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CALMODULIN REGULATES THE EXPRESSION OF CDKS, CYCLINS AND REPLICATIVE ENZYMES DURING PROLIFERATIVE ACTIVATION OF HUMAN T LYMPHOCYTES

Josep Colomer, Antonia López-Girona, Neus Agell and Oriol Bachs

Department of Cell Biology, Faculty of Medicine, University of Barcelona, Casanova 143, 08036-Barcelona, Spain

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SUMMARY: Cell cycle is regulated by the activation of complexes of cyclins and cyclin-dependent protein kinases at specific points. Quiescent cells lack both cyclins and cyclin-dependent kinases but their expression is induced after proliferative activation. Cyclin A/cdk2 complexes are involved in the onset of DNA replication whereas cyclin B/cdc2 trigger mitosis. We report here that Ca^{2+} and calmodulin regulate the expression of cdk2, cdc2, cyclin B and the proliferating cell nuclear antigen (a co-factor of DNA polymerase-δ) in human T lymphocytes. Likewise, the expression of cdk4, cyclin A and DNA polymerase-α is dependent of the sinergistic effect of both the Ca^{2+}/calmodulin and the protein kinase C pathways. Thus, calmodulin controls DNA synthesis by regulating the levels of cdk2 and proliferating cell nuclear antigen and mitosis entry by modulating the expression of cyclin B and cdc2.

The involvement of Ca^{2+} and calmodulin (CaM) in the regulation of the cell cycle is now well documented. The evidence indicate that CaM regulates the transition from G_0 to G_1 (1), the onset and progression of DNA replication (2,3) and the initiation of mitosis (4). However, little is known regarding how CaM modulates these processes.

The triggering of DNA replication has been suggested to be associated to changes in cytosolic and nuclear CaM occuring during proliferative activation. A surge of cytosolic CaM has been observed in different cellular types shortly before DNA synthesis (5-7) and an increase of nuclear CaM is produced during the G_1/S transition in proliferating rat liver cells and human T lymphocytes (8,9). This nuclear CaM binds to the nuclear matrix (9,10) which is the structure that contains the replication factories where DNA synthesis occurs (11). Isolated replication factories contain CaM and several CaM-binding proteins suggesting that CaM could be a component of these factories and thus to be directly involved in the triggering and progression of DNA synthesis (12).

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The addition of CaM antagonists to cultures of NRK cells inhibits DNA replication and the activity of DNA polymerase-α suggesting that the role of CaM on the control of DNA replication could be through the regulation of the activity of this replicative enzyme (3). However, the maximal inhibition is observed when the drugs are added to the cultures during the early/mid G1, suggesting that CaM also regulates an event occuring before the assembly of replication factories (3).

Thus, we have explored here the possibility that CaM could control the cell cycle through the regulation of the expression of cyclins or/and cyclin-dependent protein kinases (cdks) which are key regulators of the main transitions of the cell cycle (13). We also have studied whether the expression of DNA polymerase-α and the proliferating cell nuclear antigen (PCNA) (a co-factor of DNA polymerase-δ) are regulated by Ca^{2+}/CaM in proliferating human T lymphocytes.

MATERIALS AND METHODS

Cell cultures. Peripheral blood lymphocytes (PBL) were used as a source of T lymphocytes. They were obtained from buffy coats from healthy donors and cultured at 1 x 10^6 cells/ml as described (10). Cells were activated by adding Phytohaemaglutinin (PHA) at a final concentration of 90 μg/ml. At 20 h after PHA addition, the anti-CaM drugs W12 or W13 (1, 5 or 7.5 μg/ml) were added when indicated. Alternatively, PBL were stimulated with the phorbol ester TPA (1nM), with the calcium ionofor ionomycin (0.75 μM) or by both together.
Measurement of DNA synthesis. DNA synthesis was evaluated by measuring the incorporation of [*methyl*-^{3}H]-thymidine (Amersham: 3 μCi/ml) during the last 8 h of culture as described (14).
Obtention of samples. To obtain total cellular extracts (homogenates) PBL were centrifuged at 1100 x *g* for 15 min to eliminate the culture media, resuspended in 250 mM sucrose, 5 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 μg/ml aprotinin and then washed again. Finally, the cells were resuspended in a small volume of 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM PMSF, 0.5 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor and 10 μg/ml N-tosyl-L-phenylalanine chloromethyl ketone and sonicated during 15 sec.
Immunoblotting. Homogenates were separated on SDS-polyacrylamide mini slab gels (6% or 10%) and the proteins transferred to Immobilon-P strips for 2 h at 60 V. The sheets were preincubated in phosphate buffered saline, 5% defatted milk powder for 1 h at room temperature. Then they were incubated with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl with 1% bovine serum albumin and 0.5% defatted milk powder containing antibodies against cdk2 (3 μg/ml; UBI), cdk4 (6 μg/ml; UBI), cdc2 (3 μg/ml; UBI), cyclin A (1:10 dilution; mAb-JR441), cyclin B (1 μg/ml; UBI), DNA polymerase-α (1:10 dilution; UBI), PCNA (10 μg/ml; Boehringer Mannheim) or actin (1:400 dilution; ICN) for 2 h at room temperature. After washing the strips were incubated with an alkaline phosphatase coupled secondary antibody (1:5000 dilution) for 1 h and the reaction visualized.
Protein determination. The protein content was measured by the method of Bradford (15) using bovine serum albumin as a standard.

