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Treatment of human peripheral lymphocytes with concanavalin A activates expression of glutathione reductase

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A cDNA clone corresponding to mRNA present only in proliferating cells was isolated and its nucleotide sequence determined. This cDNA is 723 nucleotides long and encodes a portion of the human glutathione reductase mRNA corresponding to the amino acids 77–318 of the mature protein. Expression of glutathione reductase mRNA was undetectable in resting human T-lymphocytes and was induced shortly after cells had been triggered to proliferate by the treatment with concanavalin A. This result suggests that synthesis of glutathione reductase is generally activated in replicating cells which may indicate that this enzyme plays a functional role during cell proliferation.

Glutathione reductase; Cell cycle; cDNA; Gene expression

1. INTRODUCTION

Peripheral human T-lymphocytes in culture are non-profilerating cells which can be stimulated to divide by treatment with lectins, such as ConA or PHA. These activated lymphoblasts which are responsive to the T-cell growth factor interleukin 2 (Il2) synthesize various inducible proteins related to growth control and differentiation. In an attempt to characterize molecules which are expressed in lymphoblasts in response to treatment with ConA, we have constructed a representative cDNA expression library of lectin stimulated human T-cells and have identified individual clones with an antiserum raised against ConA stimulated lymphocytes. Here we describe the identification of a cDNA clone encoding parts of the human glutathione reductase (GR) mRNA. The level of GR mRNA expression sharply rises from undetectable levels in resting cells to appreciable concentrations shortly after ConA treatment. The induction of GR transcription is demonstrated by Northern blot analysis utilizing the GR cDNA as a probe. Several other agents which are capable of initiating growth of human lymphocytes in culture also induce the expression of the GR gene.

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Abbreviations: ConA, concanavalin A; GR, glutathione reductase; PHA, phytohemagglutinin; dCTP, deoxycytidine triphosphate; 1 × SSC, sodium chloride (0.15 M) with sodium citrate (0.015 M); SDS, sodium dodecyl sulfate

2. EXPERIMENTAL

2.1. *Construction of λ gt11 cDNA library*

Total poly $(A)^+$ RNA was prepared from concanavalin A (ConA) stimulated human lymphocytes obtained from buffy coats [1] by the guanidinium isothiocyanate method [2] followed by affinity chromatography on oligo (dT) cellulose [3]. Synthesis of cDNA and cloning into the phage expression vector λ gt11 [4] was performed by standard procedures and has been described in detail previously [5].

2.2. *Antibody screening of a human T-cell library*

Total protein extract from ConA stimulated cells (obtained from DRK-Blutspendedienst, Springe, FRG) was mixed with complete Freund's adjuvant and used to immunize rabbits according to standard protocols [26]. Antiserum was preadsorbed prior to use to excess of protein extracts of resting white blood cells and lysate of *E. coli* y 1090 in order to remove antibodies directed against non-induced T-cell proteins and *E. coli* epitopes, respectively. 5×10^4 plaque forming units of a human T-cell λ gt11 cDNA library were plated on a single 15 cm diameter dish and screened with this antiserum. Bound antibody was detected with goat anti-rabbit IgG conjugated to alkaline phosphatase (Promega, Atlanta). Staining was performed with nitro blue tetrazolium (NBT) and 4'-bromo-5'-chloroindolylphosphate (BCIP) as substrates (Sigma Corp.). Positive plaques identified on duplicate filters were purified by subcloning at lower densities.

2.3. *Nucleotide sequence analysis*

DNA inserts of recombinant phages were subcloned into the plasmid pEMBL 8 [6] for restriction analysis. For sequence determination by the dideoxy nucleotide chain termination method [7] single stranded DNA was generated by superinfection of recombinant pEMBL 8-transformed *E. coli* LK111 with the single-stranded phage f1 [6]. The nucleotide sequence was established on both strands using the appropriate unidirectional deletion clones generated with exonuclease III [8].

