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Original Citation:

Calcium modulates the cyclin D1 expression in a rat parathyroid cell line / S. Bianchi; S. Fabiani; M. Muratori; A. Arnold; K. Sakaguchi; T. Miki; M. L. Brandi. - In: BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. - ISSN 0006-291X. - ELETTRONICO. - 204:(1994), pp. 691-700.

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CALCIUM MODULATES THE CYCLIN D1 EXPRESSION IN A RAT PARATHYROID CELL LINE¹

S. BIANCHI*, S. FABIANI*, M. MURATORI*, A. ARNOLD[°], K. SAKAGUCHI§, T. MIKI#,
and M.L. BRANDI*²

*Endocrine Unit, Department of Clinical Physiopathology, University of Florence,
Florence, Italy

[°] Endocrine Oncology Unit, Massachusetts General Hospital and Harvard Medical School,
Boston, Massachusetts

§Metabolic Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases,
National Institutes of Health, Bethesda, MD

#Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD

Received August 12, 1994

We have used a rat epithelial parathyroid cell line (PT-r) to study the expression and regulation of D-type cyclins. In PT-r cells the cyclin D1 gene is the most abundantly expressed, being transcribed in at least two mRNAs whose levels oscillate during the cell cycle. We also screened a cDNA library prepared from PT-r cells with the human cyclin D1 probe and isolated its rat homologue. Cyclin D2 and D3 mRNAs are both represented in PT-r cells but the former one is only barely detectable. Moreover, the oscillation of cyclin D3 transcript is slightly delayed when compared to cyclin D1 and D2.

Since extracellular calcium inhibits parathyroid cell proliferation, we looked for the effect of the ion on the expression of cyclin D genes in PT-r cells. Increasing amounts of calcium in the incubation medium reduced the expression of rat cyclin D1 and D2. The effect appears to be cell-specific and probably mediated through the inhibition of mitogenic signalling pathways.

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Cell proliferation fate in eukaryotes is decided at a unique restriction point in G1 that in yeast has been named START (1). Extracellular factors can influence cell cycling during the first gap interval of the cycle, but once the restriction point has been passed the cell is committed to mitosis and becomes refractory to external stimuli. The ordered sequence of events of the eukaryotic cell cycle is controlled by protein complexes composed of cyclin-dependent kinases (cdks) and cyclins (reviewed in 2,3). While cdks are present almost constantly during the cell

¹EMBL DATA BANK ACCESSION NUMBER: the accession number of rat cyclin D1 cDNA sequence (RN-CCND1) is X75207.

²To whom correspondence should be sent at
Endocrine Unit, Department of Clinical Physiopathology Viale Pieraccini 6, 50139 Florence, Italy.
FAX (39)-55-641026.

0006-291X/94 \$5.00

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cycle, cyclins are usually expressed at specific points, and act as regulators of the catalytic properties of cdk's and perhaps in determining their substrate specificity (4-8).

G1 specific cyclins regulate the entrance of a quiescent cell into the proliferative state, its progression through the G1 phase, and the G1/S transition. Since their synthesis has been shown to be modulated by extracellular messengers, it was proposed that one mechanism through which external agents affect the rate of cell proliferation is by controlling G1 cyclin expression or function (3).

In mammals several putative G1 cyclins (C,D,E) have been isolated (9-15). Cyclin E has been shown to associate with and activate cdk2 (13,15,16). Its expression peaks late in G1 and the complex cdk2-cyclin E has been proposed to regulate cell progression through the G1/S boundary and the entrance into the S phase (6). Induction of cyclin E over-expression reduces both the length of G1 phase and the serum requirement for the G1/S transition (17). Moreover the physiological antiproliferative factor TGF β can prevent entry into S phase by inhibiting cyclin E's association with cdk2 (18,19).

Cyclins D (D1,D2,D3) have been cloned both in mouse and in humans (9-12). Their genes are differently expressed and regulated in various tissues (9,12,20,21) and the encoded proteins may associate with distinct cdk's (7) and may interact differently with the retinoblastoma susceptibility gene product (pRb) (22,23) suggesting that the members of cyclin D family might regulate different events of G1 progression. Cyclin D genes are highly sensitive to extracellular messengers and their mRNAs are induced by growth factors (9,20,24) or reduced by antiproliferative agents (25). Induced overexpression of cyclin Ds is able to accelerate G1 progression and to shorten the length of the cycle in many cell types (26). Furthermore, cyclin D1 was originally cloned as the putative oncogene PRAD1 (or D11S287) (10), and its deregulation has been implicated in the pathogenesis of a number of human tumors including parathyroid tumors (reviewed in 27).

