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Original Article

Hominoid triosephosphate isomerase: Regulation of expression of the proliferation specific isozyme

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Summary

Three primary isoforms of the dimeric glycolytic enzyme, triosephosphate isomerase (TPI; EC 5.3.1.1), are detected in proliferating human cells. The electrophoretically separable isoforms result from the three possible combinations of constitutive subunits and subunits expressed only in proliferating cells. Only a single primary isoform is observed in quiescent cells. The two subunits, which differ by covalent modification (s), are products of the single structural locus for this enzyme. Expression of the proliferation specific subunit (TPI-2) is detected within 6–10 hr following mitogen stimulation of quiescent human cells, requires RNA synthesis and is inhibited by agents which inhibit interleukin 2 expression or function. Only the constitutive subunit (TPI-1) is detected in proliferating cells from nonhominoid primate species. A single class of TPI mRNA, which is increased >10 fold following stimulation of quiescent cells, is detected on northern blot analysis and S1 nuclease digestion analysis of RNA from quiescent and proliferating human cells. It is similar in size to the TPI mRNA from proliferating cells of the African green monkey, a primate species not expressing TPI-2. Comparison of the structure of the TPI gene from rhesus monkey (nonexpressing species) to the gene from expressing species does not suggest a mechanism for generating TPI-2. Thus, the regulation of the expression of the hominoid restricted, proliferation specific subunit of TPI has been further defined, although the mechanism for generating TPI-2 remains elusive.

Introduction

Cells, as they progress through the cell cycle, express specific genes including oncogenes [1–5], growth factors [6, 7] and several enzymes and other gene products [8–14]. Some of these variably expressed genes, such as the oncogenes and growth factors, are believed to have a direct role in the regulation of cellular proliferation, while other gene products have a role in sustaining the cellular functions necessary for competence, progression and cell division. Definition of the regulation as well as the mechanism(s) of variable gene expression is crucial to understanding cellular growth and proliferation.

Triosephosphate isomerase (TPI; EC 5.3.1.1) exhibits a unique cell cycle dependent expression. This enzyme is a dimeric, glycolytic enzyme with a high catalytic efficiency [15] and a very slow rate of evolution [16]. It is a prevalent protein, present in

all cells examined from a wide range of species [17, 18]. TPI activity increases when mammalian cells are stimulated to proliferate and in proliferating hominoid cells, a second, electrophoretically-distinct subunit of TPI (TPI-2) is synthesized [13, 19–22]. Genetic evidence demonstrates that TPI-2 is a product of the same structural locus as the constitutive subunit, TPI-1 [19, 23]. This is consistent with the molecular data that indicates the existence of only a single functional TPI locus and that it is located on human chromosome 12 [24]. Three possible combinations of primary subunits, TPI-1/1, TPI-1/2 and TPI-2/2, are observed in proliferating cells; the level of the TPI 2/2 isozyme consitutes less than 5% of the total TPI activity and therefore is difficult to detect, even in very rapidly dividing cells. The subunits have similar molecular weights, but the TPI-2 subunit is more acidic (covalent modification of ~ 4 charge units) and more thermolabile than TPI-1 [19, 25]. The expression of TPI-2 is not a post-translational event [20] and requires both RNA and protein synthesis [21]. The expression of TPI-2 is seemingly unique among examples of expression of alternative isozymes or isoforms in that the second subunit is expressed only in proliferating cells and only in the cells of hominoid species [17, 26]. Thus TPI, a constitutively expressed housekeeping enzyme exhibiting unique proliferation specific expression, provides an interesting system for the study of the regulation of gene expression.

We have examined TPI-2 appearance, as well as the increase in TPI mRNA and total TPI enzyme activity, in mitogen stimulated human peripheral lymphocytes and growth arrested human fibroblasts. Selected cell cycle inhibitors were used to further define the signals for and timing of the appearance of TPI-2.

Methods

Cell culture – fibroblasts

The human diploid dermal fibroblast line, GM10, (12 fetal weeks) was obtained from Dr. J. Thoene (Dept. of Pediatrics, Univ. of Michigan) and maintained in RPMI 1640 (Gibco, Chagrin Falls, OH) supplemented with 10% fetal calf serum (Gibco), 50 units/ml penicillin plus 50 μg/ml streptomycin (Gibco) and 2 mM glutamine (Sigma, St. Louis, MO). Cells were split 1:3 at confluency (0.25% trypsin (Sigma) in phosphate buffered saline (PBS)) into 75 cm^2 culture flasks. Fibroblast cells were growth arrested by 11 days of incubation in media containing 0.25% fetal calf serum. This time period of incubation was necessary for the reduction of the TPI activity because of the 28–30 hour half life of TPI [20]. Cells were re-stimulated with 20% fetal calf serum, with or without various inhibitors [dexamethasone (DEX, 0.1 mM, Sigma), hydroxyurea (HU, 1 mM, Sigma), and 5-fluorouridine (FU, 0.2 mM, Calbiochem, San Diego, CA)] for indicated times, harvested, washed with PBS, and stored as pellets at −70° C.

Cell culture – lymphocytes

One unit of whole blood was collected from volunteer donors into ACD anticoagulant. The blood was centrifuged at 2000 rpm for 10 minutes at room temperature. The buffy coat was removed, diluted with PBS supplemented with 0.1% glucose, layered over Histopaque-1077 (Sigma), and centrifuged at 1400 rpm for 30 minutes at room temperature. The isolated lymphocytes were washed in mitogen-free media (RPMI 1640, 20% fetal calf serum, 50 units/ml penicillin plus 50 μg/ml streptomycin and 2 mM glutamine) and counted. Cell recovery was $1.4 \pm 0.5 \times 10^6$ cells per ml whole blood.

