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ORIGINAL ARTICLE

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Atypical protein-kinase Cζ, but neither conventional Ca^{2+}-dependent protein-kinase C isoenzymes nor Ca^{2+}-calmodulin, participates in regulation of telomerase activity in Burkitt's lymphoma cells

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Abstract *Purpose*: To clarify the role of the pathways dependent on protein-kinase C (PK-C) and Ca^{2+}/calmodulin (CaM) in the regulation of telomerase activity in Burkitt's lymphoma cells. *Methods*: Burkitt's lymphoma cells (Raji and Daudi) were treated with the PK-C inhibitor, bisindolylmaleimide (BIM), or the CaM inhibitor, trifluoperazine (TFPZ), in a dose-dependent manner and in a time-dependent manner. The activities of PK-C isoenzymes were analyzed fluorimetrically using POLARIS assay kits. CaM-kinase II activity was analyzed radiographically, using CaMK-II immunoprecipitation kinase assay kits. Telomerase activity was detected by a conventional telomeric repeat amplification protocol and Stretch PCR. The level of catalytic subunit of telomerase (hTERT) in drug-treated and nontreated cells was analyzed by flow cytometry using anti-hTERT antibody labeled with ZenonAlexa Fluor-488 IgG. Apoptosis was estimated in terms of phosphatidylserine exposure on the cell surface and DNA fragmentation. *Results*: It was found that BIM inhibited telomerase activity and this process preceded apoptosis. The subsequent addition of exogenous PK-C (mixture of isoenzymes) to the cell lysates restored telomerase activity if incubation of cells with BIM was up to 24 h. Using PK-C isoenzymes, it was established that atypical PK-Cζ, but not conventional Ca^{2+}-dependent PK-Cα, PK-Cβ or PK-Cγ, is responsible for the reactivation of telomerase in BIM-treated cells. BIM also showed a well-expressed cytotoxicity against intact leukemia cells. In contrast, the CaM inhibitor TFPZ showed the same cytotoxic effect without any influence on telomerase activity during incubation for 24 h with leukemia cells. After incubation for 48 h, TFPZ markedly suppressed telomerase activity. However, the effect followed apoptosis and appeared to be a result of cell death. The addition of exogenous CaMK-II to the cell lysates obtained from TFPZ-treated cells did not reactivate telomerase. *Conclusion*: The present study confirmed the participation of atypical PK-Cζ, but not conventional Ca^{2+}-dependent PK-C isoenzymes (α, β, γ) nor the Ca^{2+}/CaM-dependent pathway, in the regulation of telomerase activity in Burkitt's lymphoma cells.

Keywords Telomerase · Protein-kinase Cζ · Calmodulin · Burkitt's lymphoma · Bisindolylmaleimide · Trifluoperazine

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Introduction

Telomerase, a ribonucleoprotein reverse transcriptase, extending telomeres of eukaryotic chromosomes, has been found to be suppressed in normal human somatic cells and to be activated in about 90% of human cancers and immortalized cell lines [1–7]. Low levels of telomerase activity have been detected in resting lymphocytes, but stimulation of specific antigen cell surface receptors markedly increases telomerase activity [8–10]. The ability to switch telomerase on and off has potentially important implications in anticancer and antiaging therapy, but the mechanism(s) of telomerase regulation are far from established. It appears to be a multifactorial process, involving many transcriptional and post-transcriptional steps. For example, telomerase affects gene expression, various post-translational protein–protein interactions, and protein phosphorylation [11, 12].

The catalytic core of human telomerase consists of an RNA template (hTR), a catalytic protein subunit with reverse transcriptase activity (hTERT) and additional telomerase-associated proteins (hTEP1, p23, Hsp90, and dyskerin) [13–15]. Studies on the correlation between telomerase activity and the expression of hTR and hTERT have indicated that the telomerase activity strongly correlates with the abundance of mRNA for hTERT, but not with the abundance of hTR [15–18]. Cloning and characterization of hTERT have also confirmed its basic role in cell transformation [11, 12], indicating that hTERT is the key regulator of telomerase activity and its de novo expression and post-translational modifications are essential steps.

hTERT and hTEP1 are considered to be phosphoproteins and their reversible phosphorylation seems to be a prerequisite for modulation of telomerase activity [11, 12, 19, 20]. This hypothesis is based on the finding that protein phosphatase 2A (PP-2A) specifically inhibits telomerase activity in the nuclear lysates of human breast cancer and melanoma cells, suggesting that dephosphorylation locks telomerase into an inactive conformation [21, 22]. In contrast, the phosphorylation of hTERT and hTEP1 restores telomerase activity [11, 12], and it has been established recently that protein kinases Cα and Cζ (PK-Cα and PK-Cζ) are involved directly in phosphorylation of the telomerase proteins in human breast cancer and nasopharyngeal cancer cells, respectively [23–25]. Upregulation of telomerase in human lymphocytes during activation also requires PK-C-dependent activity [8, 9].

Protein-kinase B (PK-B or Akt), mitogen activated protein-kinase (MAPK) and protein tyrosine kinase (PTK) have been found to modulate telomerase activity, too [22, 26, 27]. It has been reported that hTERT associates directly with the c-ABL-protein tyrosine kinase, resulting in phosphorylation of its tyrosine residues and telomerase inhibition [26].

Another possible candidate for induction of telomerase activity is Ca^{2+}, which is widely recognized as an essential intracellular secondary messenger, regulating gene expression and cell proliferation in eukaryotic cells. It has been established that the application of calcium channel blockers or calcium chelators inhibits telomerase activity, but the mechanism(s) remains unclear [28–30]. The effects of Ca^{2+} are frequently mediated via interaction with calmodulin (CaM) and strong evidence indicates that the effects of Ca^{2+}/CaM are often achieved through the regulation of protein phosphorylation [31–33]. A family of CaM-dependent protein-kinases has been identified: myosin light chain kinase, phosphorylase kinase, CaM-kinase I (CaMK-I), CaMK-II, CaMK-III (phosphorylating elongation factor 2) and CaMK-IV [33]. Each of these enzymes may be a potential candidate for regulation of telomerase activity. Moreover, the conventional PK-C isoenzymes (α, β, γ) also require Ca^{2+} and indirectly depend on the Ca^{2+}/CaM pathway. However, little is known about the role of Ca^{2+}/CaM in telomerase regulation.

The present study was designed to investigate whether PK-C-dependent and Ca^{2+}/CaM-dependent pathways are involved in regulation of human telomerase activity in leukemia. Using Burkitt's lymphoma cell lines (Raji and Daudi) we examined the effects of bisindolylmaleimide I (BIM, a PK-C inhibitor) and trifluoperazine (TFPZ, a CaM inhibitor) on the activity of telomerase, the relationship between telomerase and the viability of leukemia cells as well as the ability of exogenous purified PK-C isoenzymes (conventional α, β, γ and atypical ζ) and CaM-kinase II to reactivate telomerase in cell protein extracts obtained from BIM-treated or TFPZ-treated cells.

Materials and methods

Preparation of cells

The human leukemia cell lines (Raji and Daudi) derived from Burkitt's lymphoma (Hayashibara Biochemical Laboratories, Okayama, Japan) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C in a humidified atmosphere containing 5% CO_2. The cell lines were a generous gift from Dr. Minowada (Hayashibara Biochemical Laboratories). The cells used for assay were in the logarithmic phase. They were sedimented by centrifugation (1000 rpm, 10 min) and washed three times with PBS (4°C) before experiments.