We have used a rat parathyroid epithelial cell line (PT-r) to study some of the molecular mechanisms that underlie parathyroid cell proliferation. We have found that parathyroid cells express all three known members of the cyclin D family but cyclin D2 mRNA is only barely detectable. Using a PT-r cDNA library we also isolated and sequenced the rat cyclin D1 cDNA, showing a high homology with the human and mouse proteins (93% and 97% respectively).

Parathyroid cell proliferation is negatively regulated by extracellular calcium both "in vivo" and "in vitro" even though the mechanisms through which the cation affects parathyroid cell growth are unclear (28,29). We demonstrated that in PT-r cells calcium ions inhibited the expression of cyclin D1 and D2 genes. These results suggest a possible mechanism through which calcium ultimately controls parathyroid cell proliferation.

MATERIAL AND METHODS

CELL CULTURES

PT-r cells were grown at 37 °C in a calcium-free mixture (1:1) of Coon's modified Ham's F12 and Dulbecco's modified Eagle's minimum essential medium (Gibco) supplemented with 5% calf serum, 1% Nutridoma-SP (Boehringer-Mannheim), 100 units per ml of penicillin, 100 μ g per ml of streptomycin, and 1.5 mM CaCl₂ (growth medium) To determine the effects

of different calcium concentrations on PT-r cells proliferation, cultures were maintained in the presence of different calcium chloride concentrations (0.05 mM, 1.5 mM, and 4 mM). Stimuli with bovine acidic fibroblast growth factor (aFGF; R&D Systems, Minneapolis MN 55413), parathyroid hormone related peptide (PTH-RP) and TPA were performed in PT-r cells kept for 24 hours in growth medium deprived of serum and nutridoma.

SYNCHRONIZATION AND [³H] THYMIDINE INCORPORATION

PT-r cells were synchronized at the G1/S boundary by sequential thymidine aphidicolin treatment (10). Cells were incubated for 12 hours in growth medium containing 2 mM thymidine (Sigma), washed three times with PBS (Phosphate-buffered saline) and incubated again with 24 μM deoxycytidine (Sigma) and 24 μM thymidine. Ten hours later cells were trypsinized and aliquoted in 24-well dishes (10⁵ cells/well), 6-well dishes (5X10⁵ cells/well), and in 10 cm dishes (10⁶/dish). Incubation with 5 μg/ml aphidicolin (Sigma) for 14 hours was followed by release from the G1/S block with four washes in PBS and incubation in complete medium. One μCi per well of [³H]-thymidine (Amersham) was added to each well of the 24-well dishes 15 minutes before each indicated time point. After 30 minutes incubation the [³H]-thymidine incorporated in the TCA precipitate was counted by liquid scintillation spectroscopy.

NORTHERN BLOTTING AND LIBRARY SCREENING

RNAs were prepared according to the guanidinium thiocyanate method, fractionated on a 1% agarose formaldehyde gels and blotted onto nylon membranes (NYTRAN S&S). Hybridizations were performed with ³²P-dCTP radiolabeled probes using either a cDNA containing the entire coding sequence for the PRAD1/human cyclin D1 or cDNAs containing the coding sequences for the mouse cyclin D2 and D3 (CYL2/3) and a cDNA probe encoding for the rat cyclophilin. Hybridizations were carried overnight at 42 °C in a solution containing 50% formamide, 5X Denhardt's, 5X SSC, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. Filters were washed at high stringency (30 min. at 60°C in 0.1X SSC/0.1% SDS) and autoradiographed.

A complementary DNA library from PT-r was prepared in λ-pCEV27 (30). Approximately 10⁶ plaques were plated and screened at high stringency conditions using the PRAD1 probe radiolabeled with ³²P-dCTP (Hybridization at 42 °C in 50% formamide, 5X SSC, 20 mM Tris.Cl pH 7.6, 2X Denhardt's, 0.1% SDS, 10% dextran sulfate; washes at 68 °C in 0.1X SSC, 0.1% SDS). About 100 positives were isolated and analyzed by restriction mapping. The cDNAs were sequenced according to the dideoxynucleotide method.

