The dual role of protein kinase C in the regulation of telomerase activity in human lymphocytes

Wei-Yun Sheng, Ya-Li Chien, Tzu-Chien V. Wang*

Department of Molecular and Cellular Biology, Chang Gung University, Kwei-San, Tao-Yuan 333, Taiwan

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Abstract Protein kinase C (PKC) has been implicated to play an essential function in the upregulation of telomerase activity in activated T cells, yet its role in the regulation of telomerase activity remains largely unknown. In this work, we present evidence that PKC activity is required both for the induction of *hTERT* expression and for the post-transcriptional control of telomerase enzyme activity in T lymphocytes. Of the several PKC isoforms present in lymphocytes, only the level of PKC-ζ was greatly increased during T cell activation, implicating that PKC-ζ may be required for the post-transcriptional control of telomerase enzyme activity in T lymphocytes.

Key words: Protein kinase C; hTERT; Telomerase; T cell activation

1. Introduction

Telomerase is a specialized reverse transcriptase that directs the synthesis of telomeric repeats (TTAGGG in human) at chromosome ends [1]. Telomerase appears to play a key role in maintaining telomere length and in replicative senescene. Normal human somatic cells express low or undetectable telomerase activity and are mortal. In contrast, moderate or high telomerase activity is present in the majority of immortal and cancer cells [2,3]. Ectopic expression of telomerase activity has been shown to extend the life-span of several normal human cells [4–8]. Together these observations support an important role of telomerase in cellular immortalization.

The minimal catalytic core of human telomerase consists of a RNA component (hTER) and of a catalytic protein subunit (hTERT) with reverse transcriptase activity [9–14]. Additional telomerase-associated proteins (hTEP1, dyskerin, p23, and Hsp90) have been identified [15–18], yet their biochemical function remains obscure. Studies on the correlation between telomerase activity and the expression of *hTER*, *hTEP1*, and *hTERT* have indicated that telomerase activity is strongly correlated with the abundance of *hTERT* mRNA [10,11]. The abundance of mRNA for *hTEP1* and hTER does not correlate with telomerase activity [15,16,19]. Together with the result that ectopic expression of hTERT in somatic cells is sufficient to restore telomerase activity [4–7,12,20], these observations demonstrate that hTERT is the key regulator of enzyme activity.

The promoter region of hTERT gene has been characterized [21–23]. Sequence analysis has revealed that the hTERT promoter contains hormone response elements and binding sites for several transcription factors including cMyc, Spl, and others [21–23], implicating that *hTERT* expression may be regulated by multiple factors. Several activators (cMyc, Spl) and repressors (WTI, Mad1, E2FI, p53 and MZF-2) for *hTERT* expression have been identified [24,25]. Exactly how these activators and repressors may function in the different types of cells to regulate the expression of *hTERT* remains largely unknown. While transcriptional regulation of *hTERT* gene expression appears to be the primary control of telomerase in human cells, several studies have indicated that protein phosphorylation can reversibly regulate the function of telomerase [26–28]. Protein kinase C (PKC)-α and PKC-ζ have been shown to control telomerase activity by phosphorylating telomerase proteins in breast cancer cells and nasopharyngeal cancer cells, respectively [27,29].

T lymphocytes require extensive cell division and clonal expansion for their functions. To achieve this, T lymphocytes may develop regulatory mechanism to overcome the problem of replicative senescence. It has been reported that telomerase activity is expressed and stringently regulated during T lymphocyte development and differentiation [30]. Telomerase activity is low or undetectable in peripheral T lymphocytes, but is upregulated several hundred-fold upon activation [30,31]. The expression of telomerase activity in activated T lymphocytes has been known to require PKC-dependent activity [32,33]. Exactly how PKC functions to upregulate telomerase activity during T cell activation is not known, however. In this work, we investigate if the expression of telomerase activity in activated T cells is mediated by the PKC-dependent transcriptional induction of *hTERT* expression or by the PKC-dependent post-transcriptional control of enzyme activity, or both.

