Specific transformation abolishes cyclin D1 fluctuation throughout the cell cycle

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

[a] *Obstetrics and Gynecology, University of Vienna, Department of Prenatal Diagnosis and Therapy, AKH-EBO-E6, Währinger Gürtel 18–20, A-1090 Vienna, Austria*
[b] *Institute of Molecular Biology, Vienna Biocenter, University of Vienna, Dr. Bohrgasse 9, A-1030 Vienna, Austria*
[c] *Institute of Molecular Genetics, Vienna Biocenter, University of Vienna, Dr. Bohrgasse 9, A-1030 Vienna, Austria*

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Abstract **We analysed cyclin D1 mRNA and protein expression in several different cell types after separating these cells according to their different cell cycle phases by centrifugal elutriation. In normal human and rat fibroblasts cyclin D1 expression is high in early to mid G1 and decreases about 6–7 fold before onset of replication. It has been demonstrated that specific transforming events, such as loss of functional retinoblastoma protein, overexpression of c-*myc*, and transfection with the human papillomavirus oncoproteins E6 and E7 cause transcriptional downregulation of cyclin D1 expression in logarithmically growing cells. We found that such transformed cells exhibit loss of the cell cycle-dependent cyclin D1 fluctuation accompanied with reduced upregulation of cyclin D1 in G1 phase. The data presented here provide the experimental support for a recently suggested model involving the function of the retinoblastoma protein in cyclin D1 cell cycle regulation.**

Key words: Cyclin D1 expression; Cell cycle; Retinoblastoma protein; Transformation

1. Introduction

Cyclin D1 activates the cyclin-dependent kinases 4 and 6 to phosphorylate the retinoblastoma protein (pRB) during G1 phase, which leads to the activation of the transcription factor E2F (for recent reviews see [1-3]). One main antagonist of this function of cyclin D1 is p16, the inhibitor of cyclin-dependent kinases 4 and 6 [4]. The recent observation that p16 is constitutively expressed throughout the ongoing cell cycle allowed us to postulate a model in which D-type cyclin expression is the main cell cycle-regulatory level of these kinase activities [5]. Accordingly, one would expect cyclin D1 expression to be high in G1 and to decrease later on in the cell cycle. Nevertheless, so far data on cell cycle regulation of cyclin D1 expression in different cell types obtained using different synchronization procedures are conflicting. (1) In HeLa cells after release from a G1/S block cyclin D1 mRNA begins to rise just after S phase, peaks around M phase and gradually declines during G1 phase [6,7]. (2) In mammary epithelial cells and normal human fibroblasts, synchronized by growth factor deprivation and readdition, cyclin D1 mRNA and protein peaks in early to mid G1 [7-9]. (3) In human urinary bladder carcinoma cells cyclin D1 peaks in early to mid G1 phase, shown in cells after release from a nocodazole block [10]. (4) This also holds true for the murine homologue of cyclin D1, CYL1, analysed after CSF-1 stimulation of quiescent mouse macrophages. Interestingly, in these cells cyclin D1 does not decrease any more as long as CSF-1 is present [11]. (5) A newly established cytofluorometric approach revealed that cyclin D1 protein is high in G1 and decreases before S in normal cells [12]. Furthermore, the authors expanded their analyses on B-cell tumours carrying the t(11;14) translocation, which juxtaposes cyclin D1 under the control of the IgH enhancer. The cyclin D1 cell cycle regulation in these tumours is identical to that in normal cells [13]. (6) On the other hand no cell cycle-dependent fluctuation of cyclin D1 expression has been observed in the human fibroblast line WI-38 released from a thymidine/aphidicolin block or in the myeloid cell line HL-60 [14,15]. These discrepancies might be related to the specific synchronisation procedures used or might be due to cell line-specific differences in cyclin D1 regulation (discussed in [15-17]). The latter must especially be considered since data have accumulated showing that specific transforming events cause downregulation of cyclin D1 expression in logarithmically growing cells. Accordingly, in the present report we analyse cyclin D1 cell cycle regulation in several cell types all separated according to the different cell cycle phases by centrifugal elutriation. We compare the cyclin D1 regulation of normal cells to that of specifically transformed cells.