RESULTS

The addition of a combination of the phorbol ester TPA plus ionomycin to cultures of human T lymphocytes induced DNA replication (Fig. 1A). On the contrary, when they

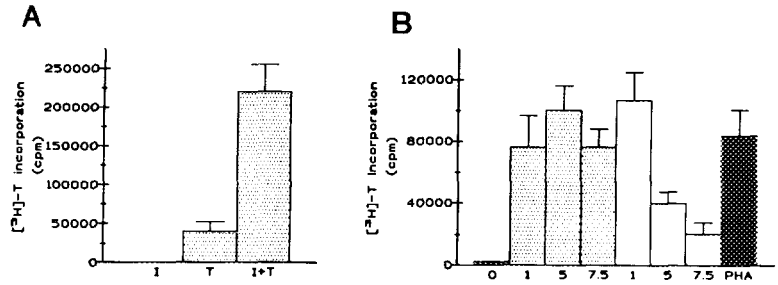

FIG. 1. **Activation of DNA synthesis in human T lymphocytes**. PBL were cultured for 72 h and [^{3}H]-thymidine incorporation into DNA was measured, as described under materials and methods. **A:** Cells were cultured in a medium containing 0.75 μM ionomycin (I), 1 nM TPA (T) or both (I+T). **B:** Cells were cultured with (PHA) or without PHA (0) in the medium and DNA replication measured at 72 h. At 20 h after PHA activation different amounts [(1) 1μg/ml, (5) 5μg/ml, (7.5) 7.5 μg/ml] of W12 (speckled bars) or W13 (white bars) were added to the cultures and DNA synthesis determined at 72 h. The values are the mean of three different experiments ± standard error.

were added separately DNA synthesis was not produced (Fig. 1A). Alternatively, PBL were proliferatively activated by the addition of PHA (Fig. 1B). To examine the effect of anti-CaM drugs on DNA synthesis, three different doses of W13 or W12 (1, 5 or 7.5 μg/ml) were added to the cultures at 20 h and the incorporation of [^{3}H]-thymidine examined at 72 h after the activation with PHA. W12 was used as a control for W13 treatment. The results revealed that W13 at 5 or 7.5 μg/ml inhibited DNA synthesis by 50 and 75% respectively whereas at these doses W12 did not show any effect.

The expression of several cdks ($p33^{cdk2}$, $p33^{cdk4}$ and $p34^{cdc2}$) during proliferative activation of PBL was determined by western blotting. As showed in figure 2A non of the cdks was present in quiescent cells (time 0). After activation with PHA, cdk2 appeared at 12 h and increased until 36 h remaining stable at least until 72 h when DNA replication is maximal. Cdk4 appeared at 24 h, increased until 48 and then remained constant until 72 h. Cdc2 started to be visible at 36 h and then increased progressively until 72 h (Fig. 2A). To analyze whether the expression of these quinases was dependent of Ca^{2+}, protein kinase C or of both, immunoblotting experiments were carried out after the addition of TPA, ionomycin or a combination of both substances to PBL cultures. As showed in figure 2B cdk2 and cdc2 were induced by ionomycin but not by TPA. The combination of TPA plus the ionofor induced the same amount of cdk2 that ionomycin alone whereas in the case of cdc2 a higher increase of this kinase was observed with the combination of both substances. Cdk4 was only expressed with the combination of TPA plus ionomycin. The effect of W13 on the expression of these cdks was also examined. The results revealed that W13 partially inhibited the expression of the three kinases although the effect on cdk2 was the most pronounced. In all these experiments a western blot using anti-actin antibodies was used as a control of the loading of the gels.

