycle by centrifugal elutriation. Cytofluorometric analyses of the DNA distributions of the separated fractions are presented on top of the figure. Western blot analysis and its quantification are presented in the middle and the lower pannel (compare Fig. 1).

protein expression determined by an induction of p16 mRNA expression in all phases of the cell cycle.

As described above it has been speculated that p16 expression could be regulated via the transcription factor E2F [12,15–17]. To ensure that fluctuations of E2F-dependent transcription throughout the cell cycle can be detected in our normal lymphocytes, we rehybridized the Northern blot shown in Fig. 3 with a thymidine kinase (TK) cDNA-probe. Expression of TK mRNA has been shown to be regulated via E2F [21] and to increase at the G1/S boundary and decrease during G2 of the normal ongoing cell cycle [22]. We demonstrated that TK mRNA is cell cycle-regulated in the same cells in which p16 expression is cell cycle-independent (Fig. 4). The main antagonist of p16 is cyclin D1, because it activates CDK4 and CDK6. Cyclin D1 mRNA expression is high in early G1 and decreases during S-phase of the cell cycle (Fig. 4).

4. Discussion

It has earlier been demonstrated that p16, the inhibitor of cyclin dependent kinase 4 and 6, is overexpressed in retinoblastoma protein-negative cells as well as in DNA tumor virus-transformed cells [6,12,13,17]. This observation and the fact that p16 protein expression increases when growth-arrested cells are restimulated [12] led to the speculation about the possible involvement of the transcription factor E2F in the regulation of the expression of this inhibitor [12,15–17]. We examined the regulation of this cyclin-dependent kinase inhibitor during the ongoing cell cycle after centrifugal elutriation. Neither p16 mRNA nor its protein expression are regulated during the cell cycle of normal phytohemagglutinin-stimulated lymphocytes, retinoblastoma protein-negative cells, papilloma virus-transformed HeLa cells, and acute promyelocytic leukemia cells. However, the fact that we did not observe cell cycle specific regulation of p16 does not exclude the possibility that E2F plays a role in the basal expression or the growth regulation of this CDK-inhibitor. There are examples for both E2F-dependent genes which are S-phase regulated during the ongoing cell cycle and for E2F-regulated genes which are not. The DNA precursor pathway enzyme thymidine kinase has been shown to be regulated via E2F [21]. Furthermore, TK expression is known to be low in Go and to dramatically increase before the onset of S-phase after restimulation (reviewed in [23]). It has

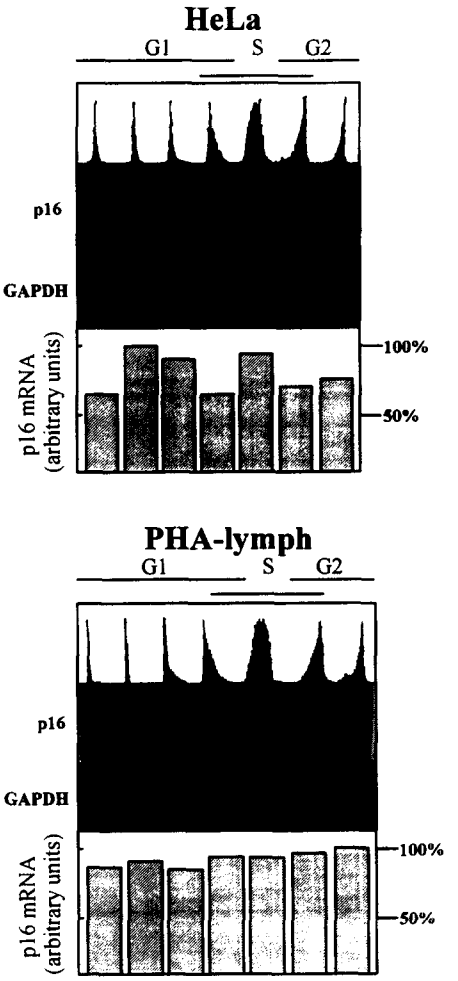

Fig. 3. Regulation of p16 mRNA expression throughout the ongoing cell cycle. Logarithmically growing phytohemagglutinin-stimulated lymphocytes (PHA-lymph) and HeLa cells were separated into fractions of different cell cycle phases by centrifugal elutriation. The fractions were cytofluorometrically analysed for DNA distribution (upper panel). mRNA of each fraction was blotted to nylon and sequentially hybridized with p16 cDNA (p16) and cDNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The Northern blot signals were densitometrically quantified and p16 mRNA expression was related to GAPDH expression (the highest value was set to 100%; lower panel).

