Cyclic AMP and Calcium Regulate at a Transcriptional Level the Expression of the CD7 Leukocyte Differentiation Antigen*

(Received for publication, April 17, 1991)

Mercedes Rincón‡, Antonio Tugores, and Miguel López-Botet
From the Sección de Inmunología, Hospital de la Princesa, Universidad Autónoma de Madrid, Madrid 28006, Spain

The CD7 surface molecule is one of the earliest leukocyte differentiation antigens expressed during T-cell ontogeny (1). CD7 antigen was formerly proposed to be a useful marker for the identification of T-cell acute lymphoblastic leukemia (2, 3). Nevertheless, recent reports have described CD7" nonlymphocytic leukemias, pointing out that the antigen may be already expressed on pluripotent bone marrow progenitors (4).

Lazarovits and Karsh (5) have reported that the CD7-specific mAb 7G5 partially inhibits the allogenic mixed lymphocyte reaction, whereas solid-phase-coupled anti-CD7 mAb has been shown to mediate comitogenic effects with anti-CD3 mAb on cultured T lymphocytes (6). So far, the biological meaning of these findings is unknown, but the potential importance of the CD7 molecule has drawn special attention since Jung et al. (7) described a patient with severe combined immunodeficiency disease (SCID) whose only known phenotypic defect was an absence of CD7 expression on peripheral blood lymphocytes.

cDNA cloning has showed that human CD7 bears significant homology with members of the immunoglobulin superfamily (8). Synthetic peptide technology and computer analysis of the primary amino acid sequence have predicted a threedimensional structure of the N terminus of CD7, identifying putative binding regions based on Igk-chain homology. These analyses suggest similarities between the CD7 transmembrane region and the fusion sequences of both human immunodeficiency virus and respiratory syncytial virus (9).

Since mitogenic activation of T-cells increases CD7 cell surface expression (10), we decided to examine the molecular mechanisms regulating CD7 expression on peripheral blood T-cells. Our results indicate that treatment with either a Ca" ionophore (A23187) or a cAMP analogue, dibutyryl cAMP (Bt,cAMP), stimulated CD7 expression on the surface of T lymphocytes, by increasing the steady-state-specific mRNA levels. The induced mRNA accumulation did not require de novo protein synthesis and appeared secondary to an increased gene transcription rather than to a modification of mRNA stability.

EXPERIMENTAL PROCEDURES

Materials—The mAbs used in the present study were 3A1 anti-CD7 (11), TS2/18 anti-CD2 (12), and Spv-Tb3 anti-CD5 (13). Phorbol 12-myristate 13-acetate (PMA), A23187 Ca" ionophore, Bt,cAMP, prostaglandin E, forskolin, cycloheximide (CHX), and actinomycin D were purchased from Sigma, phytohemagglutinin M (PHA) from Difco (Detroit, MI), [α-32P]dCTP and [α-32P]UTP from Du Pont-New England Nuclear Research, and RNase free-DNase I and Proteinase K from Boehringer Mannheim (Germany).

Cell Cultures—Peripheral blood mononuclear cells were separated from heparinized venous blood by sedimentation of Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) and cultured in RPMI 1640 (Flow, Irvine, Scotland) supplemented with 1% penicillin-streptomycin (Flow), 1% L-glutamine (Flow), and 5% fetal calf serum (Flow). This is referred to as complete medium. Peripheral blood lymphocytes were purified from mononuclear cells by removal of adherent cells on plastic Petri dishes (1 h at 37 °C in 5% CO2). T lymphocyte-enriched populations (PBTL) were obtained by passing peripheral blood lymphocytes through a nylon wool column (14).

Activation of PBTL was carried out by culturing 2 × 10^6 cells/ml in a humidified atmosphere containing 5% CO2 with (a) 0.5% (v/v) PHA, (b) anti-CD3 mAb directly bound to the plastic surface (0.5 μg/well in the 24 well plates) previously described (14), (c) A23187 calcium ionophore (1 μM), (d) PMA (5 ng/ml), and (e) Bt,cAMP (2 × 10^-4 M). In several experiments parallel cultures were carried out in round-bottomed 96-well plates, and their proliferative response to the different stimuli was determined by assessing [3H]thymidine incorporation as described (14). As previously described in detail (15), the early effect of T-cell mitogens on intracellular Ca" was analyzed.
by spectrofluorimetry. CAMP levels were measured as detailed else-where (14).