Similar experiments were carried out to study the expression of cyclins A and B. Both cyclins were absent in quiescent cells and were progressively expressed from 36 h after

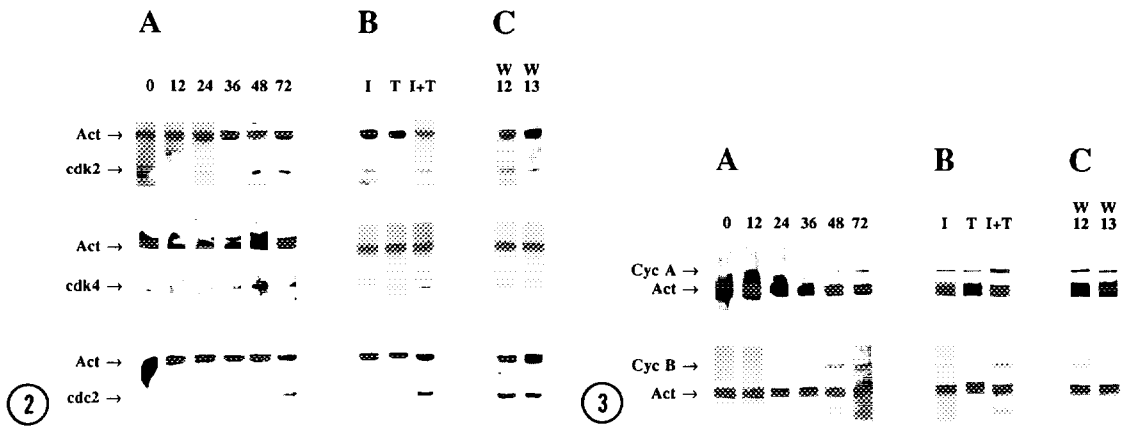

FIG. 2. **Expression of different cdks in human T lymphocytes**. Homogenates from quiescent (0) and PHA-activated PBL were prepared at different times and analyzed by western blotting using specific antibodies against actin (Act), cdk2, cdk4 or cdc2. **A:** Time course of PHA mediated expression of the different cdks. **B:** Cells were cultured in a medium containing 0.75 μM ionomycin (I), 1 nM TPA (T) or both (I+T). The homogenates were prepared at 72 h and then analyzed by western blotting. **C:** Cells were activated with PHA and at 20 h after activation W12 or W13 was added to a final concentration of 7.5 μg/ml, homogenates prepared at 72 h and then analyzed by western blotting. Actin was used as a control for the loading of the gels.

FIG. 3. **Expression of cyclins A and B in human T lymphocytes**. Homogenates from quiescent (0) and PHA-activated PBL were prepared at different times and analyzed by western blotting using specific antibodies against actin (Act), cyclin A (Cyc A) and cyclin B (Cyc B). **A:** Time course of PHA mediated expression of both cyclins. **B:** Cells were cultured in a medium containing 0.75 μM ionomycin (I), 1 nM TPA (T) or both (I+T), homogenates prepared at 72 h and then analyzed by western blotting. **C:** Cells were activated with PHA and at 20 h after activation W12 or W13 was added to a final concentration of 7.5 μg/ml. Homogenates were prepared at 72 h and then analyzed by western blotting. Actin was used as a control for the loading of the gels.

activation with PHA (Fig. 3A). Ionomycin alone induced both cyclins, whereas TPA alone only induced cyclin A at similar levels that ionomycin did. The combination of both substances produced higher levels of both cyclins (Fig. 3B). Cyclin B was dramatically inhibited by W13 whereas cyclin A was only partially affected by this anti-CaM drug (Fig. 3C).

The expression of DNA polymerase-α and PCNA was also studied. As showed in figure 4A both proteins were not present in quiescent PBL. DNA polymerase-α increased progressively from 12 to 72 h whereas PCNA started to increase at 36 h after activation. PCNA was induced by ionomycin but not by TPA whereas the combination of both substances potentiates the expression. DNA polymerase-α only was induced by the combination of both TPA plus ionomycin but not when they were added separetely (Fig. 4B). The addition of W13 decreased the expression of both PCNA and DNA polymerase-α although the effect on PCNA was more pronounced (Fig. 4C).

