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PHOSPHORYLATION OF TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE IN LEUKEMIC CELLS

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SUMMARY - Phosphorylation of terminal deoxynucleotidyl transferase within leukemic cells has been demonstrated, using ^{32}P labelling of intact cells in culture, followed by immunoprecipitation of the cellular extracts using an anti-terminal transferase antiserum. The phosphate linkage was found to involve serine and threonine residues. Purified calf thymus terminal transferase served as a substrate for cyclic AMP independent protein kinase obtained from leukemic cells. Phosphorylation in vitro of terminal transferase was accompanied by increased activity and decreased inhibition by excess ribo-ATP. These results indicate that terminal transferase is a physiologic cyclic AMP independent protein kinase substrate, and that this reaction may be important in its control.

Terminal deoxynucleotidyl transferase (TdT) (EC 2.7.7.31) is an enzyme found only in lymphoid cells at the earliest stages of differentiation and in phenotypically corresponding leukemic cells (1). Although its detailed control and function remains to be elucidated, it is thought to play a role in the generation of immunologic diversity (2). Because of the importance of protein phosphorylation as a biochemical control mechanism in general (3), and its specific involvement in malignant transformation by certain viruses (4), we have investigated this enzyme as a possible physiologic substrate of leukemic cellular protein kinase (PK) (EC 2.7.1.37).

Abreviations Used

TdT, terminal deoxynucleotidyl transferase; PK, CK and TdT K, protein, casein and TdT kinase, respectively ; SDS, sodium dodecyl sulfate; $d(pA)_{\overline{78}}$, $d(pT)_6$, polydeoxyadenylic acid with an average length of 78 nucleotides and hexamer of thymidylic acid.

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MATERIALS AND METHODS

NALM-6 cell line (TdT^+ human lymphoblastic leukemia) (5) was passaged in our laboratory in modified McCoy's 5A medium and harvested while in exponential growth. 8 x 10^8 cells were incubated in 800 ml of phosphate free medium containing 5% dialyzed fetal calf serum and 5.79 mCi $^{32}PO_4$ for 5 hours at 37°. Washed cells were homogenized in 8 mM potassium phosphate pH 7.2, 8 mM NaCl with 0.5% Triton X100. The 15,000 xg supernatant was diluted with an equal volume of 5 mM 2-mercaptoethanol and then applied to a phosphocellulose column which had been previously equilibrated with 50 mM potassium phosphate pH 6.5 buffer. The column was washed with the same buffer until A_{280} returned to baseline, then eluted with 0.3 M pH 7.2 potassium phosphate buffer. TdT activity was determined as previously described (6) using $d(pA)_{\overline{78}}$ as primer. Active fractions were pooled and dialyzed against phosphate buffered saline (PBS). Immunoprecipitation was performed by adding 100 µl of anti-TdT serum (7) to 2 ml of sample, incubating at 37° for 40 minutes, then adding 300 µl of formalin-fixed staph A cells (BRL, Inc., Gaithersburg, Md). This was incubated an additional 30 minutes at 4° and then pelleted. The resuspended sample was pelleted through a 10% sucrose cushion and then washed in PBS three times, and then resuspended in 0.0625 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, boiled 90 seconds, centrifuged and the supernatant applied to a 10% polyacrylamide gel, along with (methyl-^{14}C) methylated molecular weight standards (bovine serum albumin, ovalbumin, carbonic anhydrase and lactoglobulin A). After SDS-polyacrylamide gel electrophoresis, the gel was dried and exposed to Kodak X-Omat AR film overnight. The labelled band was then excised and the proteins eluted (8), hydrolyzed (9) with 6 N HCl for 1½ hours at 110°, dried under N_2, and dissolved in 5 to 7 µl of phosphotyrosine, phosphoserine and phosphothreonine standard solution, each 1.7 mg/ml. Phosphoamino acid analysis was performed as previously described (9) using two dimensional thin layer electrophoresis at pH 1.9, 950 V for 2 hours and pH 3.5, 1000 V for one hour. Radioactivity was detected by fluorography and quantitated by scraping each spot into a vial and counting by liquid scintillation.