Cell viability assay

The effects of BIM and TFPZ on cell viability were measured using the CellTiter-Glo luminescent cell viability assay [34]. ATP bioluminescence is used as a marker of cell proliferation and viability. Briefly, the drugs were dispensed as 10-μl aliquots into 96-well microplates. Eight independent experiments were done for each drug concentration. The cells were suspended in PBS to a concentration 5×10^5 cells/ml and were added in 90-μl aliquots to each patch. After 0–48 h incubation at 37°C in a cell incubator, CellTiter 96 kit (Promega, Madison, Wis.) was added in aliquots of 100 μl to each patch and incubated with cell suspensions for 1 h at room temperature (RT) following the procedure recommended by the manufacturer. The luminescence produced by luciferase catalyzed luciferin/ATP reaction was measured using an InterMedImmunomini NJ-2300 multiwell scanning spectrophotometer. The data were normalized to the control group. The effects of BIM and TFPZ on cell viability were calculated as the percentage decrease/increase in luminescence in comparison with the control nontreated cells.

The viability of leukemia cells in the absence and in the presence of 25 μ*M* BIM or 10 μ*M* TFPZ was analyzed also by flow cytometry using FITC-CD90

fluorescent antibody. A Beckman Coulter-Epics XL flow cytometer was used in accordance with the manufacturer's recommendations after fine adjustments for optimization. The forward-scatter and side-scatter (SS) parameters were adjusted to accommodate the inclusion of leukemia cells within the acquisition data. No cells were excluded from the analysis, and 10,000 cells were counted. Data were collected and the viable and dead cells were analyzed using XL System II software.

Phosphatidylserine (PSer) assay

Cells cultured in 96-well plates (3×10^5 cells/well, 100 μl), were incubated with BIM (25 μ*M*) or TFPZ (10 μ*M*). At each time-point the cells were collected by centrifugation (1000 rpm, 20 min) and washed twice with PBS containing 2.5 m *M* $CaCl_2$ (Annexin binding buffer). Cell suspension (100 μl) was incubated with 5 μl FITC-conjugated Annexin V (FITC-Annexin V) for 10 min at RT in the dark. The cells were washed twice with Annexin binding buffer and resuspended in 100 μl of the same buffer. FITC-Annexin V bound to PSer on the cell surface was detected spectrofluorimetrically at an emission wavelength of 535 nm (excitation wavelength 488 nm) using a Fluoromark fluorescent microplate reader (Bio-Rad, Hercules, Calif.).

DNA fragmentation assay

Cells cultured in 96-well plates (5×10^5 cells/well, 100 μl) were incubated with BIM (25 μ*M*) or TFPZ (10 μ*M*). At each time-point, the cells were collected, the cells lysed and DNA extracted using a commercially available assay kit for DNA fragmentation (Apoptosis Ladder Detection Kit, Wako) based on the method of Ioannou and Chen [35]. The extracted DNA was analyzed by electrophoresis on an agarose gel (1.5% gel density) using TBE buffer. PBS was added to control samples instead of drug. DNA from apoptotic cells formed a ladder on the agarose gel while DNA from non-apoptotic cells appeared as a single band or smeared on the agarose gel. The presence of random DNA fragmentation (a smear) is characteristic of necrosis.

Telomerase activity assay

Telomerase activity was detected using a conventional telomeric repeat amplification protocol (TRAP) and the Stretch PCR method using a telomerase assay kit (TeloChaser; Toyobo, Osaka, Japan). The products of the telomerase reaction (TRAP products) were analyzed by TBA electrophoresis in a non-denaturing 12% polyacrylamide gel. Gels were stained using a silver staining kit (Pharmacia Biotech) according to the manufacturer's instructions. As a positive control, HeLa cells possessing known telomerase activity were used. As a negative control, an aliquot of each control sample was incubated at 70°C for 10 min to inactivate telomerase. The concentration of TRAP product was detected spectrophotometrically at a wavelength of 450 nm and the results were calculated as percentages of the subsequent positive control. The results were normalized against the internal standard.

Protein phosphorylation in vitro

Cells treated with 25 μ*M* BIM (24 and 48 h) or 10 μ*M* TFPZ (48 h) were lysed and cell extracts were prepared for in vitro protein phosphorylation as described by Li et al. [23].

For PK-C-mediated protein phosphorylation, exogenous pure PK-C (mixture of isoenzymes, Sigma) or respective PK-C isoenzyme (α, β, γ, ζ, Sigma) was added to the cell protein extract and phosphorylation was carried out according to the method described by Li et al. with slight modification [23]. After 10 min incubation at 30°C, aliquots of phosphorylated cell extract were assayed for telomerase activity as described above. The PK-C activity in cell lysates was analyzed using POLARIS kinase enzyme activity kits (Chromagen) according to the manufacturer's instructions.

For CaM-mediated protein phosphorylation, exogenous pure CaMK-II (Sigma) was added to the cell protein extract and phosphorylation was carried out according to the method described by Cheng et al. [36]. After 20 min incubation at 30°C, aliquots of phosphorylated cell extract were assayed for telomerase activity. The activity of CaMK-II in cell lysates was analyzed radiographically using a CaMK-II assay kit (Upstate Biotech, Lake Placid, N.Y.). The assay is based on phosphorylation of a specific substrate peptide (KKALRRQETVDAL) by transfer of γ-phosphate of adenosine-5′-[^{32}P]triphosphate by CaMK-II. Cell extracts from drug-untreated and enzyme-untreated cells were used as controls in all kinase activity assay experiments.

Flow cytometric assay of hTERT level

Anti-hTERT antibody (rabbit, Calbiochem) was labeled using a ZenonAlexa Fluor-488 rabbit IgG labeling kit (Molecular Probes). Permeabilization of cells for the Fluor-488-conjugated anti-hTERT antibody was carried out with IntraPrep permeabilization reagent (Immunotech). The antibody–antigen interaction was detected by flow cytometry (Beckman Coulter-Epics XL). Data were collected and analyzed using XL System II software. No cells were excluded from the analysis, and 5000 cells were counted. Data are presented as a dot plot of Fluor-488 fluorescence (SS, *y*-axis, vs Fluor-488-conjugated antibody, *x*-axis) with quadrant markers drawn to distinguish the cells containing different levels of antibody–antigen complexes (Fig. 4). Figure 4a *A*

corresponds to cells containing maximum (control) levels of hTERT, Fig. 4a *B* corresponds to cells containing moderate or low levels of Fluor-488-conjugated hTERT antibody and therefore expressing moderate or low levels of hTERT, and Fig. 4a *C* corresponds to cells without fluorescent marker and therefore without hTERT (spontaneous cell fluorescence). To the right of each histogram are shown Fluor-488 fluorescent curves. Normal lymphocytes (derived from clinically healthy blood donors, 38–40 years of age), known to express very low levels of hTERT (predominantly in T cells), were used as negative controls.

