Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 4991–4995, June 1993
Biochemistry

Maturation stage and proliferation-dependent expression of dUTPase in human T cells

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ABSTRACT **We have developed a database of lymphoid polypeptides detected by two-dimensional polyacrylamide gel electrophoresis to aid in studies of leukemogenesis and of mutation affecting protein structure. In prior studies, we observed a 19-kDa phosphopolypeptide which was induced with proliferation in mature T cells and constitutively expressed in immature thymocytes. In this report we describe the identification of this polypeptide as the phosphorylated form of dUTPase (EC 3.6.1.23), following cDNA cloning of the gene, based on a partial amino acid sequence of the phosphopolypeptide. Studies of the expression and phosphorylation of dUTPase in human T cells indicate that accumulation and phosphorylation of dUTPase in mature T cells occur in a cell cycle-dependent manner. Interestingly, noncycling immature thymocytes express constitutively high levels of phosphorylated and unphosphorylated dUTPase. These results suggest an important role for dUTPase in immature thymocytes that is independent of proliferation.**

A regulated sequence of gene expression occurs following activation of mature lymphoid cells and their progression through the cell cycle (1–3). However, less is known regarding the manner in which proteins associated with proliferation or specifically with DNA synthesis and repair are regulated during lymphoid differentiation. That certain genes responsible for DNA replication or repair are differentially expressed during lymphoid maturation is suggested by restriction of somatic rearrangement and hypermutation in T-cell receptor (TCR) and/or immunoglobulin genes to specific lymphoid maturational stages (4). We have recently identified by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) a small subset of proteins which are induced with proliferation in mature T cells and constitutively expressed in noncycling immature thymocytes. This group includes proliferating-cell nuclear antigen (PCNA) and oncoprotein 18 (Op18) (5–7). The high constitutive expression of some, but not all, proliferation-related proteins in noncycling immature thymocytes suggests that immature thymocytes have a requirement for specific proteins that are cell cycle-regulated in mature cells that is independent of proliferation.

Deoxyuridine triphosphate nucleotidohydrolase (dUTPase, EC 3.6.1.23) prevents synthesis of uracil-substituted DNA, by keeping dUTP levels low (8–10). It has been hypothesized that a high dUTP level overwhelms the uracil excision/repair process (11), leading to cell death (12). Recently, Canman *et al.* (13) demonstrated that resistance to fluorodeoxyuridine-induced DNA damage correlated with elevated dUTPase activity and failure of cells to accumulate dUTP. Additionally, inhibition of the enzyme has been suggested to induce programmed cell death during development in *Drosophila* (14).

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In the course of systematic studies of qualitative and quantitative variation in protein patterns of expression in relation to both lymphoid maturation and cell cycle progression, undertaken in part to identify normal physiological variation distinct from protein expression due to mutation, we previously described a 19-kDa phosphopeptide, designated polypeptide 1077, a member of the above-mentioned subset of proteins which was induced with proliferation in mature T cells and constitutively expressed in immature thymocytes (5). In this report, we describe the identification of this polypeptide as a phosphorylated form of dUTPase, which has been suggested to be the enzymatically active form (15). We have also identified a nonphosphorylated form of dUTPase, which has allowed studies of the expression and phosphorylation of dUTPase in human T cells. Our data indicate that, whereas the accumulation and phosphorylation of dUTPase in mature human T cells occur in response to mitogenic stimulation, immature thymocytes express high levels of phosphorylated and unphosphorylated dUTPase, suggesting a requirement on the part of immature T cells to maintain a high level of DNA fidelity that is independent of proliferation.††

