Cloning and Expression of a cDNA for Human Thioredoxin*

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Thioredoxin is the best representative enzyme of a group of proteins, widely distributed and possessing dithiol-disulfide oxidoreductase activity. We have constructed a cDNA library from messenger RNAs isolated from a lymphoblastoid B cell line (Epstein-Barr virus-immortalized normal human lymphocytes). Screening of this library with synthetic oligonucleotide probes, constructed from the NH₂-terminal amino acid sequence of a protein produced by this line, allowed us to identify a full-length cDNA clone coding for human thioredoxin. The open reading frame (315 nucleotides long) codes for a protein of 164 amino acids (excluding the initial methionine). This protein possesses the highly conserved enzymatic active site common to plant and bacterial thioredoxins: Trp-Cys-Gly-Pro-Cys (amino acids 30-34). These data provide for the first time the complete primary sequence of a thioredoxin of mammalian origin.

Recombinant human thioredoxin, expressed in Escherichia coli, possesses a dithiol-reducing enzymatic activity as assayed on mammalian and plant substrates. It is able to reduce the interchain disulfide bridges of murine pentameric IgM and porcin insulin and also to activate vegetal NADP-malate dehydrogenase.

Studies of human thioredoxin mRNA expression and regulation in immunocompetent cells of human origin indicate that the protein is weakly expressed in resting lymphocytes and monocytes, but the level of human thioredoxin mRNA transcription is quite important in activated monocytes and established dividing human cell lines.

Thioredoxin is an oxidoreductase enzyme of 12,000 daltons, containing a dithiol-disulfide active site (1, 2). It is ubiquitous and found in many organisms, from plants and bacteria to mammalians. It has been identified originally in Escherichia coli as a hydrogen donor for ribonucleotide reductase and deoxyribonucleotide synthesis (1). In higher organisms, the thioredoxin system (which includes NADPH as a donor, thioredoxin reductase, and thioredoxin) seems to participate as a general dithiol-disulfide oxidoreductase in the cells. Multiple in vitro substrates for thioredoxin have been identified so far, such as ribonuclease, choriogonadotropins, proteolytic enzymes (3), coagulation factors (4), glucocorticoid receptor (5), and insulin (6, 7). The reduction of the latter is used as a classical activity test. If thioredoxins have been extensively studied biochemically and functionally, the only cDNA sequence reported yet is from Anabaena 7119 (8).

During the course of studies focused on activation of immune cells via "hormones" called interleukins, we have reported in a previous paper the biochemical purification of a novel protein (9) produced by an EBV human lymphoblastoid B cell line, named 3B6 (10). This 3B6 protein has been purified biochemically to homogeneity, and a high-NH₂-terminal amino acid sequence has been obtained. In this paper, using synthetic oligonucleotide probes constructed from the protein sequence, we report the successful cloning of a full-length cDNA coding for the previously purified protein. We also present evidence indicating that this new molecule is a human thioredoxin based upon protein homologies and biological activity of the recombinant protein.

No immunological role for thioredoxin has been identified so far. However, our results demonstrate a regulation of human thioredoxin message: RNA expression in various immunocompetent cells, such as lymphocytes and monocytes, and in several cell lines of human origin.

EXPERIMENTAL PROCEDURES

RNA Extraction

Total RNA was extracted from various lymphoid cells using the guanidinium/cesium chloride (CsCl) method (11) slightly modified as follows. Briefly, 50 x 10⁶ cells in 75 μl of saline buffer were lysed in 0.5 ml of 6 M guanidinium isothiocyanate, 5 mM citrate, 100 mM 2-mercaptoethanol, and 0.5% Sarkosyl NL30 solution. After sonication for 10 s, the cell extract was layered over 0.5 ml of a 5.7 M CsCl, 250 mM sodium acetate, 100 mM EDTA solution. It was then ultracentrifuged for 90 min at 25 000 x g in a TL 100.2 rotor (Beckman). The pellet was washed in 30% deionized formamide, 70% ethanol; dried and resuspended in Tris/EDTA/sodium dodecyl sulfate (SDS). It was kept precipitated in ethanol and sodium acetate.

