Terminal deoxynucleotidil transferase is a nuclear PKC substrate

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Abstract Protein phosphorylation is the regulatory mechanism of many cellular events in response to changes in metabolic activity and environmental conditions. Seeing that PKC and TdT levels in cells are both regulated by PMA, we sought particularly intriguing to investigate TdT phosphorylation in vivo, utilizing KM-3 cells, a TdT-positive human pre-B cell line treated with PMA and in vitro, employing purified PKC and human recombinant TdT. Our data show that TdT is a substrate for PKC activity, suggesting that TdT phosphorylation could play a key role in the pathway affecting the control of gene transcription and protein synthesis during lymphoid cells differentiation.

Key words: PKC; Terminal transferase; PMA; Phosphorylation; Differentiation

1. Introduction

Terminal deoxynucleotidyl transferase (TdT) is a 'creative' DNA polymerase found only in lymphoid cells at early stages of differentiation and in corresponding leukemic cells [1,2]. It has long been thought to play a role in the generation of somatic diversity in primitive T and B cells [3], without a general mutagenic effect [4]. Recent gene disruption experiments in mice have shown that TdT is responsible for creating N region diversity [5,6] in immunoglobulin and T cell receptor genes. It has been previously proved that in KM-3 cells the TdT synthesis can be downregulated by phorbol ester treatment [7,8]. The complete amino acid sequence of human TdT has been obtained by DNA-cloning and shows that the first 17 N-terminal residues contain a potential nuclear localization sequence and phosphorylation sites [9]. Experiments on labeling of human lymphoblastoid cells with [^{32}P]phosphate showed that TdT can be phosphorylated by a cAMP-dependent protein kinase with an increase of TdT activity [10]. In vitro phosphorylation of TdT by the catalytic subunit of cAMP-dependent protein kinase results in phosphorylation within the first 17 N-terminal residues [11]. Protein kinase C (PKC) might be involved in the regulation of TdT since it is present and operates in the nucleus of most cell types [12–15]. PKC is physiologically regulated by diacylglycerol and Ca^{2+} and can be activated by phorbol esters [16–19]. In this study, we have demonstrated in vitro phosphorylation of human recombinant TdT by purified PKC and in vivo phosphorylation of TdT in KM-3 cells after treatment by PMA, thus, raising the possibility that PKC might modulate TdT function and/or localization.

2. Materials and methods

Enzymatically active human recombinant TdT (predominantly 58-kDa species) was purified from recombinant baculovirus-infected insect cells [20]. Protein A sepharose-conjugated monoclonal antibody against TdT protein was from Supertech (Rockville, MD). Peroxidase-conjugated secondary antibodies and normal goat serum were from Litton Bionetics (Kensington, MD). [γ-^{32}P]ATP (5000 Ci/mmol) and γ-$^{32}PO_4$ orthophosphate in aqueous solution (HCl-free, 10 mCi/ml) were obtained from Amersham (UK).

2.1. Cell culture

KM-3 cell line is a TdT-positive human pre-B cell line originally described by Schneider et al. [21]. Cells were grown in RPMI-1640 medium supplemented with 10% FBS, 4 mM L-glutamine, 100 mM Na-pyruvate and 25 mM HEPES. Cell cultures at a density of 2.5×10^5/ml, with >98% viability by Trypan blue exclusion test, were used for experiments. For induction experiments, the cells were suspended in complete medium in the presence of 20 μM PMA prepared as previously described [8] and cultured for a maximum of 4 h.

2.2. TdT in vivo phosphorylation

3×10^7 of untreated, PMA-treated and PMA-treated KM-3 cells in the presence of 50 μM bisindolylmaleimide hydrochloride as PKC inhibitor [22] were incubated in 3 ml DMEM phosphate-free medium containing 10% FCS and 0.5 mCi/ml/1×10^6 $^{32}PO_4$ for 4 h at 37°C. Washed cells were homogenized in 1 ml P_i 0.2 M (pH 7.4). Immunoprecipitation was performed by adding to 2 ml of sample 10 μl of conjugated protein A sepharose gel monoclonal antibody antihuman TdT, at the concentration of 2 mg/ml, and then incubated at cold room overnight. The immunoprecipitate where washed after spinning. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), consequently, the same amount of proteins has been electrophoresed as standard procedure. After SDS-polyacrilamide gel electrophoresis, the samples were electroblotted on nitrocellulose. Nitrocellulose paper was treated as described [23] for immunological detection of TdT and autoradiographed on Kodak X-OMAT film.

2.3. TdT in vitro phosphorylation

10 μg recombinant TdT proteins were phosphorylated with 0.07 U PKC at 30°C for 10 min in the presence of 5 mM $MgCl_2$, 3 mM dithiothreitol, 100 μM vanadate, 250 μM $CaCl_2$, 100 μg/ml phosphatidylserine and 4 μg/ml diacylglycerol or 20 μM PMA as PKC activator. As specific PKC inhibitor, we used 50 μM bisindolylmaleimide hydrochloride. The reactions were stopped with appropriate volumes of 4 × sample buffer (0.25 M Tris-HCl (pH 6.8), 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.005% Bromophenol blue), boiled for 5 min and electrophoresed on 10% acrylamide/ 0.1% SDS according to Laemmli (1970). The proteins were electroblotted on nitrocellulose, stained with Ponceau S and autoradiographed on Kodak X-OMAT film. PKC was obtained from rat brain essentially as described by Kikkawa et al. [24], omitting the final chromatographic step.