2. Materials and methods

2.1. Cells, elutriation and FACS analysis

All used cells and cell lines were obtained from the American Type Culture Collection. The cell growing conditions have been described in detail [18]. All cultures were routinely screened for the absence of mycoplasma. c-*Myc* overexpressing Rat1 cells were kindly provided by M. Eilers. These cells express a protein containing the hormone-binding domain of the human oestrogen receptor fused to the 3′ end of human Myc. The function of this overexpressed Myc is oestrogen-dependent [19]. Cells were fractionated by centrifugal elutriation as previously described [4,5]. Each separated fraction was monitored by a PAS-II flow cytometer (Partec) using 6 μmol/l 4,6-diamidino-2-phenylindol-dihydrochloride to stain DNA.

2.2. Western blot analysis

Protein extraction and Western blot analysis were performed as described [20]. For immunodetection an anti-pRB antibody (Pharmingen, 15126E), an anti-human cyclin D1 antibody (Pharmingen, 14726E) and an anti-mouse cyclin D1 antibody (Progen, DCS-6) were used. In the used cells both cyclin D1 antibodies have been shown not to cross-react with D2 or D3 cyclins (see also [9,10]). Absolute quantification of the signals was performed: each blot was originally exposed for different time periods, and we chose exposures of comparable intensity, which still represented the linear range. The

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

**O.P. and T.S. contributed equally to this work.

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signals were scanned densitometrically, and the obtained values were each divided by the exposure time and normalized to μg loaded protein.

2.3. Thymidine kinase assay

Thymidine kinase activity in vitro was measured by conversion of radioactive thymidine to thymidine monophosphate [18]. Enzyme activities were normalized to total protein concentration, determined using the BioRad protein assay reagent with bovine serum albumin as a standard.

2.4. Northern blot analysis

RNA preparation and Northern blot procedure are described in [20]. The probes for hybridization were full length human and rat cyclin D1 cDNA and a 1300 bp cDNA fragment of the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Quantification of the signals was performed as described for Western analyses and cyclin D1 mRNA expression was normalized to GAPDH expression.

3. Results

3.1. Expression of cyclin D1 throughout the ongoing normal cell cycle

To investigate the normal cyclin D1 cell cycle regulation, we chose non-transformed diploid human and rat fibroblasts. Since differences in the genomic organisation of the human and the rodent homologues of cyclin D1 have been described ([21] and references therein), we wanted to compare cyclin D1 cell cycle regulation in normal human and rodent cells. Logarithmically growing cells (Table 1) were separated by centrifugal elutriation. About half a million cells per separated fraction were cytofluorometrically analysed for DNA distribution (upper panels in Fig. 2–5). As indicated by the cytofluorometric data, the separation quality of the elutriations of human and rat fibroblasts (Figs. 2 and 3) was very comparable (see also Fig. 5). The first fractions contained more than 89% G1 cells, the best S phase fractions represented over 75% S phase cells, and the amount of G2 cells (also including mitotic cells) in the end fractions was about 76%. To further confirm that the separation quality was comparable and to ensure that the separated cells did not exhibit considerable perturbations of metabolic functions, we analysed thymidine kinase (TK) activity of each fraction. TK activity has been shown to increase at the G1/S boundary throughout the ongoing cell cycle [18,22]. In both cell types the cyclin D1 protein level was high in mid G1 and decreased about 6–7 fold before S, right at the time when TK activity increased (Figs. 2 and 3A). To obtain more information about the purity of the different elutriated G1 fractions we analysed the status of pRB phosphorylation