Construction and Screening of cDNA Library

Poly(A)+ RNA from the 3B6 cell line was isolated by oligo(dT)-8 Trisacryl M (IBF) chromatography of the total RNA (12). The cDNA library was constructed by the method of Gubler and Hoffman (13) using Amersham's cDNA synthesis system. The EcoRI sites were methylated with EcoRI methylase (New England Biolabs), and EcoRI probes labeled with [γ-³²P]ATP (14) using a kit (IBF) and used for hybridization.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s) J04026.

The abbreviations used are: EBV, Epstein-Barr virus; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
linkers (New England BioLabs) were ligated onto the ends. The cDNAs were digested with EcoRI (Amersham Corp.) and size fractionated by Sephadex G-100 chromatography. The first two-thirds of the eluate were then ligated into EcoRI-digested GT10 and packaged using a Vector Cloning System (Stratagene) according to the manufacturer's instructions. After infecting and plating Escherichia coli c608 Tri/FT, 10 random recombinant phages were obtained. After amplification, the screening was performed at 10° plaques/plaque; and the phages were transferred on duplicate sheets of nylon membrane (Hybond N, Amersham Corp.), processed, and prehybridized for 2 h at 42°C in 0.25 mM DDT, and 1.0 mM sodium azide; Buffer B: 50 mM Tris/HCl (pH 7.5) and 50% SDS + 1% SDS + 0.25 M NaCl in Buffer B was applied. Thioredoxin was eluted with about 0.2 M NaCl; fractions were pooled (285 ml) and dialyzed against Buffer C. The dialyse was clarified by centrifugation at 100,000 × g for 30 min and applied to a second DEAE-Sepharose column (25 × 5 cm) equilibrated with Buffer C. A 3-liter gradient of 0-0.5 M NaCl in Buffer C was applied. Thioredoxin eluted at 125-150 mM NaCl; fractions were pooled (500 ml) and titrated to pH 7.5 with 1 M Tris base, and DTT was added to 0.5 mM. The solution was concentrated to 20 ml by ultrafiltration using Diaflo YM-5 membranes (Amicon), filtered with Millex-GV 0.22-μm filter units (Millipore), and applied to a 38 × 3-cm column of Ultrogel AcA54 (Pharmacia LKB Biotechnology Inc.) equilibrated with Buffer D. Thioredoxin-containing fractions were pooled (50 ml), sterile-filtered using Millex-GV 0.22-μm filter units, and stored at -80°C.

**Protein Determination**

The concentration of purified protein was determined by measuring the absorbance at 280 nm using a Hewlett-Packard 8450 spectrophotometer. A molar absorption coefficient of 280 nm of 7.14 cm^-1 (based on a M, of 11,700) was determined by qualitative amino acid analysis as outlined by Allen (18). The S value corresponds to A 1.0% 6.10 ± 1 cm at 280 nm.

**Amino Acid Analysis**

Protein samples were hydrolyzed in constant boiling HCl in N2-flushed evaporated tubes in the presence of phenol (1 mg/ml) and 100 mM ethanethiol. Hydrolysates were analyzed on a Beckman 6300 amino acid analyzer.

**Analytical Separation Methods**

Electrofocusing was carried out on thin-layer polyacrylamide gels (Pharmacia LKB Biotechnology Inc., Ampholine PAG plates, pH 3.5-9.5) according to the manufacturer's instructions. SDS-PAGE was carried out on 15% (w/v) polyacrylamide gels using the buffer system described by Laemmli (19).

**Thioredoxin Assay**

Three assays were performed to evaluate the dithiol-disulfide oxidoreductase activity of recombinant human thioredoxin.

**DTT-dependent Activation—IgM Mudra**

Anti-IgM Mudra were derived from mice immunized intraperitoneally with an IgM antibody-secreting murine hybridoma and purified by 50% ammonium sulfate precipitation, followed by gel filtration chromatography on a Pharmacia LKB Biotechnology, Inc. S300 column. Thioredoxin (1 mM) was used by 100 mM DTT for 1 h at 20°C and then microdialyzed for 1 h on a Millipore filter (0.25-μm pore size) against 80 mM HEPES, 10 mM EDTA buffer.