MATERIALS AND METHODS

Lymphocyte and Thymocyte Preparation, Cell Culture, and Metabolic Labeling. Human peripheral blood lymphocytes (PBLs) were prepared from venous blood of healthy volunteers and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. PBLs were mitogenically stimulated with anti-CD3 (OKT3 ascites, 1:200) or with phytohemagglutinin (PHA). In some experiments, partial cell cycle synchronization was achieved by adding 10 mM hydroxyurea for 24 hr following OKT3 stimulation, and PBLs were cultured for an additional 20 hr to allow cells to accumulate at the G_1/S boundary. Cells were washed three times with medium and returned to the original flask with fresh OKT3 added. Three hours following release of the block at G_1/S, nocodazole (100 ng/ml) was added for 20 hr to block cells in G_2 and M. Nocodazole was removed by washing cells three times with medium. Thymocyte subpopulations were isolated (16) from thymic tissue obtained from children undergoing cardiac surgery. Immature $CD4^+CD8^+$ (double-positive) and mature $CD4^+$ or $CD8^+$ (single-positive) thymocytes were obtained by negative selection for CD28 or CD1, respectively, as CD28 is expressed at high levels only in single-positive thymocytes and CD1 is expressed at high levels only in double-positive

Abbreviations: PHA, phytohemagglutinin; 2-D, two-dimensional; PVDF, poly(vinylidene difluoride); PCNA, proliferating-cell nuclear antigen; PBL, peripheral blood lymphocyte; TCR, T-cell receptor.

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††The sequence reported in this paper has been deposited in the GenBank data base (accession no. L11877).

thymocytes (16, 17). The human leukemia T-cell line Jurkat was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. For metabolic labeling, cells (10^7 per ml) were suspended in phosphate-free or methionine-free RPMI 1640 containing [^{32}P]orthophosphate (carrier-free, 400 μCi/ml, Amersham; 1 μCi = 37 kBq) for 2 hr or [^{35}S]methionine (250 μCi/ml, Amersham) for 4 hr, respectively, at 37°C.

2-D PAGE. Analytical 2-D PAGE using carrier ampholyte-based isoelectric focusing was performed as described (18), with pH 4–8 Resolyte [2% (vol/vol); Hoefer]. Whole cell lysates were prepared (1.8–3 × 10^6 cells) with 30 μl of lysis solution [9.5 M urea/2% (vol/vol) 2-mercaptoethanol/2% (vol/vol) Nonidet P-40/50 mM NaF/0.1 mM Na_3VO_4]. Focusing was for 16 hr at 1000 V. The second-dimension separation was in an SDS/11.4–14% polyacrylamide gradient gel. After silver staining, 2-D gels were digitized and polypeptide spots were quantitated as described (19). 2-D gels of metabolically labeled cells were dried and placed in storage phosphor imaging cassettes for 40–86 hr (20). Digitized images were obtained with a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA) and spot quantitation was done with software (IMAGEQUANT,) supplied by the manufacturer.

Protein Structural Studies. Polypeptide 1077 was purified by preparative 2-D PAGE using an immobilized pH gradient-based isoelectric separation in the first dimension (21). Following electrophoresis, proteins were electrophoretically transferred (1.3 mA/cm^2 for 2 hr) to poly(vinylidene difluoride) (PVDF) membranes (Immobilon P, Millipore) with a semidry blotting system (SemiPhor, Hoefer). Transfer buffer was 50 mM sodium borate (pH 9.0) containing either 20% (vol/vol) methanol (anode) or 5% (vol/vol) methanol (cathode). Polypeptide 1077 was localized by brief staining with Coomassie blue and excised. Protein was eluted from PVDF membranes with 5% Tween 20 in 10 mM sodium phosphate (pH 7.0) for 4 hr at 37°C (J. Leykem and J.R.S., unpublished work). Protein was precipitated with 15% (wt/vol) trichloroacetic acid overnight at 4°C. Precipitated protein was washed three times with acetone at −20°C. Tryptic digestion was performed with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) (1:20 enzyme/substrate weight ratio) in 100 mM ammonium bicarbonate (pH 8.2) at 37°C. After 4 hr, a second aliquot of trypsin was added. Digestion was stopped after a total of 20 hr. Tryptic peptides in 0.1% trifluoroacetic acid in water (solvent A) were separated on a C_{18} column (2.1 × 150 mm, Vydac, Hesperia, CA) with a linear gradient from 5% to 60% solvent B (0.07% trifluoroacetic acid in 80% acetonitrile) in 55 min at a flow rate of 150 μl/min. Detection was at 210 nm and fractions were collected manually. Selected peptides were subjected to N-terminal sequencing with a gas-phase sequencer (model 470A or 473A, Applied Biosystems).