T-cell blasts were obtained upon stimulation of PBTL for 4 days with PHA (0.5% v/v) at 10^6 cells/ml in complete medium. Thereafter, cells were washed and grown for 4–5 days with recombinant interleukin-2 (IL-2) (10 units/ml), kindly provided by Hoffman-La Roche (Basel, Switzerland). T-cell lines CEM, Jurkat, and PBL, were grown in complete medium.

**Flow Cytometry Analysis**—As previously described (14), cells were incubated with hybridoma supernatants containing mAb (100 ml/2.5 × 10^5 cells), washed, and further incubated with fluorescein isothiocyanate-labeled goat anti-mouse F(ab')2 (Dakopatts, Copenhagen, Denmark). Samples were analyzed by flow cytometry (EPICS-C; Coulter Scientific, Harpenden, United Kingdom).

**RNA Isolation and Northern Blot Analysis**—Total cellular RNA was extracted as described (16) and quantified by absorbance at 260 nm. RNA (10 mg per lane) was denatured, electrophoresed through 2.2 M formaldehyde/1% agarose gels and blotted onto Biodyne nylon membranes (Pall Corp., Glen Cove, NY) as described (17). Filters were hybridized overnight, at 42 °C, under standard conditions (50% formamide, 5 × Denhardt's solution, 0.1% sodium dodecyl sulfate, 24 mM phosphate buffer, pH 7.5 × SSC (1 × SSC = 0.15 M NaCl, 15 mM trisodium citrate), and 250 μg/ml denatured salmon sperm DNA), with DNA probes radiolabeled by the random primer method (18). Autoradiographic band intensities were quantified using a 300-A Computing Densitometer (Molecular Dynamics, Sunnyvale, CA).

The CD7 probe was a full length cDNA cloned in pHS3 vector, kindly provided by B. Seed (Boston, MA) (6). The β-actin probe was a HinfIBamHI fragment (0.6 kb) from the 3'-untranslated region of the β-actin cDNA (19).

**Run-on Transcription Assays**—Nuclear run-on analyses were performed according to a method previously described (20). Briefly, nuclei from 4 × 10^7 peripheral blood lymphocytes were incubated for 30 min at 30 °C with 100 μg of RNA polymerase II in 10 μM ATP, 5 μM GTP, and 10 μM [γ-32P]UTP. After DNase I and proteinase K treatment, the reaction products were extracted with phenol/chloroform and precipitated. Samples were spun through a 2.2 M formaldehyde/l% agarose gels and blotted onto Biodyne nylon membranes (Pall Corp., Glen Cove, NY) as described (17). Filters were hybridized overnight, at 42 °C, under standard conditions (50% formamide, 5 × Denhardt's solution, 0.1% sodium dodecyl sulfate, 24 mM phosphate buffer, pH 7.5 × SSC (1 × SSC = 0.15 M NaCl, 15 mM trisodium citrate), and 250 μg/ml denatured salmon sperm DNA), with DNA probes radiolabeled by the random primer method (18). Autoradiographic band intensities were quantified using a 300-A Computing Densitometer (Molecular Dynamics, Sunnyvale, CA).

**RESULTS AND DISCUSSION**

**CD7 Surface Expression Is Induced by Bt,cAMP and the A23187 Calcium Ionophore in Human T Lymphocytes**—We analyzed by flow cytometry the expression of the CD7 antigen during T-cell activation. Human T lymphocytes were purified from peripheral blood and incubated for 24 h in the presence of different mitogenic stimuli. Both PHA and soluble-phase-coupled anti-CD3 induced an up-regulation of CD7 cell surface expression (Table I) in agreement with previous reports (10).