For in vivo and in vitro experiments, the same antibodies were used. Mouse monoclonal and rabbit polyclonal antihuman TdT antibodies were prepared and their specificity established as described [25,26]. Protein A sepharose monoclonal anti-TdT specificity has been verified

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in our experiments and as previously reported [25–28]. We did not obtain cross-reactions with other proteins (data not shown).

3. Results and discussion

Since TdT and PKC colocalize in the interchromatin domains ([12,15,23,29–33] and pers. data), we tested TdT as a substrate for PKC. The results obtained by in vitro phosphorylation of recombinant human TdT with partly purified PKC is shown in the autoradiography in Figs. 1 and 2. The Ponceau S-stained nitrocellulose suggests that recombinant human TdT is predominantly 58-kDa species (Fig. 1). A trace of 56-kDa species present probably represents translation at an alternate start site on the TdT mRNA in the baculovirus expression system. The autoradiogram (Fig. 1) shows that both 58- and 56-kDa peptides are phosphorylated by PKC. Some minor autophosphorylation bands are also present, including the 80-kDa native PKC, as revealed in both gel lanes. We have also carried out in vitro phosphorylation of calf thymus TdT with PKC and found that besides the 58- and 56-kDa peptides, lower molecular weight species of calf thymus TdT as well as the α- and β-subunits of the low molecular weight form of calf thymus TdT are also phosphorylated by PKC (data not shown). These

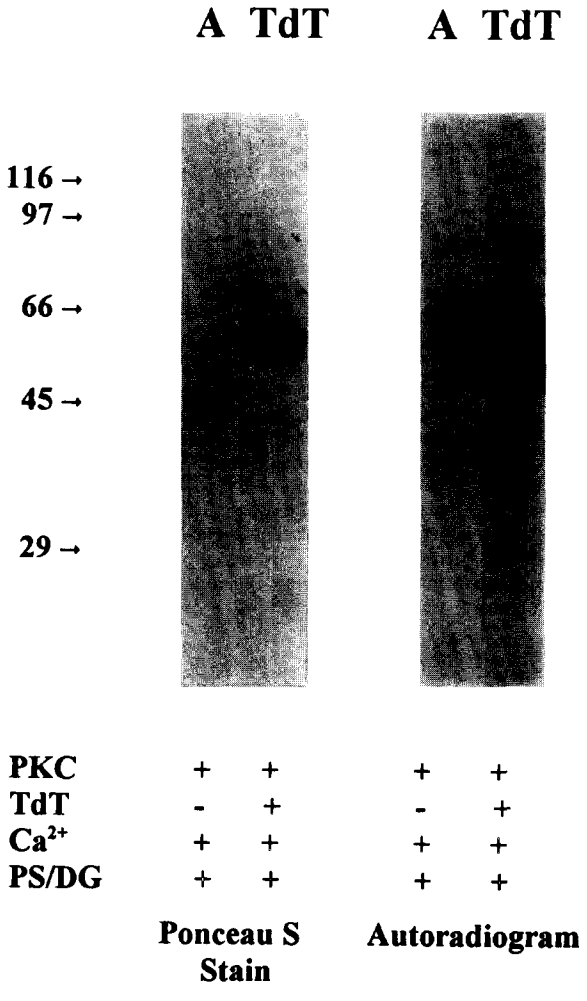

Fig. 1. In vitro phosphorylation of recombinant human TdT by PKC. (A) Autophosphorylation lane containing complete mixture except TdT. Molecular weight markers are in kDa.

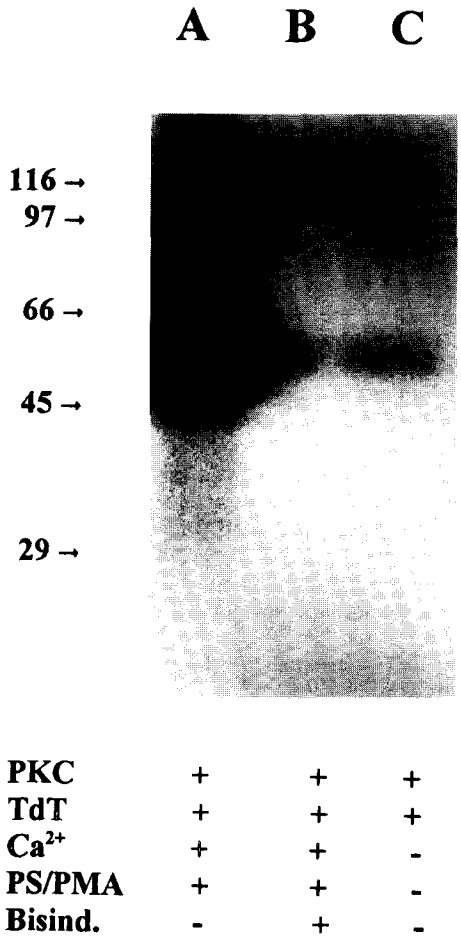

Fig. 2. In vitro phosphorylation assay. Lane A shows a over-phosphorylated band (58 kDa) undetectable in the presence of PKC inhibitor (lane B). Lane C documents a sample containing only PKC and TdT. Molecular weight markers are expressed in kDa.