For Western blotting, cellular polypeptides were separated by 2-D PAGE and transferred to PVDF membranes. Immunodetection was with a rabbit antiserum to vaccinia virus dUTPase, followed by goat anti-rabbit IgG-horseradish peroxidase conjugate (Sigma). The F2L gene product of vaccinia virus (22) has been determined enzymatically to be a dUTPase (N.A.R., unpublished results). To obtain antiserum, the entire vaccinia dUTPase gene was expressed as a part of a fusion with 37 kDa of the amino portion of the bacterial TrpE protein (23) and the fusion protein was used for immunization (N.A.R., unpublished results).

cDNA Cloning and DNA Sequencing. A cDNA library of the leukemic T-cell line HSB-2 constructed in the CDM7 plasmid vector (24) was screened for polypeptide 1077 cDNA clones. The 5′-end-labeled probe used for screening was a mixture of 29-mer synthetic oligonucleotides of 64 degenerate sequences, TTT(or C)-TAT(or C)-CCI-GAG(or A)-ATI-GAG(or A)-GAG(or A)-GTI-CAG(or A)-GC, which take into account all possible codons for Phe-Tyr-Pro-Glu-Ile-Glu-Glu-Val-Gln-Ala. To minimize the degeneracy of the oligonucleotide mixture, we used inosine (I) as a spacer whenever the third base of a codon could be one of three or four bases. Two positive clones, 1077-1 and 1077-2, were identified and inserts were subcloned into pBluescript SK(+) and M13 vectors. Sequence analysis was by the dideoxy chain-termination method with the universal and T7 primers. As sequence information was obtained, additional synthetic oligonucleotides were constructed and used as primers.

Northern blot analysis was performed as described (25). For hybridization, the 1.0-kb insert of dUTPase cDNA clone 1077-2 was labeled by nick-translation (GIBCO/BRL) with [α-^{32}P]dCTP.

RESULTS

Purification and Partial Internal Amino Acid Sequencing of Polypeptide 1077. The location and level of polypeptide 1077 relative to other landmark polypeptides in 2-D gels of PBLs activated with OKT3 for 3 days are indicated in Fig. 1. Polypeptide 1077 was purified from Jurkat cells by preparative 2-D PAGE. Direct N-terminal microsequencing failed to yield sequence information, indicating that the polypeptide has a blocked N terminus. To obtain internal amino acid sequence information, polypeptide 1077 was eluted from PVDF membranes and digested with trypsin. Sequence information was obtained for 74 aa in six tryptic peptides (Fig. 2). A search of the Protein Identification Resource sequence database (Release 28.0) revealed a remarkable 68% identity (84% similarity) of the sequence of these six peptides with the deduced sequence for the F2L gene product of vaccinia virus (22). Enzymatic characterization of recombinant F2L gene product has shown that the protein is a dUTPase (N.A.R., unpublished results).