**Activation of Protein kinase C (PKC) associated with an increase of intracytoplasmic Ca^2+ concentration [Ca^{2+}], are events known to be physiologically triggered through the CD3/T-cell receptor complex (CD3-TCR). As previously reported (22, 23) stimulation of PBTL with soluble anti-CD3 mAb raised the [Ca^{2+}], up to 3-fold the basal levels as measured by spectrofluorimetry (not shown). To determine the nature of the intracellular signals involved in the regulation of CD7 expression, we comparatively analyzed the effects of either PKC activation or the increase of [Ca^{2+}]. Treatment of T-cells for 24 h with the A23187 Ca^{2+} ionophore markedly enhanced the surface expression of CD7 (Table I). In contrast, PMA caused a down-regulation of CD7, as previously reported by others (24). The mitogenic effect and the increase of [Ca^{2+}] triggered by different stimuli which enhanced CD7 expression were compared. As shown in Table II, the latter event appeared unrelated to the proliferative response since it was induced by either soluble anti-CD3 mAb or concentrations of A23187 that did not trigger a significant mitogenic response. Moreover, the CD7 up-regulation was detectable at concentrations of A23187 that induced an early increase of [Ca^{2+}], comparable to that mediated by soluble anti-CD3 mAb. Similarly, Ware et al. (25) have recently shown that submitogenic concentrations of ionomycin up-regulate CD7 expression by increasing gene transcription. In contrast to the action of T-cell mitogens, the ionophore mediated a transient effect which was completely inhibited by cyclosporin A, suggesting the involvement of additional signals in mitogen-dependent up-regulation of CD7 expression. Since cAMP plays a role as a second messenger in different cellular systems, and, moreover, we and others have previously shown that stimulation with either anti-CD3 mAb (26, 27) or PHA (28) increases the intracellular cAMP, [cAMP], in T-cells, we analyzed the possible participation of the cyclic nucleotide in the up-regulation of CD7. Interestingly, T-cell stimulation with Bt,cAMP also induced an overexpression of the molecule (Table I and Fig. 1), comparable to that obtained with A23187. A different pattern of response to the same stimuli, observed for the CD2 molecule, is shown for comparison (Table I). As displayed in Fig. 1, the increased expression of CD7 in the presence of A23187 or Bt,cAMP was detectable after 24 h, being more evident after 48 h of culture. Moreover, the combination of

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>CD7 ΔMFI</th>
<th>CD2 ΔMFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA (0.5%)</td>
<td>66 ± 21</td>
<td>88 ± 45</td>
</tr>
<tr>
<td>Anti-CD3 mAb (2 μg/ml)</td>
<td>88 ± 44</td>
<td>52.9</td>
</tr>
<tr>
<td>A23187, 0.5 μM</td>
<td>200 ± 47</td>
<td>17.1</td>
</tr>
<tr>
<td>A23187, 0.1 μM</td>
<td>192 ± 40</td>
<td>20.0</td>
</tr>
<tr>
<td>A23187, 0.05 μM</td>
<td>57 ± 11</td>
<td>1.2</td>
</tr>
</tbody>
</table>

The percentage of positive cells was calculated by subtracting the fluorescence detected in the negative control (P3X63).

AFTER incubation of the cells with different reagents, changes in linear mean fluorescence intensity (MFI) were calculated as follows: MFI = MFI (stimulated cells) – MFI (untreated cells).

**Table II**

**Upregulation of CD7 expression in PBTL does not correlate with their proliferative response**

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>Δ[Ca^{2+}]</th>
<th>ΔCD7 expression</th>
<th>[H]Thymidine incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA (0.5%)</td>
<td>66 ± 21</td>
<td>88 ± 45</td>
<td>52.9</td>
</tr>
<tr>
<td>Anti-CD3 mAb (2 μg/ml)</td>
<td>88 ± 44</td>
<td>52.9</td>
<td></td>
</tr>
<tr>
<td>A23187, 0.5 μM</td>
<td>200 ± 47</td>
<td>17.1</td>
<td></td>
</tr>
<tr>
<td>A23187, 0.1 μM</td>
<td>192 ± 40</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>A23187, 0.05 μM</td>
<td>57 ± 11</td>
<td>1.2</td>
<td></td>
</tr>
</tbody>
</table>

The data represent the increment of [Ca^{2+}], attained over the basal levels.