Immunoblot analysis of hTERT levels

The cells were treated with BIM for 24 h. Aliquots of 4×10^5 cells were lysed in TeloChaser buffer containing a protease and phosphatase inhibitor cocktail (Toyobo) for 30 min at 4°C, centrifuged at 12,000 *g* for 20 min at 4°C and the supernatant was dissolved 1:1 in 2× Laemmli sample buffer (1.1 *M* Tris-HCl, pH 6.0, 3.3% SDS, 22% glycerol, 10% β-mercaptoethanol, 0.001% bromphenol blue). Samples containing equal amounts of protein were heated at 95°C for 10 min and were applied to a 5% stacking 4–12% resolving SDS-polyacrylamide gel. Electrophoresis was carried out in two steps: at 80 V for 15 min and 120 V for 2 h at RT (about 22°C). BioRad Kaleidoskope protein standards (Melville, N.Y.) were also applied for comparison. After electrophoresis the separated protein fractions were transferred to a Hybond-P PVDF membrane (Amersham Bioscience, Piscataway, N.J.) using an XCell II Blot Module (Novex). The transfer was carried out at 35 V for 18 h at 4°C. The membranes were cut at 45 kDa (for β-actin) and 127 kDa (for hTERT) molecular weight levels. A double antibody procedure was used to detect the proteins. The membranes were incubated under agitation for 1 h at RT in a blocking solution (PBS containing 5% dry skimmed milk and 0.1% Tween-20), then at RT for 1 h in anti-hTERT (rabbit, Calbiochem, diluted 1:1000) or anti-β-actin monoclonal antibodies (mouse, Calbiochem; 1:20,000). The antibody solution was removed and the membranes were washed three times with PBS containing 0.1% Tween-20 (PBS-T). The membranes were then incubated for 1 h at RT under agitation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma, 1:5000) for anti-hTERT and goat anti-mouse IgM (Sigma, 1:2000) for anti-β-actin antibody. Chemiluminescence was detected immediately using an ECL Advance Western blotting detection kit (Amersham Bioscience).

Statistical analysis

One-way analysis of variance (ANOVA) was used followed by Bonferroni's test for significant differences. Statistical significance was defined at $P<0.05$. The statistical procedures were performed using GraphPad InStat software. Data are expressed as mean ± SD.

Reagents

All reagents of analytical grade were obtained from Amersham Pharmacia Biotech. Becton Dickinson (Franklin Lakes, N.J.), Gibco BRL (Rockville, Md.), Pharmacia Biotech (Piscataway, N.J., Sigma Chemical Company (St. Louis, Mo.), Toyobo Company (Osaka, Japan), and Wako Pure Chemical Industries (Osaka, Japan).

Results and discussion

Strategies targeting telomerase regulation are usually directed to DNA or mRNA and use either cytotoxic drugs or antisense oligonucleotide substances against the RNA template. However, there are increasing amounts of data on the involvement of post-translational processes and factors in the regulation of telomerase activity, indicating that the protein part of telomerase may be a key target for its control. In this context, protein phosphorylation is an important post-translational mechanism commonly used in modulation of protein structure and function [12, 19]. It has been reported in human breast cancer, nasopharyngeal cancer and melanoma cell lysates that telomerase activity is markedly inhibited in the presence of PP-2A or nonspecific alkaline phosphatase [19–22] and this effect is prevented by the PP-2A inhibitor or exogenous purified PK-C [19, 23–25]. These results suggest a model in which telomerase exists in two different conformations that can be switched on and off by reversible phosphorylation and dephosphorylation [12]. It is accepted that dephosphorylation locks telomerase into an inactive conformation and is responsible for its negative control, while protein phosphorylation is able to restore telomerase activity and is essential for its activation [12]. However, this model remains controversial following recently reported data indicating that telomerase activity is suppressed by c-Abl tyrosine kinase-mediated phosphorylation of its hTERT catalytic subunit [26].

The present study was designed to clarify the validity of this model, investigating the role of PK-C and CaM-dependent protein kinases in the regulation of human telomerase activity in Burkitt's lymphoma cells.

PK-C and CaM were inhibited by their respective inhibitors—BIM and TFPZ. In preliminary experiments, the dose-dependent and time-dependent effects of BIM and TFPZ on cell viability were examined. As shown in Fig. 1a, BIM showed well-expressed cytotoxic activity against leukemia cells at concentrations in the range 25–50 μM per 5×10^5 cells/ml and with incubation times of 12 h and longer. TFPZ possessed a similar cytotoxicity at concentrations in the range 5–10 μM per 5×10^5 cells/ml and with incubation times of 12 h and

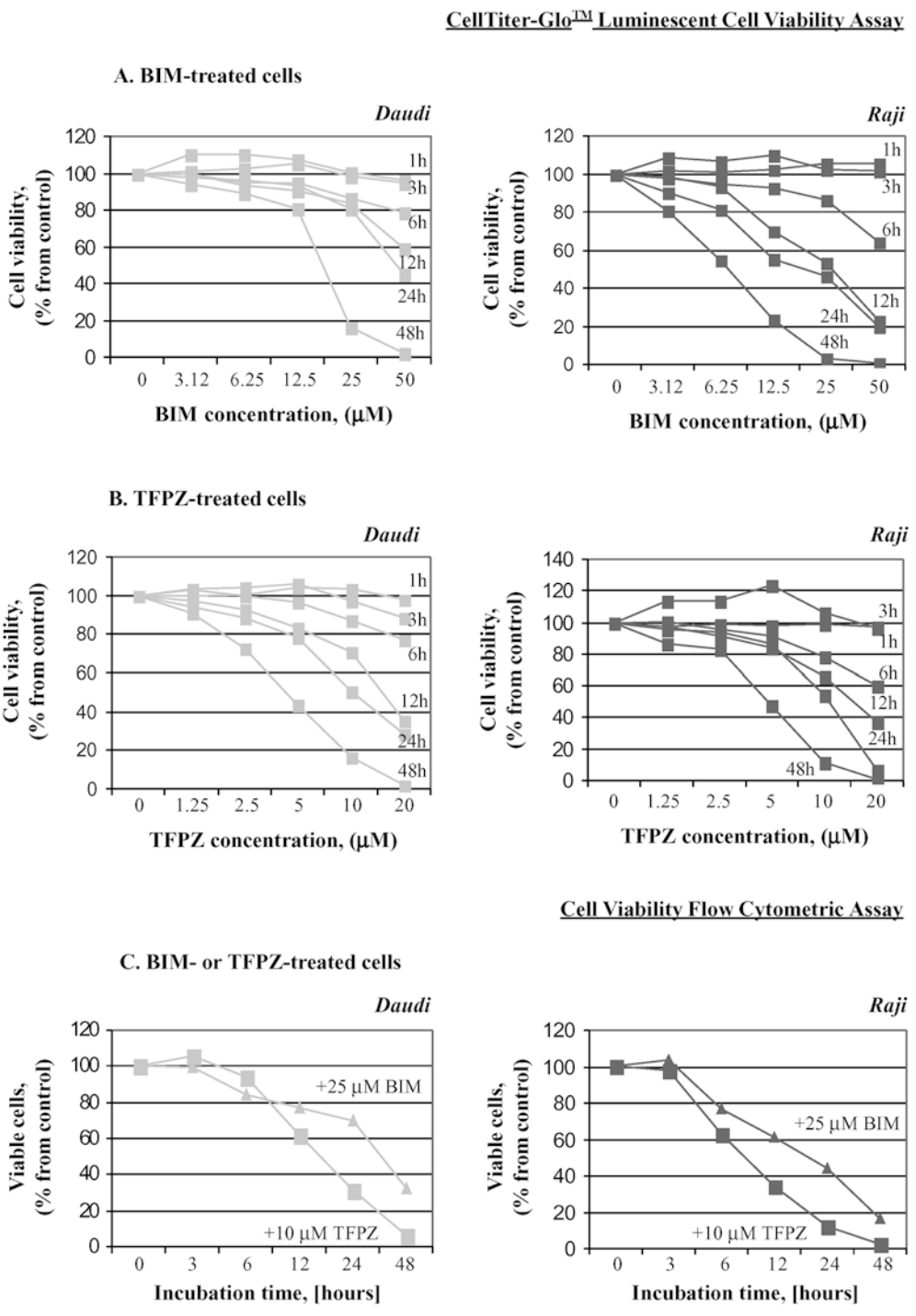


Fig. 1a–c Dose-dependent and time-dependent effects of BIM and TFPZ on the viability of human Burkitt's lymphoma cells. Daudi and Raji cells (5×10^5/ml) were cultured in the absence (control) and in the presence of BIM or TFPZ. Cell viability was determined by the CellTiter-Glo luminescent cell viability assay using ATP bioluminescence as a marker of cell viability (**a**, **b**) or by flow cytometry **c**. The results are presented as percentages in relation to control values, and are the means from eight (**a**, **b**) or three (**c**) independent experiments. The SD did not exceed 15% of the respective mean values

