Microinjected glutathione *S*-transferase Yb subunits translocate to the cell nucleus

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We have previously shown that a 30 kDa DNA-binding protein isolated from rat cell nuclei exhibits the chemical and immunological properties of glutathione *S*-transferase Yb subunits [Bennett, Spector & Yeoman (1986) *J. Cell Biol.* **102**, 600–609]. It was of interest, therefore, to determine whether Yb subunits isolated from rat liver nuclei would return to nuclear fractions upon reintroduction to cell cytoplasm via red-blood-cell-mediated fusion. Labelled Yb subunits were associated with nuclear fractions 60 min after cell fusion. The microinjected protein remained associated with the nuclei for 18 h and was not extractable with low-salt washes. In addition, injected Yb subunits were found to equally distribute between extractable (56%) and residual (44%) nuclear fractions. These experiments demonstrate that glutathione *S*-transferase Yb subunits isolated from nuclei rapidly translocate to nuclei upon reintroduction into cell cytoplasm.

INTRODUCTION

Recently, we have identified a rat liver DNA-binding protein, protein BA (Catino *et al.*, 1978), as a member of the glutathione *S*-transferase family (Bennett *et al.*, 1986). The glutathione *S*-transferases are a family of proteins which catalyse the transfer of glutathione to a large variety of electrophilic compounds, exhibit Δ^5 -3-oxosteroid isomerase activity, selenium-independent glutathione peroxidase activity and the ability to bind a large number of nonsubstrate ligands (Chasseaud, 1979; Jakoby & Habig, 1980; Mannervik, 1985). Glutathione *S*-transferases are dimeric proteins composed of homo- and heterodimers. Rat liver contains at least ten isoenzymes and six distinct types of subunits (Mannervik *et al.*, 1985).

Nonhistone protein BA was first identified in studies comparing chromatin-associated proteins in normal and transformed cells. Protein BA was found to be a major protein in normal hepatocytes and lymphocytes, but was undetected in chromatin isolated from transformed cells (Yeoman *et al.*, 1973, 1975, 1978). The chromatin-associated form of protein BA (Catino *et al.*, 1978) was identified as a glutathione *S*-transferase Yb subunit (Bennett *et al.*, 1986). The soluble, nucleoplasmic protein BA (Bennett *et al.*, 1982) was found to be a mixture of Ya, Yb, and Yc glutathione *S*-transferase subunits (Bennett *et al.*, 1986). Antibodies generated against Yb subunits, isolated from rat liver nuclei, stained both the cytoplasm and nuclei in rat liver cryosections and a normal rat liver cell line (Bennett & Yeoman, 1985; Bennett *et al.*, 1986). Furthermore, the antibodies stained discrete interchromatinic nuclear domains which were also demonstrated to contain U-snRNP (Bennett & Yeoman, 1985; Bennett *et al.*, 1986). As a first step in the investigation of the role of glutathione *S*-transferase in the cell nucleus, we microinjected Yb subunits isolated from rat liver nuclei into the cytoplasm of a cell line

which lacked Yb subunits in their chromatin, the Walker 256 cell line (Yeoman *et al.*, 1975). The localization and the stability of the microinjected protein were examined in the present study.

EXPERIMENTAL PROCEDURES

Isolation and radiolabelling of proteins

Glutathione *S*-transferase Yb subunits were isolated from saline/EDTA (0.075 M-NaCl/0.025 M-EDTA, pH 8.0) extracts of rat liver nuclei as previously described (Bennett *et al.*, 1982). Those fractions from Ultrogel AcA 44 column chromatography that were enriched in Yb subunits were used for microinjection experiments (see lane *a* of Fig. 2). Crystallized bovine serum albumin was purchased from Miles Laboratories. Proteins were iodinated by the lactoperoxidase–glucose oxidase method (Marchalonis, 1969) using Enzymo Beads (Bio-Rad Laboratories). Protein (20–490 μ g) was labelled with 1 mCi of Na¹²⁵I for 45 min at 20 °C. Labelled proteins were dialysed twice against 1 litre of 10 mM-sodium phosphate/150 mM-NaCl/10 mM-KI, pH 7.4, and four times against 1 litre of 10 mM-sodium phosphate/150 mM-NaCl, pH 7.4. Final specific activities ranged from 5×10^5 to 5×10^6 c.p.m./ μ g of protein. Protein concentrations were determined as described by Bradford (1976).

Red-blood-cell-mediated microinjection

Human red blood cells were loaded with proteins by the ‘pre-swell’ method (Schlegel & Rechsteiner, 1978). Loaded cells were extensively washed with Ca²⁺/Mg²⁺-free Hanks’ balanced salt solution (Gibco). Loaded red blood cells were fused with Walker 256 cells as described by Schlegel & Rechsteiner (1978). The fusion mixture contained 10^9 loaded red blood cells, 10^7 Walker 256 cells and 1000 haemagglutinating units of u.v.-inactivated

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; Ya, Yb and Yc, 25.6 kDa, 27.0 kDa and 28.0 kDa monomers of glutathione *S*-transferase; U-snRNP, uridylic-acid-rich-RNA-containing small nuclear ribonucleoprotein particles.