Identification of Polypeptide 1077 as dUTPase. An oligonucleotide mixture (29-mers) coding for 10 aa of tryptic peptide T5 (Fig. 2) was used to screen an HSB-2 cDNA library. Two clones, 1077-1 and 1077-2, were identified as having 0.6-kb and 1.0-kb inserts, respectively. The complete coding and 5′ noncoding region of each insert was sequenced. The cDNA codes for a 141-aa polypeptide (Fig. 3). The translated amino acid sequence contained an exact match for the six peptides whose sequences had been determined. The calculated molecular mass of the polypeptide is 16.6 kDa, with a predicted isoelectric point of 8.06. While the discrepancy between the

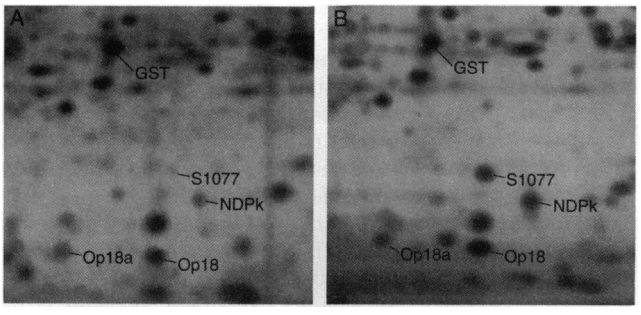

FIG. 1. Proliferation-related expression of polypeptide 1077 in PBLs as determined by 2-D PAGE. Freshly isolated PBLs (*A*) were treated with OKT3 (anti-CD3) for 3 days (*B*). Polypeptides were separated by 2-D PAGE and visualized by silver staining. Shown is a section of 2-D gels from approximately 26 kDa (top) to 17 kDa (bottom) and pI 5.6 (left) to pI 6.5 (right). Indicated are polypeptides in this region of the gel which we have identified by microsequencing. Op18 and one of its major phosphorylated forms (Op18a) and nucleotide diphosphate kinase A (NDPk) are proliferation-related polypeptides; glutathione *S*-transferase π (GST) does not change significantly with proliferation.

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                             T1_______________→        T2______
Human      1          M Q L R F A R L S E H A T A P T R G S A R A A G Y D
Vaccinia          M N I N S P V R F V K E T N R A K S P T R Q S P Y A A G Y D
                  ____________________→       T3__________________
Human     27      L Y S A Y D Y T I P P M E K A V V K T D I Q I A L P S G C Y
Vaccinia          L Y S A Y D Y T I P P G E R Q L I K T D I S M S M P K F C Y
                  →                           C1________________→ T4__→
Human     57      G R V A P R S G L A A K H F I D V G A G V I D E D Y R G N V
Vaccinia          G R I A P R S G L S L K G . I D I G G G V I D E D Y R G N I
                                                                T5____
Human     87      G V V L F N F G K E K F E V K K G D R I A Q L I C E R I F Y
Vaccinia          G V I L I N N G K C T F N V N T G D R I A Q L I Y Q R I Y Y
                  ______________________→
Human    117      P E I E E V Q A L D D T E R G S G G F G S T G K N
Vaccinia          P E L E E V Q S L D S T N R G D Q G F G S T G L R
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FIG. 2. Alignment of polypeptide 1077 amino acid sequence with that of the F2L gene product (dUTPase) of vaccinia virus. Arrows indicate the five tryptic and one chymotryptic peptides for which a complete or partial amino acid sequence was obtained.

apparent molecular mass of polypeptide 1077 (19 kDa) observed on 2-D gels and the theoretical (16.6 kDa) could be due to posttranslational modification, it is not due to phosphorylation (see below). Taking into account the fact that polypeptide 1077 has a blocked N-terminus and is phosphorylated at a single site, the predicted pI of the modified polypeptide is 6.12, in close agreement with pI 6.0 observed in 2-D gels. The complete predicted amino acid sequence (Fig. 2) shows 63% identity with dUTPase of vaccinia virus, with allowance for a single amino acid gap in the viral protein, and 35% identity with *Escherichia coli* dUTPase (26). While this manuscript was in preparation McIntosh *et al.* (27) reported the sequence of human dUTPase. Our cDNA sequence (GenBank accession no. L11877) is identical to the first 572 nt of their sequence (GenBank accession no. M89913). The 5′ flanking sequence is 2 nt longer and the 3′ noncoding sequence of the 1.0-kb cDNA clone 1077-2 is about 160 nt longer than the sequence of McIntosh *et al.* (27). A limited sequence for 52 nt at the 3′ end of clone 1077-2 was obtained and contained the polyadenylylation signal sequence AATAAA and terminated with a 14-nt poly(A) tail (data not shown).