EXPRESSION OF CYCLIN D1-D3 IN PT-r CELLS AND CLONING OF RAT CYCLIN D1

We have used cDNA probes containing the entire coding region of human cyclin D1 (PRAD1 clone P1-4 see ref. 10) and mouse cyclin D2 and D3 (CYL2,3) to look for the presence of cyclin D mRNAs in PT-r cells. Northern blot analysis was performed using about 30 μg of total RNA extracted from asynchronously growing cells and the same volume of pooled resuspended RNA was loaded in every lane. As shown in Fig. 1, rat cyclin D1 gene was the most abundantly expressed being transcribed in at least two major transcripts of about 4.8 Kb and 1.8 Kb. In asynchronously growing PT-r cells cyclin D2 mRNA is only barely detectable with two faint bands at 4.7 Kb and 6,5 Kb (Fig.1). Cyclin D3 gene is transcribed as a single mRNA of about 3 Kb whose abundance is relatively low if compared to the 4.8Kb transcript of rat cyclin D1 (Fig.1). We conclude that unlike many other cell lines, rat parathyroid cells do express all three genes of the cyclin D family, albeit with considerable variability among their mRNA levels.

A PT-r cDNA library was constructed in the plasmid composite vector λ-pCEV27 (30). About 10⁶ plaques were screened at high stringency using the human cyclin D1/PRAD1 cDNA

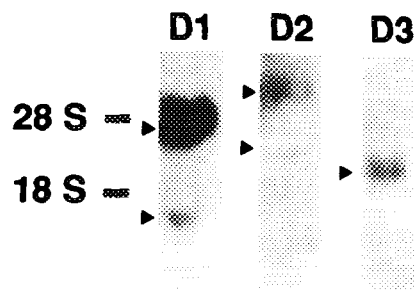
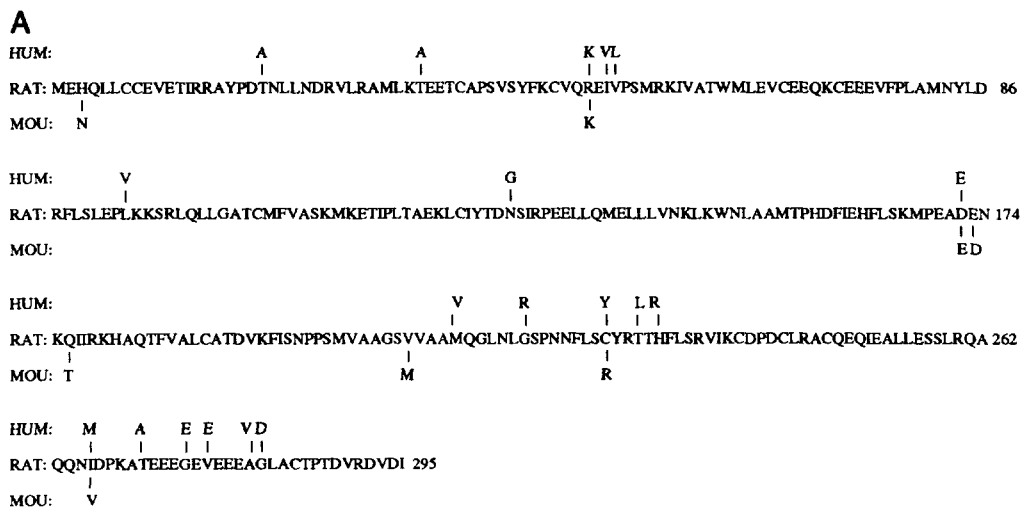


Fig.1. Expression of cyclin D genes in PT-r cells. The same volume of resuspended total RNA extracted from asynchronously growing PT-r cells was loaded in every lane (about 30 μ g). The blots were probed using either the coding region of human cyclin D1/PRAD1 or the coding regions of the mouse cyclins D2, D3.