Using the same buffer, 2 μl of 1.7 μM IgM and 0.1, 1, and 5 μl of a 1 mM solution of reduced thioredoxin were mixed for overnight reaction at 20°C. SDS-PAGE analysis for protein size determination was performed upon nonreducing conditions using a 2-10% acrylamide gel and silver staining as previously reported by Laemmli (19) and Merrill et al. (20).

**DTT-dependent Activation of Corn Leaf NADP-Malate Dehydrogenase—Corn leaf NADP-malate dehydrogenase (21) and spinach thioredoxin were purified as described previously (22). Enzyme solutions contained 3 μM Tris-HCl (pH 7.2), 0.3 mM DTT, 0.15 mM NADP-malate dehydrogenase, and either thioredoxin or human thioredoxin as indicated. After a 30 min incubation at 20°C, a 0.1 μl aliquot was used to determine the activity of NADP-malate dehydrogenase, followed by changes in absorbance at 340 nm as described earlier.

**Catalytic Activity of Human Thioredoxin on DTT-dependent Insulin Reduction (6)**—Comparison was made between recombinant human thioredoxin and commercial E. coli thioredoxin (IMCO). In a total volume of 1 ml, the reaction medium contained 100 μM phosphatase buffer (pH 7.1), 0.13 μM porcine insulin (Novo Pharmaceutical Industry, Paris, France), and thioredoxin as indicated. Each reaction was performed using protein purified as described elsewhere.
was initiated by adding 0.5 μM DTT to the cuvette, and absorbance at 650 nm was immediately recorded at 30°C.

RESULTS

Isolation of cDNA Clones

A λGT10 library constructed from the poly(A)+ RNA of 3B6 lymphoid cells was screened with two synthetic oligonucleotide probes deduced from the NH₂-terminal amino acid sequence of the 3B6 protein (Fig. 1). Among 48,000 clones screened, seven hybridized with both probes. Both clones have an identical sequence, which is shown in Fig. 2. This sequence is 500 base pairs long excluding the poly(A) tail and has a single reading frame. The first ATG codon located at nucleotide 64 from the 5′-end is followed by a 312-nucleotide-long open reading frame ending with an in-frame termination codon TAA at position 379 and is therefore able to code for a 104-amino acid protein. The classical polyadenylation site, AATAAA, is located 102 nucleotides downstream from this TAA codon. An EcoRI-DraI restriction fragment from clone W1 was used to probe a Northern blot of the original 3B6 RNA (Fig. 3). One single band was observed, indicating that a single species of mRNA of 550 base pairs is present in these cells.

Analysis of Deduced Amino Acid Sequence

Comparison of the deduced amino acid sequence with the sequence information available from the biochemical analysis of the 3B6 protein showed that the previously determined NH₂-terminal 21 amino acids were located within this reading frame, immediately after the first methionine codon of the cDNA clone (Fig. 2). The theoretical M, of 11,880 and the pl of 4.88 were in good agreement with the experimental values previously found for the 3B6 protein (9). No potential glycosylation sites were detected. The molecule contained 5 cysteine residues. Computer search against the National Biomedical Research Foundation DNA sequence data bank revealed no homology with any known gene, but comparison at the

![Fig. 1. Sequences of synthetic oligonucleotides used for cDNA library screening.](image)

![Fig. 2. Nucleotide sequence of human thioredoxin cDNA and derived amino acid sequence.](image)
protein well characterized in plants and bacteria. The homology was absolute over a highly conserved five amino acid sequence, Trp-Cys-Gly-Pro-Cys, referred to as the active site characteristic of all thioredoxins previously described. A few other conserved amino acids were found apart from the active site: a shorter region of homology is found upstream, Val-Asp-Phe, and an alanine and downstream a proline. No significant homology with vegetal thioredoxin from *Anabaena* 7119 (8) and spinach chloroplast (3) or bacterial thioredoxin from *Corynebacterium nephridii* (23) and *E. coli* (24) could be determined elsewhere in the molecule. In contrast, many homologies were noticed with a partial amino acid sequence of rat hepatoma thioredoxin (25) at the NH₂ terminus (Fig. 4).

