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Electrostatic Association of Glutathione Transferase to the Nuclear Membrane

*EVIDENCE OF AN ENZYME DEFENSE BARRIER AT THE NUCLEAR ENVELOPE**

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The possible nuclear compartmentalization of glutathione *S*-transferase (GST) isoenzymes has been the subject of contradictory reports. The discovery that the dinitrosyl-diglutathionyl-iron complex binds tightly to Alpha class GSTs in rat hepatocytes and that a significant part of the bound complex is also associated with the nuclear fraction (Pedersen, J. Z., De Maria, F., Turella, P., Federici, G., Mattei, M., Fabrini, R., Dawood, K. F., Massimi, M., Caccuri, A. M., and Ricci, G. (2007) *J. Biol. Chem.* 282, 6364–6371) prompted us to reconsider the nuclear localization of GSTs in these cells. Surprisingly, we found that a considerable amount of GSTs corresponding to 10% of the cytosolic pool is electrostatically associated with the outer nuclear membrane, and a similar quantity is compartmentalized inside the nucleus. Mainly Alpha class GSTs, in particular GSTA1-1, GSTA2-2, and GSTA3-3, are involved in this double modality of interaction. Confocal microscopy, immunofluorescence experiments, and molecular modeling have been used to detail the electrostatic association in hepatocytes and liposomes. A quantitative analysis of the membrane-bound Alpha GSTs suggests the existence of a multilayer assembly of these enzymes at the outer nuclear envelope that could represent an amazing novelty in cell physiology. The interception of potentially noxious compounds to prevent DNA damage could be the possible physiological role of the perinuclear and intranuclear localization of Alpha GSTs.

Glutathione *S*-transferases (GSTs)[2] are a superfamily of enzymes that protect the cell from toxic endogenous or xenobiotic compounds. Eight different gene-independent, isoenzymatic classes have been found in mammals, and two of these, Alpha and Mu classes, are abundantly expressed in rat liver, where they represent 43 and 56%, respectively, of the entire pool of cytosolic GSTs (1). Beside their catalytic competence to conjugate GSH to many toxic substances having an electrophilic center, these enzymes also display peroxidase activity and ligandin properties (2, 3). In hepatocytes, Alpha GSTs efficiently trap a natural nitric oxide carrier, the dinitrosyl-diglutathionyl-iron complex (DNDGIC), thus preventing the irreversible inhibition of glutathione reductase caused by this compound (4). Preliminary evidence was also reported that GSTs could be associated with the nuclear membrane or compartmentalized in nuclei. The presence of GSTs on intracellular membranes is not a new finding. MGST1 is a peculiar trimeric microsomal integral membrane isoenzyme discovered and characterized many years ago (5). GSTA4-4, a specific isoenzyme able to detoxify hydroxyalkenals, displays a widespread mitochondrial, peroxisomal, and cytosolic localization, but the plasma membrane also binds detectable amounts of this enzyme (6). Furthermore, the tight association of Alpha and Mu class GSTs with the microsomal membrane fraction of rat liver was demonstrated by Morgenstern *et al.* (7), and about 2% of the cytosolic GSTA1-1 has been found in the microsomal membrane of sheep liver cells (8). Immunohistochemical evidence suggested the presence of nuclear Alpha and Pi class GSTs in different human tissues, but these studies did not quantify the specific isoenzymes involved (9, 10). Other investigations claimed either the presence or the absence of nuclear compartmentalization of GSTs. In particular, McCusker *et al.* (11) did not report any detectable nuclear GST activity, whereas Soboll *et al.* (12), using a nonaqueous technique of cell fractionation, found that both Alpha and Mu GSTs are present in the nucleus. Other studies reported a nuclear localization of GSTs (13–15), but the identification of the specific isoenzyme(s) involved in this association and their quantification are uncertain.

This study reveals for the first time that beside a significant amount of Alpha GSTs inside the nucleus, an equivalent amount is found in electrostatic association with the outer nuclear membrane. This particular modality of interaction has been detailed in cells and model systems using confocal microscopy, immunostaining experiments, and molecular modeling.

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[2] The abbreviations used are: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DNDGIC, dinitrosyl-diglutathionyl-iron complex; GSNO, *S*-nitrosoglutathione; GUV, giant unilamellar vesicle; LUV, large unilamellar vesicle; NBD-Cl, 4-chloro-7-nitro-2,1,3-benzoxadiazole; NBD-HEX, 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol; HPLC, high pressure liquid chromatography; ITO, indium tin oxide.