longer (Fig. 1b). The cytotoxic effects of both substances were estimated using ATP bioluminescence as a marker of cell viability. The effects of BIM and TFPZ on the viability were also investigated by flow cytometry (Fig. 1c). At the selected concentration (10 μ*M*), TFPZ showed slightly greater cytotoxicity than BIM (25 μ*M*), especially with incubation times in the range 12–48 h.

The cytotoxic effects of both drugs were better manifested against Raji cells than Daudi cells, suggesting that Raji cells are more sensitive to PK-C inhibitors or CaM inhibitors. This difference could be due to a multitude of factors, including: (1) different intracellular concentration of the drugs between the two cell lines; (2) possible differences in accumulation, influx or efflux of the drugs; and (3) differential metabolism or protein binding of the drugs within the cells. It may also be possible that the lower mRNA expression levels of c-*myc* and c-*fgr* protooncogenes in Raji cells than in Daudi cells are also one of the potential reasons for the higher sensitivity of Raji cells to BIM and TFPZ [37, 38]. It has

been established that both protooncogenes are antiapoptotic signals and their mRNA sensitizes cells against cytotoxic agents [38, 39]. Moreover, overexpression of c-*myc* is able to induce hTERT expression [40].

Proceeding from the dose-dependent and time-dependent effects of BIM and TFPZ on cell viability, in the experiments directed to the determination of telomerase activity we used, respectively, 25 μ*M* BIM or 10 μ*M* TFPZ per 5×10^5 cells/ml and incubation times in the range 6–48 h. It is well known that at these concentrations BIM and TFPZ to a great extent inhibit PK-C and CaM-dependent protein-kinases, respectively [41, 42].

BIM inhibited telomerase activity in a time-dependent manner in Daudi cells and Raji cells (Fig. 2a). The suppression of telomerase was manifested even after 12 h of incubation with BIM and the inhibitory effect increased with increasing incubation time. The concentration of TRAP products had decreased by about 40% in Daudi cells and by about 55% in Raji cells after 24–48 h incubation with drug, and the value was similar to that for the negative control for telomerase activity. In an analogous manner to cell viability, telomerase was more sensitive to BIM in Raji cells than in Daudi cells.

In contrast, TFPZ did not show antitelomerase activity even after 24 h incubation with cell suspensions (Fig. 2b). TFPZ inhibited telomerase after 48 h incubation and its effect seemed to be similar in both cell lines—the levels of TRAP products decreased by about 40% in Daudi cells and by about 45% in Raji cells.

The results obtained are in agreement with previously published data indicating that treatment with PK-C inhibitors or calcium-channel blockers suppresses telomerase activity in cancer cells [9, 24, 25, 28, 43, 44].