**Recombinant Protein Purification**

Recombinant human thioredoxin was expressed in *E. coli* W3110cl (see “Experimental Procedures”) as a soluble protein accounting for 15% of the total proteins present in induced cells (Fig. 5, lane A). The protein was purified with an overall yield of 40%, representing a recovery of 6 mg/g of cells, wet weight. The protein migrated as a single band on SDS-PAGE (Fig. 5, lane C) with a M₀ of 12,000. The protein was monomeric under native conditions as judged by its elution behavior on Ultrogel AcA 54. Purity of the preparation was also indicated by the observation of a single band on isoelectric focusing (Fig. 5, lane D) with an isoelectric point of 4.7. The experimentally determined pI had the same value as that estimated from the amino acid composition. Amino acid analysis indicated excellent correlation between the experimental and the theoretical amino acid compositions (data not shown). Sulphhydryl analysis by modification with dithiothreitol and iodoacetamide followed by amino acid analysis indicated the presence of 5 cysteine residues/molecule as predicted by the cDNA sequence.

**Biological Characterization**

Since the major function of thioredoxins is a dithiol-disulphide oxidoreductase enzymatic activity, we tried at first to reduce an immunological substrate and next to confirm the thioredoxin specificity by classical tests.

**IgM Reduction**—We thought that pentameric IgM, which contains 26 interchain disulfide bridges, might constitute a potential substrate for thioredoxin. Three different doses of reduced recombinant human thioredoxin were mixed with a constant concentration of IgM and incubated overnight at room temperature (see “Experimental Procedures”). The products resulting from the reaction were analyzed by SDS-PAGE under nonreducing conditions (Fig. 6). With the highest dose of recombinant human thioredoxin (600-fold molar excess of recombinant human thioredoxin/IgM, i.e., 23-fold excess of disulfide bridges), four major bands were identified corresponding to 70 kDa (heavy chain IgM, μ), 25 kDa (light chain IgM = L), 27 kDa (dimeric human thioredoxin), and 13 kDa (monomeric human thioredoxin). A minor band at 95 kDa (μL) was also seen (Fig. 6, lane D). With intermediate doses of recombinant human thioredoxin (5-fold excess of human thioredoxin/IgM disulfide bridges), the pentameric IgM at 95 kDa and heavy chain dimers of IgM at 140 kDa (μL) were predominant (Fig. 6, lane E). The four major bands seen with the highest dose were not so intense. With the lowest dose of recombinant human thioredoxin (a 0.5 ratio of human thioredoxin/IgM disulfide bridges), the pentameric IgM was left intact (Fig. 6, lane F).

**DTT-linked Activation of NADP-Malate Dehydrogenase**—A common and specific assay to test the thioredoxin activity was then used. A dose-dependent curve of human and spinach thioredoxin on the DTT-dependent activation of corn leaf NADP-malate dehydrogenase is shown in Fig. 7. Although at equivalent concentrations, recombinant human thioredoxin was less efficient than spinach thioredoxin, it was able nevertheless to activate strongly NADP-malate dehydrogenase at higher concentrations (80 μM). At low concentrations of recombinant human thioredoxin (1–2 μM), a lag of 10 min

**Fig. 3. Analysis of 3B6 total RNA by Northern blotting.** RNA extracted from the 3B6 lymphoblastoid B cell line was hybridized with the EcoRI-DraI fragment from the W1 clone used as a probe. The first lane shows the RNA size markers synthesized by SP6 RNA polymerase, and the second lane shows the single 3B6 mRNA species which is at the 550-base level.

**Fig. 4. Comparison of different thioredoxin protein sequences from various origins.** Human, thioredoxin from the 3B6 cell line; *Rat*, thioredoxin from hepatoma; *Spinach*, thioredoxin type m from chloroplasts; *Anabaena*, thioredoxin from strain 7119. The different sequences are compared to human thioredoxin, and the homologies are boxed. The conserved active site is shaded. NH₂ terminal sequence homologies between inferior organisms are not shown for clarity.
Fig. 5. SDS-PAGE and isoelectric focusing of human thioredoxin. Lanes A–C refer to SDS-PAGE carried out on a 15% (w/v) polyacrylamide gel. Lane A, whole cell extract from E. coli cells thermally induced at 42°C; lane B, molecular mass standards from top to bottom (phosphorylase b 93 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) carbonic anhydrase (31 kDa), soybean trypsin inhibitor (22 kDa and lysozyme 14.4 kDa); lane C, purified human thioredoxin. Lanes D and E refer to isoelectric focusing on a thin-layer polyacrylamide gel. Lane D, purified thioredoxin; lane E, standard proteins (the isoelectric points are indicated to the right). Both the SDS-PAGE and isoelectric focusing gels were stained with Coomassie Blue.

was observed in the time course of NADP-malate dehydrogenase activation (data not shown). The apparent $K_a$ values computed from these reactions were 70 and 4 $\mu M$ for human thioredoxin and spinach thioredoxins, respectively.