See footnote Table I. PBTL were incubated either alone or in the presence of the different stimuli for 72 h in 96-well microtiter plates (1.5 × 10^5 cells/well) and labeled with [H]thymidine during the last 18 h. Data represent the mean of triplicate samples. [H]Thymidine incorporation on unstimulated cultures was 1119 cpm. S.D. of triplicates was in every case <10% of the mean.

As shown in Table II, the latter event appeared unrelated to the proliferative response since it was induced by either soluble anti-CD3 mAb or concentrations of A23187 that did not trigger a significant mitogenic response. Moreover, the CD7 up-regulation was detectable at concentrations of A23187 that induced an early increase of [Ca^{2+}], comparable to that mediated by soluble anti-CD3 mAb. Similarly, Ware et al. (25) have recently shown that submitogenic concentrations of ionomycin up-regulate CD7 expression by increasing gene transcription. In contrast to the action of T-cell mitogens, the ionophore mediated a transient effect which was completely inhibited by cyclosporin A, suggesting the involvement of additional signals in mitogen-dependent up-regulation of CD7 expression. Since cAMP plays a role as a second messenger in different cellular systems, and, moreover, we and others have previously shown that stimulation with either anti-CD3 mAb (26, 27) or PHA (28) increases the intracellular cAMP, [cAMP], in T-cells, we analyzed the possible participation of the cyclic nucleotide in the up-regulation of CD7. Interestingly, T-cell stimulation with Bt,cAMP also induced an overexpression of the molecule (Table I and Fig. 1), comparable to that obtained with A23187. A different pattern of response to the same stimuli, observed for the CD2 molecule, is shown for comparison (Table I). As displayed in Fig. 1, the increased expression of CD7 in the presence of A23187 or Bt,cAMP was detectable after 24 h, being more evident after 48 h of culture. Moreover, the combination of
The elevation of [Ca\(^{2+}\)] has already been described to induce the expression of certain surface antigens such as 4F2, a molecule associated with cell growth and activation (29), whereas the action mediated by Bt\(_2\)cAMP was particularly striking. Although the elevation of cAMP has been shown to induce the expression of different genes, including human proenkephalin (30), vasoactive intestinal polypeptide (31), α-gonadotropine (32, 33), and rat somatostatin (34), CD7 constituted the first example of a T-cell membrane molecule positively regulated by the cyclic nucleotide. So far, the increase of cAMP, induced by cAMP analogues or reagents that activate adenylate cyclase has been found to inhibit proliferation (14, 35, 36) as well as IL-2 production (37, 38) and IL-2 receptor (CD25) expression (14) in mitogen-stimulated human T-cells. This inhibition appeared to be a consequence of a direct effect of cAMP on early signal transduction, during the activation process (14). Herein, we demonstrate that cAMP by itself can enhance the expression of certain cell surface antigens, and thus it might play a more complex regulatory role in T-cells. Our data are in line with similar observations preliminarily reported by others (39, 40).

The hypothesis that cAMP-dependent up-regulation of CD7 expression might be involved in the suppressive effect mediated by the cyclic nucleotide on T-cell function can be considered since recent studies by Emara et al. (41, 42) showed that cross-linking of cell-bound anti-CD7 mAbs could inhibit the mitogenic response in T-cells, and suggested a suppressive role for the putative natural ligand. Nevertheless, that possibility appears uncertain since both events do not correlate. In fact, mitogenic stimuli (PHA and anti-CD3 mAb) and Ca\(^{2+}\) ionophores up-regulate as well CD7 expression but trigger T-cell proliferation. In addition, anti-CD7 mAbs has been also reported to costimulate T-cell activation induced via CD3-TcR (6). Furthermore, we have previously shown that cAMP inhibits early stages of T-cell receptor-mediated signal transduction (14) that precede de novo gene expression. Whether the dual effects of cAMP may reflect the participation of different protein kinase A isozymes is uncertain.