as radiolabeled probe and more than 100 positives were isolated. Restriction analysis showed the presence of at least three distinct inserts and clones pCEVD1.1 (insert of about 2.2 Kb), pCEVD8.6 (insert of about 1.5 Kb) and pCEVD13.8 (insert of about 4.0 Kb) were chosen for sequencing. All three inserts appeared to be full transcripts with poly-A tails, identical 5' ends and the same open reading frame of 885 nucleotides, encoding a predicted protein of 295 amino acids, which is highly homologous to the human and mouse cyclin D1 (93% and 97% identity, respectively) (Fig. 2A). The entire sequence of the pCEVD8.6 insert, which corresponds to the 1.8 Kb transcript detected in Northern blots, is shown in Fig. 2B. Similarly to the 1.7 Kb transcript of the human cyclin D1 (10), a canonical polyadenylation signal (AAUAAA) is missing in the small transcript of the rat cyclin D1. Compared to pCEVD8.6 the insert of clone pCEVD13.8, which represents the 4.8 Kb band detected in Northern blotting, has a different 3' non coding region that has been produced by a further transcription and a different polyadenylation pattern: a recognizable "consensus" (AAUAAA) is in this case present 14 bases upstream of the poly-A tail. The cDNAs contained in clones pCEVD1.1 and pCEVD13.8 have identical 3' termini but a sequence of about 1.8 Kb has been spliced out in the 3' untranslated region of the former one. However, in Northern blotting, we could not detect any mRNA of comparable molecular size to the pCEVD1.1 cDNA.

DEPENDENCE OF CYCLIN D_s EXPRESSION ON CELL CYCLE PHASES AND EXTRACELLULAR CALCIUM CONCENTRATION

The expression of cyclin D genes has been reported to oscillate during the cell cycle being maximal in G1, although patterns may vary among different cell lines and in response to different extracellular factor (9-12,20). In PT-r cells cyclin D1 and D2 mRNAs are low during S phase and fluctuated slightly in advance when compared to cyclin D3 mRNA. The results are shown in Fig. 3A,B and the amount of total RNA loaded was compared using a rat cyclophilin cDNA probe.



B

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CCGTCGACGGCCATTACCAATCGCGAAACCGGCGCAGTAGCAGAGAGCTGCAGACTCCGCGCG
CTCCGGAGACCGGTCTGATAGCGCGAAGCAGCGTCAGCAGACACCGGAGCCCAACCCGG
ACCACAGCCCTCCCCAGGCGGCGCGCATGGAACACCAGCTCTGTGCTGGAAAGTGGAGACC
ATCCGCGCGCGCTACCTGACACCAATCTCCTCAACGACCGGGTCTGCGAGCCATGCTTAAGA
CTGAGGAGAACTGCGCGCCCTCCGTTTCTACTTCAAGTGCCTGCAGAGGGAGATTGTGCCATC
CATGCGGAAATCGTGGCCACCTGGATGTAGAGGTCTGCGAGGAGCAGAAAGTGGAAAGAGG
AGGTCTTCCCGTGGCCATGAACTACCTGGACCGGTCTTGTCTCTGGAGCCCTGAAGAAGAG
CCGCTGACAGCTTCTGGGGCCACCTGCATGTTGCTGGCCCTTAAGATGAAGGAGACCATTC
CTGACTGCCGAGAAAGTGTGCATCTACACTGACAACTCTATCCGCCCGAGGAGTGTCTGCAAA
TGGAAGTGTCTTGGTGAACAAGCTCAAATGGAACCTGGCCGCACTGACTCCCCACGATTTTCAT
CGAACACTTCTCTCCAAAATGCCAGAGCGGATGAGAAACAACAGATCATCCGCAACATGC
ACAGACCTTTGTGCCCTCTGTGCCACAGATGTGAAGTTCATTTCCAAACCCACCTCCATGGTG
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TCTGTACCGCACAACGCACTTCTTCCAGAGTCATCAAGTGTGACCCGGACTGCCTCCGTG
CCTGCCAGGAACAGATTGAAGCCCTTCTGGAGTCAAGCCTGCGCCAGGCCAGCAGAACATCG
ATCCCAAGGCCACCGAGGAGAAAGGGAAAGTGGAGGAAGAAGCTGGTCTGGCTTGACACCC
ACCGAGCTCCGAGATGTGGACATCTGAGGGCCACCGCAGGCGGGAGTCACCAAGTAGTGGCA
TCGGAAAGAGAAAGGAGCCAGCCGGTGTCTCTGACGAGGTCCCCTTGGGATGTGTGTGTAC
CAGAAGGGGAAGTTTGTCTCTTGTGTGTTGTTTTCTTAATCTTCTCCCTTCTATCTGATT
TAAGCAAAAGAGAAAAAAATACCTGAAAGCTGTCTTAAAGAGAGAGAGAGATAGAATTC
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AGAAGGATTTTGTATCTTTATGTGTTAAGAAAAAAAGCTTAAGAAACATTGCTTT 1454
bp