2. Materials and methods

2.1. Chemicals, enzymes, antibodies, and oligonucleotides

RPMI medium 1640, Hanks' balanced salt solution (HBSS), Superscript II reverse transcriptase, TRIzol reagent, antibiotics, and phytohemagglutinin (PHA) were purchased from Gibco-BRL. RNase inhibitor, bisindolylmaleimide I (BIM 1), and Gö6976 were purchased from CalBiochem. Co., San Diego, CA, USA. Taq DNA polymerase was from Viogene Co., Taipei, Taiwan. Antibodies against individual PKC isoform and secondary antibody were from Santa Cruz Biotech. The sequence and source of TS, CX, LT5, LT6, K136 and K137 oligonucleotides were previously described [29]. Gel electrophoresis

*Corresponding author. Fax: 886-3-211-8700.
E-mail address: tcvwg@mail.cgu.edu.tw (T.-C.V. Wang).

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reagents were from Bio-Rad. All other chemicals were from Sigma Chemical Co.

2.2. Cell culture

Blood was collected in heparinized tubes, and mononuclear cells were isolated by density gradient centrifugation using Ficoll-Hypaque (Ficoll-Paque, Pharmacia Biotech. Co., Uppsala, Sweden). These peripheral blood mononuclear cells were cultured in RPMI medium 1640 containing 20% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 U/ml streptomycin and 0.25 μg/ml amphotericin B) for 24 h. Floating cells were collected and used as the source of peripheral blood lymphocytes (PBL). PBL were cultured at a density of 1×10^6 cells/ml in RPMI medium 1640 using 24-well plates. When required, PHA, BIM I, and Gö6976 were added at 1:50, 5 mM, and 2.5 mM, respectively. Cells were grown at 37°C in a humidified incubator containing 5% CO_2.

2.3. Telomerase activity assay

Cell extracts were obtained as described [34]. A polymerase chain reaction (PCR)-based telomeric repeat amplification protocol (TRAP) [2] was used for assaying telomerase activity. The condition for PCR amplification and analysis of PCR products by electrophoresis on polyacrylamide gel was as described [34].

2.4. Determination of hTERT mRNA expression by reverse transcriptase PCR

Total RNA was extracted by TRIzol isolation reagent following manufacturer's instruction. The primers used were: LT5 and LT6 for *hTERT*, and K136 and K137 for GAPDH. The condition for reverse transcriptase PCR for *hTERT* mRNA was predetermined with serial dilutions of total RNA such that the amount of RNA used in the reaction was in the linear range of the assay. In brief, reverse transcription was performed by addition of 30 ng of total RNA, 10 pmol of each primer, 4 U of reverse transcriptase, 10 U of RNase inhibitor, and 2 U of Taq polymerase to a 30 μl of buffer containing 20 mM Tris, pH 8.2, 50 mM KCl, 0.25 mM dNTPs, 0.1% bovine serum albumin, and 1 mM $MgCl_2$. The reaction mixtures were incubated at 42°C for 1 h, followed by heat inactivation at 94°C for 3 min. PCR amplification was carried out by 31 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 1.5 min. The PCR products were separated by electrophoresis in 5 or 8% polyacrylamide gels and analyzed as described [29].

2.5. Western blot analysis of PKC isoforms

Western blot analysis was performed as previously described [29].

3. Results

3.1. PKC is required for the induction of hTERT expression

Since hTERT appears to be the key regulator of telomerase activity and is known to be upregulated during T cell activation [31], our first question is to address if PKC activity is required for the induction of *hTERT* expression. PBL were treated with PHA to stimulate T lymphocyte proliferation and the expression of *hTERT* and telomerase activity was followed. As shown in Fig. 1, an increase of *hTERT* mRNA was detectable as early as 6 h after the addition of PHA to the cells. Expression of *hTERT* peaked at 1–2 days and then slowly decreased to the basal level. The expression of telomerase activity began to increase after about 12–18 h and peaked at 2–3 days following the addition of PHA to the cells. After 3 days, telomerase activity began to level off and then decreased slowly. In the absence of PHA, there was no detectable increase of telomerase activity or *hTERT* RNA expression over 2 days (data not shown). These results are in general agreement with the results obtained by others that the expression of telomerase activity in activated T cells is accompanied with the induction of *hTERT* expression [31].