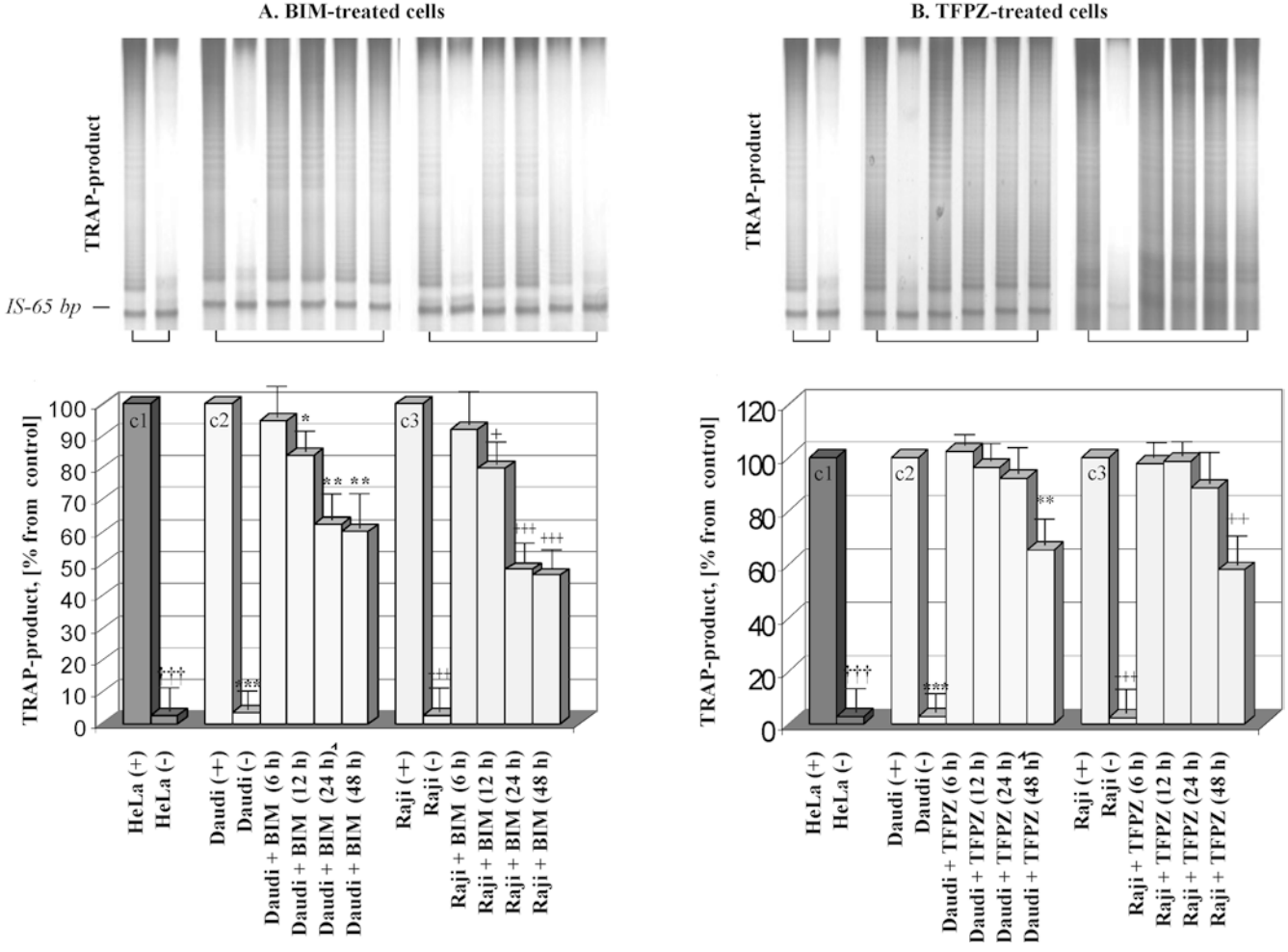


Fig. 2 Effects of BIM and TFPZ on the telomerase activity of human Burkitt's lymphoma cells. Raji and Daudi cells (5×10^5/ml) were cultured in the absence (control) and in the presence of 25 μ*M* BIM or 10 μ*M* TFPZ, respectively. Telomerase activity was assayed in cell lysates by a conventional telomeric repeat amplification protocol (TRAP) (*IS* internal standard, 65 bp). The concentrations of TRAP products were determined spectrophotometrically at 450 nm and are presented as percentages in relation to the respective control (*c1*, *c2*, *c3*). The absolute values of absorbance at 450 nm for *c1*, *c2* and *c3* were 1.635 ± 0.158, 1.740 ± 0.109 and 2.105 ± 0.314, respectively. The results are presented as means ± SD from six independent experiments. *HeLa(+)* positive control; *HeLa(−)* negative control, cell lysate treated at 70°C for 10 min; *Daudi(+)* *Raji(+)* cell lysates obtained from BIM-untreated and TFPZ-untreated cells; *Daudi(−)* *Raji(−)* cell lysates obtained from BIM-untreated and TFPZ-untreated cells incubated at 70°C for 10 min. $^{\dagger\dagger\dagger}P<0.001$ vs *c1*, $^{*}P<0.05$ vs *c2*, $^{**}P<0.01$ vs *c2*, $^{***}P<0.001$ vs *c2*, $^{+}P<0.05$ vs *c3*, $^{++}P<0.01$ vs *c3*, $^{+++}P<0.001$ vs *c3*