Lymphocytes were seeded at $1–2 \times 10^6$ cells/ml into the above media containing 20 mg/ml phytohemagglutinin-M (PHA, Difco, Detroit, MI) and/or 20 ng/ml phorbol 12-myristate 13-acetate (TPA, Sigma). Cells were stimulated/inhibited for indicated times, harvested, washed with PBS, and stored as pellets at −70° C.

Protein/enzyme determinations

Cell extracts were obtained by freeze/thawing as

described by Decker and Mohrenweiser [19]. The supernatants were stored at −70° C. Total protein was determined using the BioRad Protein Assay Kit [27]. TPI, lactate dehydrogenase (LDH, E.C. 1.1.1.27), and glucosephosphate isomerase (GPI, E.C. 5.3.1.9) enzyme activities were determined as described by Fielek and Mohrenweiser [28]. Units of activity are μmol of product formed per minute. The relative distribution of the activity in the TPI 1/1 and TPI 1/2 isozymes was assessed by staining native polyacrylamide gels as described by Decker and Mohrenweiser [17]. Approximately equal units of total TPI activity were routinely loaded in each lane of the gels. Each gel was stained for several time periods to ensure that the relative staining intensity of each isozyme was a measure of relative differences in enzyme activity.

RNA determinations

RNA was extracted from frozen cell pellets of peripheral human lymphocytes, PHA stimulated human lymphocytes, human lymphoblasts, human fibroblasts and African green monkey kidney fibroblasts (vero, from M. Levine, Department of Human Genetics, University of Michigan) in guanidinium isothiocyanate as described by Manitias *et al.* [29]. RNA concentration was determined by spectrophotometric measurements.

Total glyoxal treated RNA [30] was used for both dot blot and northern blot analyses. Total RNA was electrophoretically separated and then transferred to GeneScreen*Plus* as recommended by Du Pont New England Nuclear (Boston, MA). The northern and dot blots were hybridized with a nick translated human TPI cDNA probe (pHTP15a) [31], a gift of Dr. L. Maquat, Dept. of Human Genetics, Roswell Park Memorial Institute, Buffalo, New York.

S1 nuclease experiment

A probe was constructed from the human TPI cDNA clone. The 936 bp EcoRI fragment corresponding to the entire TPI coding region (718 bp), except for 32 bp of the 3′ end of the coding region and including 218 bp of the pKT218 vector (see Fig. 2 of reference 31 for a detailed restriction site map of the cDNA) was the probe. This probe extends into exon 7 (the most 3′ exon) of the gene, thus it covers all of the known intron/exon junctions of the human gene [24]. The fragment was isolated from low melting point agarose (BRL) by melting the agarose at 60° C and then extracting the sample several times with an equal volume of phenol followed by ethanol precipitation.

The EcoRI fragment was dephosphorylated with calf intestinal alkaline phosphatase (BRL), then labeled using T4 polynucleotide kinase (BRL) and g-^{32}P-ATP (Amersham, Arlington Heights, IL). This labeled fragment was hybridized to 10 μg of each of various total RNA samples at 57° C overnight in 80% formamide. The duplex was then digested with S1 nuclease (Promega) as described by Weaver and Weissman [32]. Briefly, 30 units of S1 nuclease was added to the DNA – RNA hybridization mix and incubated for 30 minutes at 37° C. Nucleic acids were precipitated from the hybridization mixture, resuspended in 80% formamide, denatured (3 minutes at 90° C) and separated on a denaturing (6 M urea) 6% polyacrylamide gel (1500 volts for 3–8 hours). After electrophoresis, an autoradiogram of the gel was made by an overnight exposure at −70° C.

Results

Time course of TPI-2 appearance

The time course of appearance of TPI-2 and the role of several cellular growth factors in regulating TPI-2 activity were studied in human fibroblasts. An erythrocyte hemolysate and lysates from proliferating human lymphyblastoid cells and African green monkey kidney fibroblasts were routinely included as reference samples in each isozyme analysis. Two differences in the isozyme pattern are noteworthy in comparing the human samples in lanes 1 and 5 of Fig. 1. The four additional bands of TPI activity migrating immediately anodal of the primary TPI 1/1 isozyme band in the erythrocyte