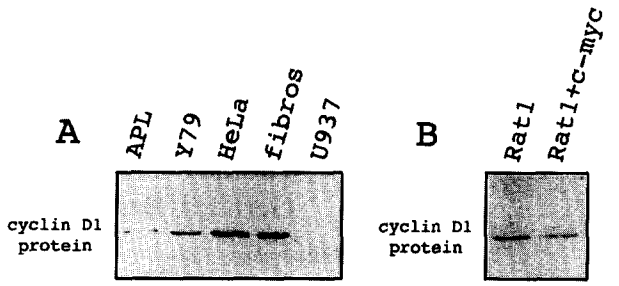


Fig. 1. Cyclin D1 protein levels in different human (A) and rat (B) cells. Protein of the different cell types was denatured and reduced. It was run on a 12.5% SDS-PAA gel and transferred to nitrocellulose by electroblotting. Blots were stained with Ponceau-S to confirm equal amounts of loaded protein. After blocking overnight at 4°C, immunodetection was performed using anti-cyclin D1 antibody. Signals were developed using enhanced chemilumescence. APL, acute promyelocytic leukaemia cells; Y79, retinoblastoma cells; HeLa, cervix carcinoma cells, papillomavirus-transformed; fibros, normal diploid fibroblasts; U937, histiocytic lymphoma cells; Rat1, normal diploid fibroblasts; Rat1+c-myc, c-*myc* overexpressing Rat1-MycER cells.

in normal Rat1 cells. pRB is known to be phosphorylated in mid G1 (reviewed in [3]). Our results, that phosphorylated pRB occurs for the first time in fraction 2, demonstrated that the first early G1 fraction does not contain significant contamination by later G1 cells (Fig. 3B). We further showed that the cell cycle-dependent fluctuation of cyclin D1 protein in these normal cells is paralleled by an identical mRNA oscillation (Figs. 2 and 3A and Fig. 5). Taken together our data confirm that cyclin D1 mRNA and protein peaks in early to mid G1 and decreases before S throughout the ongoing cell cycle of normal cells and that expression of the human and rodent homologue of cyclin D1 oscillates identically during the cell cycle.

3.2. Expression of cyclin D1 throughout the cell cycle of transformed cells

Recently, data have accumulated describing a transcriptional downregulation of cyclin D1 expression in specifically transformed cells. First, cells lacking functional pRB have been reported to express lower levels of this cyclin than their normal counterparts. It has been demonstrated that these cells do not require cyclin D1 function in G1 for proper cell cycle progression [23-26]. Direct proof for the role of pRB in regulating cyclin D1 expression has been obtained by transfection of a reporter gene under control of the cyclin D1 promoter,

Table 1. Cyclin D1 abundance in logarithmically growing normal and transformed cells

Cell type and origin	G1 (%)	S (%)	G2/M[a] (%)	pRB protein	Cyclin D1 protein	Cyclin D1 mRNA
Normal						
Human fibroblasts	63	22	15	+	+++	++
Rat1 fibroblasts	59	28	13	+	+++	++
Transformed						
Human Y79 retinoblastoma cells	44	37	19	−	+	+
Human HeLa cervix carcinoma cells[b]	46	34	20	+	++	ND
Human acute promyelocytic leukaemia cells	44	41	15	+	±	ND
Human U937 histiocytic lymphoma cells	48	37	15	ND	−	−
Rat1 fibroblasts; c-*myc* overexpressing	58	30	12	+	++	+

[a]DNA distributions were determined by flow activated cell analyses. The values are means of three independent determinations.
[b]Human papillomavirus 18 (E6 and E7)-transformed cells.
+++, detection of a very strong signal; ++, detection of a strong signal; +, detection of a weak signal; ±, detection of a very weak signal; − no signal detected; ND, not determined.