2.4 *Northern blot analysis*

Total RNA was prepared from stimulated and non-stimulated

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human lymphoblasts as described before [2]. Following glyoxylation and electrophoresis on 1% agarose gels, RNA was blotted onto Gene Screen filters as described [9]. The GR-cDNA insert was excised from pEMBL8 with *Eco*RI, and labelled with ^{32}P-dCTP by nick-translation (Kit, Amersham Buchler, FRG). The labelled probe was hybridized at 42°C in a solution containing 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 50% formamide, 5 × Denhardt's solution, 1% SDS, 0.1% sodium pyrophosphate, and denatured salmon sperm DNA (100 μg/ml). Filters were washed in 2 × SSC, 0.5% SDS at 65°C for 1 h, followed by washing in 0.1 × SSC, 0.1% SDS at 55°C for 1 h, and was then exposed on X-ray film at −80°C. The S6 cDNA probe [5] was used as control on the same filters after removal of the first probe.

2.5. *Southern blot analysis*

10 μg of human genomic DNA were digested with various restriction enzymes according to the procedures recommended by the supplier (Boehringer, FRG), separated on 0.8% agarose gels, transferred to nitrocellulose and hybridized with labelled GR cDNA according to standard procedures [3] as described above.

3. RESULTS

3.1. *Isolation and identification of a cDNA clone encoding the human glutathione reductase*

A cDNA expression library of ConA stimulated human lymphoblasts was constructed in the phage vector λ gt11. In order to identify gene sequences which were expressed in lymphocytes upon ConA treatment, we raised antiserum against stimulated T-cells in rabbits. To eliminate antibodies directed against constitutively expressed antigens, such as housekeeping and structural proteins, the antiserum was preabsorbed to native protein extracts from non-stimulated peripheral white blood cells. The expression library was screened with preadsorbed antiserum and individual positive clones were isolated. The primary criteria for a detailed examination of the isolated cDNA clones was the induction of their corresponding mRNAs in ConA treated T-cells (see Discussion below). Accordingly, one cDNA clone was further analysed and its nucleotide sequence determined. As shown in Fig. 1, the clone contains 726 nucleotides and has an open reading frame throughout the cloned fragment. The computer assisted search of the EMBL data bank revealed that the deduced amino acid sequence of the cDNA clone was entirely identical to amino acids 77–318 of the human glutathione reductase (EC 1.6.4.2.) [10–13]. This enzyme constitutes a ubiquitous FAD-containing protein which catalyzes the reduction of oxidized glutathione at the expense of NADPH. The partial sequence represented by our cDNA isolate encompasses the FAD and the NADPH binding domains, two important functional regions of

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GR-CDNA

                                                30
GAA TTC ATG CAT GAT CAT GCT GAT TAT GGC TTT CCA AGT TGT GAG GGT AAA TTC AAT
Glu Phe Met His Asp His Ala Asp Tyr Gly Phe Pro Ser Cys Glu Gly Lys Phe Asn

                                                90
CGT GTT ATT AAG GAA AAG CGG GAT GCC TAT GTG AGC CGC CTG AAT GCC ATC TAT CAA
Arg Val Ile Lys Glu Lys Arg Asp Ala Tyr Val Ser Arg Leu Asn Ala Ile Tyr Gln

                                               150
AAT CTC ACC AAG TCC CAT ATA GAA ATC ATC CGT GGC CAT GCA GCC TTC ACG AGT GAT
Asn Leu Thr Lys Ser His Ile Glu Ile Ile Arg Gly His Ala Ala Phe Thr Ser Asp

                                               210
AAG CCC ACA ATA GAG GTC AGT GGG AAA AAG TAC ACC GCC CCA CAC ATC CTG ATC GCC
Lys Pro Thr Ile Glu Val Ser Gly Lys Lys Tyr Thr Ala Pro His Ile Leu Ile Ala

                                               270
GGT GGT ATG CCC TCC ACC CCT CAT GAG AGC CAG ATC CCC GGT GCC AGC TTA GGA ATA
Gly Gly Met Pro Ser Thr Pro His Glu Ser Gln Ile Pro Gly Ala Ser Leu Gly Ile

                                               330
AGC GAT GGA TTT TTT CAG CTG GAA GAA TTG CCC GGC CGC AGC GTC ATT GTT GGT GCA
Ser Asp Gly Phe Phe Gln Leu Glu Glu Leu Pro Gly Arg Ser Val Ile Val Gly Ala

                                               390
TAC ATT GCT GTG GAG ATG GCA GGG ATC CTG TCA GCC CTG GGT TCT AAG ACA TCA CTG
Tyr Ile Ala Val Glu Met Ala Gly Ile Leu Ser Ala Leu Gly Ser Lys Thr Ser Leu