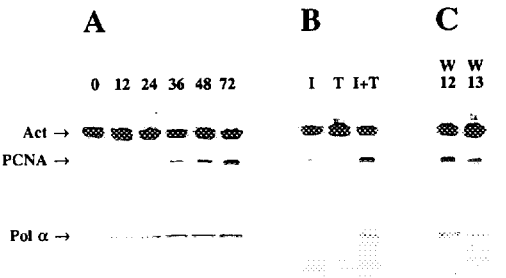

FIG. 4. **Expression of PCNA and DNA polymerase-α in human T lymphocytes.** Homogenates from quiescent (0) and PHA-activated PBL were prepared at different times and analyzed by western blotting using specific antibodies against PCNA and DNA polymerase-α (Pol α). **A:** Time course of PHA mediated expression of both proteins. **B:** Cells were cultured in a medium containing 0.75 μM ionomycin (I), 1 nM TPA (T) or both (I+T). The homogenates were prepared at 72 h and then analyzed by western blotting. **C:** Cells were activated with PHA and at 20 h after activation W12 or W13 was added to a final concentration of 7.5 μg/ml. Homogenates were prepared at 72 h and then analyzed by western blotting. Actin was used as a control for the loading of the gels.

DISCUSSION

The onset of DNA replication is triggered by the activation of cyclin A/$p33^{cdk2}$ complexes during the G1/S boundary. These activated complexes phosphorylate nuclear substrates involved in DNA synthesis (16). The best known of these substrates is the replication protein A (RPA) which is a ssDNA binding protein involved in the unwinding of DNA and the activation of DNA polymerase-α (16,17). $p33^{cdk2}$ Also binds to cyclins E and D and participate in the progression of G1 (13). Quiescent human T lymphocytes lack both cyclin A and $p33^{cdk2}$ but their expression is induced after proliferative activation. We report here that $p33^{cdk2}$ is regulated by Ca^{2+} and CaM as indicated by its maximal induction with submitogenic levels of ionomycin and by the inhibition of its expression when cells were treated with W13. We also show that the sinergistic effect of the Ca^{2+}/CaM plus the protein kinase C pathways are necessary for the expression of cyclin A. These results suggest that the role of CaM in regulating DNA synthesis when quiescent lymphocytes are induced to enter the cell cycle is through the control of the $p33^{cdk2}$ levels.

The expression of PCNA is also regulated by Ca^{2+} and CaM. PCNA is a co-factor of DNA polymerase-δ which increases the processivity of this enzyme and is essential for the synthesis of the leading and lagging strands of DNA (17). Also the expression of DNA polymerase-α is inhibited by W13 although in this case the Ca^{2+}/CaM pathway is not the only contributor since for its expression the sinergistic action of TPA and ionomycin is required. The expression of the late G1 genes, including those coding for the replicative enzymes, seems to be under the control of the products of the supressor genes as retinoblastoma (Rb). In quiescent cells the Rb protein (pRb) is not phosphorylated and associates to the E2F transcription factor. After activation by growth factors pRb is phosphorylated by cdks and dissociates from E2F. The released E2F can then form complexes with other proteins,

including cyclin A, which bind to specific sites in the promoters of the late G1 genes and induce the transcription of these genes (16). The addition of anti-CaM drugs to cell cultures inhibits DNA replication and also the phosphorylation of pRb, suggesting that CaM could regulate the expression of the genes encoding for the replicative enzymes by modulating the phosphorylation level of pRb (18). Thus, the role of CaM on the expression of PCNA could be a consequence of its effect on the expression of $p33^{cdk2}$ and subsequently on the phosphorylation of pRb.

Cyclin B and $p34^{cdc2}$ form complexes which are responsible for the triggering of mitosis (16). We show here that both proteins were induced by the Ca^{2+} ionofor (although not maximaly) and that the induction was inhibited by W13 indicating that the role of CaM in regulating mitosis entry could be through the control of cyclin B and $p34^{cdc2}$ levels. However, the decrease of cyclin B by W13 could be a consequence of the blockade of DNA replication since inhibition of DNA replication blocks cyclin B expression (19). These results are in agreement with those recently reported indicating that in *Aspergilus nidulans* CaM regulates the triggering of mitosis by modulating the activity of $p34^{cdc2}$ (20).

The results reported here suggest that in addition to a possible role of Ca^{2+}/CaM in the function of replication factories these complexes have also an important role on the transition from quiescence to the cell cycle by controling the expression of some key regulators of the cell cycle as cdks and cyclins.

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