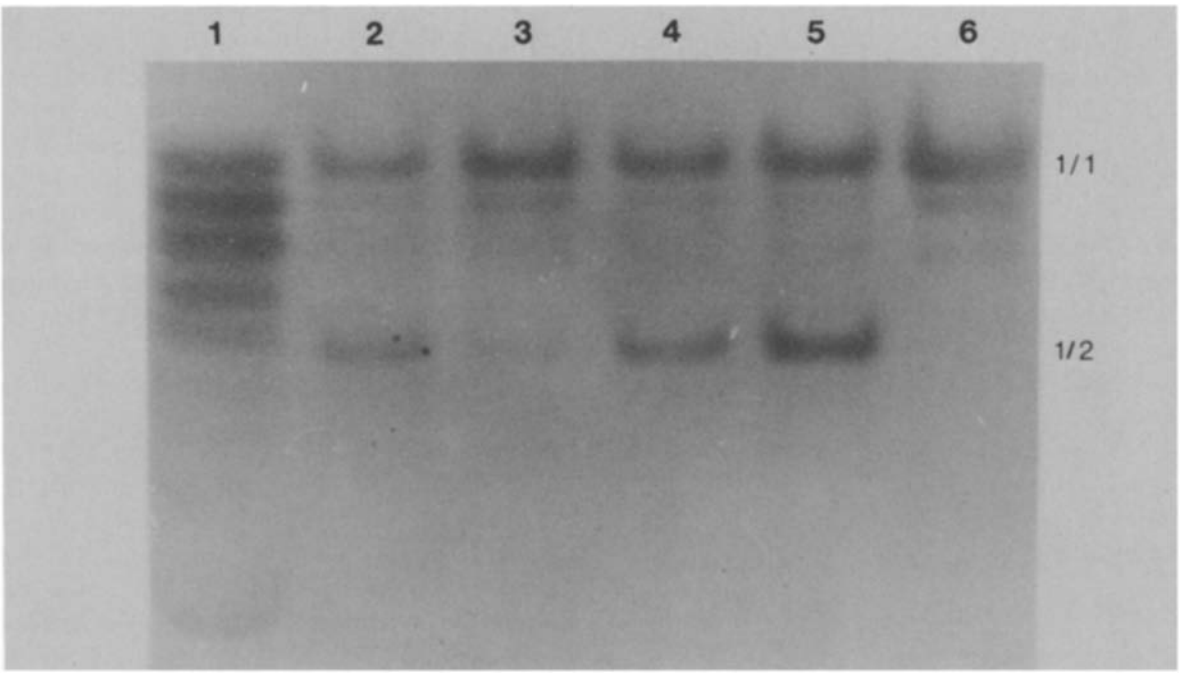

Fig. 1. Time course of TPI-2 appearance following serum stimulation of human fibroblasts. Human fibroblasts were rendered quiescent by serum deprivation for 11 days. The fibroblasts were restimulated by the addition of serum. Cell extracts were electrophoresed on a native polyacrylamide gel, which was then stained for TPI activity. Lane 1 – erythrocyte hemolysate standard, lane 2 – log phase human fibroblasts, lane 3 – human fibroblasts following 11 days of serum deprivation, lane 4 – serum restimulated quiescent human fibroblasts (24hr), lane 5 – human lymphoblast, lane 6 – vero fibroblast.

sample are the isozyme forms generated via the spontaneous deamidation of the two labile asparagine residues in each subunit [33]. Only the first of the deamidation products is marginally detectible in the proliferating lymphoblast sample. The second observation is the existence of a prominent isozyme (designated TPI 1/2) in the lymphoblastoid cell sample that does not comigrate with any of the isozyme forms of the erythrocyte sample. An additional, more anodal migrating isozyme can be detected in human lymphoblastoid cell samples if the staining time is increased (see Fig. 1 of reference 17). The isozyme ratio (TPI 1/1 to TPI 1/2) shifted during the period necessary for the cells to reach quiescence, with most of the residual activity associated with the TPI 1/1 isozyme in confluent fibroblasts maintained in 0.25% FCS for 11 days (lanes 2 and 3). The deamidated forms of the enzyme are also more prominent in the lysate from the quiescent cells. The level of total TPI activity was reduced to 30% of the initial activity during this interval (Table 1). As both TPI-1 and TPI-2 have the same rates of degradation [20], the change in isozyme profile reflects the continued synthesis of TPI-1, while TPI-2 synthesis was 'turned off' dur-

Table 1. Enzyme activity in serum deprived human fibroblasts

Days in 0.25% FCS	Activity[a]		
	TPI	LDH	GPI
0	1.964	0.035	0.010
5	1.342	0.016	0.006
11	0.652	0.008	0.003
10 hrs in 20% FCS[b]	0.960	0.017	0.006
48 hrs in 20% FCS[b]	1.510	0.021	0.008

[a] μmol product formed/min/10^7 cells.

[b] Cells had been grown in 0.25% FCS for 11 days before the addition of fresh media containing 20% FCS. FCS = Fetal Calf Serum; TPI = Triosephosphate Isomerase; LDH = Lactate Dehydrogenase; GPI = Glucosephosphate Isomerase.