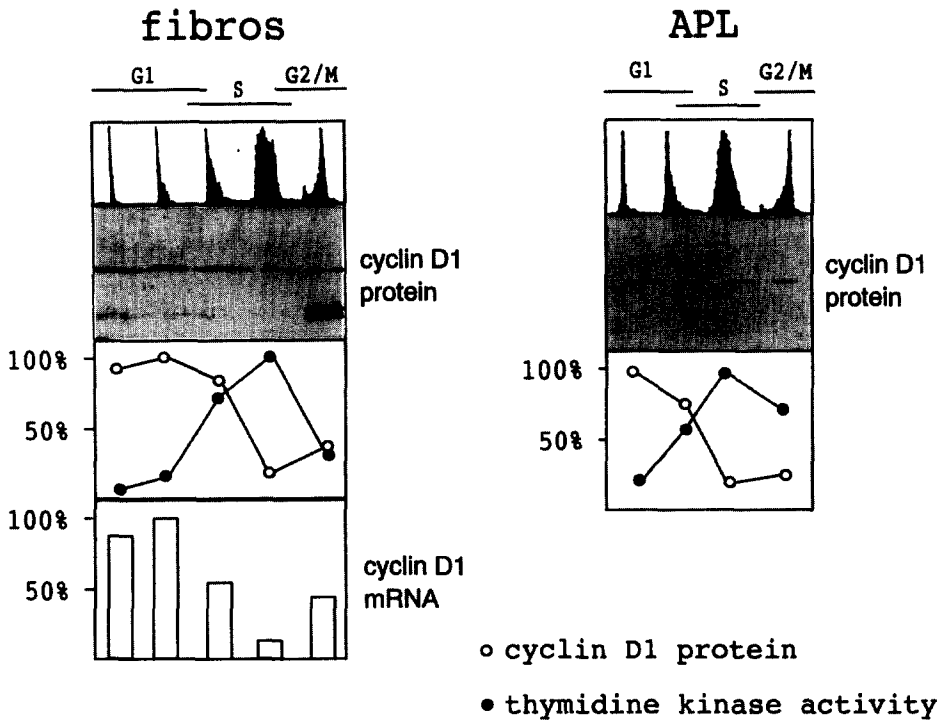
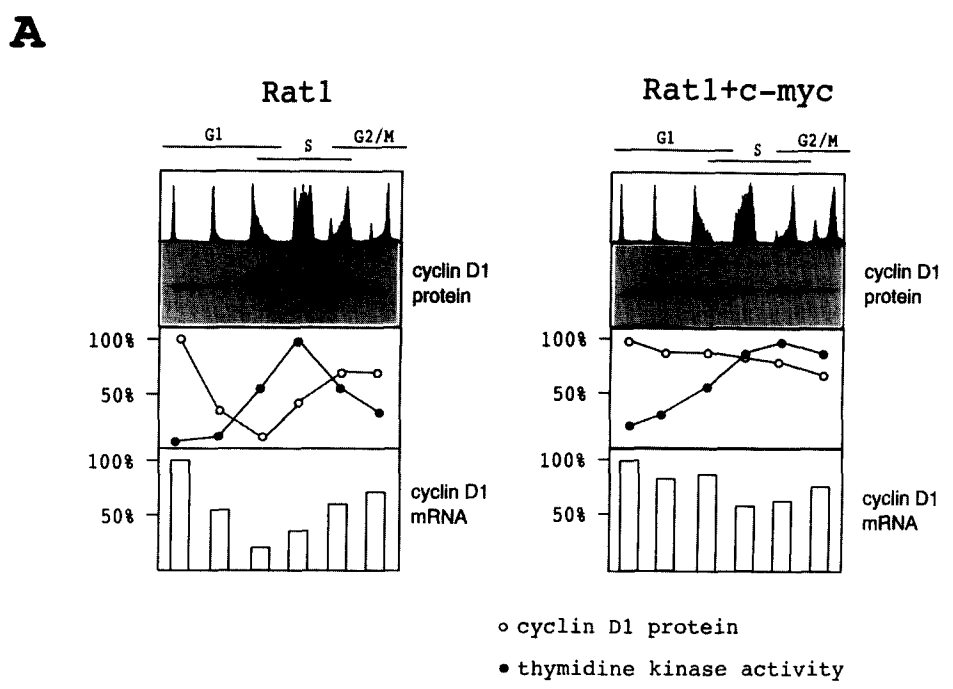