                                               450
ATA CGG CAT GAT AAG GTA CTT AGA AGT TTT GAT TCA ATG ATC AGC ACC AAC TGC ACG
Ile Arg His Asp Lys Val Leu Arg Ser Phe Asp Ser Met Ile Ser Thr Asn Cys Thr

                                               510
GAG CTG GAG AAC GCT GGC GTG GAG GTG CTG AAG TTC TCC CAG GTC AAG GAG GTT AAA
Glu Leu Glu Asn Ala Gly Val Glu Val Leu Lys Phe Ser Gln Val Lys Glu Val Lys

                                               570
ACT TTG TCG GGC TTG GAA GTC AGC ATG GTT ACT GCA GTT CCC GGT AGG CTA CCA GTC
Thr Leu Ser Gly Leu Glu Val Ser Met Val Thr Ala Val Pro Gly Arg Leu Pro Val

                                               630
ACC ATG ATT CCA GAT GTT GAC TGC CTG CTC TGG GCC ATT GGG CGG GTC CCG AAT ACC
Thr Met Ile Pro Asp Val Asp Cys Leu Leu Trp Ala Ile Gly Arg Val Pro Asn Thr

                                               690
GAC CTG AGT TTA AAC AAA CTG GGG ATT CAA ACC GAT GAC AAG GGT CAT ATC ATC GTA
Asp Leu Ser Leu Asn Lys Leu Gly Ile Gln Thr Asp Asp Lys Gly His Ile Ile Val