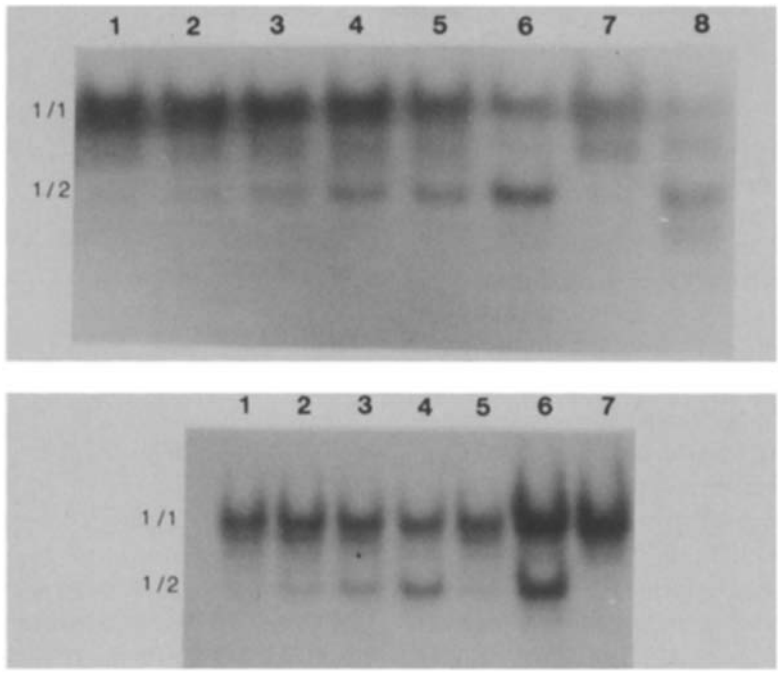

Fig. 2. Time course of TPI-2 appearance in stimulated lymphocytes. Peripheral human lymphocytes were stimulated with phytohemagglutinin (PHA). Cell extracts were electrophoresed through a non-denaturing gel and the gel was stained for TPI activity. A. Lane 1 – erythrocyte hemolysate standard, lane 2 – 0 hours after PHA addition, lane 3 – 6 hours after PHA addition, lane 4 – 12 hours after PHA addition, lane 5 – 24 hours after PHA addition, lane 6 – 66 hours after PHA addition, lane 7 – vero fibroblast, lane 8 – human lymphoblast, B. Lane 1 – 0 hours after PHA addition, lane 2 – 12 hours after PHA addition, lane 3 – 24 hours after PHA addition, lane 4 – 48 hours after PHA addition, lane 5 – 48 hours unstimulated, lane 6 – human lymphoblast, lane 7 – vero fibroblast.

TPI-1, while TPI-2 synthesis was 'turned off' during this time period. Stimulation of the quiescent fibroblasts by the addition of medium containing 20% FCS resulted in a two to three fold increase in total TPI activity (Table 1) and increased prominence of the TPI 1/2 isozyme within 24 hours (Fig. 1, lanes 3 and 4). No indication of a proliferation specific isozyme is observed in proliferating fibroblasts from the African green monkey (vero) (lane 6).

Purified growth factors have been reported to stimulate an increase in TPI activity in quiescent rat cells [34]. A 50–80% increase in TPI activity was observed when quiescent human fibroblast cells were stimulated with fibroblast growth factor (FGF) or epidermal growth factor (EGF) (data not shown), while, as noted above, the TPI activity was increased 2.5 times at 24 hours and 3-fold at 48 hours following stimulation with FCS. Thus, FGF and EGF were much less effective than 20% FCS in stimulating the increase in TPI activity in serum deprived human fibroblasts. The FGF and EGF stimulated increase in TPI activity was also associated primarily with the TPI-2 subunit.

TPI-2 activity was detectable by 6 hours and constituted a significant portion of the total TPI activity by 18 hours following PHA stimulation of human peripheral lymphocytes (Fig. 2). Equal amounts of total TPI activity were loaded in each lane in Fig. 2, thus, the apparent decline of the TPI 1/1 isozyme reflects TPI-2 synthesis and the increased contribution of TPI-2 to the total TPI activity rather than a decrease in the TPI 1/1 activity. The ratio of the activity associated with the TPI heterodimer (TPI 1/2) to the TPI 1/1 homodimer following 48 hours of PHA stimulation of lymphocytes (Fig. 2b lane 4) was approximately 1:1. This was similar to the relative distribution of the two

isoforms observed in a rapidly dividing human lymphoblast cell line (TK6) (Fig. 2b lane 6 or lane 5 of Fig. 1). TPI 1/2 was barely visible in lymphocytes after 48 hours in RPMI media supplemented with FCS, but without the addition of mitogen (Fig. 2b lane 5). The isozyme distribution in these cells was similar to that observed at the initiation of the incubation (Fig. 2b lane 1).

Increased TPI activity, expressed on a per cell basis, was detected at 6–8 hours after initiation of stimulation and continued to increase for the duration of the experiment (Table 2), attaining a level three to four fold over TPI activity in non-stimulated lymphocytes. The basal level of activity in non-stimulated cells was approximately 5×10^{-7} units/cell. TPI activity increased to 18×10^{-7} units/cell at 48 hours after PHA addition. While TPI enzyme activity per cell increased during this time, enzyme activity per μg of total cellular protein remained relatively constant after an initial increase occuring within the first 6–8 hours of mitogen stimulation. Thus, the increase in TPI activity was similar to the increase of total protein observed in these cells. The changes in GPI and LDH enzyme activities followed the same pattern as TPI activity; they began to increase 6–8 hours after PHA addition and continued to increase through the remainder of the experiment. This increased LDH activity was associated with a change from predominantly the muscle type (LDHA) isozyme to predominantly the heart type (LDHB) isozyme (not shown). The phorbol ester, phorbol 12-myristate 13-acetate, either alone or in combination with PHA did not stimulate TPI-2 accumulation or the increase in TPI activity (data not shown).

Table 2. Enzyme activity in mitogen stimulated peripheral lymphocytes

Hours[b]	Activity[a]			Total protein $\mu g/10^7$ cells
	TPI	LDH	GPI	
0	5.2	0.05	0.49	20.81
6	14.8	0.52	1.60	74.15
12	10.7	0.52	1.41	61.26
18	12.9	0.61	1.25	64.25
22	11.2	0.70	1.27	67.64
26	12.5	0.94	1.52	78.11
30	14.0	1.24	1.61	80.31
36	12.9	1.06	1.34	72.17
48	18.1	2.77	2.11	114.32

[a] μmol product formed/min/10^7 cells.
[b] hours following addition of phytohemaggutinin M. TPI = Triosephosphate Isomerase; LDH = Lactate Dehydrogenase; GPI = Glucosephosphate Isomerase.