Fig. 2. Cell cycle regulation of cyclin D1 expression in normal human fibroblasts (fibros) and in human acute promyelocytic leukaemia cells (APL). Logarithmically growing cells were separated into fractions of different cell cycle phases by centrifugal elutriation. The fractions were cytofluorometrically analysed for DNA distribution (upper panel) and for cyclin D1 protein expression (compare Fig. 1). The Western blot signals were quantified densitometrically and related to each other by setting the highest value within one cell type at 100%. The fractions were also analysed for thymidine kinase activity. Enzyme activity is presented in pmol TMP formed/mg protein/h, highest value set at 100%. Absolute values of the different cells cannot be compared, since the presented blots represent different exposure times. For quantitative comparison see Figs. 1 and 5. In the different fractions of the normal fibroblasts cyclin D1 mRNA expression was analysed by Northern blot and related to glyceraldehyde-3-phosphate dehydrogenase expression (the highest value was set at 100%; lower panel, fibros).

together with the *Rb* gene, into pRB-deficient cells: expression driven by the exogenous as well as the endogenous cyclin D1 promoter was induced via pRB [27]. Secondly, cell transformation by transfection with DNA tumour virus oncoproteins, such as SV40 large T antigen, adenovirus E1A, and papillomavirus E6/E7, also causes downregulation of cyclin D1 expression and makes the cell cycle progression cyclin D1-independent [24,28,29]. Finally, high and continuous overexpression of the proto-oncogene c-*myc* has also been shown to downregulate cyclin D1 expression [30,31]. Accordingly, we wanted to investigate whether this downregulation in transformed cells occurs to the same extent in all cell cycle phases and therefore does not cause alterations in cyclin D1 cell cycle fluctuation, or whether this downregulation is accompanied by changes in cyclin D1 cell cycle regulation. To this end, we decided to analyse the pRB-negative cell line Y79, human papillomavirus 18 (E6 and E7)-transformed HeLa cells and c-*myc* overexpressing Rat1 fibroblasts. During logarithmic growth all these cells exhibit lower levels of cyclin D1 protein and mRNA than normal fibroblasts do (Table 1, Fig. 1). In our analysis we included acute promyelocytic leukaemia (APL) cells as a control for cells with very low cyclin D1 expression, which are transformed independently from any of the transformation events described above (Table 1, Fig. 1). Since in these APL cells cyclin D1 expression was almost undetectable, we had to draw larger and therefore fewer fractions after centrifugal elutriation to ensure a sufficient number of cells to obtain cyclin D1 Western blot signals (Fig. 2). In all other transformed cell types the separation quality of the centrifugal elutriations was very comparable to each other and to normal cells, as indicated by flowcytometric data (see also Fig. 5) and by TK activity results (Figs. 2, 3A and Fig. 4).

The cyclin D1 protein oscillation is lost throughout the ongoing cell cycle of pRB-negative Y79 cells, papillomavirus-transformed HeLa cells and c-*myc* overexpressing Rat1 cells (Figs. 3A, 4 and Fig. 5). Cyclin D1 mRNA does not fluctuate during the cell cycle of c-*myc* overexpressing Rat1 cells (Fig. 3A) or Y79 cells (Fig. 4). Absolute quantitative comparison revealed that whereas the level of cyclin D1 expression is comparable in the S phase fractions of normal, pRB-negative, papillomavirus-transformed and c-*myc* overexpressing fibroblasts, the upregulation of cyclin D1 in G1 is greatly reduced in the transformed cells (Fig. 5). These data strongly suggest that lower cyclin D1 expression in these logarithmically growing transformed cells is caused by a loss of the transcriptional upregulation of this cyclin in G1.

Quantitative comparison further revealed that cyclin D1 expression in acute promyelocytic lymphocytes is lower in all cell cycle phases, but that these cells also express a G1-specific 6–7-fold upregulation of this cyclin (Figs. 2 and 5; APL). The observed low expression of cyclin D1 in this cell type might be due to its haematopoietic origin, rather than to its transformation status. It has earlier been shown that specific haematopoietic cells do not express cyclin D1 ([12,23] and references therein). These analyses revealed that most of the haematopoietic cell types which express cyclin D1 were of the myeloid lineage whereas cyclin D1 expression was not detectable in lymphoid cells. One could speculate that the low cyclin