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Fig.2. Sequence of rat cyclin D1. **A.** Amino acids sequence of the rat cyclin D1 protein is compared to the human (10) and the mouse (9) homologs. **B.** Nucleotide sequence of the rat cyclin D1 cDNA (clone 8.6). Start codon (position 153-155) and stop codon (1038-1040) are underlined.

Parathyroid cell proliferation has been shown to be negatively modulated by calcium and increasing amounts of calcium in the medium inhibit PT-r cell growth (28,29). These observations prompted us to investigate if calcium could influence the expression of rat cyclin D genes in PT-r cells. The cells blocked at the G1/S boundary by sequential treatment with

thymidine and aphidicolin were released from the block and incubated in complete medium containing either 0.05 mM, 1.5 mM, or 4 mM calcium chloride. Samples were lysed at 4, 8, and 12 hours and the total RNA extracted. Northern blot hybridizations were performed using either PRAD1 or mouse CYL2 and CYL3 as cDNA radiolabeled probes. The results reported in Fig. 4 indicate that expression of rat cyclin D1 and D2 was inhibited by calcium both at 8 and 12 hours while cyclin D3 expression was unaffected. Differences in the amount of specific mRNA at different times could be correlated to the cell cycle phases at which the cells have been lysed. The same experiment was performed using a preosteoclastic cell line (FLG29.1) but we could not observe any similar effect (data not shown).

THE EFFECTS OF CALCIUM ON CYCLIN D1 EXPRESSION ARE INDIRECT

To further investigate calcium's effect on cyclin D1 expression we decided to incubate PT-r cells in media deprived of growth factors and nutrients, but containing different calcium concentrations (0.05 mM, 1.5 mM, and 4 mM). After 24 hours starvation cells were harvested and lysed for total RNA preparation. As shown in Fig. 5A, cyclin D1 mRNA levels were almost undetectable with no differences in the amount of transcripts at different extracellular