Calf thymus TdT was purified by the method of Chang and Bollum (10) to a specific activity of 23,300 U/mg. TdT activities in experiements using this preparation were performed by adding 10 µl of sample to 100 µl of assay mixture containing 200 mM potassium cacodylate pH 7.2, 1mM $CoCl_2$, 1mM 2-mercaptoethanol, 10 µM $d(pT)_6$ and 1 mM (^3H) dCTP (35,400 cpm/assay tube). This mixture was incubated for 10 minutes at 37° and then spotted in its entirety onto Whatman GF/B filter discs, washed with 5% trichloroacetic acid, 1% sodium pyrophosphate, and then 95% ethanol, and counted by liquid scintillation. Activities are presented as cpm incorporated per reaction tube during the incubation period. Cyclic AMP independent casein kinase was extracted from leukemic cells and purified through DEAE cellulose and Sephacryl S-200 chromatography steps as previously described (11,12). Protein kinase assays and phosphorylation reactions were performed as previously described, using reaction mixtures containing 20 mM (γ-^{32}P) ATP, 50 mM Tris-HCl pH 7.5, 3 mM $MgCl_2$ and casein 5 mg/ml, histone (Sigma II-AS) 2 mg/ml, or purified calf thymus TdT either 1 mg/ml for assays or at higher concentrations as indicated for some experiments. Column fractions were assayed in 80 µl reaction assays, containing 40 µl aliquots of each fraction and 180,000 cpm of (γ-^{32}P) ATP. The reaction mixtures were incubated for 10 minutes at 31°, and terminated by spotting 50 µl onto Whatman No. 3MM filter paper discs, which were then washed with 10% trichloroacetic acid, 1% sodium pyrophosphate, then 95% ethanol and then ether. The paper discs were dried and counted by liquid scintillation, and results presented as cpm incorporated into acid-insoluble material during the incubation period. Affinity chromatography of TdT was performed using a 0.7 x 10.5 cm oligo $(dT)_{12-18}$ cellulose column (13), which was equilibrated and exhaustively washed

with 50 mM Tris-HCl pH 7.5, 0.05% 2-mercaptoethanol, 5% glycerol. TdT was eluted with 1.0 M KCl, with continuous monitoring at A_{280} for protein.

RESULTS AND DISCUSSION

NALM-6 lymphoblastic leukemic cells were labelled with $^{32}PO_4$, and TdT was recovered from the lysate by phosphocellulose chromatography followed by immunoprecipitation, using a monospecific rabbit antiserum (7). SDS-polyacrylamide gel electrophoresis followed by fluorography revealed a major labelled band of MW 59,000 (Fig. 1) corresponding to the high molecular weight form of TdT characteristic of human leukemic cells (14). The phosphoprotein was recovered and subjected to phosphoamino acid analysis, which disclosed 85% phosphoserine and 15% phosphothreonine.

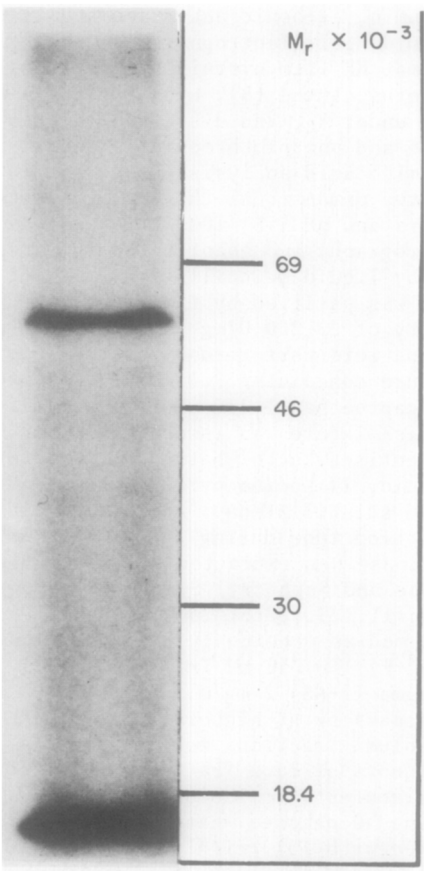

Figure 1 - In vivo phosphorylation of terminal transferase. TdT immunoprecipitated from ^{32}P labelled NALM-6 cells were subjected to SDS-polyacrylamide gel electrophoresis and fluorography. Positions of (methyl-^{14}C) methylated molecular weight standards are indicated by scale at right.