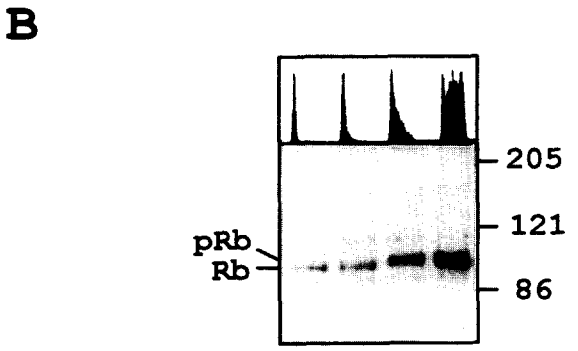


Fig. 3. Cell cycle regulation of cyclin D1 expression in normal and c-*myc* overexpressing Rat1 fibroblasts. Rat1 and Rat1-MycER were separated by centrifugal elutriation. (A) Cyclin D1 protein expression in each fraction was determined by Western blot analysis and compared to thymidine kinase activity (compare Fig. 2). mRNA of each fraction was blotted to nylon and sequentially hybridized with cDNA probes specific for cyclin D1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The Northern blot signals were densitometrically quantified and cyclin D1 mRNA expression was related to GAPDH expression (the highest value was set at 100%; lower panel). Absolute values of the different cells cannot be compared. For quantitative comparison see Figs. 1 and 5. (B) Status of retinoblastoma protein phosphorylation in the different G1 fractions of elutriated Rat1 cells. Protein extracts of the first four Rat1 elutriation fractions presented above (A) were investigated by Western blot analysis using anti-retinoblastoma protein antibody. pRb, phosphorylated forms of retinoblastoma protein.

D1 expression in our APL cells might be due to a relative abundance of lymphoid progenitors in this line. In this context, it might be important to note that another promyelocytic leukaemia cell line (HL-60) has also been shown to express cyclin D1 [15], whereas we did not detect cyclin D1 expression in the monocytic cell line U937 (Table 1, Fig. 1).

Taken together, our data indicate that certain transformation events, such as loss of pRB, c-*myc* overexpression, and DNA tumour virus transfection, share the capacity to abolish the cell cycle-dependent cyclin D1 oscillation.

4. Discussion

We analysed cyclin D1 mRNA and protein fluctuations throughout the ongoing unperturbed cell cycle of different cell types after centrifugal elutriation. In normal cells cyclin D1 mRNA and protein expression is high in G1 and decreases 6–7 fold at the G1/S boundary. This regulation is identical in human and rodent cells. In the present report it is demonstrated for the first time that cells transformed via loss of pRB, papillomavirus transfection or c-*myc* overexpression exhibit loss of this cell cycle-dependent cyclin D1 fluctuation accompanied with reduced upregulation of cyclin D1 in G1 phase. Our data make it possible to map the effects of the studied transforming events on cyclin D1 regulation to the G1 phase of the continuous cell cycle. The observed alterations are no general effect of transformation since acute promyelocytic leukaemia cells (this report) and e.g. urinary bladder carcinoma cells [10] exhibit the normal cyclin D1 cell cycle regulation. We used HeLa, Y79, and APL cells to investigate