EXPERIMENTAL PROCEDURES

Materials—6-(7-Nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX), dinitrosyl-diglutathionyl-iron complex (DNDGIC), and *S*-nitrosoglutathione (GSNO) were synthesized as described previously (16, 17). 1-Chloro-2,4-dinitrobenzene (CDNB) and polyclonal antibodies against Alpha and Mu GSTs were from Calbiochem. Fluorescein-conjugated goat anti-rabbit IgG was from Vector Laboratories, Burlingame, CA. Recombinant human GSTA1-1, GSTM2-2, and GSTP1-1 were expressed in *Escherichia coli* and purified as described previously (17–19). All other materials were of reagent grade.

Cells—CCRF-CEM cells (human T-lymphoblastic leukemia) were grown as described previously (16). Hepatocytes were prepared from Wistar male rats as described in the accompanying paper (4). Experiments were carried out in accordance to the ethical guidelines for animal research (Communication of the Italian Ministry of Health).

Preparation of Subcellular Fractions—Subcellular fractions were prepared and characterized as reported in the accompanying paper (4) except that two parallel procedures were used; one procedure used a saline isotonic solution (0.05 M KCl, 0.04 M KH_2PO_4, and 0.1 M sucrose, pH 7.4), and the other procedure used a pure 0.25 M sucrose without salts.

Purification of Weakly and Tightly Bound GSTs—The nuclear fraction obtained from 10 g of liver was washed three times with 20 ml of 0.25 M sucrose and resuspended in 20 ml of 0.25 M sucrose containing 10 mM potassium phosphate buffer, pH 7.4. The suspension was rapidly centrifuged at 1000 × *g*, and the procedure was repeated. Further rapid extractions did not increase the amount of GSTs in the supernatant. The collected supernatants were concentrated and represent the "weakly bound GSTs." The pellet was again resuspended in the same buffer solution and incubated under gentle agitation for 1 h. This procedure was repeated three times. The collected supernatants were concentrated and represent the "tightly bound GSTs." Both weakly and tightly bound GSTs were purified by affinity chromatography through a column (1 × 4 cm) of glutathione-Sepharose matrix (21). The first eluate was again passed through the column to retain quantitatively the Alpha GSTs.

GST Activity—The standard test for glutathione transferase activity contained 1 mM GSH and 1 mM CDNB in 1 ml of 0.1 M potassium phosphate buffer, pH 6.5. An isotonic enzymatic test for GST was also performed using standard phosphate-buffered saline buffer, pH 6.5, containing 1 mM GSH and 1 mM CDNB. The activity was followed spectrophotometrically at 340 nm. Activity of nuclear GST was measured by diluting aliquots of a nuclear suspension in the standard assay mixture or using the isotonic test described above. After 2 min of preincubation, the time course of the reaction was linear. A similar procedure was used for activity determination in the mitochondria and microsome and lysosome fractions. With these activity measurements, it is not possible to distinguish whether the activity is because of GSTs inside the intact structures or due to GSTs bound to the outside of the membranes.

SDS-PAGE and Immunoblot Analysis—Protein samples were analyzed by SDS-PAGE, visualized with Coomassie Brilliant Blue R-250, and transferred to Hybond-ECL nitrocellulose membranes (GE Healthcare). The proteins were immunoblotted with either a polyclonal anti-Alpha GST antibody or a polyclonal anti-Mu GST antibody.

EPR Spectroscopy—EPR measurements were made as described previously (4).

HPLC Analysis of GSTs—The GSTs extracted from nuclei were resolved on a reverse-phase (C18, 4.6 mm × 250 mm) column, essentially as reported by Yeh *et al.* (1).

Immunofluorescence Studies—The nuclei pellet was fixed in 4% freshly depolymerized paraformaldehyde in 0.25 M sucrose for 4 h at 4 °C. After extensive washings in 0.25 M sucrose, floating pellets were incubated with a polyclonal anti-Alpha GST diluted 1:100 in 0.25 M sucrose overnight at 4 °C and washed again in 0.25 M sucrose for 20 min. Pellets were then incubated with fluorescein-conjugated goat anti-rabbit IgG diluted 1:200 in 0.25 M sucrose, for 1 h at room temperature, washed in 0.25 M sucrose, and finally mounted on slides with Vectashield (Vector Laboratories). Slides were observed in a laser scanning confocal microscope (Nikon), and micrographs were digitally captured.

Fluorescence Labeling of GSTA1-1 and GSTM2-2—1 mM NBD-Cl was reacted with 10 μM purified human GSTA1-1 or GSTM2-2 in 0.1 M potassium phosphate buffer, pH 6.0. After 2 h the excess of reagent was removed by G-25 Sephadex chromatography, and the modified enzymes were analyzed spectrophotometrically. The selective alkylation of enzyme cysteines was confirmed on the basis of the diagnostic absorption peak centered at about 430–440 nm.