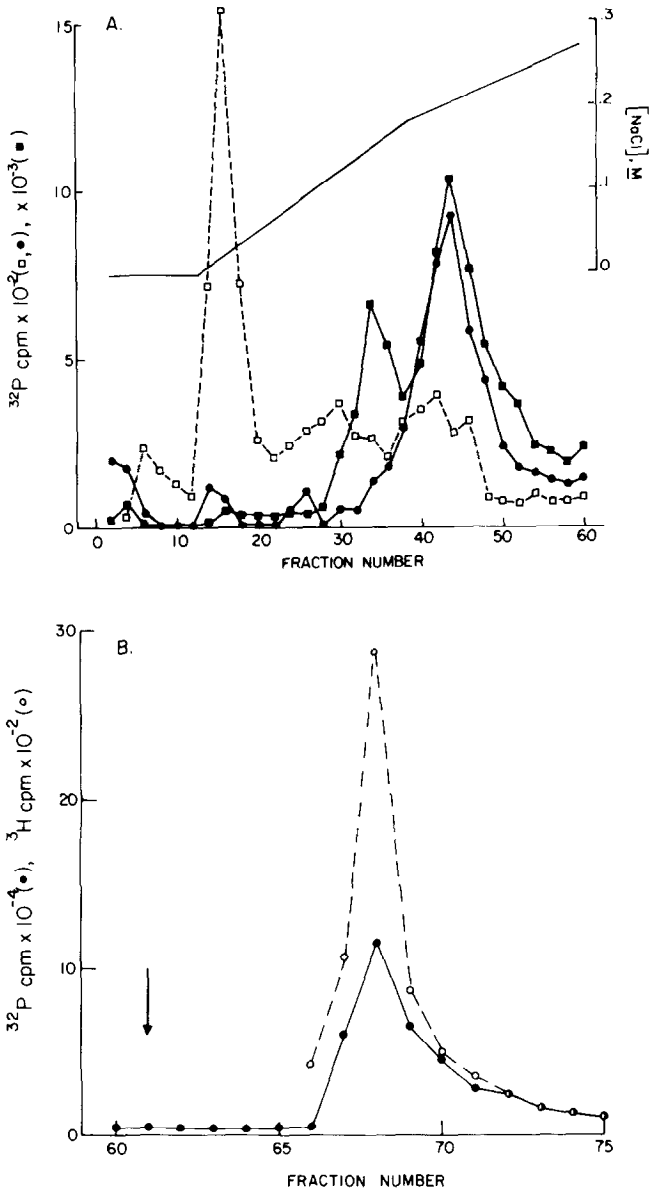

Figure 2 - In vitro phosphorylation of terminal transferase.

A. Dialyzed extract from human T-lymphoblast cell line CEM was chromatographed using a 0.8 x 10 cm DEAE-cellulose column eluted with a gradient of 0 to 0.3 M NaCl in a total of 160 ml of 5 mM potassium phosphate pH 7.5, 10% glycerol, 0.1% 2-mercaptoethanol and 3 ml fractions collected (11,12). PK activities were determined and are presented as described in Materials and Methods using as substrates (□) histone with 1 mM cAMP, (■) casein and (●) TdT. The position and slope of the eluting salt gradient is indicated by the diagonal line read from the right hand scale. Coelution of TdT K and the major CK peak is apparent.

B. Six mg. of calf thymus TdT which had been phosphorylated using 300 µl of leukemic cell extract and (γ-^{32}P) ATP (5.4 x 10^8 cpm) was applied to an oligo $(dT)_{12-18}$ cellulose column and eluted with 1.0 M KCl (arrow) after the column had been exhaustively washed with the equilabration buffer. Protein containing fractions of 1 ml were assayed for TdT activity (○) using $d(pT)_6$ as primer and (^{3}H)dCTP as the labeled triphosphate, and for total ^{32}P cpm present per 25 µl aliquot (●). Coelution of phosphorylated product and TdT activity is apparent.

In order to elucidate the enzymology of the observed intracellular phosphorylation, purified calf thymus TdT was used as a substrate in the following experiments. The preparation was phosphorylatable *in vitro* by crude and partly purified human leukemic cell PK preparations. TdT kinase (TdT K) activity coeluted from DEAE-cellulose (Fig. 2A), as well as Sephacryl S-200 (data not shown), along with cAMP independent casein kinase (CK) (11,12). The identity of the phosphorylated product as TdT was confirmed by their coelution from an oligo-dT cellulose column (13) (Fig. 2B). TdT K activity was much more prone to inhibition by high salt concentrations than CK (83% inhibition of TdT phosphorylation as compared to 40% inhibition of casein phosphorylation by 0.25 M NaCl). When partly purified and dialyzed CK was tested with various substrates at 1 mg/ml, TdT K activity, relative to CK, was 1.31, compared to 0.14 relative activity for lysozyme and no observable activity with bovine serum albumin. TdT phosphorylation by leukemic cell extracts and semi-purified CK was not stimulated by cAMP.

In order to determine if phosphorylation of TdT influences its activity, the effect of preincubation with PK and rATP upon TdT activity was examined. As shown in Figure 3, phosphorylation of TdT was associated with increased activity, particularly in the presence of high concentrations of rATP, as occur intracellularly. rATP has been shown to be competitively incorporated into the growing deoxyribonucleotide chain (15). This incorporation has been suggested to lead to decreased ability of the chain to accept additional deoxynucleotide residues, particularly after two rATPs have been added (15). The slopes of greater than one observed on the modified Hill plot (16) (Fig. 3) are consistent with this concept of chain termination requiring addition of more than one rATP in a cooperative fashion. In countering the inhibitory effect of physiologic concentrations of rATP, phosphorylation might exert an important regulatory effect upon TdT activity. Further studies will be necessary to elucidate the effect of phosphorylation upon TdT kinetics in detail.