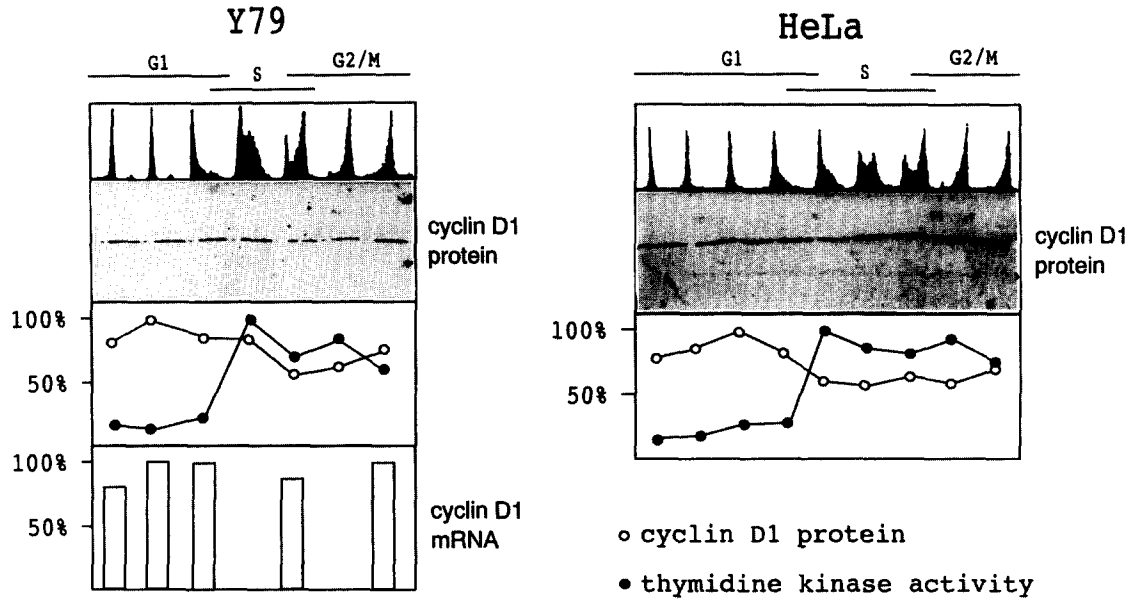


Fig. 4. Cell cycle regulation of cyclin D1 expression in human pRB-negative Y79 cells and in human papillomavirus-transformed HeLa cells. Cells were separated by centrifugal elutriation. Cyclin D1 protein expression in each fraction was determined by Western blot analysis and compared to thymidine kinase activity (compare Fig. 2). mRNA of different fractions of elutriated Y79 cells was blotted to nylon and sequentially hybridized with cDNA probes specific for cyclin D1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The Northern blot signals were densitometrically quantified and cyclin D1 mRNA expression was related to GAPDH expression (the highest value was set at 100%; lower panel, Y79).

the consequences of the according transformation events for cyclin D1 cell cycle expression, since those cell lines were already analysed for cyclin D1 regulation during random growth [8,15,23]. The Rat1-MycER cells enabled us to selectively connect the observed alterations of cyclin D1 cell cycle regulation to the function of the proto-oncogene c-*myc* [19,30,31]. The detailed description of the regulation of p16 and pRB expression, as well as of E2F-dependent transcription in the used cell lines [4,5,8,15,22,23,31,33] further facilitated the conclusion of a causal connection between the transformation events the cell lines were derived from, and cyclin D1 deregulation.

Our data very strongly support a recently suggested model for cyclin D1 cell cycle regulation [24,27]: transcription from the cyclin D1 promoter is driven, directly or indirectly, by the underphosphorylated form of pRB, present in early and mid G1. Inactivation of pRB via phosphorylation in late G1 would shut off cyclin D1 transcription. Our observed cell cycle-dependent fluctuation of cyclin D1 expression in normal cells is in perfect agreement with this hypothesis. It has further been concluded that tight complex formation with a viral oncoprotein would occupy the available pRB pockets in G1, thereby preventing pRB's normal activating role for cyclin D1 expression. In line with this model, one would expect that cyclin D1 expression can also not be induced in the G1 phase of pRB-negative cells. Our quantitative comparison revealed that whereas the levels of cyclin D1 protein and mRNA expression in S phase of normal fibroblasts, pRB-negative, and DNA tumour-virus transformed cells are comparable, the observed G1 induction of cyclin D1 expression in normal fibroblasts is greatly diminished in these transformed cells (Fig. 5). This observation can be perfectly explained by the postulated model. In this context it is important to note that very recent data demonstrated that pRB-deficient cells do not express lower levels of cyclin D1 than their normal counterparts, indicating that loss of pRB is necessary but not sufficient to downregulate cyclin D1 expression [26,32].