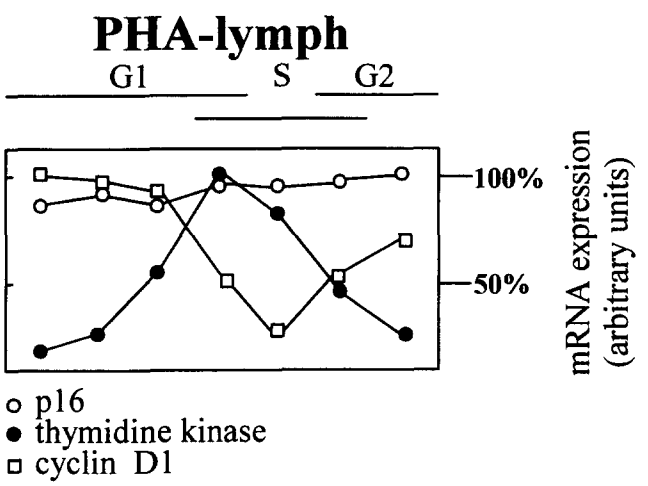

Fig. 4. mRNA expression of p16, thymidine kinase and cyclin D1 throughout the cell cycle of normal lymphocytes. The expression of these genes has been normalized to the levels of GAPDH mRNA expression. The highest value within each analysed gene has been set to 100% (values between different genes are not comparable).

recently been demonstrated that TK mRNA as well as protein expression is S-phase regulated throughout the normal ongoing cell cycle [22,24]. On the other hand, the oncogene c-*myc* has also been shown to be regulated via the transcription factor E2F [25]. Although c-*myc* expression is highly upregulated during restimulation from Go to logarithmic growth, it is continously expressed in proliferating cells in a cell cycle-independent manner [26].

The interplay of cyclins, cyclin-dependent kinases, the inhibitors of these kinases, and pRb enables the cell cycle clock to control its own forward progress. One described circuit, which is responsible for the regulation of the transition of cycling cells over the restriction point in mid to late G1, involves the D type cyclins, CDK 4 and CDK6, and the specific inhibitor of these two kinases, p16. CDK4 as well as CDK6 phosphorylate pRb and thereby activate the transcription factor E2F, to promote S-phase-specific gene expression (reviewed in [5,14,15]). The expression of these cyclin-dependent kinases is constant throughout the cell cycle; their regulation occurs at other levels. Binding of D-type cyclins to these CDKs as well as phosphorylation of these CDKs by the cyclin-dependent kinase activating kinase (CAK) induce their activites. Binding of p16 to CDK4 and/or CDK6 inhibits their association with D-type cyclins and thereby inactivates these kinases (reviewed in [5] and [27]). The question arising is, how are the activities of CDK4 and CDK6 restricted to a cell cycle period from mid to late G1? The earlier observation that p16 is growth-regulated induced speculations about the following model of CDK4/CDK6 regulation during the ongoing cell cycle [12,15–17]: Cyclin D is transcriptionally upregulated in early G1 and subsequently activates CDK4 and CDK6 to phosphorylate pRb. Phosphorylated Retinoblastoma protein releases a transcription factor that induces p16 expression. p16 causes dissociation of the cyclin D/CDK complexes by binding to the kinases. Thereafter, cyclin D, not longer protected by association with CDK4/CDK6, is degraded. This model suggested that CDK4 and CDK6 activities are switched on by the transcriptional upregulation of D-type cyclins in G1, and are switched off by the transcriptional upregulation of the cyclin-dependent kinase inhibitor p16. In accord with this model, one would expect to detect a sharp increase of p16 expression in late G1. The data we present in this report do not support this model, since we did not observe any cell cycle-dependent regulation of p16 expression throughout the ongoing cell cycle.

When p16 is not regulated during the cell cycle, how are the activites of CDK4 and CDK6 restricted to G1? We favour a model in which the main cell cycle regulators of these kinase activities are the D-type cyclins (compare Fig. 4). Analysing expression during the ongoing cell cycle after centrifugal elutriation, we observed high levels of cyclin D1 in early G1 and a very sharp decrease of its expression at the G1/S boundary. Our results are in agreement with earlier data on cyclin D1 expression during restimulation of arrested normal diploid fibroblasts: The level of cyclin D1 was low in quiescent cells, and in early G1 the protein was synthesized rapidly and accumulated steadily. As cells entered S-phase cyclin D1 disappeared from the nucleus and the total abundance of the protein decreased [28]. In the same cells, in which we observed the G1-phase specific cyclin D1 expression, p16 was constantly expressed over the cell cycle, whereas the E2F-dependent transcription of thymidine kinase is highly increased at the G1 to S-phase transition. Accordingly, we suggest that as long as high levels of D-type cyclins are expressed in the cell, their antagonist p16 cannot bind to CDK4 and/or CDK6 and thereby inactivate them. During that time E2F-dependent transcription is induced, as detectable by TK mRNA expression. After the G1/S boundary mRNA transcription of cyclin D is switched off and p16 protein can inhibit the kinase activities. This suggestion is in perfect agreement with our earlier observation that E2F-dependent transcription of thymidine kinase and dihydrofolate reductase is induced during a short and very distinct time period, ranging from late G1 to mid S. During S-phase this E2F-dependent transcription already decreases again [24]. However, although our proposed model can explain how the interactions between p16, D-type cyclins, and CDK4/CDK6 could be cell cycle-regulated, the G1/S-regulation of E2F-dependent transcription is more complicated, since another complex formed between CDK2 and cyclin E also phosphorylates pRb in late G1 and thereby activates E2F [5,15,27].

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