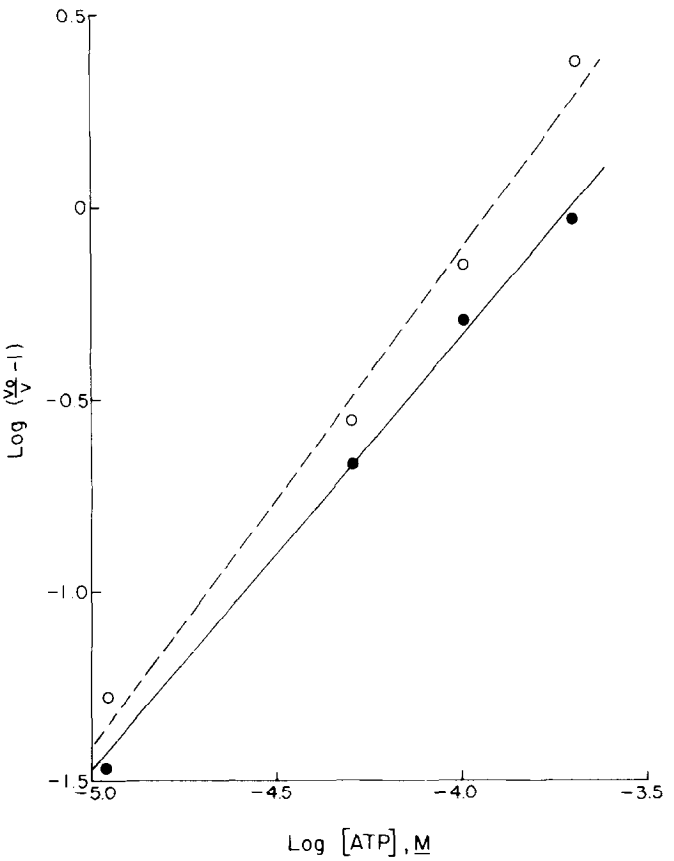

Figure 3. Effect of phosphorylation upon TdT activity. Purified TdT was exposed to phosphorylating conditions using unlabelled 20 μM ATP and either active (●) or previously boiled (○) leukemic cell extracts as the protein kinase source, for 10 minutes at 31°. Aliquots of 10 μl were withdrawn from these mixtures and then assayed for TdT activity in the presence of increasing concentrations of additional ATP, up to 200 μM. The effect of ATP upon TdT reaction velocities (V), relative to those without additional ATP (V_0), are shown using the modified Hill plot of Loftfield and Eigner (16). The lines were fit by linear regression, with calculated slopes of 1.29 for non-phosphorylated and 1.14 for phosphorylated TdT. Using K_m (dCTP) = 4.0×10^{-4} M, the K_i for competitive inhibition by ATP was calculated to be 2.7×10^{-6} M for unphosphorylated TdT and 1.9×10^{-5} M for phosphorylated TdT.

These results thus suggest a mechanistic link between two enzymatic systems, each of which is of largely unknown function but both of which have prominent associations with leukemia. Beyond the well known role of TdT as a "marker" for particular types of leukemia (1), and its likely role in the generation of immunologic diversity (2), it should be recognized that the subpopulation it defines is apparently critical in the expression of certain types of leukemogenic stimuli (17). The possibility that TdT itself has an important functional role in leukemo-

genesis contributed to our interest in studying its phosphorylation. Although phosphorylation by transforming gene products has been associated with induction of malignancy, the phosphorylation here observed appeared to be attributable to previously described (11,12) and widely distributed PKs rather than such gene products. The interpretation that the CKs studied in vitro are responsible for the observed intracellular phosphorylation is highly likely on the basis of the chromatographic studies and phosphoamino acid analysis described above, and the subcellular distribution of CK previously noted. The role of these cyclic AMP independent CKs within cells is nonetheless of interest, as they are present in high levels in leukemic cells, especially within the nuclei (11). Few other natural substrates of these enzymes have been identified or suggested. These include RNA polymerase II (18), non-histone chromatin associated proteins (19) and ribosomal proteins of some cell types (20). It thus seems possible that TdT phosphorylation is part of a coordinated general pathway pertaining to control of gene transcription and protein synthesis, which is appropriately activated in proliferating normal and malignantly transformed cells.

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