Inhibition studies

The effects of agents that interfere with cell division at different stages of the cell cycle on the FCS stimulated accumulation of TPI activity in quiescent fibroblasts are presented in Table 3. The inhibitors were added concurrently with FCS. Dexamethasone had its most significant effect at 24 hours, inhibiting 90% of the FCS response; the activity was also reduced at 48 and 60 hours. Hydroxyurea did not significantly affect the increase in TPI activity at the 24 hour time point but did inhibit the expected accumulation at 60 hours, probably reflecting decreased metabolic activity in cells where cell division is inhibited. The inhibition of the increase in TPI was reflected in the absence of the expected increase in the TPI 1/2 isozyme relative to the level of the TPI 1/1 isozyme. Fluorouridine, which inhibits RNA synthesis [35] (DNA synthesis would be inhibited at longer in-

Table 3. Triosephosphate isomerase activity in stimulated human fibroblasts

Treatment	Activity[a]	
	24 hours[b]	60 hours[b]
0.25% FCS	0.570	0.560
20% FCS	1.380	1.460
20% FCS + DEX	0.720	1.000
20% FCS + HU	1.420	1.140
20% FCS + FU	0.770	0.660

[a] μmol product formed/min/10^7 cells.
[b] Hours after addition of 20% FCS following 12 days of 0.25% FCS. FCS = Fetal Calf Serum; PHA = Phytohemagglutinin-M; DEX = Dexamethasone; HU = Hydroxyurea; FU = 5-fluorouridine.

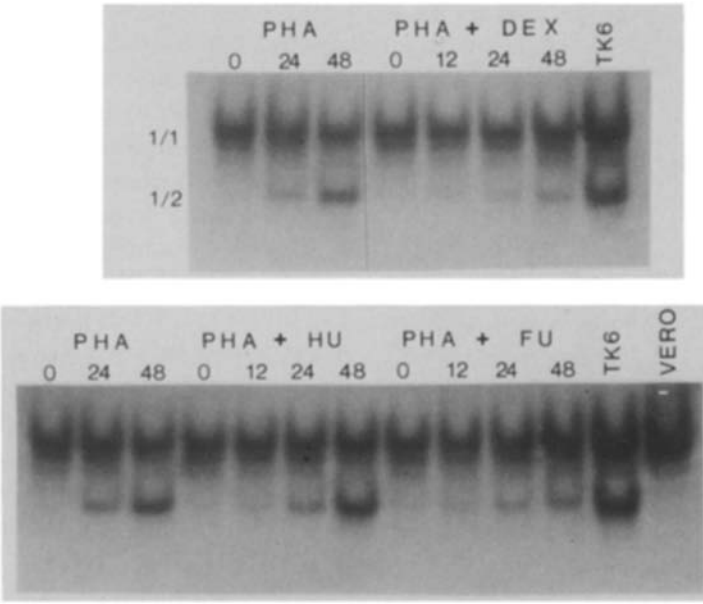

Fig. 3. Effects of inhibitors on TPI accumulation following mitogen stimulation of human lymphocytes. Peripheral lymphocytes were stimulated with phytohemagglutinin (PHA) 2.5 hours following addition of the inhibitor. Cells were harvested 0, 12, 24, or 48 hours after PHA stimulation. Cell extracts were electrophoresed on a native polyacrylamide gel, which was then stained for TPI activity. Abbreviations: PHA = Phytohemagglutinin-M, DEX = Dexamethasone, HU = Hydroxyurea, FU = 5-Fluorouridine, TK6 = human lymphoblast, Vero = African green monkey fibroblast.

cubation times), inhibited > 80% of the expected response to FCS. The incomplete effectiveness of FU reflects the time necessary for equilibration with the cellular uridine pool [36].

The effects of the inhibitors on TPI activity and TPI-2 appearance in stimulated lymphocytes were similar to the results observed in the fibroblast experiments. TPI activity increased 2.5-fold by 12 hours following mitogen addition and remained constant throughout the remainder of the time course in this experiment. Treatment of lymphocytes with mitogen and dexamethasone prevented the increase in TPI activity observed with mitogen alone. At 48 hours following PHA addition, TPI activity in DEX plus PHA treated cells, was only 30% of the activity in PHA alone treated cells and the increase in TPI-2 activity observed following PHA stimulation was almost completely inhibited by DEX treatment (Fig. 3). In hydroxyurea plus PHA treated lymphocytes, TPI activity at 12 and 24 hours was > 70% of the level of activity in cells treated with PHA alone. The increase in TPI-2 activity was not diminished by the addition of HU to the mitogen stimulated cells (Fig. 3). Twelve hours following addition of PHA to fluorouridine treated cells, TPI activity was less than 50% that of PHA alone treated cells and was less than 30% of the activity in the mitogen cells at 48 hours. There was only a minimal appearance of TPI-2 in FU plus PHA treated cells. The level of TPI-2 in these cells was similar to the levels seen in cells with media alone (non-stimulated cells).