Liposome Preparation—Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Large unilamellar vesicles (LUVs) were prepared according to a procedure reported previously (22). Briefly, lipids were dissolved in a chloroform/methanol solution (1:1 v/v), and the solvents were evaporated under reduced argon atmosphere until a thin film formed. Complete evaporation was ensured by applying a rotary vacuum pump for at least 2 h, and the film was hydrated with a 0.25 M sucrose solution. After vigorous stirring and 10 freeze and thaw cycles, the liposome suspension was extruded 31 times through two stacked polycarbonate membranes with 100-nm pores (Avestin, Ottawa, Ontario, Canada). Final phospholipid concentration was determined by the Stewart method (23). Anionic liposomes were formed by egg phosphatidylcholine, egg phosphatidylglycerol, and cholesterol (8:1:1 molar ratio) to reproduce the charged lipid and cholesterol content of the nuclear membrane (24). Neutral liposomes were formed by phosphatidylcholine and cholesterol (9:1 molar ratio).

Giant unilamellar vesicles (GUVs) were prepared by the electroformation method (25). Lipids were dissolved in a chloroform/acetonitrile solution (95:5 v/v) at a 5 mM total concentration; 0.25 ml of this solution was deposited on a 25 × 30-mm indium tin oxide (ITO)-covered glass slide, and spin-coated at 600 rpm for 5 min. Complete solvent evaporation was ensured by applying a rotary vacuum pump for at least 2 h. A 15 × 15 × 1.5-mm electroformation chamber was formed by separating the lipid covered ITO electrode by another bare ITO glass slide with a poly(dimethylsiloxane) spacer. The chamber was filled with a 0.25 M sucrose solution, and a 1.1-V (peak to peak), 10-Hz potential was applied to the two ITO electrodes, using a

TABLE 1

Subcellular localization of GST activity

Activity measurements were done on intact organelles, isolated in pure sucrose (0.25 M) or in 0.1 M sucrose, 0.05 M KCl, and 0.04 M KH_2PO_4, pH 7.4, using the isotonic assay medium described under "Experimental Procedures."

Cell components	GST (units/g tissue)	
	Sucrose	Sucrose + potassium phosphate buffer
Cytosol	101 ± 8	110 ± 10
Nuclei	16 ± 2	8 ± 2
Mitochondria	1.6 ± 0.4	0.7 ± 0.3
Lysosomes	2.9 ± 0.7	1.6 ± 0.2
Microsomes	4.2 ± 1	2.1 ± 0.4

Wavetek 182-A function generator. After 1.5 h the potential was switched to 3 V, 4 Hz for 10 min to favor detachment of GUVs from the electrode. The solution contained in the electroformation chamber was gently removed, and aliquots were deposited on silanized microscope slides for observation in the confocal microscope. The lipid compositions of GUVs were the same employed for LUVs, except that 1% rhodamine-labeled phosphatidylethanolamine was included to allow visualization of the liposomes by confocal microscopy.

Electrostatic Calculations—Molecular structures of human and rat GSTs were derived from the following Protein Data Bank entries: 2GSD (human A1-1), 2GTU (human M2-2), 6GSS (human P1-1), 1EV4 (rat A1-1), and 1B4P (rat M2-2). Protein charges were calculated by using the PDB2PQR software (26), and Poisson-Boltzmann calculations of electrostatic potential were performed with the APBS program (27), with 161 × 161 × 161 grid points, a 110-Å coarse grid and an 83-Å fine grid dimension, dielectric constants equal to 2 and 78.54 for protein and water, respectively, and Debye-Hückel boundary conditions. Protein dipoles were estimated by employing the Dipole server, and molecular graphics were realized with the MOLMOL (28) and Chimera software (29).

Confocal Imaging—Confocal images were acquired with a confocal laser scanning microscope, Nikon PCM 2000 (Nikon Instruments) equipped with Spectra Physics Ar ion laser (488 nm, 514 nm) and He-Ne laser (543.5 nm) sources. A 60×/1.4 oil immersion objective was used for the observations.