GAA TTC
Glu Phe
```

Fig. 1. The nucleotide sequence and predicted amino acid sequence of the partial GR cDNA clone. The cloned *Eco*RI fragment shown here represents coding information for the amino acids 77–318 of the human glutathione reductase.

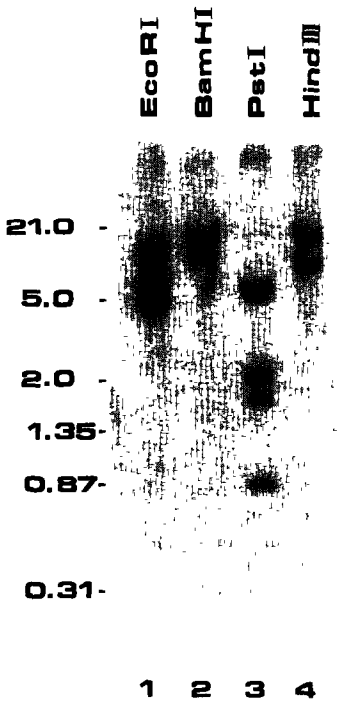

Fig. 2. Southern blot analysis of the human gene encoding glutathione reductase. The DNA was digested with the indicated restriction enzymes. The filter was hybridized with ^{32}P labelled GR cDNA probe (sp. act. 1×10^8 cpm/μg). Exposure time on X-ray film was 48 h.

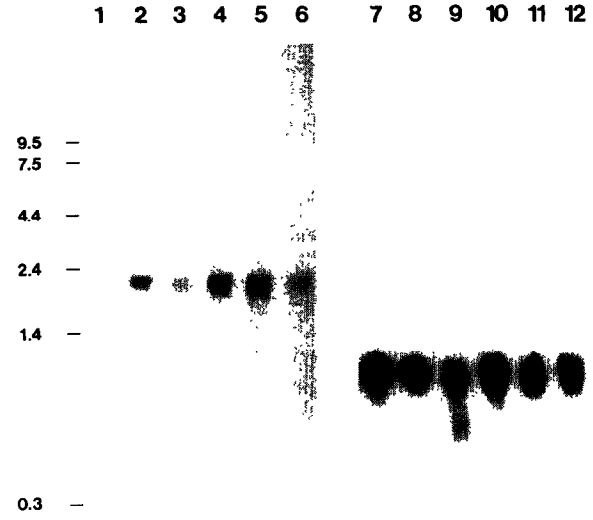

Fig. 3. Determination of GR mRNA levels in human lymphoblasts in culture stimulated with ConA. Stimulation periods were: minus ConA (lane 1), 1 h (lane 2), 3 h (lane 3), 5 h (lane 4), 8 h (lane 5), and 24 h (lane 6). The probe was GR cDNA (lanes 1–6) and S6 cDNA encoding the ribosomal protein S6 (lanes 7–12 [5]) used as control on the same blot. Approximately equal amounts of RNA were loaded in each lane. The specific activities of both hybridization probes were also similar.

the enzyme [10]. Using the GR cDNA clone as probe for Southern blot analysis on human genomic DNA, a simple pattern of hybridizing signals was detected in digests with various restriction enzymes (see Fig. 2). The low complexity of DNA fragments detected with the GR probe may indicate that glutathione reductase is encoded by a unique gene in the human genome.

3.2. *Expression of GR mRNA is activated in ConA stimulated lymphoblasts*

To ascertain that the GR cDNA clone represents an mRNA species that is induced in ConA treated lymphoblasts but is not present in non-proliferating, peripheral white blood cells, RNA was isolated from resting human lymphocytes and from cells harvested at various time points after stimulation with ConA. RNA samples were subjected to Northern blot analysis using the GR cDNA as a probe. As shown in Fig. 3, non-stimulated cells virtually contain no GR mRNA but start to express GR mRNA within 1 h upon the addition of ConA. GR mRNA continues to accumulate for at least 8 h and then returns to low levels after approximately 24 h. The expression of mRNA coding for the ribosomal protein S6 which was used as a control for a constitutively expressed mRNA was found to remain unaltered. When non-lymphatic tissue culture cells such as the human fibroblast line A375, erythroleukemia cells K562, and the human B cell line Molt47 were analyzed, expression of high levels of GR mRNA was observed in proliferating cultures. However, when cells were grown to high densities to induce contact inhibition, only low levels of GR mRNA were detected (data not shown). In order to test whether induction of GR mRNA was specific for the stimulation with ConA, the effect of other agents known to activate lymphocytes were also investigated. As shown in Table I, expression of GR mRNA was always induced when cells had been triggered to proceed through the cell cycle irrespective of the inducing agent. Although the phorbolester TPA alone did not activate GR expression, a slight cooperative effect with the weak inducer PHA was observed. That IL-2 by itself cannot stimulate resting T-cells to grow, is probably due to the lack of IL-2 receptors on these cells and it is only after the initial stimulation of receptor synthesis by ConA that the cells become responsive.

4. DISCUSSION

Our general approach to isolate cDNAs from lectin stimulated human T-lymphocytes that represent mRNAs which are specifically induced in activated cells and are not present in resting lymphocytes yielded a partial cDNA clone coding for the ubiquitous enzyme glutathione reductase. This enzyme belongs to a group of flavin-containing disulfide oxidoreductases which also includes the dihydrolipoamid dehydrogenase [14], thioredoxin reductase [15] and mercuric reductase [16]. The human glutathione reductase originally isolated

Table I

Induction of GR mRNA

Probe	Inducing agent					
	ConA	PHA	TPA	PHA + TPA	ConA + IL2	IL2
Glut.-reduct. (CA5)	+ +	+	–	+ +	+ +	–
Rib. protein (S6)	–	–	–	–	–	–

from erythrocytes [10,17], leucocytes [18] and platelets [19], is the best characterized of these proteins. Its amino acid sequence has been determined [10–13] and its structure has been established at a resolution of 1.54 Å [20].

Glutathione reductase catalyzes the reduction of oxidized glutathione with NADPH. It thereby maintains a high ratio of reduced to oxidized glutathione in the cell, probably in order to protect proteins from damage by oxidation [21]. Since glutathione is an antioxidant and eliminates free radicals from the cell, it has been proposed to play a biological role in the ageing process [22]. Here, we report that glutathione reductase mRNA is virtually undetectable in non-proliferating cells and that it only accumulates after induction of cell division. This effect is not specific for a particular cell type nor does it require a specific inducing agent. It has been shown previously that the enzyme activity of glutathione reductase fluctuates periodically during the cell cycle of sea urchin eggs [23]. Its highest activity is actually observed during the phase of DNA synthesis [24]. It is therefore interesting to speculate that glutathione reductase is not only involved in generating reduced glutathione as a protective measure but that it may also play a major role in the supply of hexose precursors for the synthesis of nucleotides via the generation of NADP. It has been shown in Ehrlich ascites tumor cells that the hexose monophosphate pathway is indeed largely dependent on and regulated by the availability of NADP which in turn is generated by glutathione reductase [25]. The isolation of a cDNA probe for the human glutathione reductase provides a useful tool to study the interesting regulation of this gene in more detail. The observation of the cell cycle controlled synthesis of glutathione reductase suggests a functional role which awaits further elucidation.

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