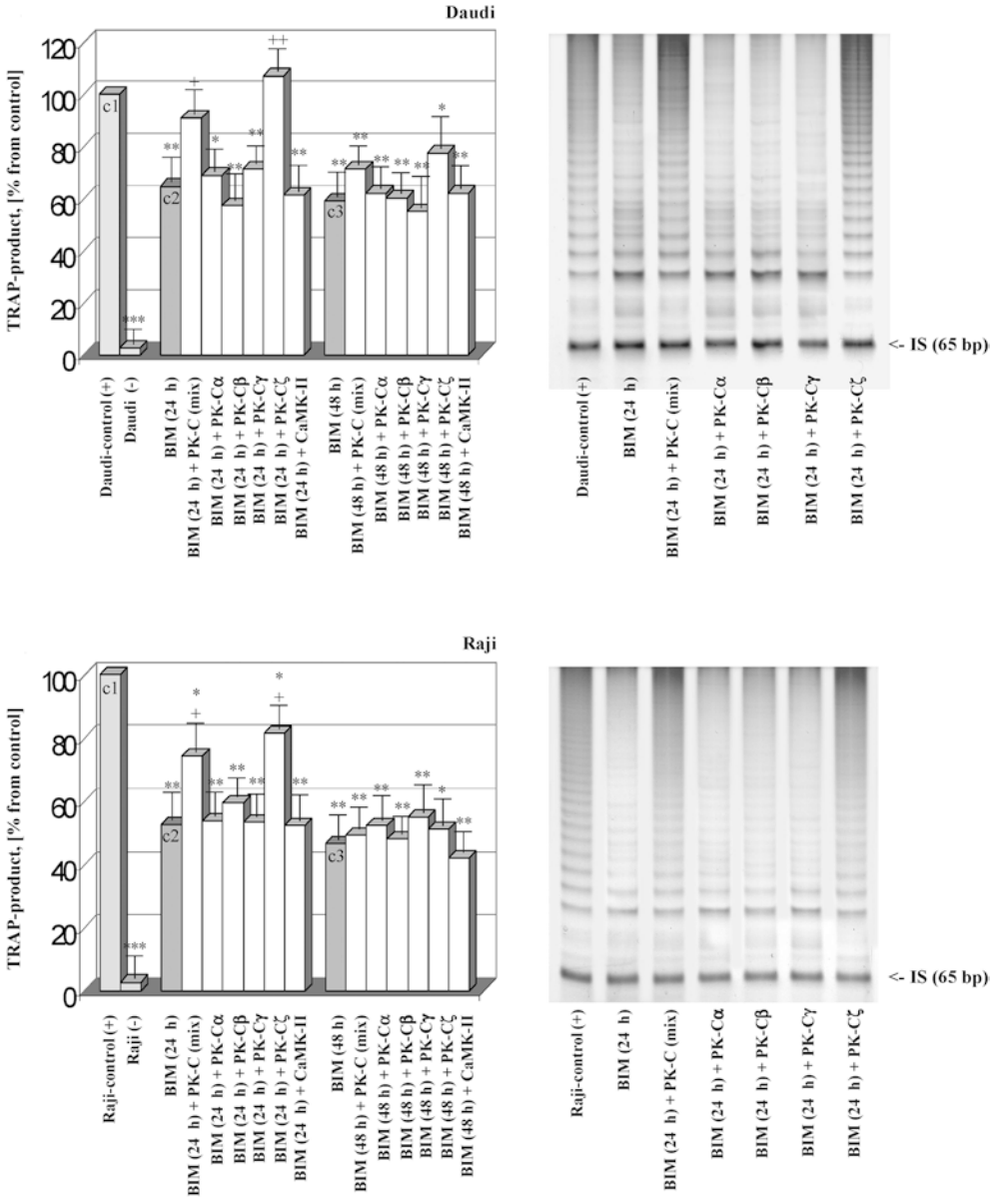


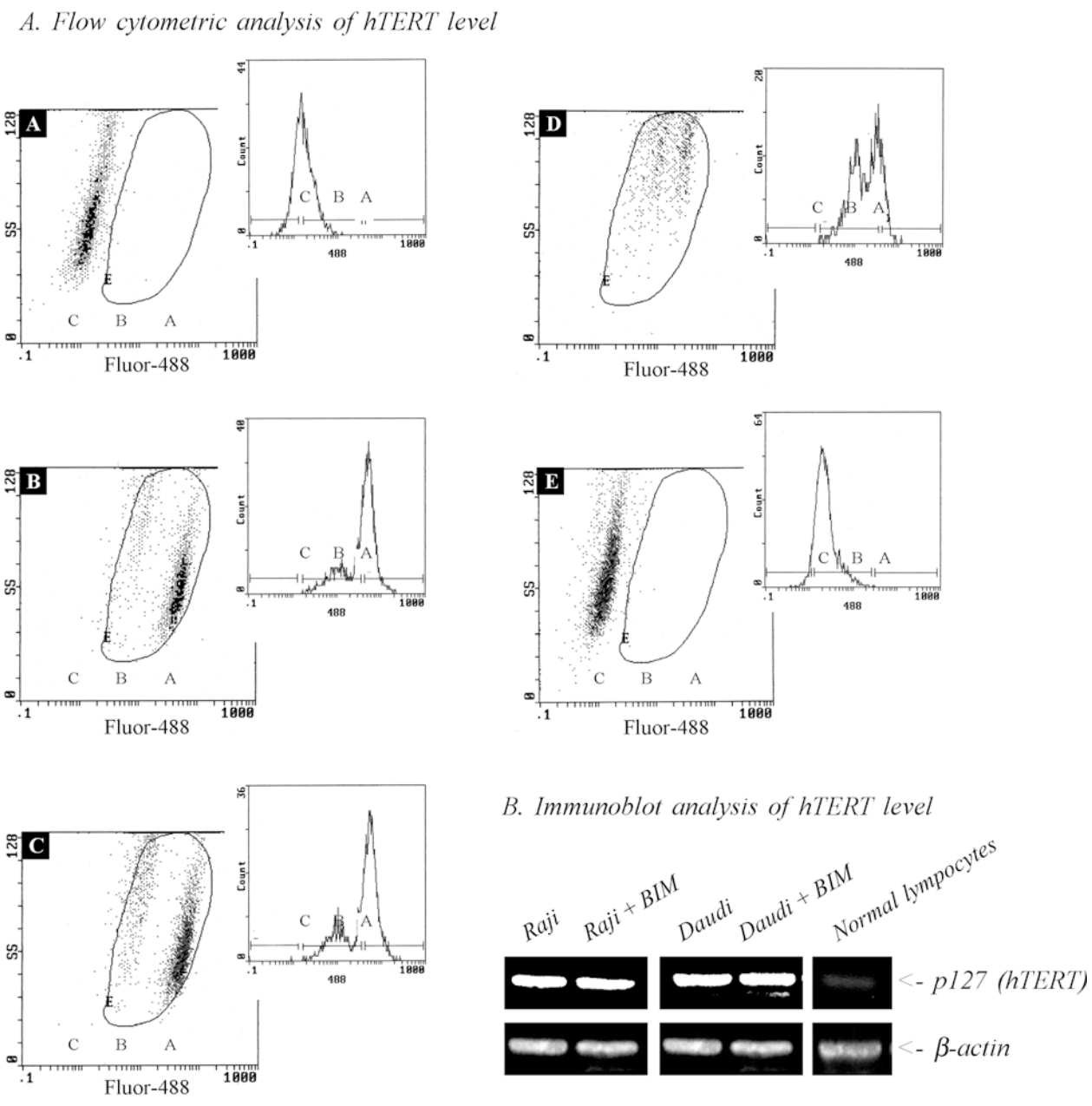
Fig. 3 Reactivation of telomerase by in vitro PK-C- or CaMK-II-catalyzed phosphorylation of cell extracts obtained from BIM-treated Burkitt's lymphoma cells. Daudi and Raji cells 5×10^5/ml) were cultured in the absence (control) or the presence of 25 μ*M* BIM for 24 or 48 h. Cell extracts were obtained and phosphorylated in vitro by exogenous purified PK-C isoenzymes or CaMK-II as described in Materials and methods. Telomerase activity was assayed in cell extracts by a conventional telomeric repeat amplification protocol (TRAP) (*IS* internal standard, 65 bp). The concentrations of TRAP products in each extract were determined spectrophotometrically at 450 nm and were calculated as percentages in relation to control (*c1*). The absolute values of absorbance at 450 nm for *c1* in Daudi and Raji cells were 1.609 ± 0.157 and 2.248 ± 0.257, respectively. The results are presented as means ± SD from five independent experiments. *Daudi(+) Raji(+)* cell extracts obtained from BIM-untreated cells; *Daudi(−) Raji(−)* cell extracts obtained from BIM-untreated cells and incubated at 70°C for 10 min. $^*P<0.05$ vs *c1*, $^{**}P<0.01$ vs *c1*, $^{***}P<0.001$ vs *c1*, $^{+}P<0.05$ vs *c2*, $^{++}P<0.01$ vs *c2*, $^{\dagger}P<0.05$ vs *c3*

Table 1 Activity of exogenous kinases (PK-C isoenzymes, CaMK-II) in cell lysates obtained from BIM-treated and TFPZ-treated Burkitt's lymphoma cells. The endogenous levels of the respective kinase activity were subtracted from the results. The concentration of exogenously added protein kinases was 1 n*M*

Cells	+ PK-C-mix (mP)[a]	+ PK-Cα (mP)[a]	+ PK-Cβ (mP)[a]	+ PK-Cγ (mP)[a]	+ PK-Cζ (mP)[a]	+ CAMK-II (cpm)[b]
Daudi	182 ± 31	228 ± 24	195 ± 32	248 ± 27	235 ± 18	120,803 ± 22,474
Daudi + BIM (24 h)	165 ± 27	197 ± 23	190 ± 17	211 ± 14	215 ± 23	118,422 ± 28,003
Daudi + BIM (48 h)	171 ± 22	185 ± 35	174 ± 28	225 ± 21	205 ± 14	107,663 ± 12,890
Daudi + TFPZ (48 h)	158 ± 35	207 ± 26	201 ± 22	198 ± 34	241 ± 27	101,552 ± 15,848
Raji	210 ± 28	233 ± 25	263 ± 39	215 ± 25	248 ± 26	152,088 ± 29,570
Raji + BIM (24 h)	187 ± 35	218 ± 28	235 ± 28	194 ± 21	222 ± 29	141,446 ± 20,983
Raji + BIM (48 h)	190 ± 25	204 ± 16	241 ± 27	185 ± 17	218 ± 15	147,702 ± 31,005
Raji + TFPZ (48 h)	184 ± 27	207 ± 22	218 ± 20	197 ± 23	237 ± 33	127,403 ± 25,809

[a]Fluorescence polarization
[b]Radioactivity of [^{32}P] incorporated into CaMK-II substrate peptide during a 20-min incubation

A discrepancy was observed between the effects of the two drugs on cell viability and telomerase activity (Figs. 1 and 2). BIM and TFPZ inhibited the viability of leukemia cells more than telomerase activity, which was probably a result of the semiquantitative nature of the TRAP assay and nonlinear dependence of telomerase



activity on the number of viable cells used in the non-radioactive TRAP analyses.

Since the effects of BIM and TFPZ on cell signaling are mediated directly through PK-C- and CaM-dependent protein kinases, respectively, we estimated the potential of both enzymes to restore the telomerase activity in BIM-treated and TFPZ-treated cells. It was found that the addition of exogenous PK-C (mixture of isoenzymes) to the cell protein extracts obtained from cells treated with BIM for 24 h restored the telomerase activity substantially in Daudi cells and to a lower extent in Raji cells (Fig. 3). Using pure PK-C isoenzymes it was observed that atypical PK-Cζ, but not conventional Ca^{2+}-dependent PK-C isoenzymes (α, β, γ), is responsible for telomerase reactivation. However, in cell extracts obtained from cells treated with BIM for 48 h, exogenous PK-C (mixture of isoenzymes) and pure PK-Cζ had little effect on telomerase activity—TRAP products remained at relatively low levels and a slight but non-significant increase was observed only in Daudi cells after the addition of PK-Cζ. The activity of exogenous PK-C isoenzymes in cell lysates was found to be relatively high in both drug-treated and untreated cells (Table 1). Since BIM did not affect the levels of hTERT in Raji cells or Daudi cells (Fig. 4), its effect on telomerase activity can be considered to be mediated through PK-C-dependent post-translational regulation of hTERT or through alteration of another subunit of telomerase.