To explore if PKC activity is required for the induced expression of *hTERT*, the effects of PKC inhibitors on the expression of telomerase activity and *hTERT* were examined in PHA-treated PBL. As shown in Fig. 2, the increased expression of *hTERT* and telomerase activity was completely inhibited when PKC inhibitor (BIM I or Gö6976) was added simultaneously with the PHA, indicating that the induced expression of *hTERT* requires PKC activity.

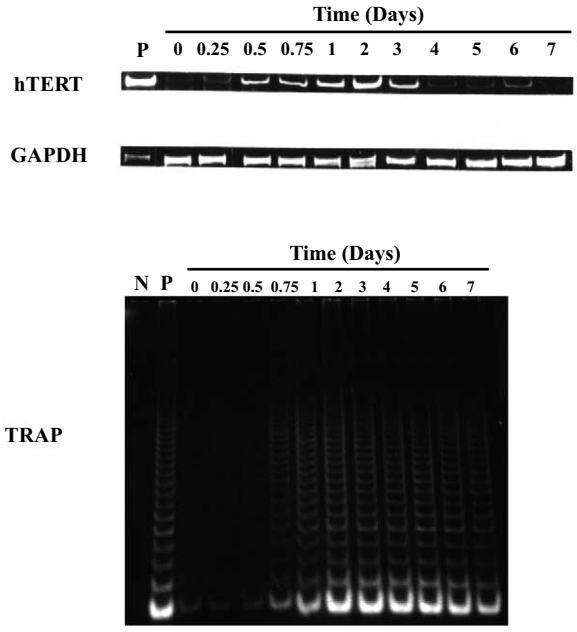

Fig. 1. Kinetics of *hTERT* expression and telomerase activation in PHA-treated PBL. PBL were treated with PHA for the indicated times and assayed for the expression of *hTERT* mRNA and telomerase activity as described in Section 2. The expression of GAPDH mRNA, served as a control, was also included. Lane P is a positive control using cell extract obtained from telomerase-positive HL-60 cells. Lane N is a negative control with no cell extract.

3.2. PKC is also required for post-transcriptional control of telomerase activity

In view of the reports that protein phosphorylation can reversibly regulate the function of telomerase [26–29], we ask if PKC activity is also required for post-transcriptional control of telomerase enzyme activity. PBL were treated with PHA to induce the expression of *hTERT* and PKC inhibitors

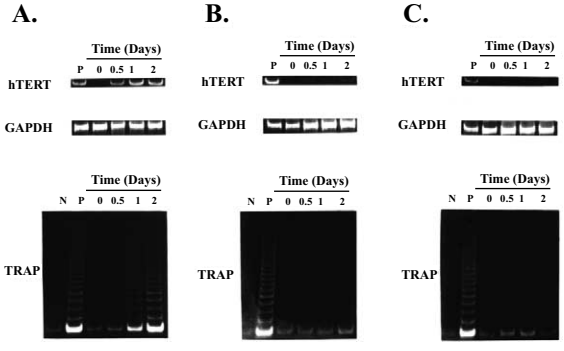

Fig. 2. Effect of BIM I and Gö6976 on the expression of *hTERT* and telomerase activity in PHA-treated PBL. PBL were treated with PHA and PKC inhibitor (BIM I or Gö6976) simultaneously for the indicated times and assayed for the expression of *hTERT* mRNA and telomerase activity as described in Section 2. Panels: A, no inhibitor; B, plus BIM 1; C, plus Gö6976.