**Induced Surface Expression of the CD7 Molecule Correlates with Increased Specific mRNA Levels**—Resting PBTL were treated for different times with either A23187 or Bt\(_2\)cAMP, and total RNA was extracted from each sample. Northern blot hybridization studies were performed to compare the levels of CD7 mRNA under the different culture conditions. As shown in Fig. 2, low levels of a specific 1.3-kb transcript were detected in resting T-cells and markedly increased upon stimulation with either Bt\(_2\)cAMP or A23187. CD7 mRNA levels detectably raised within the first h of treatment, reaching a maximum after 12 h and persisting for at least 48 h. The calcium ionophore synergized with Bt\(_2\)cAMP to yield a 10-fold induction as compared to the basal mRNA amount (data not shown). These data strongly suggested that both the effect mediated by a different cAMP analogue, 8-Br-cAMP (10\(^{-3}\) M), and by reagents able to activate adenylate cyclase increasing the [cAMP], to 20-30 pmol/2 × 10\(^{6}\) cells such as prostaglandin E\(_\text{2}\) (10\(^{-6}\) M) or to 12-20 pmol/2 × 10\(^{6}\) cells such as forskolin (5 × 10\(^{-6}\) M), as assessed by specific radioassay. Under such conditions we observed an increase of CD7 expression after treatment with 8-Br-cAMP (ΔMFI-14), prostaglandin E\(_\text{2}\) (ΔMFI>13), or forskolin (ΔMFI>9) during 20 h, thus supporting that the effect was due to the increase of [cAMP].

To determine whether CD7 could be up-regulated on preactivated T-cells we analyzed the effect of either Bt\(_2\)cAMP or calcium ionophore on T lymphoblasts. As shown in Table III, both stimuli were able to induce an overexpression of the antigen in normal proliferating T-cells. In contrast, no changes were detected in the different leukemia T-cell lines of both immature (CEM) or mature (JK, PEER) phenotypes which we examined (Table III). Thus, the regulation of CD7 in T-cell lines appeared to be different from that of normal T-cells.

![Fig. 2. Effect of Bt\(_2\)cAMP and A23187 on the levels of CD7 mRNA. PBTL (2 × 10\(^{6}\) cell/ml) were incubated for several time intervals in the presence of Bt\(_2\)cAMP (2 × 10\(^{-4}\) M) (A) or Ca\(^{2+}\) ionophore A23187 (1 μM) (B). Total RNA (10 μg/lane) was isolated and analyzed by Northern blot, as described under "Experimental Procedures," sequentially hybridizing with radiolabeled CD7 and β-actin cDNA probes.](image-url)
reagents increased CD7 expression by acting through different complementary pathways. We analyzed as well the accumulation of CD7-specific mRNA in the leukemic T-cell line Jurkat that, as shown above, did not increase the surface expression of CD7 upon stimulation. The steady-state levels of CD7 mRNA detectable in untreated cells were not significantly modified by A23187 and were minimally increased by Bt,cAMP, as compared to the effect observed on PBTL, further suggesting that CD7 is differently regulated in T-cell lines (Fig. 3).

Cyclic AMP and Calcium Activate CD7 Gene Transcription without Modification of the mRNA Stability—A number of distinct molecular mechanisms could account for the accumulation of CD7 mRNA in T lymphocytes. To analyze whether the cAMP- and calcium-dependent induction mechanisms of CD7 gene expression are mediated by changes in the specific mRNA stability, we comparatively estimated the half-life of the CD7 mRNA. Resting T-cells were incubated either with medium, Bt,cAMP, or A23187. After incubation for 12 h, the transcription inhibitor actinomycin D was added to the culture medium, and total RNA was isolated at different time intervals. Northern blot (Fig. 4A) and densitometric analysis (Fig. 4B) revealed that both Bt,cAMP- and Ca\textsuperscript{2+} ionophore-induced CD7 mRNA levels displayed a similar half-life (t\textsubscript{1/2} = 5 h), shorter than that observed for unstimulated T-cells (t\textsubscript{1/2} = 7 h). The half-life of CD7 mRNA appears to be relatively long as compared with that observed for oncogenes and lymphokines (t\textsubscript{1/2} = 30 min) (43), but fits into the average half-life (t\textsubscript{1/2} = 8 h) reported for randomly chosen mammalian mRNAs (44).