RESULTS

GST Activity in Subcellular Fractions of Rat Hepatocytes—Many peripheral membrane proteins can easily be detached with mild treatments like increasing the ionic strength. As most standard procedures for the isolation of subcellular components include the use of buffered solutions, some of the electrostatic protein-membrane interactions occurring in intact cells may be lost. Thus, in our study on the cellular compartmentalization of GSTs, we used only an isotonic solution of sucrose (0.25 M) for homogenization of the liver, as well as for isolation of the subcellular components. In addition, we adopted a particular GST activity determination on intact organelles (see "Experimental Procedures") that avoids inactivation caused by sonication steps or by detergent extractions. As shown in Table 1, crude nuclear, lysosomal, mitochondrial, and microsomal fractions all contain GST activity. The highest activity was recovered in the nuclear pellet that contained an amount corresponding to about 15% of the overall cytosolic GST activity. Interestingly, about 50% of the GST activity of the fractions is lost when the subcellular components are isolated in isotonic saline solution (Table 1), suggesting that about half of the GST could be electrostatically bound. Because of the considerable amount of GST activity associated with the nuclear pellet, only this specific subcellular fraction was further studied and characterized.

GST Activity in Hepatocyte Nuclear Pellet—The nuclear pellet from rat liver, isolated after homogenization in 0.25 M sucrose (1:10), was washed three times with 10 volumes of sucrose 0.25 M to remove any contamination of the cytosolic GSTs. Optical microscopy showed that the nuclei were intact. The nuclear pellet resuspended in 0.25 M sucrose contained a total of 16 GST units per g of tissue. After treatment of nuclei with 10 volumes of 10 mM potassium phosphate buffer or 20 mM NaCl in the presence of 0.25 M sucrose, about 8 units were released into the surrounding solution, confirming the presence of GSTs electrostatically bound to the nuclei (weakly bound GST). No additional release was observed after increasing the concentration of the buffer up to 0.1 M. The remaining activity associated with the nuclear pellet, termed tightly bound GST (about 8 units), could only be partially extracted by multiple and prolonged incubations (1 h each) with 10 volumes of 0.25 M sucrose in 10 mM potassium phosphate buffer. Sonication treatment of the nuclear pellet or the use of detergents like Triton X-100 even at low concentrations (1%) caused partial and irreversible inactivation of the enzyme. The use of concentrated carbonate did not increase the rate and extent of the extraction, indicating that the tightly bound GST did not behave like a peripheral protein (8). The most likely localization of the tightly bound GST is the nuclear interior, as indicated by confocal microscopy (see below).

Further evidence of the presence of equivalent levels of GSTs associated with the nucleus was provided by EPR spectroscopy. It has been demonstrated that the paramagnetic species DNDGIC binds tightly to Alpha and Mu GST with 1:1 stoichiometry, giving an EPR spectrum different from that of the free complex (30). Titration of GSTs with DNDGIC therefore allows quantification of GSTs. Experiments performed on nuclei isolated in 0.25 M sucrose or in saline solution are shown in Fig. 1 where the loss of activity because of the binding of DNDGIC is also reported. Starting from a rat liver of 10 g (51 mg of total GSTs), the weakly bound GST was about 5 mg and a similar amount was recovered as tightly bound GST.

GSTA1-1 and GSTA2-2 Are Involved in the Nuclear Association—The extracts containing the solubilized weakly and tightly bound GSTs contain other proteins as well. In fact, they display specific activities of ~3 units/mg that rise to 16 units/mg after glutathione-Sepharose affinity chromatography. SDS-PAGE, performed after the affinity step, indicates that these samples have similar protein composition (Fig. 2*A*) with a main component at about 25 kDa and a minor component of 24 kDa (about 20%). The immunoblot in Fig. 2*B* shows that only the major component at 25 kDa cross-reacts with the anti-Alpha GST antibody, whereas neither the 25-kDa nor the 24-kDa component react with the anti-Mu GST antibody. The minor component, which neither belongs to the Alpha nor to the Mu class, has not yet been further analyzed. To identify the specific Alpha isoenzyme(s) involved in the weak and tight association, a simple reversed phase HPLC analysis was performed, accord-

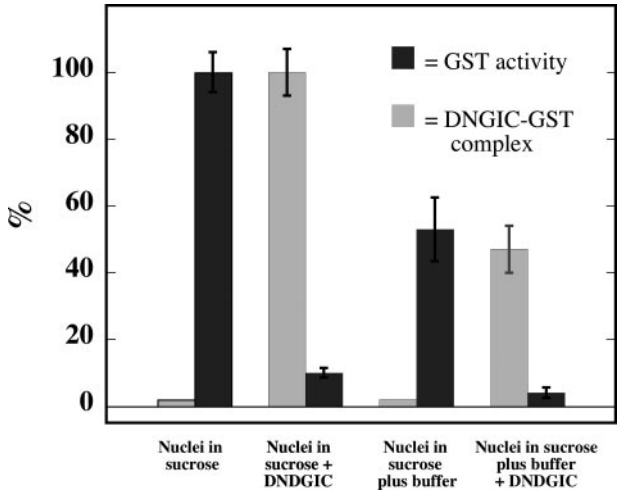

FIGURE 1. **Titration of the nuclear GST with DNDGIC.** The nuclear pellet isolated in 0. 25 M sucrose is incubated with 10 volumes of 10 μM DNDGIC at pH 7.4. After removal of the free complex by G-25 Sephadex chromatography, GST activity under isotonic conditions and EPR quantifications are performed as described under "Experimental Procedures." The same experiment is performed with the nuclear pellet isolated in 10 mM potassium phosphate buffer, pH 7.4, and 0.25 M sucrose. Data are the means ± S.D. of three experiments.