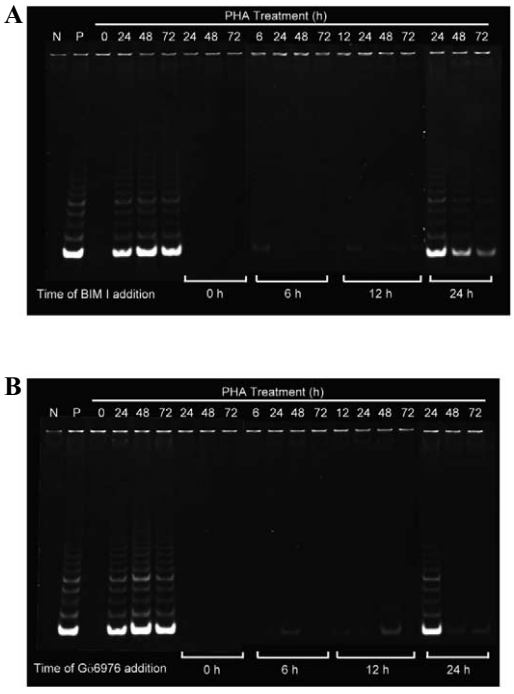

Fig. 3. Effect of delayed addition of PKC inhibitors on the telomerase activity of PHA-treated PBL. PBL were treated with PHA and PKC inhibitor (BIM I or Gö6976) was then added at the indicated times. Telomerase activity was assayed by TRAP as described in Section 2. Panels: A, plus BIM 1; B, plus Gö6976.

were then added to examine their effects on the expression of telomerase activity. As shown in Fig. 3, when PKC inhibitors were added 6 or 12 h after PHA addition, telomerase activity was not activated in the following days. However, the *hTERT* mRNA was readily detectable at these times and continued to be expressed in the following days (Fig. 4). When PKC inhibitors were added 24 h after PHA addition, telomerase activity was not further increased. Instead, the telomerase activity was reduced in the following days (Fig. 3). Accompanied with the decrease of telomerase activity, the expression of *hTERT* mRNA was also reduced (Fig. 4).

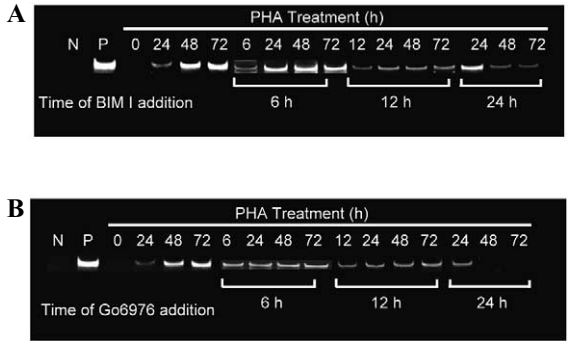

Fig. 4. Effect of delayed addition of PKC inhibitors on the expression of *hTERT* in PHA-treated PBL PBL were treated with PHA and PKC inhibitor (BIM I or Gö6976) was then added at the indicated times. The expression of *hTERT* mRNA was assayed by reverse transcriptase PCR as described in Section 2. Lane P is a positive control using cell extract obtained from telomerase-positive HL-60 cells. Lane N is a negative control with no cell extract. Panels: A, plus BIM 1; B, plus Gö6976.

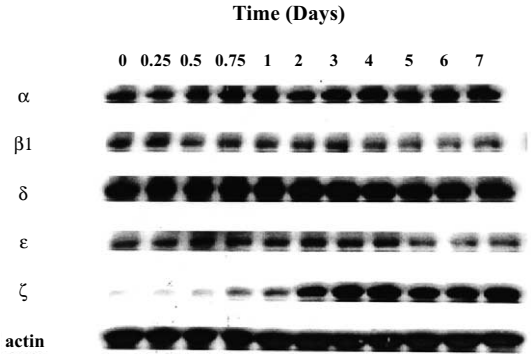

Fig. 5. Level of PKC isoforms in PHA-treated PBL. PBL were treated with PHA and cultured for the indicated times. The level of PKC isoforms was monitored by Western blot as described in Section 2. The level of actin, served as an internal control, was included.

3.3. *Level of individual PKC isoforms in PHA-treated lymphocytes*

Since specific PKC isoform is employed by different cancer cells to control telomerase activity in vivo [27,29], we seek to understand which PKC isoform(s) may participate in the control of telomerase activity in lymphocytes. The level of PKC isoforms was monitored by Western blot during T cell activation and the results are shown in Fig. 5. The level of PKC-α, -β1, -δ, or -ε was little changed after the PHA-stimulation. In contrast, the expression of PKC-ζ was greatly increased. A significant increase of PKC-ζ began to be detected at about 18 h after PHA addition, which coincided with the increased expression of telomerase activity seen in Fig. 1.