Since mRNA stabilization was not a mechanism leading to the increased expression of CD7, we attempted to determine whether an enhancement in the transcriptional rate of the gene could be involved. For this purpose, nuclei isolated from T-cells incubated for 3 h in the presence of medium, Bt,cAMP, or A23187 were subjected to run-on analysis. 32P-Labeled primary transcripts were hybridized to filters containing a full-length CD7 cDNA cloned into the pH3M plasmid. Nuclear run-in assays showed a low transcription rate of the CD7 gene in nonstimulated T-cells. Upon stimulation with either Bt,cAMP or A23187, the transcription rate of the CD7 gene was induced 2- and 3-fold, respectively (Fig. 5). These results indicated that, at least, one of the mechanisms involved in regulating CD7 expression is an increase in the transcription rate of the gene. Although we could not detect any differences between the cAMP- and calcium-dependent transcription rates, it is likely that different transcription factors are activated through each pathway. Recently, a genomic clone of the CD7 gene has been isolated and several cis regulatory elements such as a NF-kB site, a G-C box, a TRE, and an AP2 site have been proposed within the 5' upstream region of the gene (45). Although NF-kB has been described to be activated by PKC through the release of a specific inhibitor in the cytoplasm (I-kB)(46), cDNA cloning has shown a phosphorylation site for PKA that may enhance its DNA binding avidity (47). Like phorbol esters and cAMP (48), free Ca\textsuperscript{2+} can also induce
Cyclic AMP and Calcium Induce CD7 Gene Transcription

- - - - - A23187
- - - - - Bt2 cAMP
- - - - - + CHX

CD7

β-actin

FIG. 6. Effect of protein synthesis inhibition on Bt2cAMP- and A23187-induced CD7 gene expression. PBTL were preincubated for 30 min in the presence or absence of CHX (10 μg/ml). Thereafter, cells were treated with either medium alone, Bt2cAMP (2 × 10^-4 M) or A23187 (1 μM) for 12 h. Then, RNA was isolated and analyzed by Northern blot (10 μg/lane) by sequentially hybridizing with radiolabeled CD7 and β-actin cDNA probes.

Among the multiple mechanisms that may underlie the lack of responsiveness of the leukemic cell lines to A23187 and Bt2cAMP, in terms of CD7 expression, the possibility that the DNA binding activity of transcription factors regulated and/or activated by cAMP or Ca^2+ might be already induced has been considered. Preliminary data indicate that the binding activity of nuclear factor CREB, as analyzed by electrophoretic mobility shift assays, is constitutively activated in Jurkat cells as compared to PBTL.

CAMP- and Calcium-mediated Induction of CD7 Gene Expression Do Not Require de Novo Protein Synthesis—To better understand the regulation of the CD7 gene expression we investigated its protein synthesis requirements. To this end, PBTL were cultured alone or stimulated either with Bt2cAMP or A23187 for 12 h. Parallel samples were preincubated with CHX for 30 min, this protein synthesis inhibitor being present in the culture medium during the stimulation period. As shown in Fig. 6, CHX did not abrogate the increase of CD7 mRNA induced by Bt2cAMP and calcium ionophore. By contrast, a superinduction of the specific mRNA levels in the presence of CHX may involve translational control. The regulation of CD7 expression during T-cell development from lymphoid progenitors and the role played by Ca^2+ and cAMP need to be explored. In this report, we propose that the overexpression of CD7 upon activation of T-cells is probably contributed by the increase of [Ca^2+]i, associated to this process. Furthermore, an increase of the cyclic nucleotide has been reported to occur upon mitogenic stimulation via the T-cell receptor (26, 27) and, thus, might also participate in the regulation of CD7 expression, mediating a synergistic effect with Ca^2+ as shown in the present study. Although the precise role of CAMP in T-cell activation is uncertain, the cyclic nucleotide could be involved in the regulation of CD7 gene expression during thymic and peripheral stages of development.

REFERENCES


A. Tugores, unpublished data.
Cyclic AMP and Calcium Induce CD7 Gene Transcription