Thioredoxin-catalyzed Reduction of Insulin by Dithiothreitol—In order to confirm the specificity of our recombinant protein, we examined the reduction of insulin, which is a classical assay for thioredoxins. For this purpose, we compared the activities of E. coli thioredoxin with recombinant human thioredoxin. The reduction of insulin disulfide bridges by DTT at pH 7.0 was determined with different concentrations of thioredoxins. As seen in Fig. 8, both thioredoxins displayed the same activity: the rates of insulin reduction were 1.7 $\Delta A_{405}$/min/mg of thioredoxin.

**Human Thioredoxin mRNA Expression and Regulation in Immunocompetent Cells**

The expression of human thioredoxin mRNA in various cells of lymphoid or related origin was studied by Northern blot analysis using two different probes; an EcoRI-DraI fragment of clone W1 or the synthetic oligonucleotide EW2. As shown in Fig. 9, human thioredoxin mRNA was present in all human T and B cell lines, as well as in the monocytic and erythroid lines tested. However, whereas it was high in the EBV 3B6 and 721 lines, it was lower in the Burkitt lines Daudi and Raji, indicating a different expression level in the different cell lines. The same Northern blot hybridized with a $\beta$-actin riboprobe revealed the same level of mRNA in all the cells tested (data not shown).

We next investigated the human thioredoxin mRNA expression level, during activation in vitro by various stimulating agents, in lymphocytes and monocytes (Fig. 10). The steady level of mRNA in these freshly isolated cells was quite low. Mitogenic stimulation for 24 h by phytohemagglutinin of peripheral blood lymphocytes increased the expression of human thioredoxin mRNA. The activation of monocytes by bacterial lipopolysaccharide or phorbol myristate acetate, a known activator of protein kinase C, increased the level of human thioredoxin mRNA from 1 to 18 h of stimulation. Phorbol myristate acetate-activated 3B6 cells also showed an increased level of human thioredoxin expression.

Finally, as seen in Fig. 11, the human thioredoxin probes (both the cDNA and the oligonucleotide probes) did not hybridize with RNA extracted from murine lymphoid lines (EL4 and CTL L2). In contrast, the gibbon ape lymphoid line MLA 144 contained a mRNA species of equivalent size which positively hybridized with the human thioredoxin probes.

**DISCUSSION**

Thioredoxin is the best representative enzyme of a group of widely distributed proteins possessing dithiol-disulfide ox-
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FIG. 8. Thioredoxin-catalyzed reduction of insulin by dithiothreitol. The absorbance at 650 nm is plotted against time. A, E. coli thioredoxin (TRX)-dependent insulin reduction. □, without thioredoxin; ●, with 2.5 μM thioredoxin; ■, with 5 μM thioredoxin B, human thioredoxin (HTR)-dependent insulin reduction. □, without human thioredoxin; ●, with 3 μM human thioredoxin; ■, with 5 μM human thioredoxin; ○, with 7 μM human thioredoxin.

FIG. 9. Human thioredoxin mRNA expression in various lymphoid cell lines. Northern blot analysis was performed using the synthetic oligonucleotide EW2 under low stringency conditions (6 × SSC, 42 °C). Lanes 1–3, T cell lines HSB2, Jurkat, and 8166, respectively; lanes 4–6, EBV B cell lines 3B6, 721, and YB4, respectively; lanes 7 and 8, Burkitt lymphoma-derived B cell lines Raji and Daudi, respectively; lane 9, monocytic cell line U937; lane 10, multipotent erythroid cell line K562.