TPI mRNA studies

A single primary species of TPI mRNA was detected in human lymphocytes by northern blot analysis of RNA, although it is not possible to exclude the existence of additional mRNA species of similar size. This mRNA was approximately 1.2 kb in both dividing and non-dividing cells. This is the same size as previously reported [31] and is consistent with the size expected given the structure of the

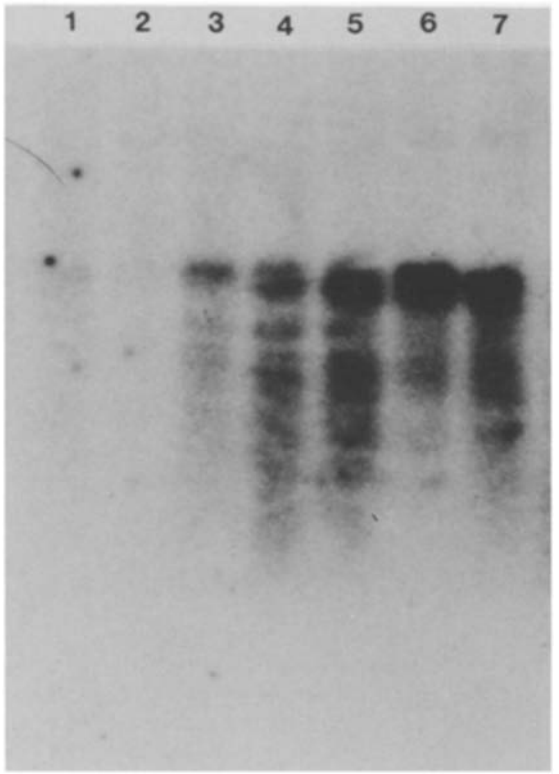

Fig. 4. TPI mRNA accumulation in mitogen stimulated lymphocytes. Total RNA was isolated from phytohemagglutinin (PHA) stimulated human lymphocytes. The RNA was treated with glyoxal, subjected to electrophoresis through a 1.1% agarose gel, transfered to GeneScreen*Plus* and hybridized with the human TPI cDNA probe. Shown is an overexposure of the autoradiogram. Lane 1 – 0 hours after PHA addition, lane 2 – 4 hours after PHA addition, lane 3 – 8 hours after PHA addition, lane 4 – 12 hours after PHA addition, lane 5 – 18 hours after PHA addition, lane 6 – 24 hours after PHA addition, lane 7 – 50 hours after PHA addition.

human [24] and rhesus genes [37]. TPI mRNA was barely detectable in RNA isolated from nondividing cells. Fig. 4 shows an overexposed autoradiograph, where the TPI mRNA in unstimulated cells (0 time, lane 1) can be visualized. The additional, smaller mRNAs are presumably partially degraded TPI mRNA molecules and are only observed upon the 4–5 fold overexposure of the autoradiograph necessary to detect a band in lane 1. At 8 hours following PHA stimulation of lymphocytes (lane 3) the level of TPI mRNA has increased at least several fold. The TPI mRNA levels continue to increase, compared to total RNA, through the last time point in this experiment, which is 50 hours.

Only a single size TPI mRNA species was detectable in human fibroblasts and it is the same size as observed in lymphoblasts. The pattern for TPI mRNA appearance during serum deprivation and subsequent serum restimulation of human fibroblasts was similar to that observed for the TPI 1/2 isozyme. The level of TPI mRNA in quiescent fibroblasts was significantly reduced relative to confluent cells and was almost undetectable in quiescent cells compared to the level in proliferating cells. At 20 hours following FCS stimulation, TPI mRNA was at least twice the level detected in confluent cells and more than 10 times the level in quiescent cells. The TPI mRNA returned to a level comparable to confluent cells at 36 and 52 hours following addition of FCS to quiescent fibroblasts. The cell growth factors, EGF and FGF, modulated TPI mRNA accumulation to an extent which was similar to the increase in TPI activity.

The various agents which inhibited the mitogen stimulated increase in TPI activity and the expression of TPI-2 also prevented the mitogen stimulated accumulation of TPI mRNA. Analysis of dot blots of total RNA from stimulated lymphocytes, probed for TPI mRNA, showed that the TPI mRNA level was less in cells treated with DEX plus PHA than in cells treated with PHA alone and was only slightly increased over that detected in unstimulated cells. HU plus PHA treatment did not significantly affect TPI mRNA levels but FU plus PHA drastically reduced TPI mRNA accumulation compared to TPI mRNA levels in cells treated with PHA alone. The pattern was exactly that observed for TPI 1/2 appearance.

DNA synthesis, as measured by [^{3}H]-thymidine incorporation into trichloroacetic acid precipitated material, began at 30–36 hours following mitogen stimulation (data now shown).

S1 nuclease mapping

An autoradiogram of the S1 nuclease digestion pattern obtained with the EcoRI fragment of the cDNA is seen in Fig. 5. The EcoRI probe hybrid-