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Sendai virus (Bennett *et al.*, 1983). Unfused red blood cells were separated from Walker 256 cells by layering over 50 % fetal calf serum as previously described (Bennett *et al.*, 1983). This procedure was repeated until unfused red blood cells comprised less than 0.5 % of the total number of cells.

Cellular fractionation

After the microinjection procedure, cells were either fractionated as described below or plated in 75 cm² flasks and incubated at 37 °C for the indicated times. Cells were scraped from the culture dish, washed twice with phosphate-buffered saline at 4 °C and homogenized in 25 mM-citric acid with a Teflon/glass homogenizer at 4 °C. All subsequent manipulations were performed at 4 °C. The cellular homogenate was centrifuged at 1000 *g* for 10 min to obtain a crude nuclear pellet. Nuclei were resuspended in 0.34 M-sucrose/5 mM-MgCl₂/5 mM-Tris/HCl (pH 7.4)/1.0 mM-PMSF, layered over a cushion of 0.88 M-sucrose/5 mM-MgCl₂/5 mM-Tris/HCl (pH 7.4)/1.0 mM-PMSF and centrifuged at 1500 *g* for 10 min. Purified nuclei were sequentially extracted with: (1) 0.075 M-NaCl/0.025 M-EDTA (pH 8.0)/1.0 mM-PMSF; (2) 10 mM-Tris/HCl (pH 8.0)/1.0 mM-PMSF; (3) 8.5 M-urea/1 % 2-mercaptoethanol/50 mM-sodium phosphate (pH 7.6)/1.0 mM-PMSF. Radioactivity in each cellular fraction was determined with a Beckman Gamma 4000 counter.

RESULTS

Subcellular distribution of microinjected glutathione S-transferase

The subcellular distribution of microinjected nuclear glutathione S-transferase Yb subunits was determined by cellular fractionation. The microinjection protocol required a 20 min incubation period at 37 °C to fuse red blood cells with tissue culture cells (Schlegel & Rechsteiner, 1978) followed by several differential centrifugation steps at 4 °C to remove unfused red blood cells, requiring a total time of 1 h. Immediately after the microinjection procedure 85 % of the labelled protein was associated with nuclei (Table 1). At 18 h after microinjection 94 % of the labelled protein was associated with the nuclear fraction. The nuclear association was not due to unfused loaded red blood cells because the

Table 1. Subcellular distribution of microinjected nuclear Yb subunits

Immediately after the microinjection procedure, or 18 h after microinjection of ¹²⁵I-Yb subunits, 2 × 10⁶ cells were fractionated as described in the Experimental procedures section. These results are the result of three determinations from two separate experiments.

Subcellular fraction	Time after microinjection ...	¹²⁵ I-labelled Yb subunits (% of total)	
		1 h	18 h
Total cell		100	100
Cytoplasm		14.8 ± 8.1	6.1 ± 3.2
Nucleus		85.2 ± 8.1	93.9 ± 3.2

Table 2. Solubility of microinjected nuclear Yb subunits from isolated nuclei

At the indicated times, nuclei were isolated from microinjected cells and sequentially extracted with the buffers listed below. Total radioactivity associated with the nuclei was between 40000 and 80000 c.p.m. Results are the average of three determinations from two separate experiments. N.D., value not determined.

Nuclear extract	Time after microinjection ...	¹²⁵ I-labelled Yb subunits (% of total)	
		1 h	18 h
0.075 M-NaCl/0.025 M-EDTA		1.0 ± 0.2	1.9 ± 0.8
10 mM-Tris/HCl		0	2.0 ± 0.5
8.5 M-Urea/50 mM-sodium phosphate/1 % 2-mercaptoethanol		N.D.	51.4 ± 3.4
Residue		99.0 ± 0.3	45.6 ± 4.4

contaminating red blood cells account for less than 5 % of the total radioactivity. The nuclear localization of the Yb subunits was not an artifact of cellular fractionation, because ¹²⁵I-labelled bovine serum albumin microinjected under identical conditions was found predominantly in the cytoplasm (> 60 %; results not shown). We cannot rule out the possibility that a portion of the microinjected Yb subunits may be associated with the nuclear envelope, since 30 % of the radioactivity in the nuclear pellet was solubilized by homogenization in 10 mM-Tris/HCl/1 % deoxycholate (pH 7.4).