The extension product ladder for BIM-treated Daudi cells (Fig. 3) shows that the processivity of telomerase was also altered, with multiple extension products

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Fig. 4 **a** Flow cytometric analysis of hTERT levels in BIM-treated and non-treated Burkitt's lymphoma cells. *A* Spontaneous fluorescence of non-treated Raji cells; *B* non-treated Raji cells containing Fluor-488-conjugated anti-hTERT antibody (control); *C* BIM-treated Raji cells containing Fluor-488-conjugated anti-hTERT antibody; *D*, *E* for comparison, histograms of drug-treated leukemia cells expressing moderate levels of hTERT (*D*) and non-treated normal lymphocytes expressing negligibly low levels of hTERT (*E*). *Y*-axis, side scatter (SS); *X*-axis, Fluor-488 fluorescence. *Quadrant A* Cells containing maximum (control) levels of Fluor-488-conjugated anti-hTERT antibody and therefore expressing maximum level of hTERT; *quadrant B* cells containing moderate or low levels of Fluor-488-conjugated anti-hTERT antibody and therefore expressing moderate or low levels of hTERT; *quadrant C* cells without fluorescent marker and therefore without hTERT (spontaneous cell fluorescence). To the right of each histogram are shown curves of Fluor-488 fluorescence. The results are representative of both Raji and Daudi cells. **b** Immunoblot analysis of hTERT levels in BIM-treated cells. The cells were treated with BIM (25 μ*M*) for 24 h. Cell lysates were obtained and immunoblot analysis of hTERT was carried out as described in Materials and methods using anti-hTERT antibody. Cell lysates, obtained from non-treated normal lymphocytes, were used as a controls for the specificity of anti-hTERT antibody. Blots from one typical experiment were presented

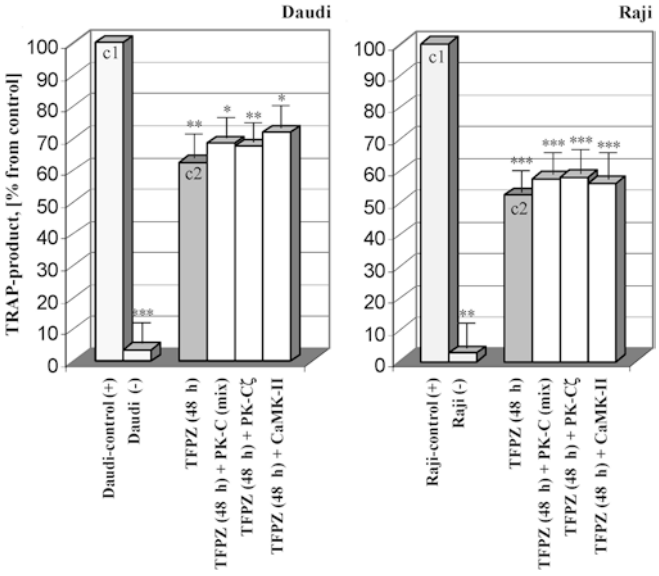

Fig. 5 Reactivation of telomerase by in vitro PK-C-catalyzed or CaMK-II-catalyzed phosphorylation of cell extracts obtained from TFPZ-treated Burkitt's lymphoma cells. Daudi and Raji cells (5×10^5/ml) were cultured in the absence (control) and in the presence of 10 μ*M* TFPZ for 48 h. Cell extracts were obtained and phosphorylated in vitro by exogenous purified PK-C isoenzymes or CaMK-II as described in Materials and methods. Telomerase activity was assayed in cell extracts by a conventional telomeric repeat amplification protocol (TRAP) (*IS* internal standard, 65 bp). The concentrations of TRAP products in each extract were determined spectrophotometrically at 450 nm and were calculated as percentages in relation to the control (*c1*). The absolute values of absorbance at 450 nm for *c1* in Daudi and Raji cells were 1.609 ± 0.157 and 2.248 ± 0.257, respectively. The results are presented as the means ± SD from four independent experiments. *Daudi(+) Raji(+)* cell extracts obtained from TFPZ-untreated cells; *Daudi(−) Raji(−)* cell extracts obtained from TFPZ-untreated cells and incubated at 70°C for 10 min. $^*P<0.05$ vs *c1*, $^{**}P<0.01$ vs *c1*, $^{***}P<0.001$ vs *c1*

deviating from the six-base ladder in the low part of the ladder. This feature is completely restored following the addition of the PK-C-mix or PK-Cζ, but not following the addition of any of the other isoforms. This indicates that this effect may be due to a direct effect on some components of the telomerase complex, and that the modulation of the enzyme by PK-C may regulate processivity more than it regulates activity. Such an effect was not found in BIM-treated Raji cells or in TFPZ-treated cells.

It is well known that PK-C-dependent and CaM-dependent pathways are closely related, that PK-C inhibitors can indirectly influence CaM-dependent protein kinases, and that CaM inhibitors can influence Ca^{2+}-dependent PK-C isoenzymes. To avoid such crosstalk and side effects of BIM or TFPZ and to clarify the potential of other protein-kinases in telomerase induction, we investigated the possibility that exogenous CaMK-II could reactivate telomerase in the same cell extracts (the activity of CaMK-II in cell lysates is presented on Table 1). As can be seen from Fig. 5, exogenous CaMK-II did not restore telomerase activity in cell lysates obtained from BIM-treated or TFPZ-treated Daudi or Raji cells.

Summarizing, the present study provided direct proof that the PK-C-dependent pathway (in particular atypical PK-Cζ), but not the Ca^{2+}/CaM-dependent pathway, is a major activator of telomerase in Burkitt's lymphoma cells.

There are some discrepancies between our results and previously published results [21, 25]. Yu et al. have reported that exogenous PK-C almost completely reactivates telomerase in nasopharyngeal cancer cells obtained after 3 days incubation with BIM [25]. Similar effects have been observed by Li et al. in PMC42 breast cancer cells treated with PP-2A [21]. It has also been found that the inhibition of telomerase activity by PK-C inhibitors in nasopharyngeal cancer cells appears to be specific since treated cells are mostly viable (i.e., greater than 75%) and still retain a significant capacity for protein synthesis [24]. We established that: (1) exogenous purified PK-C (ζ isoenzyme or mixture of isoenzymes) only poorly reactivated telomerase in cells treated for 48 h with BIM; (2) TFPZ strongly inhibited telomerase in Raji and Daudi cells after 48 h incubation, but exogenous PK-Cζ and CaMK-II did not restore telomerase activity; and (3) the viability of leukemia cells strongly decreased during 48 h of incubation with PK-C or CaM inhibitor—about 80% for BIM and almost completely for TFPZ (Fig. 1). It seems that in Burkitt's lymphoma cells the effect of BIM on telomerase activity is mediated by PK-C (in particular ζ isoenzyme) as shown in cell suspensions incubated for up to 24 h. Long-term treatment of leukemia cells with BIM or TFPZ (48 h and more) appeared to induce other mechanism(s) of telomerase regulation which are PK-C-independent and CaMK-II-independent. To clarify this discrepancy, which was probably a result of