4. Discussion

Possible involvement of PKC in the upregulation of telomerase activity in activated T cells may include: (i) phosphorylation of telomerase proteins, (ii) increased expression of telomerase genes through PKC-dependent signal pathway, and (iii) both of the above postulates. In this work, we have presented evidence to indicate that the increased expression of telomerase activity during T cell activation requires PKC-dependent activity to induce the expression of *hTERT*. It is likely that the early induction of *hTERT* expression during T cell activation is mediated through PKC-dependent signal pathway to induce the expression of *cMyc*, since it is known that *cMyc* is one of the earlier genes that are induced following T cell activation [35] and that overexpression of cMyc is able to induce the expression of *hTERT* [36–38]. Activation of pre-existing PKC isoforms is likely to account for the induced expression of *hTERT*. Rapid translocation of specific PKC isoforms to membrane has been observed in T cells stimulated to proliferate by various agents [39]. At present, it is not yet possible to correlate the activation of any specific PKC isoform(s) with the induction of *hTERT*.

In addition to a role in the induction of *hTERT* expression, PKC-dependent activity is also required for the post-transcriptional control of telomerase enzyme activity in T lymphocytes. Such a conclusion is based on the observations that no activation of telomerase activity was observed when PKC inhibitors were added at 6 or 12 h after PHA treatment (Fig. 3). Under this condition, the expression of *hTERT* mRNA was either little changed or continued to increase in the following

days (Fig. 4). The lack of activated telomerase activity in the presence of *hTERT* expression suggests that PKC activity is also required for the post-transcriptional control of telomerase. A role of PKC in the control of telomerase activity has been demonstrated to phosphorylate telomerase proteins [26–29]. We suggest that PKC also participates in the phosphorylation of telomerase proteins in T lymphocytes.

A non-transcriptional mechanism of regulating telomerase has also been described previously for $CD4^+$ T cells activated by anti-CD3 [40]. Phosphorylation of hTERT protein was observed and is thought to account for the increased telomerase activity under this condition. It is likely that PKC participates in the phosphorylation of hTERT during this activation event, although definitive proof of such a postulate awaits further confirmation.

While the delayed addition of PKC inhibitors did not appear to inhibit the expression of *hTERT* in PBL treated with PHA for 6–12 h, an inhibition of *hTERT* expression was observed in PBL treated with PHA for 24 h (Fig. 4). Although it is possible that T lymphocytes activated for different lengths of time may employ different mechanism to express *hTERT* and therefore, display differential sensitivity to the inhibition by PKC inhibitors. At present, we are at a loss trying to understand these puzzling data.

Finally, we have attempted to understand which PKC isoform participates in the post-transcriptional control of telomerase activity. Among the several PKC isoforms detected in lymphocytes, only PKC-ζ is greatly increased following PHA stimulation (Fig. 5). The increased expression of PKC-ζ appears to coincide with the increase of telomerase activity. This result plus the fact that the expression of telomerase activity was considerably lagged behind the induction of *hTERT* (Fig. 1) suggest that the post-transcriptional control of enzyme activity probably does not employ the pre-existing PKC isoforms, rather, the increased PKC-ζ may be required to perform such an function. However, the inhibition of telomerase activity by Gö6976 (Fig. 3B) casts some doubts about the validity of this deduction. This drug is known to be relative specific for inhibiting calcium-dependent PKC isoforms (PKC-α, -β, and -γ) in vitro [41]. If such a specific inhibition also occurs in the treated cells, the observed inhibition of telomerase by this drug would argue that the calcium-dependent PKCs are also involved in the control of telomerase activity. Perhaps, the post-transcriptional control of telomerase activity in T lymphocytes may require the coordinated action of several PKC isoforms. Attempts to specifically inhibit individual PKC isoform during T cell activation using antisense oligonucleotides were unsuccessful, because these primary cells were refractory to DNA transfection. Therefore, while our data are consistent with a role of PKC-ζ in the post-translational control of telomerase activity, we can not exclude the possibility that other PKC isoform(s) also participates in the post-transcriptional control of telomerase activity in T lymphocytes.

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