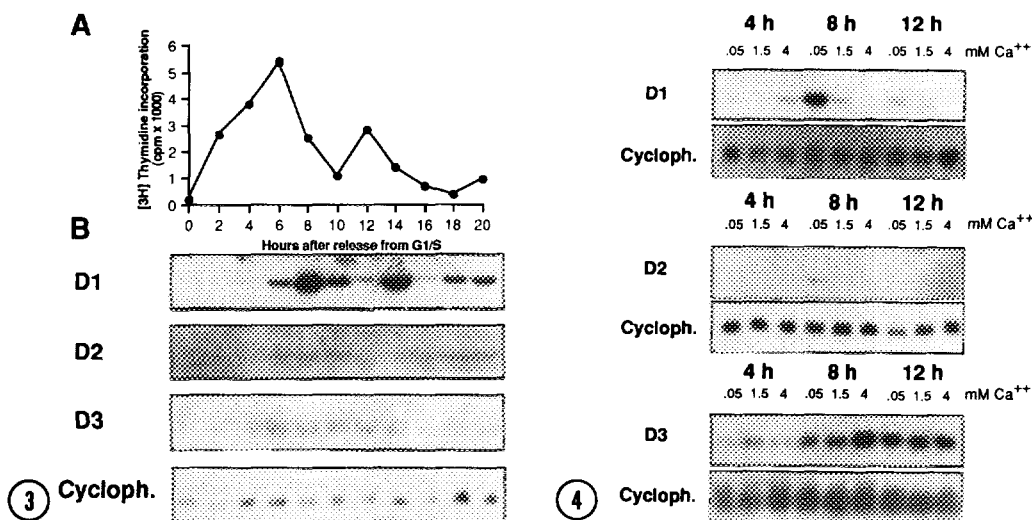


Fig.3. Dependence of cyclin Ds expression from cell cycle phases. **A.** [³H]-thymidine incorporation curve of PT-r cells released at the G1/S boundary. **B.** Fluctuation of rat cyclin D1, D2, and D3 mRNAs. RNA samples were obtained from cells lysed at every indicated time point after the release from the G1/S block and Northern blots were hybridized either with PRAD1 or Cyl2 and Cyl3 radiolabeled probes. The transcripts of rat cyclin D1 and D2 shown are the ones at 4.8 Kb and 4.7 Kb, respectively. Total RNA loaded was quantified by rat cyclophilin hybridizations.

Fig.4. Dependence of cyclin Ds expression from extracellular calcium concentration. RNAs were extracted 4, 8, and 12 hours after the G1/S block release from PT-r cells incubated at different concentrations of calcium chloride in the media (0.05 mM, 1.5 mM, and 4 mM). Northern blots hybridized with PRAD1, Cyl2, and Cyl3 cDNA probes were normalized using rat cyclophilin cDNA probe. The 4.8 Kb transcript of rat cyclin D1 and the 4.7 Kb transcript of rat cyclin D2 are represented.

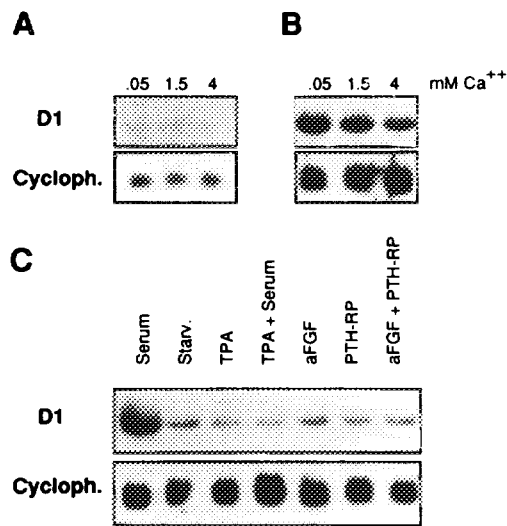


Fig.5. Effects of mitogenic agents on cyclin D1 expression in PT-r cells. Northern blot analysis of **A**, 24-hour starved cells incubated at different extracellular calcium concentrations, **B**, cells kept for 24 hours in growth medium (1.5 mM calcium chloride) without serum and Nutridoma, maintained for 6 additional hours in the starvation medium but at different calcium chloride concentrations (0.05 mM, 1.5 mM, and 4 mM) and then stimulated with 5% calf serum for 4 hours, **C**, RNAs extracted from PT-r starved cells or from cells incubated for a period of 4 hours with serum and nutrients, TPA (10^{-7} M), aFGF (10nM), and PTH-RP (100 nM).

calcium. Conversely, when PT-r cells were starved, kept for additional 6 hours in the starvation medium but at different calcium concentrations (0.05 mM, 1.5 mM, and 4 mM) and then stimulated by 5% calf serum for 4 hours, a higher cyclin D1 transcription was found in the presence of low extracellular calcium (Fig.5B).

PT-r cells produce acidic fibroblast growth factor (aFGF) and bear the receptor for aFGF (31). Since both the growth factor production and the receptor expression on the cell surface are negatively regulated by extracellular calcium we tested if aFGF had any effect on cyclin D1 transcription. Rat parathyroid cells kept for 24 hours in growth medium without growth factors and nutrients were stimulated by 10 nM aFGF for 4 hours. As reported in Fig.5C, serum deprivation decreased the amount of cyclin D1 transcript and aFGF did not restore the conditions of serum stimulated cells.

Parathyroid hormone related peptide (PTH-RP) is a mitogenic peptide involved in hypercalcemia of malignancy syndrome (32). PT-r cells produce PTH-RP and extracellular calcium has a negative effect on its secretion (27,28). We found that stimulation of starved cells with 100 nM PTH-RP for 4 hours, either alone or in association with aFGF, was unable to produce any change in the synthesis of cyclin D1 mRNA (Fig .5C).