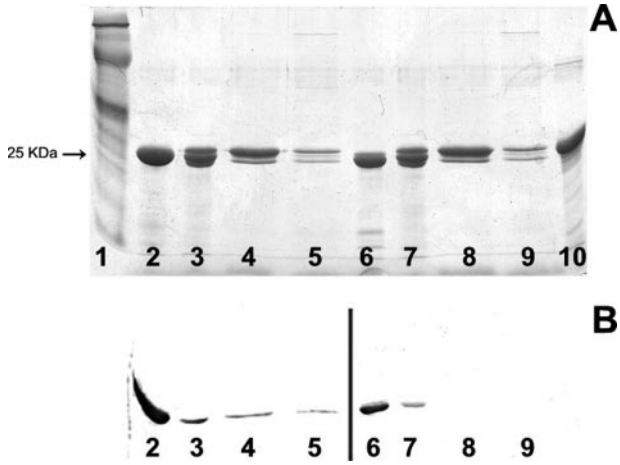

FIGURE 2. **SDS-PAGE and immunoblotting.** *A,* SDS-PAGE. *Lane 1,* protein markers; *lane 2,* purified human GSTA1-1; *lanes 3* and *7,* purified pool of cytosolic GSTs isoenzymes; *lanes 4* and *8,* weakly bound GST; *lanes 5* and *9,* tightly bound GST; *lane 6,* purified human GSTM2-2; *lane 10,* GSTP1-1 used as negative control. *B,* immunoblotting using anti-Alpha GST (*left side*) and anti-Mu GST (*right side*).

ing to the procedure described by Yeh *et al.* (1). The weakly bound GSTs are mainly represented by GSTA1-1, GSTA2-2, and GSTA3-3, approximately with the same relative abundance as found in the cytosol. Conversely, the tightly bound GSTs are mainly represented by GSTA1-1 and GSTA3-3 (Fig. 3).

Direct Observation of the Association of Alpha GSTs to the Nuclear Membrane—Direct evidence of intranuclear and perinuclear association of Alpha GSTs is provided by confocal fluorescence microscopy using an anti-Alpha GST antibody and a secondary fluorescent antibody. All nuclei display intense staining at the periphery but also inside the nuclei (Fig. 4*A*). NBDHEX, a specific fluorescent probe for Alpha GST, gives further confirmation. It has been demonstrated recently that this fluorescent compound, able to trigger apoptosis in human tumor cells, acts as a strong inhibitor of Alpha, Pi, and Mu GSTs