FIG. 10. Human thioredoxin mRNA expression during activation. Northern blot analysis was performed using the cdNA fragment EcoRI-DraI under high stringency conditions (4 × SSC, 65 °C). Lanes 1 and 2, 4-h phorbol myristate acetate-activated and unstimulated 3B6 RNAs, respectively; lanes 3 and 4, resting and phytohemagglutinin-activated peripheral blood lymphocytes, respectively; lane 5, resting monocytes; lanes 6–8, 1-, 4-, and 18-h lipopolysaccharide-activated monocytes, respectively; lanes 9 and 10, 4- and 18-h phorbol myristate acetate-activated monocytes, respectively.

idoreductase activity (thioredoxin and glutaredoxin systems) (26, 27). Although partial amino acid sequences of animal thioredoxins have been reported previously (25, 28, 29), no cloning of mammalian thioredoxin cdNA or gene has been published yet. We present in this paper the complete amino acid sequence deduced from a cdNA clone of a human thioredoxin. This human enzyme possesses the completely conserved five-amino acid active site. Some surrounding residues which may be of importance for the secondary structure are also conserved. Apart from this region, the protein homology with thioredoxins from various origins is variable. This homology is found to be strong with the incomplete sequences available for rat and rabbit thioredoxins (28, 29). In contrast, a weak homology with vegetal (spinach (30)) and bacterial (C. nephridii (23), E. coli (24)) thioredoxins is observed. Interestingly, the human thioredoxin enzymatic activity is similar to the spinach and bacterial thioredoxins, suggesting that the only requirement in the oxidoreductase activity is the active site. At the nucleotide sequence level, however, interspecies mRNA analysis by Northern blot shows a strong cross-hybridization between human and gibbon, but none with mouse. These findings are in support of a nucleotide homology between man and ape.

The partial amino acid sequence previously reported (9) has shown that the mature human thioredoxin protein starts with a valine. According to the sequence of the cdNA, this
valine follows just a methionine. This implies that the translation product is processed. Several mechanisms are possible. One could be the elimination of the NH$_2$-terminal methionine. This hypothesis is supported by the nucleotide environment of the ATG codon which is in good agreement with Kozak's (31) consensus sequence for an initiation codon. A second possibility could be the existence of an additional NH$_2$-terminal peptide: this peptide could correspond either to a leader peptide allowing the transport of the protein to a specific cellular compartment or to a preprotein possessing or not the biological activity.

The enzymatic oxidoreductase activity of recombinant human thioredoxin produced in E. coli is detected in a wide spectrum of substrates from different origins. Recombinant human thioredoxin can reduce murine IgM disulfide bridges. It displays the same activity as vegetal thioredoxin on plant substrates, although with stoichiometric differences. Finally, it can also catalyze porcine insulin reduction by DTT. It seems therefore that many proteins with disulfide bridges may be potential substrates for thioredoxins. This enzymatic family, with highly conserved active site and molecular weight through evolution, is most probably of major importance for the cells. The principal cellular localization and the major substrates are not really defined. An interesting observation was that of the formation, upon oxidation, of human thioredoxin dimers. Indeed immunoblots, using antibodies against a human thioredoxin NH$_2$-terminus synthetic peptide, were positive with crude extracts from lymphoid human cell lines, revealing a monomeric (13 kDa) and a dimeric (26 kDa) band of human thioredoxin (data not shown). This indicates that an equilibrium between monomeric reduced human thioredoxin and dimeric oxidized human thioredoxin may exist physiologically.

Messenger RNA coding for human thioredoxin is detected in most lymphoid and hematopoietic human cell lines tested. This indicates that immortalized cells, which divide actively, have a high level of expression of the human thioredoxin protein. In contrast, resting cells such as lymphocytes or monocytes, freshly isolated from the circulating blood, express human thioredoxin mRNA very weakly. Upon activation, these cells transcribe substantial amounts of human thioredoxin mRNA. These results are in good agreement with previous histo-immunologic studies, using antibodies against rat thioredoxin, which did not detect the presence of the enzyme in resting lymphocytes (32).

The high level of human thioredoxin expression, in dividing lymphocytes, which enabled us to isolate this protein from an EBV B cell line, may suggest some important immunological role for the enzyme. We now have the tools (human genetic probes, recombinant purified protein and antibodies) to examine further these questions in the human.

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