Identification of the Nonphosphorylated Form of dUTPase. It has been suggested that phosphorylation of dUTPase is necessary for enzymatic activity (15). To determine the identity of the nonphosphorylated form in 2-D gel polypeptide patterns, we used a rabbit antibody to recombinant

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      *
  1:  CGCTCGCCTTCTGGCTCTGCATGCCCTGCCTCTGAAGAGACACCCGCCATTTCACCCA

 59: GTAAGCGGGCCCGGCCTGCGAGGTGGGCGGC ATG CAG CTC CGC TTT GCC CGG
                                     met gln leu arg phe ala arg

111: CTC TCC GAG CAC GCC ACG GCC CCC ACC CGG GGC TCC GCG CGC GCC
     leu ser glu his ala thr ala pro thr arg gly ser ala arg ala
                                     *
156: GCG GGC TAC GAC CTG TAC AGT GCC TAT GAT TAC ACA ATA CCA CCT
     ala gly tyr asp leu tyr ser ala tyr asp tyr thr ile pro pro

201: ATG GAG AAA GCT GTT GTG AAA ACG GAC ATT CAG ATA GCG CTC CCT
     met glu lys ala val val lys thr asp ile gln ile ala leu pro

246: TCT GGG TGT TAT GGA AGA GTG GCT CCA CGG TCA GGC TTG GCT GCA
     ser gly cys tyr gly arg val ala pro arg ser gly leu ala ala

291: AAA CAC TTT ATT GAT GTA GGA GCT GGT GTC ATA GAT GAA GAT TAT
     lys his phe ile asp val gly ala gly val ile asp glu asp tyr

336: AGA GGA AAT GTT GGT GTT GTA CTG TTT AAT TTT GGC AAA GAA AAG
     arg gly asn val gly val val leu phe asn phe gly lys glu lys

381: TTT GAA GTC AAA AAA GGT GAT CGA ATT GCA CAG CTC ATT TGC GAA
     phe glu val lys lys gly asp arg ile ala gln leu ile cys glu

426: CGG ATT TTT TAT CCA GAA ATA GAA GAA GTT CAA GCC TTG GAT GAC
     arg ile phe tyr pro glu ile glu glu val gln ala leu asp asp

471: ACC GAA AGG GGT TCA GGA GGT TTT GGT TCC ACT GGA AAG AAT TAA
     thr glu arg gly ser gly gly phe gly ser thr gly lys asn

516: AATTTATGCCAAGAACAGAAAACAAGAAGTCATACCTTTTTCTTAAAAAAAAAAAAAA
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FIG. 3. Sequence of isolated polypeptide 1077 cDNA inserts. Initiator and terminator codons are underlined. Stars indicate the beginning of clone 1077-2 (position 1) and 1077-1 (position 180).

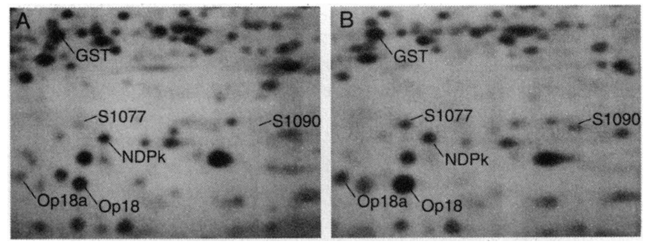

FIG. 4. Expression of dUTPase in mature (single-positive) thymocytes (*A*) and immature (double-positive) thymocytes (*B*). S1077 is the phosphorylated form of dUTPase, whereas S1090 is the nonphosphorylated form. Other abbreviations are defined in the legend to Fig. 1.