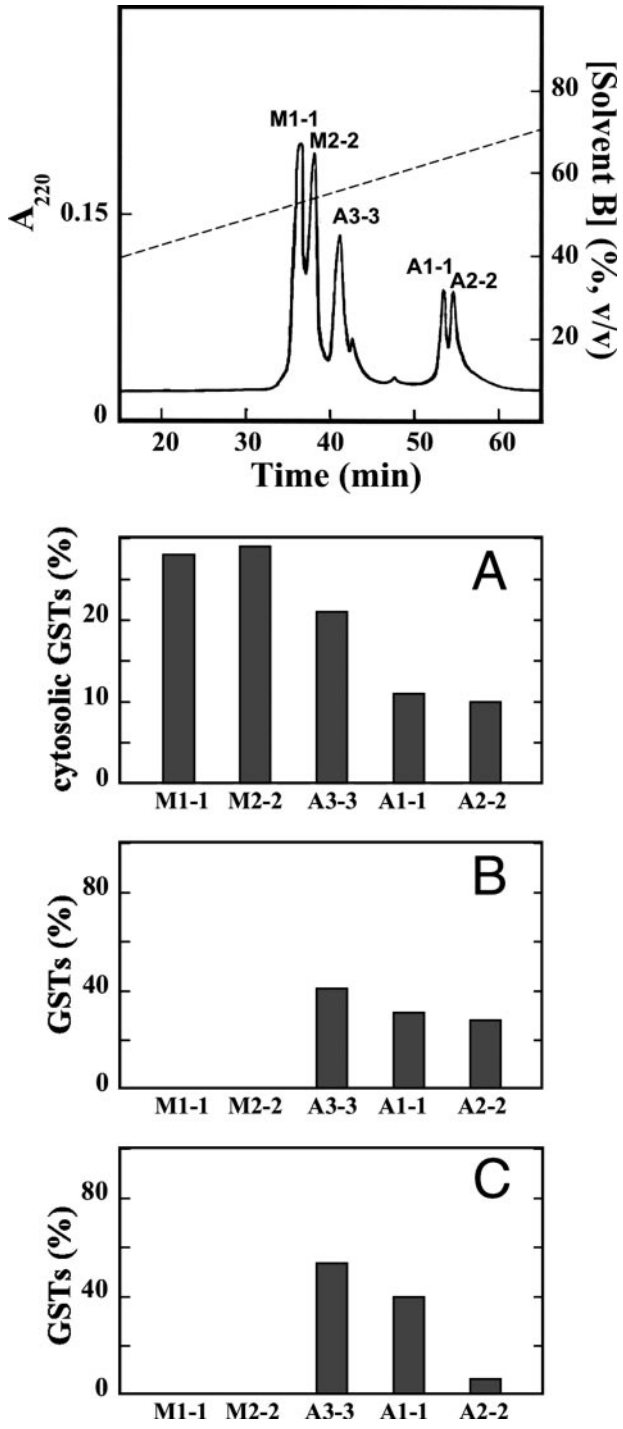

FIGURE 3. **HPLC analysis.** Tightly and weakly bound GSTs, extracted as reported under "Experimental Procedures," were purified through affinity chromatography and analyzed by reverse phase HPLC as described previously (1). Identification of GST isoenzymes were made according to Ref. 1. *Upper panel,* representative chromatogram of the cytosolic GSTs, previously purified through two subsequent affinity chromatography steps on glutathione-Sepharose matrix (21) (recovery ≥93%). *A,* relative amounts of the five cytosolic isoenzymes present in the GST pool. Isoenzymes below 2% are not reported. The total amount of cytosolic GSTs is 5.1 mg/g liver. *B,* relative amounts of isoenzymes of the weakly bound GSTs. The total amount of the weakly bound GSTs is 0.5 mg/g liver, corresponding to 10% of the cytosolic GSTs. *C,* relative amounts of isoenzymes of the tightly bound GSTs. The amount of the tightly bound GSTs after partial extraction (60%) is 0.3 mg/g liver, corresponding to 6% of the cytosolic GSTs. The nomenclature used for rat GST isoenzymes is in accordance with the one proposed recently (20). Percentages represent the mean of three experiments.