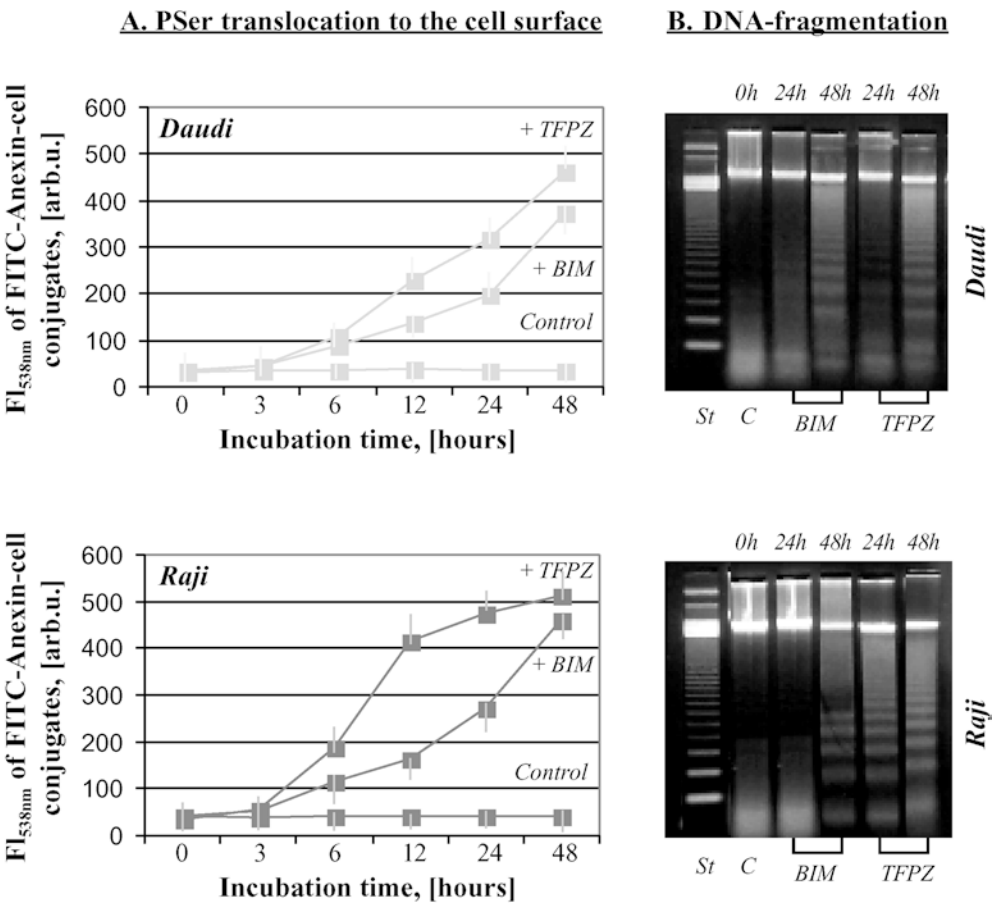

Fig. 6a, b Induction of apoptosis in BIM-treated and TFPZ-treated Burkitt's lymphoma cells. **a** PSer exposure on the cell surface. Raji and Daudi cells were cultured in the absence (*Control*) and in the presence of 25 μ*M* BIM or 10 μ*M* TFPZ for 0–48 h. FITC-Annexin V bound to cell surface PSer was detected spectrofluorimetrically. The data presented are the means ± SD from four independent experiments. **b** DNA fragmentation. Raji and Daudi cells were cultured in the absence (*C*) and in the presence of 25 μ*M* BIM or 10 μ*M* TFPZ for 0–48 h. DNA was extracted and DNA fragmentation was detected by gel electrophoresis (*St* standard marker, *C* control nontreated cells)

Table 2 Correlation between viability, telomerase activity and induction of apoptosis in BIM-treated and TFPZ-treated Burkitt's lymphoma cells. The correlation coefficients were calculated for all parameters at 25 μ*M* BIM and 10 μ*M* TFPZ

Parameters	BIM		TFPZ	
	r	*P* value	*r*	*P* value
Cell viability vs apoptosis	−0.90136	< 0.01	−0.98570	< 0.01
Cell viability vs telomerase activity	0.89069	< 0.01	0.68807	< 0.05
Apoptosis vs telomerase activity	−0.98291	< 0.05	−0.73994	< 0.05

different experimental protocols, we examined the possibility that BIM and TFPZ induce apoptosis in Raji and Daudi cells. The correlation between telomerase activity, cell viability and development of apoptosis in BIM-treated and TFPZ-treated cells were also determined.

PSer translocation from the inner to the outer monolayer of the plasma membrane of apoptotic cells was used as a criterion for development of apoptosis [44, 45]. It is well known that FITC-labeled Annexin V specifically recognizes PSer-Ca^{2+} complexes and is widely used to detect early apoptotic cells by flow cytometry or spectrofluorimetry [45–47]. Treatment of Raji and Daudi cells with the same doses of drugs as used in the telomerase assays markedly increased the concentrations of FITC-Annexin V bound to the cell surface (Fig. 6). TFPZ had a greater effect than BIM. The exposure of PSer on the surface of TFPZ-treated Raji cells increased markedly even after 12 h incubation. The same level of PSer on the surface of BIM-treated Raji cells was observed after 48 h incubation. In Daudi cells, exposure of PSer on the cell surface was also higher after treatment with TFPZ than after treatment with BIM.

It was also found that TFPZ increased binding of FITC-Annexin V to a greater degree in Raji cells than in Daudi cells ($P < 0.001$ for 3–24 h incubation; $P > 0.05$ for 48 h). A similar effect was obtained with BIM, but the difference between the cell lines was statistically significant only for the 48-h incubation ($P < 0.01$). These results are consistent with recent reports indicating that CaM antagonists significantly enhance PSer exposure

on the cell surface of leukemia cells as well as PSer synthesis de novo, which is considered a marker of development of apoptosis [44, 48]. It has also been found that the presence of PK-C inhibitors in cell cultures for more than 24 h triggers apoptotic death [49, 50]. Both BIM and TFPZ also induced DNA fragmentation in leukemia cells during 48 h incubation—a characteristic of the final stage of apoptosis (Fig. 6). However, it is thought that during incubation for 24 h or less, PK-C inhibitors do not disrupt the plasma membrane, a signal which is known to switch on apoptosis [51]. We also found that about 40–70% of leukemia cells were viable after 24 h incubation with BIM (Fig. 1c), whereas treatment with TFPZ more strongly decreased the proportion of viable cells—to about 10% of the control level in Raji cells and about 30% in Daudi cells (Fig. 1c).

The development of apoptosis explains, at least partially, the differences in telomerase activity and the potential for its reactivation by exogenous PK-C in cells treated with BIM for 24 and 48 h. Presumably, in the time interval 0–24 h the effect of BIM on telomerase occurs via PK-C inhibition and the development of apoptosis is not a limiting factor for the enzyme activity as was observed after 48 h. The exogenous PK-C (ζ isoenzyme and mix) was able to restore telomerase activity in cells treated with BIM for 24 h, and the levels of TRAP products reached the initial control levels in non-treated cells (Fig. 3). However, in cells treated with BIM for 48 h, the exogenous PK-C-mix or Cζ could not restore telomerase activity, probably because of the impossibility of overcoming the effect of apoptosis on telomerase. In contrast, the effect of TFPZ on telomerase activity even during 24 h incubation seemed to relate mainly to apoptotic cell death and protein degradation, and was not a result of a CaM-mediated mechanism. As described above, exogenous CaMK-II did not restore telomerase activity in cells treated with TFPZ for 24 and 48 h.

A very good negative correlation between telomerase activity and development of apoptosis in both BIM-treated and TFPZ-treated Burkitt's lymphoma cells was calculated ($r = -0.98$ and $r = -0.74$, respectively; Table 2). There was also a good positive correlation between telomerase activity and cell viability ($r = 0.89$, $P < 0.05$, in BIM-treated cells; $r = 0.69$, $P < 0.05$, in TFPZ-treated cells). A very good negative correlation was determined between cell viability and development of apoptosis ($r = -0.90$, $P < 0.01$, in BIM-treated cells; $r = -0.98$, $P < 0.01$, in TFPZ-treated cells). Since the correlation between telomerase activity and cell viability and apoptosis was higher in BIM-treated cells than in TFPZ-treated cells, one may speculate that PK-C-dependent mechanism(s) play a more important role in telomerase regulation than CaM-dependent mechanism(s). The inability of exogenous Ca^{2+}-dependent PK-C isoenzymes (α, β, γ) to restore telomerase activity in BIM-treated cells confirms this suggestion.

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