vaccinia virus dUTPase in Western blots. PBLs were treated with OKT3 for 2 days and then incubated with [^{35}S]methionine. Two polypeptides of similar apparent size were detected on the immunoblot (data not shown). Thus the discrepancy between the apparent size of phosphorylated dUTPase observed on 2-D gels and that predicted from the cDNA is not due to phosphorylation. The major spot had a pI similar to that of polypeptide 1077 whereas the minor form had a more basic pI which was in close agreement with the calculated pI for an N-terminally blocked, nonphosphorylated form of dUTPase. Subsequent detection of ^{35}S-labeled polypeptides on the membrane revealed the comigration of ^{35}S-labeled polypeptide 1077 and anti-dUTPase immunoreactivity. Comparison of the ^{35}S-labeled immunoblot with silver-stained ^{35}S-labeled 2-D gel patterns allowed us to identify the nonphosphorylated form of dUTPase as spot 1090 (Fig. 4) on silver-stained gels in our database of human lymphoid proteins.

We then determined the stoichiometry of phosphorylation of dUTPase based on quantitation of both forms in silver-stained gels of PBLs and thymocyte subpopulations. Freshly isolated PBLs expressed very low or undetectable levels of nonphosphorylated and phosphorylated dUTPase (Table 1). A large amount of dUTPase was expressed after 72 hr of activation of PBLs with either OKT3 or PHA, and 64–73% of dUTPase was in the phosphorylated form. In a time-course experiment, dUTPase levels reached a maximum by 96 hr of PHA activation (Table 1). The fraction of dUTPase in the phosphorylated form was >50%. Virtually all the dUTPase existed as the phosphorylated form after 48 hr of PHA activation. Further, we could determine that the high levels

Table 1. Expression of phosphorylated (P) and nonphosphorylated (NP) forms of dUTPase in activated PBLs and thymocyte subpopulations

Cell type	Treatment	Time, hr	n	Integrated intensity, OD × mm²		
				P	NP	% P
PBL	None		6	0.009*	0.004*	—
	OKT3	72	2	1.525	0.840	64
	PHA	72	4	0.750	0.283	73
	PHA	24	1	nd	nd	—
		48	1	0.673	0.030	96
		72	1	0.475	0.168	74
		96	1	0.713	0.616	54
		120	1	0.194	0.256	43
		144	1	0.240	0.069	78
Immature thymocytes	None	—	4	0.301	0.211	59
Mature thymocytes	None	—	4	0.089	0.048	65

nd, Not detected.
*Both forms of dUTPase were not detected in five of six samples.

Table 2. Analysis of dUTPase *de novo* synthesis during cell cycle progression

OKT3	Total hours in culture	Addition	Time, hr	% cells		[^{35}S]Methionine-labeled dUTPase*			
				S	G_2 + M	P†	NP†	Total†	% P
−	0		—	6.2	3.2	1.0	0.8	1.8	56
+	2		—	7.8	5.1	1.2	1.1	2.3	52
+	44		—	14.3	9.8	21.1	25.1	46.2	46
+	44	HU	20	9.1	5.4	17.5	14.2	31.7	55
+	47	−HU	3	18.3	6.3	8.0	12.5	20.5	39
+	67	NOC	20	12.0	14.2	10.3	10.4	20.7	50
+	70	−NOC	3	11.0	8.7	5.5	6.9	12.4	44

PBLs were incubated in the continuous presence of OKT3 for the indicated times. To achieve partial cell cycle synchronization, 10 mM hydroxyurea (HU) was added 24 hr following OKT3 stimulation and PBLs were cultured for an additional 20 hr to allow cells to accumulate at the beginning of S phase. Cells were washed with medium and returned to the original flask with fresh OKT3 added. Three hours after removal of hydroxyurea, nocodazole (NOC, 100 ng/ml) was added for 20 hr to block cells in G_2 plus M phase. At the indicated times, an aliquot of cells was removed and incubated with [^{35}S]methionine for 4 hr. ^{35}S-labeled polypeptides were separated by 2-D PAGE and incorporation of radioactivity was determined.