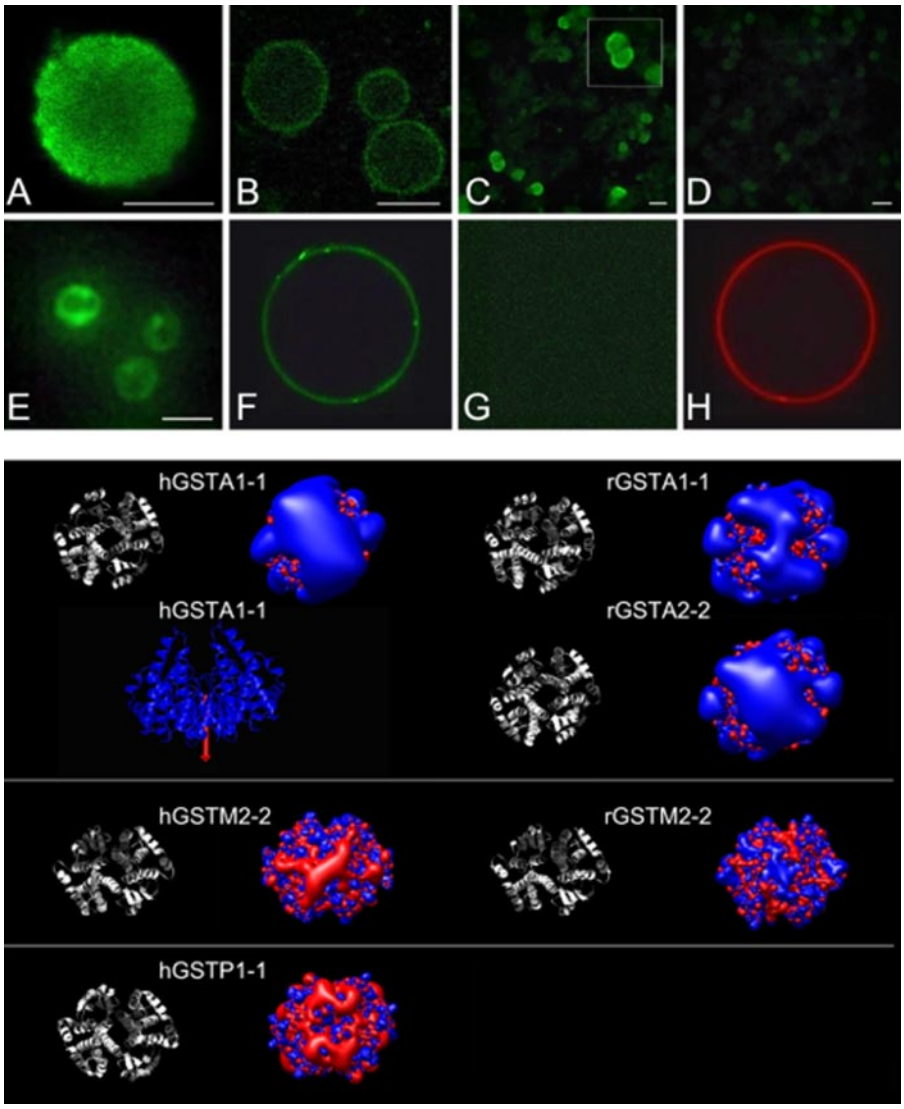

FIGURE 4. **Immunofluorescence, fluorescence label experiments, and electrostatic calculations.** *Upper panel, A,* high power oil-immersion confocal micrograph of nuclei pellets immunoreacted with anti-Alpha GST, diluted 1:100, followed by fluorescein-conjugated secondary antibody (for details see "Experimental Procedures"). *Bar,* 5 μm. *B,* nuclei pellets incubated with NBDHEX. *Bar,* 10 μm. *C,* intact rat hepatocytes incubated with NBDHEX. *Bar,* 12 μm. The *inset* shows a 2-fold magnification. *D,* rat hepatocytes, treated as in *C* and exposed to 1 mM GSNO for 1 h. *Bar,* 20 μm. *E,* low power confocal micrograph of CEM cells incubated with NBDHEX. *Bar,* 20 μm. *F,* GUVs after interaction with GSTA1-1 labeled with NBD-Cl. *G,* as in *F* after interaction with GSTM2-2 labeled with NBD-Cl. *H,* GUVs control showing only rhodamine fluorescence. *Lower panel,* molecular models represent the crystal structures of GSTA1-1, GSTM2-2, and GSTP1-1 and their isopotential surfaces corresponding to +3 kT/e (in *blue*) and −3 kT/e (in *red*). Only GSTA1-1 shows a significant positive potential on the surface of the dimer located on the protein side opposite to the active sites. In the model of hGSTA1-1, a dipolar moment of 1000 Debye was calculated and is represented as a *red arrow* inside the dimer.

by binding tightly to the active site of these enzymes (31). Interaction of this compound with Mu and Pi GSTs causes a dramatic loss of its intrinsic fluorescence, whereas the fluorescence spectrum is almost unchanged when NBDHEX binds to Alpha GST ($K_D = 5.3 \times 10^{-6}$ M) (31). Incubation of intact nuclei with 10 μM NBDHEX causes a distinct accumulation of fluorescence at the nuclear membrane and also faint fluorescence inside the nucleus (Fig. 4*B*). When the same experiment was performed with nuclei extensively washed with 10 mM potassium phosphate buffer, only faint intranuclear fluorescence was observed (not shown).

Electrostatic Binding of Alpha GST to the Nuclear Membrane; Reality or Artifact?—The data reported above prove that a part of the intracellular GST pool is associated with the nucleus. However, they do not exclude the possibility that this association might not be present in the intact hepatocyte but is induced artificially by the homogenization in sucrose in the absence of buffer or other inorganic salts. Convincing evidence that this interaction really occurs in intact cells is achieved using NBDHEX as a fluorescent intracellular marker for Alpha GSTs. NBDHEX not only specifically labels GSTs but also accumulates in the cell within a few minutes (32). After exposure of rat hepatocytes to 0.1 mM NBDHEX, faint fluorescence is visible in the cytosol, but also a strong staining of the nuclei, mainly localized on the nuclear envelope (Fig. 4*C*). To verify if NBDHEX labels selectively GSTs and to prove the absence of nonspecific interactions with the nuclear membrane, rat hepatocytes were incubated with 1 mM GSNO after treatment with NBDHEX. As shown in the accompanying paper (4), this compound causes the formation of about 0.15 mM of DNDGIC inside the cell. As DNDGIC binds to Alpha GSTs with an affinity a thousand times higher than NBDHEX ($K_D = 10^{-10}$ M), the iron complex will displace this fluorescent label. The fluorescence observed after exposure to NBDHEX and localized near the nuclear envelope fades almost completely after 1 h of incubation with 1 mM GSNO (Fig. 4*D*). The absence of nonspecific binding of NBDHEX to the membrane was also demonstrated by exposing a human tumor cell line (CEM) to NBDHEX. These tumor cells do not express Alpha or Mu GSTs but exclusively the Pi class GSTP1-1. After incubation with 50 μM NBDHEX, the nuclei appear like black holes, whereas a detectable fluorescence is visible in the cytosol, indicating that NBDHEX enters the cells (Fig. 4*E*).