Recently in parathyroid cells a transmembrane receptor capable of responding to extracellular calcium and linked to changes in phosphoinositide turn-over has been cloned (33). It is probably through the activation of the "calcium-sensing receptor" that high extracellular calcium leads to the activation of protein kinase C (PKC). To test the possible involvement of the

calcium dependent kinase in the inhibition of cyclin D1 transcription we incubated for 4 hours PT-r starved cells with the PKC activating agent 12-myristate-13-acetate (TPA; 10^{-7} M) and found that this treatment led to a further decrease in cyclin D1 expression (Fig. 5C). Moreover TPA abolished the serum induced transcription of cyclin D1 gene (Fig. 5C).

DISCUSSION

The importance of the cyclin D gene products on cell cycle regulation is supported by the high conservation that they maintain among species. In accord with this concept we found that rat cyclin D1 is highly homologous to the previously identified mouse and human proteins. We also found that all three known members of the cyclin D family are transcribed in PT-r cells albeit to different extent. Cyclin D1 mRNA is the most represented.

Parathyroid cell proliferation has been found to be negatively affected by elevated extracellular calcium, both "in vivo" and "in vitro". In asynchronously growing PT-r cells a dose dependent decrease in [3 H]-thymidine incorporation was observed with increasing ionized calcium concentration in the media (28). Present data indicate that in PT-r cells the expression of cyclin D1 is decreased by increasing extracellular calcium concentrations. The effect is detectable both at 8 hours and 12 hours after the release from the G1/S block. While cyclin D2 transcription is also modulated by calcium, its lower amounts of mRNA make it more difficult to follow its changes through the cell cycle. Cyclin D3 mRNA expression is not influenced by differences in extracellular calcium concentrations.

Previous reports have shown that the dose-dependent decrease in [3 H] thymidine uptake caused by extracellular calcium is detectable at concentrations of the cation higher than 0.1 mM: at lower values a decrease in the maximal proliferation rate was observed (28). In agreement with those findings, PT-r cells doubled faster when incubated at 1.5 mM extracellular calcium chloride than at 0.05 mM, even though cyclin D1 transcription was higher at the latter concentration. The longest generation time, however, was detected at 4 mM (data not shown). Since transient increases in intracellular calcium have been detected in proliferating eukaryotic cells at the G1/S and G2/M boundaries and early in G1(34), the above results could be interpreted as different opposing effects that the cation may have on various cell cycle transitions.

The observation that calcium inhibitory actions seem confined in G1 and the lack of effect of extracellular calcium on cyclin D1 expression in the absence of growth factors could be interpreted as an indirect effect of the cation on cell proliferation through modulation of mitogenic signalling pathways. In PT-r cells, the aFGF autocrine system could be a potential mediator of the calcium effects on cyclin D1 transcription since there is an increase in the production of the growth factor and higher expression of the receptor on the cell surface at lower calcium concentrations. However we have shown here that externally added aFGF does not restore cyclin D1 expression to the level of the serum stimulated cells. This is in agreement with previous report in which the aFGF autocrine system appears not to be the sole regulator of parathyroid cell growth. The calcium dependent kinase activator, 12-myristate-13-acetate

(TPA), is a broadly used differentiating agent and known to down-regulate some of the growth factor tyrosine kinase receptors. In parathyroid cells PKC might be activated by the stimulation of a transmembrane calcium-sensing receptor. The observation that TPA can further decrease cyclin D1 expression in starved cells could indicate an implication of PKC in the calcium mediated control of PT-r proliferation possibly through the down-regulation of growth factor receptor signalling.

In conclusion, the negative effect of calcium on parathyroid cell proliferation appears ultimately mediated through an inhibition of cyclin D1 expression. Further studies dissecting the various cell cycle events will clarify the mechanism by which calcium regulate parathyroid cell growth.

ACKNOWLEDGMENTS

We thank Dr. C.J. Sherr for kindly providing us with the cDNAs for mouse cyclin D2 and D3. The work was supported by the Progetto CNR 91.00436.PF40 and by the Associazione Italiana per la Ricerca sul Cancro (to M.L.B.); by Grants DK11794 and CA55909 from the NIH (USA) and by an American Cancer Society Faculty Research Award (to A.A.).

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