*Values are given for phosphorylated (P), nonphosphorylated (NP), and total.

†(PhosporImager signal/hr of exposure) × 10^{-3}.

of phosphorylated dUTPase previously reported in immature thymocytes (5) compared with mature thymocytes were associated with high overall levels of the protein and were not the result of differential stoichiometry of phosphorylation between immature and mature thymocytes (Fig. 4 and Table 1). Northern blot analysis of immature thymocyte and mature T-cell mRNA further substantiated this conclusion. A 1.1-kb dUTPase mRNA was detected in immature double-positive thymocytes, whereas no message was detectable in mature single-positive thymocytes or in freshly isolated PBLs (data not shown).

***De Novo* dUTPase Synthesis and Stoichiometry of Phosphorylation in Relation to Cell Cycle Progression.** To determine the extent to which *de novo* synthesis and phosphorylation of dUTPase were modulated during transition of cells through the cell cycle, PBLs were partially synchronized with a combination of hydroxyurea and nocodazole (Table 2). PBLs were stimulated with OKT3 for 24 hr and subsequently treated with hydroxyurea, which blocks cells at the G_1/S boundary. Following removal of hydroxyurea, cells were treated with nocodazole to arrest them in G_2 plus M phase. A representative experiment is shown in Table 2. In freshly isolated PBLs there was low to undetectable synthesis of dUTPase. A high level of dUTPase synthesis was not observed until 44 hr of OKT3 treatment. Synthesis was not inhibited when hydroxyurea was also present during the last 20 hr of a 44-hr OKT3 treatment. Hydroxyurea inhibits ribonucleoside diphosphate reductase, a key enzyme involved in dNTP synthesis. Following removal of hydroxyurea, cells progressed into S phase and *de novo* dUTPase synthesis was diminished within 3 hr. Subsequent treatment with nocodazole, a microtubule-depolymerizing drug, resulted in accumulation of cells in G_2 plus M phase, and dUTPase synthesis was diminished further. No significant differences were observed in the proportion of newly synthesized dUTPase that was in the presumably active phosphorylated form as cells progressed through the cell cycle.

DISCUSSION

We have previously described a group of polypeptides, including the phosphorylated polypeptide 1077, that are constitutively expressed in immature thymocytes but not in mature T cells (5). In this report we have identified polypeptide 1077 as the phosphorylated form of dUTPase. Lirette and Caradonna (15) have shown that herpes simplex virus infection results in dephosphorylation of dUTPase which correlates with decreased dUTPase activity. They concluded that phosphorylation of eukaryotic dUTPase is essential for its activity. Noteworthy is the recent finding that *E. coli* cells transfected with plasmids carrying the human dUTPase gene express elevated dUTPase activity (27). These results can be interpreted to indicate that, if phosphorylated dUTPase is the active form, *E. coli* cells possess a dUTPase protein kinase activity. Alternatively, if both forms of dUTPase are active, enzyme activity may be modulated by interaction of either form with a regulatory protein such as the 61-kDa dUTPase inhibitor expressed in early *Drosophila* development (14). The occurrence of phosphorylated dUTPase at low or undetectable levels in mature T cells is not the result of differential phosphorylation stoichiometry but appears to be related to reduced overall levels of dUTPase in mature T cells. Mitogenic stimulation of mature T cells with either OKT3 or PHA results in expression of dUTPase, predominantly in the phosphorylated form, which reaches a maximum by days 3–5. Analysis of dUTPase *de novo* synthesis in relation to progression through the cell cycle reveals that dUTPase expression occurs in late G_1 phase. As cells progress through S and into G_2 plus M phase, the level of *de novo* synthesis decreases substantially. Interestingly, a similar pattern of dUTPase activity has been reported in higher plants (28). Undifferentiated resting root meristem cells have high dUTPase levels independent of cell proliferation, whereas dUTPase levels in proliferating root meristem cells increase in a cell cycle-dependent manner.