results induce to suppose that PKC phosphorylates TdT at multiple sites, suggesting the presence of several phosphorylation sites along the peptide [9,28]. The autoradiogramm reported in Fig. 2, lane A displays the TdT in vitro phosphorylation experiments in the presence of 20 μM PMA instead of DG. Under this condition, is possible to observe a strong phosphorylated 58-kDa band corresponding to TdT protein. Lane B shows the phosphorylation assay in the presence of bisindolylmaleimide hydrochloride as specific PKC inhibitor. Lane C reports TdT phosphorylation assay performed in absence of Ca^{2+}, phospholipids and PMA. In these experimental conditions, both lane B and C do not show significant TdT phosphorylation processes. PMA-treated KM-3 lymphoblastic leukemic cells were labeled in vivo with $^{32}PO_4$. TdT was recovered from the lysate by immunoprecipitation, using a specific monoclonal antibody Protein A-conjugated (Fig. 3). SDS-polyacrylamide gel electrophoresis followed by autoradiography revealed a major labeled band of 58 kDa corresponding, as observed in vitro phosphorylation, to the high molecular weight form of TdT (Fig. 3, section B, lane a). Untreated (lane b) and PMA-treated samples cultured in the presence of a PKC inhibitor (lane c) did not show phosphorylated peptides. In

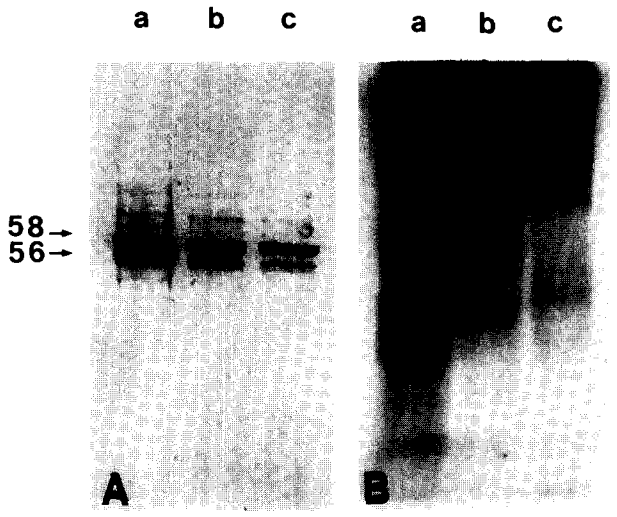

Fig. 3. (A) Immunoblotting analysis showing the presence of 58- and 56-kDa TdT in PMA-treated cells (a), untreated KM-3 cells (b) and in KM-3 cells treated with PMA and bisindolylmaleimide (c). (B) In vivo phosphorylation of KM-3 cells upon PMA treatment. It is possible to observe a phosphorylated 58-kDa TdT in PMA-treated KM-3 cells (a). Untreated cells do not show phosphorylated peptides (b). KM-3 cells treated with both PMA and Bisindolylmaleimide did not show the presence of phosphorylated TdT (c). Molecular weight markers are expressed in Kda.

Fig. 3, section A, we report Western blot analysis of the same samples reported in section B. Lines a–c display the presence of 56- and 58-kDa TdT protein. It has been recently proved that PKC can be translocated, after induction of inositol lipid breakdown, to the nucleus [31,32] and can be downregulated in KM-3 cells after 24 h of PMA treatment (pers. data). Among the diversity of nuclear targets of PKC, beside histones, lamins have been reported to be phosphorylated [33,34]. It is interesting that the sites of laminin phosporylation by PKC are the same in vivo an in vitro. DNA polymerase α has also been reported to be phosphorylated in vitro by PKC [35] and in vivo, the enzyme undergoes phosphorylation dependent on phosphatidyl inositol mono and diphosphate [36]. Other nuclear enzymes affecting DNA structure and function, such as DNA topoisomerase II, could be substrates for PKC [37] as well as the nucleoside phosphatase that mediates mRNA transport out of the nucleus [38]. The role of PKC in T cell activation has recently been described and this contributes to understanding the involvement of PKC in specific biological processes, including regulation of the immune responses [39]. Terminal transferase plays a key role in the immune differentiation events and has been described that extracellular stimuli as phorbol ester modulate the presence of TdT in prelymphocytes and can induce the PKC translocation into the nucleus, the conventional site of TdT localization. The data reported in this paper prove that the terminal transferase is a biological nuclear substrate for PKC- mediated phosphorylation process. Therefore, we propose that the phosphorylation of TdT might be an early step in the overall process of lymphoid differentiation. Since the protein kinase C consists of multiple isoenzymes with possibly distinctly different biological functions, more extensive efforts are being made for full understanding the specific PKC isoform involved in TdT phosphorylation process.

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