Our data that c-*myc* causes a decrease of cyclin D1 in logarithmically growing cells are in agreement with results of other groups: more detailed molecular analyses have revealed that repression of cyclin D1 by c-*myc* does not require association of Myc and Max in vivo, occurs at the level of transcription and is mediated by core elements of the cyclin D1 promoter. Interestingly, the authors showed that a small domain in the amino terminus of c-*myc* with structural similarity to the amino terminus of E1A is critical for repression of cyclin D1 [30,31]. The exact molecular mechanism of how overexpression of c-*myc* causes downregulation of cyclin D1 is still unclear. It has recently been demonstrated that, unlike oncogenes of DNA tumour viruses, constitutive expression of Myc does not relieve the requirement for cyclin D1 function [33]. These data allow the conclusion that Myc's role in D1 regulation is different from that played by DNA tumour virus oncogenes. Nevertheless, since very recently published data demonstrate that pRB phosphorylation is a downstream target of c-*myc* [33], the possibility that pRB is involved in the cascade of cyclin D1 downregulation via c-*myc* has still to be kept in mind.

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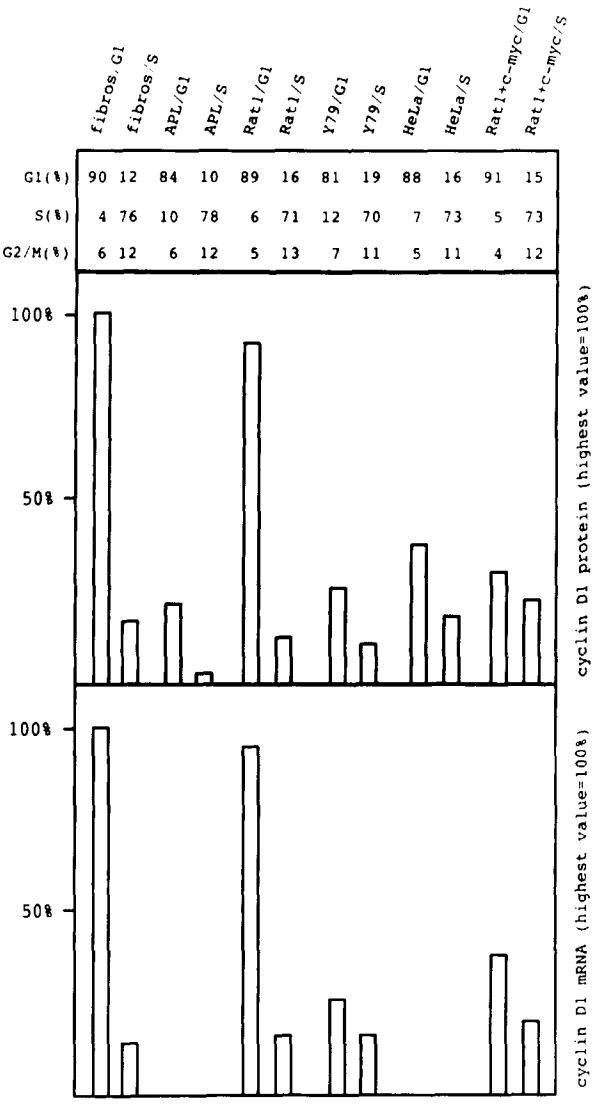

Fig. 5. Quantitative comparison of cyclin D1 protein and mRNA abundance in G1 and S phase of different normal and transformed cells. Cells were separated according to their cell cycle phases by centrifugal elutriation. In these fractions cyclin D1 protein expression was determined by Western blot analysis and related to μg loaded protein (middle panel). Cyclin D1 mRNA expression in the different fractions was quantified via Northern blot analyses and related to GAPDH expression (lower panel; for details of the method see section 2). Within each cell type the G1 fraction with the highest cyclin D1 expression level was compared to S phase fraction with the lowest cyclin D1 expression. The DNA distributions of the analysed fractions are presented in the upper panel. fibros, normal human fibroblasts; APL, acute promyelocytic leukaemia cells; Rat1, normal diploid fibroblasts; Y79, retinoblastoma cells; HeLa, cervix carcinoma cells, papillomavirus-transformed; Rat1+c-myc, *c-myc* overexpressing Rat1-MycER cells.

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