dUTPase is a member of a relatively small subset of proteins induced with proliferation in mature T cells that we have found to be constitutively expressed in immature thymocytes. The enzyme is thought to have two major functions in nucleotide metabolism: hydrolysis of dUTP (*i*) provides dUMP for dTMP production by thymidylate synthase and subsequent synthesis of dTTP and (*ii*) reduces the intracellular concentration of dUTP, thereby maintaining a low dUTP/dTTP ratio and minimizing misincorporation of uracil into newly replicated DNA. Incorporation of uracil into DNA elicits an excision repair mechanism involving uracil N-glycosidase, DNA polymerase I, and DNA ligase that leads to the transient appearance of small DNA fragments (Okazaki fragments). The presence of high levels of dUTP, such as would exist when dUTPase levels are low, results in continued misincorporation of uracil during the polymerase/ligase phase, potentially overwhelming the repair process.

The functional significance of the constitutive expression of dUTPase in immature thymocytes is not immediately apparent. However, it might be speculated that dUTPase expression could be coupled to the process of V(D)J recombination by which thymocytes rearrange their TCR genes during development. Many double-positive thymocytes have

not yet completed productive TCR gene rearrangement and are therefore still undergoing V(D)J recombination. The joints created between individual genetic elements frequently contain nucleotides not present in the germ line (29). Random insertion of nucleotides at joints is known as N-nucleotide addition, and is thought to be catalyzed by terminal deoxynucleotidyltransferase, which is present in immature T- and B-cell progenitors (30). Immature T and B cells are known to contain very high levels of dNTPs, perhaps to assist in the process of N-nucleotide addition. Thus, dUTPase could function to maintain low levels of dUTP, in order to prevent misincorporation of uracil into DNA. The expression of an enzyme involved in maintaining DNA fidelity in immature thymocytes suggests that other DNA repair enzymes may also be expressed. We have recently shown that PCNA is also expressed at high constitutive levels in immature thymocytes (6). PCNA is an auxiliary protein of DNA polymerase δ (31) and is thought to be involved in leading-strand elongation during DNA replication (32). Recent evidence suggests that PCNA is also required for DNA excision repair (33).

The mechanism by which dUTPase expression is differentially regulated in mature and immature T cells is not known. Regulation of expression may involve either an increased transcription rate, or posttranscriptional regulation, or both. There are several well-characterized examples of gene-specific regulation by each means in thymic development (34, 35). Indeed, recent studies have shown that for any given gene, the mechanisms which regulate its expression in thymocytes may differ from those which control its expression in mature T cells (34, 35). McIntosh *et al.* (27) have reported the presence of major 1.5- and 2.3-kb mRNAs in various human tissues and a minor 1.0-kb message. The smaller message may correspond to the predominant 1.1-kb species we find in immature human thymocytes. The variable abundance of dUTPase message among tissues and the relative abundance of individual species within tissues (27) suggest that multiple forms of dUTPase may exist whose expression is tissue-specific. Alternatively, different-length transcripts may reflect alternative splicing or the presence of multiple promoters providing differentiation- and tissue-specific control but still code for the same protein, as is observed for $p56^{lck}$ (36, 37). Consistent with the existence of multiple dUTPases is the observation that whereas malignancies of the brain (neuroblastoma) and hematopoietic system (lymphoid and myeloid leukemias and lymphomas) express abundant dUTPase, malignancies of the lung, breast, and colon have little or no dUTPase with physiochemical properties (pI and apparent molecular weight) of the dUTPase described in this report (unpublished observations).

We thank Drs. J. Maybaum, E. Radany, B. Richardson, D. Fox, and E. M. McIntosh for stimulating discussion and comments. This work was supported in part by Department of Energy Grant 87ER 60533 (J.R.S. and J.V.N.) and National Institutes of Health Grants CA32146 and CA26803 (S.M.H.).

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