Microinjected glutathione S-transferase localizes to the nuclear residue fraction

Previous experiments have demonstrated that 50–75 % of the nuclear glutathione S-transferase Yb subunits were soluble in 0.075 M-NaCl/0.025 M-EDTA (pH 8.0) (Catino *et al.*, 1978; C. F. Bennett, unpublished work). However, less than 2 % of the radioactivity found in the nuclear fraction of microinjected cells was extracted by the 0.075 M-NaCl/0.025 M-EDTA or 10 mM-Tris/HCl (pH 8.0) washes (Table 2). These results were unexpected, because the Yb subunits microinjected into the Walker 256 cells were isolated from NaCl/EDTA extracts of rat liver nuclei. Thus, soluble Yb subunits may become tightly associated with chromatin in cells which have previously been shown to lack chromatin-associated Yb subunits (Yeoman *et al.*, 1975). Extraction of nuclei prepared from microinjected cells with 8.5 M-urea/1 % 2-mercaptoethanol/50 mM-sodium phosphate (pH 7.6) solubilized 52 % of the labelled protein. Those conditions have previously been shown to extract Yb subunits from chromatin (Catino *et al.*, 1978; Bennett *et al.*, 1982).

Stability of microinjected ¹²⁵I-labelled glutathione S-transferase

The stability of ¹²⁵I-labelled glutathione S-transferase Yb subunits was examined by determining the radioactivity associated with the microinjected cells at various times following microinjection (Fig. 1). The microinjected Yb subunits exhibited a half-life of 140 h in the Walker 256 cells. Confirmation that the radioactivity associated with the cells was still associated with intact glutathione

Fig. 1. Stability of ^{125}I -labelled glutathione *S*-transferase Yb subunits microinjected into Walker 256 cells

Walker 256 cells were microinjected with Yb subunits isolated from rat liver nuclei and labelled with ^{125}I as described under 'Experimental procedures'. At the indicated times after microinjection, cells were harvested by mild trypsinization, washed in medium containing 5% fetal calf serum, and the radioactivity associated with the cell pellets was determined in a Beckman model 4000 gamma counter. Results are the average of two separate experiments.

Fig. 2. Degradation of ^{125}I -labelled Yb subunits microinjected into Walker 256 cells

Walker 256 cells were microinjected with ^{125}I -labelled Yb subunits isolated from rat liver nuclei, as described under 'Experimental procedures'. At 0 h (lane b), 3 h (lane c), 6 h (lane d), 18 h (lane e) and 36 h (lane f) cells were scraped from the culture dish and precipitated with acetone. Total cellular proteins were separated by SDS/polyacrylamide-gel electrophoresis, the gel was dried and exposed for autoradiography. Lane (a) represents the ^{125}I -labelled Yb subunits used for the microinjection studies.

contaminated (< 10%) with Ya subunits (Fig. 2, lane a). There was a minor accumulation of a 14 kDa degradation product with time (Fig. 2). However, more than 85% of the radioactivity was associated with the Yb subunit 36 h after microinjection (Fig. 2, lane f). From these experiments it can be concluded that the radiolabel associated with nuclear fractions was covalently attached to intact Yb subunits.

DISCUSSION

Previous studies have suggested that glutathione *S*-transferases were primarily cytoplasmic enzymes (Fleischner *et al.*, 1977; Chasseaud, 1979; Jakoby & Habig, 1980; Redick *et al.*, 1982; Mannervik, 1985). However, there have been reports that the nucleus does contain glutathione *S*-transferase (Campbell *et al.*, 1980; Redick *et al.*, 1982; Bennett *et al.*, 1986). This controversy may result from a differential localization of individual subunits or their respective dimers. The present microinjection studies support our immunolocalization studies with the glutathione *S*-transferase Yb subunit antibodies, by establishing that upon reintroduction to cell cytoplasms, they localize to nuclear fractions. At the present time it is not known if cytoplasmic and nuclear Yb subunits are identical proteins in a state of equilibrium between the two cellular compartments, or if nuclear and cytoplasmic Yb subunits are distinct proteins, which are antigenically related. Clearly, the glutathione *S*-transferases are small enough to diffuse through nuclear pores (Bonner, 1978).

These studies show that the Yb subunits isolated from rat cell nuclei rapidly return to the cell nucleus of cells in culture when they are introduced to the cytoplasm of cells by red-blood-cell-mediated fusion. Furthermore, approximately half of the injected Yb subunits (44%) can be found within the nuclear residue fraction.

These studies also demonstrate that Yb subunits are very stable within nuclei of Walker cells, i.e. they have an apparent half-life of 140 h. This value is 2.5-fold longer than the 55 h half-life reported for ligandin (YaYc) (Arias *et al.*, 1976). It compares favourably, however, with the apparent half-life of 120 h for ^{125}I -labelled high-mobility group (HMG1) protein injected into HeLa cell nuclei by Rechsteiner & Kuehl (1979). It seems reasonable to conclude, therefore, that the absence of endogenous Yb subunits from Walker cell nuclei does not result from an enhanced degradation of the protein. Furthermore, microinjection of 1.5×10^6 Yb subunit molecules per cell had no effect on cell growth or DNA synthesis when compared with cells injected with bovine serum albumin (results not shown). Thus, microinjected Yb subunits are not cytotoxic.

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