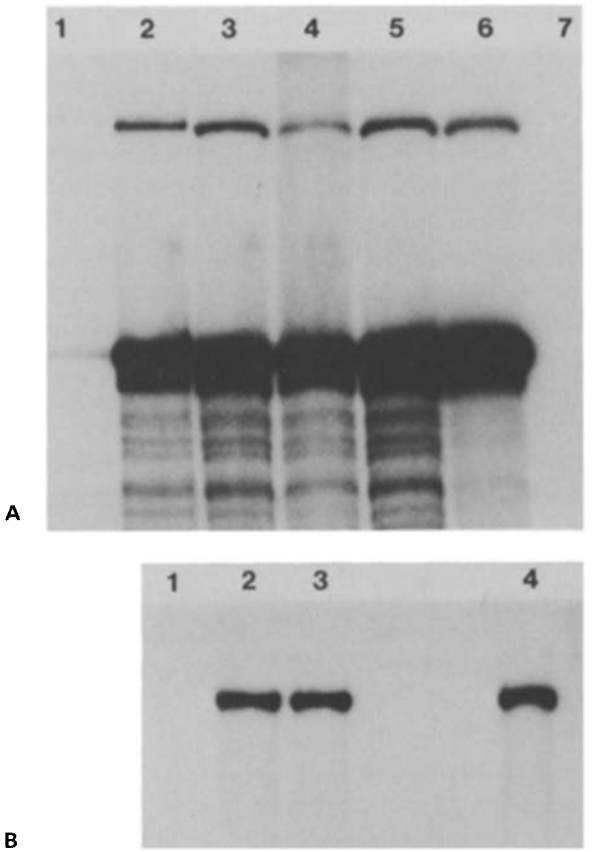

Fig. 5. S1 nuclease digestion analysis of TPI mRNA. Total RNA isolated from a series of cells was hybridized to the EcoRI probe, the non-hybridizing segments were digested with S1 nuclease and the samples electrophoresed as described in Methods. A. Lane 1 – unstimulated peripheral human lymphocytes, lanes 2 and 3 are 24 and 48 hours after PHA stimulation of peripheral human lymphocytes, lane 4 – human lymphoblasts, lane 5 – human lymphoblasts from the TPI Manchester variant, lane 6 – human lymphoblast, lane 7 – no RNA control. B. Lane 1 – no RNA control, lane 2 – 48 hours after PHA stimulation of human lymphocytes, lane 3 – human lymphoblast, lane 4 – vero fibroblast. The dark band in each figure is the protected fragment of the EcoRI probe (718 bp).

izes with the entire coding region expected in the mature TPI mRNA, except for the 32 bases at the 3′ end of the coding sequence, which are lacking in this probe. In panel A, lanes 1, 2, and 3 correspond to S1 nuclease digestions of RNA isolated from peripheral human lymphocytes of the same individual at zero time (lane 1) and 24 hours (lane 2) or 48 hours (lane 3) after initiation of mitogen (PHA) stimulation. Panel A is an overexposure of the film (as compared to panel B) in order to detect the band in lane 1. As seen in panel B, the multiple minor bands are not observed without the overexposure. These minor bands are presumably associated with hybridization to partially degraded mRNAs and/or 'nibbling' by the nuclease. The level of TPI mRNA dramatically increased in stimulated cells as was observed by northern analysis of the cells from the time course study. However no primary bands were observed in the proliferating cells which were not also present in the mRNAs from cells prior to mitogen stimulation. Lanes 4, 5, and 6 are S1 digestions of RNAs isolated from several rapidly dividing transformed human lymphoblastoid cell lines, cells which express high levels of TPI-2. Fig. 5B lane 4 is the result of an S1 digestion of vero RNA hybridized with the human EcoRI cDNA fragment. The single protected fragment is the same size as is observed in RNA from human cells. These data would exclude significant differences in mRNA structure associated with differential mRNA splicing between proliferating and nonproliferating human cells and also between human and vero cells.

Discussion

The single structural locus for TPI located on human chromosome 12 [24] encodes two electrophoretically distinct peptides [19, 23]. The expression of two subunits from a single gene for TPI is in contrast to several other glycolytic enzymes e.g. lactate dehydrogenase, aldolase and pyruvate kinase where the different primary isoforms arise from distinct genes in a gene family [38–40] although pyruvate kinase also expresses two isozymes from each of the structural loci [38]. The TPI-1 and TPI-2 peptides (subunits) differ by one or more covalent modifications involving approximately four charge units [20] which results in differences in thermostability [19, 21] but no-kinetic properties [25]. These structural differences responsible for the expression of the TPI-2 subunit are independent of the spontaneous deamidations of the labile asparagine residues [17, 20]. The TPI-1 subunit is observed in a wide range of cells [18]

while TPI-2 is only observed in proliferating cells [19, 21, 22] derived from hominoid species (from human to gibbon) [17, 26]. The proliferation specific, hominoid restricted subunit is detected in Chinese hamster ovary cells (which express only a constitutive hamster isozyme) which contain human chromosome 12 as the only human chromosome [41]. Thus, at least one of the critical pieces of information for the expression of the proliferation specific isozyme must be resident in the gene. That is, irrespective of the mechanism (alternative mRNA splice sites, amino acid residues which are potential substrates for modification, or some other mechanism) the primary transcript of the structural locus or subsequently the gene product must be the substrate for the modification.

The TPI-2 subunit of TPI was detectable within 6–10 hours following mitogen stimulation of human peripheral lymphocytes or serum restimulation of quiescent human fibroblasts. Total TPI activity increased 2.5 to 3.0-fold during the first 8–24 hours of stimulation with most of the increase associated with TPI-2. TPI-2 increased from almost undetectable levels (< 5% of total activity) in quiescent peripheral lymphocytes to 30–35% of the total activity in lymphocytes stimulated for 24 hrs with mitogen [20]. Only the constitutive TPI subunit was detectable in mitogen stimulated rabbit lymphocytes or proliferating fibroblasts from the African green monkey (Vero) or other lower primates [17, 26].

As previously noted [21], TPI-2 expression was dependent upon RNA synthesis. This would be consistent with the absence of a precursor-product relationship between the subunits [20], unless expression of TPI-2 was dependent upon *de novo* synthesis of an unidentified modifier enzyme which must also be located on human chromosome 12 to be consistent with the data from the human/hamster hybrid cell study. The expression of TPI-2 before DNA synthesis would be consistent with an absence of significant inhibition of TPI-2 expression by hydroxyurea, an agent which blocks DNA synthesis [42]. The absence of a requirement for DNA synthesis plus the observation that TPI-2 expression occurs concurrently with TPI-1 expression and is reversible argues against either gene duplication or rearrangement as a mechanism for TPI-2 expression.

The inhibition of the mitogen stimulated increase in total TPI activity and TPI-2 expression by DEX has several implications. DEX presumably acts by inhibiting interleukin-2 (IL-2) production or interleukin-2 receptor expression [5, 43]. Thus, the increase in TPI activity and TPI-2 expression require early events in the T-cell activation sequence which either involve IL-2 or are induced by IL-2 [6]. Inhibition of TPI-2 expression by cyclosporin A (Mohrenweiser, unpublished) would be consistent with the requirement for interleukin-2 function [5, 7, 43]. Additionally, the inhibition of both TPI-2 expression and the increase in total TPI activity suggests that either these events are linked or that they are both responding to similar activation signals. The expression of TPI-2 during cellular proliferation has characteristics that are similar to the expression of several oncogenes and growth factors [3, 44, 45], except that TPI-2 results from apparently altered expression of an already active gene instead of expression of a previously inactive gene. It is noted that the apparent increase in TPI mRNA following stimulation of quiescent cells (approximately 10-fold or greater) is larger than the increase in total TPI activity (2–4 fold) in either lymphocytes or fibroblasts.

Although the regulation of expression of TPI-2 must be associated with early events in the G_1 phase of cell proliferation [46], the mechanism for generating the second subunit from the single structural locus is still undefined. Previous data have eliminated a precursor-product relationship between the subunits [20] and the subunits do not differ significantly in molecular weight [20, 25]. The requirement for *de novo* protein synthesis for expression of the TPI-2 subunit [21] would be consistent with the requirement for mRNA synthesis and also the absence of a precursor-product relationship between the subunits. The requirement for mRNA synthesis would seem to also exclude, with the exception of a mechanism involving a linked (on human chromosome 12) modifier enzyme, differential modification during nascent peptide synthesis. Additionally, neither subunit is phosphorylated [47, 48], thus differential phosphorylation does

account for the charge difference and preliminary results indicate that neither subunit is sulfated [48].

A relatively common mechanism for generating two (or more) proteins from a single structural locus involves differential processing of the primary RNA transcript [49]. Many of these gene products exhibit tissue specific [50–53] and/or developmental stage specific [54–57] expression. Thus, differential splicing can be a regulated process. However, TPI-2 expression exhibits neither developmental nor tissue specificity. Rather, it is regulated by, or a product of, cellular proliferation, and its expression is restricted to hominoid species. Only a single size species of TPI mRNA was detected in either quiescent or proliferating cells and this mRNA was 1.2 kb in both TPI-2 expressing and nonexpressing cells. Similarly, only a single TPI mRNA species could be detected by S1 nuclease analysis of RNA isolated from proliferating human cells, and the protected fragments were the same size in both quiescent and proliferating cells using a probe that includes all of the known splice junctions and all but 32 nucleotides at the 3′ end of the coding region of the cDNA [37]. Vero cells do not express the TPI-2 subunit, thus, if differential mRNA processing is the mechanism for generating the TPI-2 subunit, vero should also exhibit, along with the sample from quiescent cells, an S1 nuclease digestion pattern for TPI mRNA which is less complex than the pattern observed in the proliferating human lymphoblasts. This was not observed. Additional preliminary results with a probe that encompasses the 3′ untranslated portion of the gene and the 3′ half of the coding region, including 310 nucleotides that overlap the EcoRI fragment cDNA probe, provide no indication of the existence of different or multiple mRNA species in proliferating and nonproliferating human cells or differences in the mRNAs of human and vero cells in the region of the translation termination site or the 3′ untranslated region (Old, unpublished observations). No evidence of an additional, alternative exon has been identified in either the sequence or restriction enzyme site map of the human [24] or rhesus [37] TPI genes.

The functional TPI genes from two primate species, rhesus (*Macaca mulatta*) which does not express a TPI-2 subunit and chimpanzee (*Pan troglodytes*) which expresses TPI-2, have been recently cloned and sequenced ([37] L. Craig, pers. commun). The human gene has also been previously sequenced [24]. The nucleotide sequence of the coding regions of rhesus and chimpanzee TPI genes are 98% identical and between rhesus and human the identity in nucleotide sequence is 97%. No amino acid differences are noted in comparing the derived enzyme sequences from chimp and human while the derived rhesus enzyme sequence differs from the chimp and human sequences by two residues. The enzyme from rhesus has an asparagine residue instead of a serine at position 20 and a glutamic acid at position 198 instead of an aspartic acid. Although the serine in the hominoid enzyme is a potential site for several different covalent modifications, two of these (phosphorylation and sulfation) have already been excluded. Additionally, the presence of the serine does not correlate with the species restricted expression as a serine residue exists at this position in the enzyme of several other species, including the chicken [58], which does not express a second isozyme [17].

Inspection of the chimp gene provides no evidence for the existence of additional exons which could be possible substrates for alternative processing and comparison of the nucleotide sequence of the gene from chimp and rhesus does not indicate sequences that would be suggestive of potential alternative splice sites in the chimp gene that are absent in the rhesus gene. These observations would be consistent with the absence of multiple mRNAs that are of sufficient difference to be detected by S1 nuclease mapping.

Thus, although further insight into the regulation of the expression of the hominoid restricted, proliferation specific isozyme of TPI is presented, the mechanism for generation of the TPI-2 subunit remains elusive.

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