Interaction of GSTs with Model Membranes—Liposomes were used to confirm the electrostatic association of Alpha GSTs to the nuclear membrane. Addition of increasing amounts of GSTA1-1 to a liposome suspension (lipid concentration 40 μM) in the absence of salts (pH is set to 7.4 by suitable

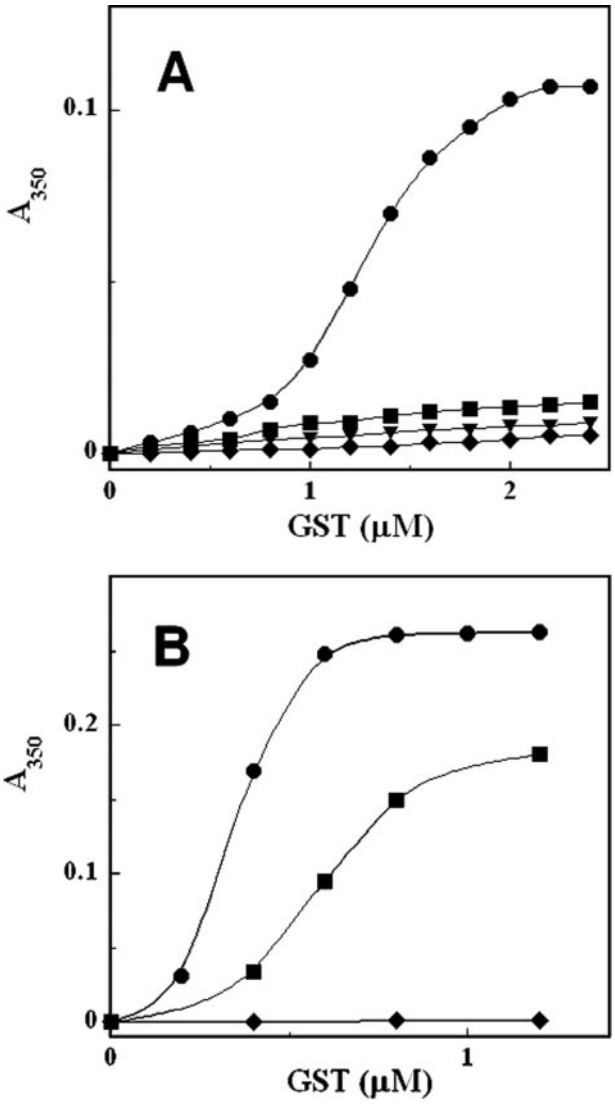

FIGURE 5. **Electrostatic association of GSTA1-1, GSTM2-2, and GSTP1-1 to liposomes as followed by turbidimetric measurements.** *A,* anionic liposomes (40 μM phospholipids) were incubated with variable amounts of purified human GSTs at pH 7. 4. Turbidity change was monitored at 350 nm. ●, GSTA1-1; ■, GSTM2-2; ◆, GSTP1-1; ▼, GSTA1-1 in the presence of 10 mM NaCl. *B,* the same experiment as in *A*, performed at pH 5.4.

addition of KOH) causes visible turbidity of the solution, clearly indicating a protein-membrane interaction (Fig. 5). A similar behavior is commonly observed when polycations associate electrostatically to vesicles containing acidic lipids, because this interaction perturbs the stability of the colloidal liposome suspension by shielding the electrostatic repulsion between liposomes (33). Very likely this is also the case with GSTA1-1, which has an isoelectric point of 8.9 (34), and which at pH 7.4 has a total charge of +10 (per dimer), as calculated with the PDB2PQR software (26). The hypothesis that vesicle aggregation is induced by protein binding to the membrane and that the origin of this interaction is essentially electrostatic is confirmed by the absence of aggregation with neutral liposomes, lacking anionic lipids (data not shown). Interestingly, at the same pH value this phenomenon is completely absent with GSTM2-2 and GSTP1-1 (Fig. 5*A*), indicating a specific interaction with Alpha GST, in agreement with the isoenzyme composition of the nuclear GST fractions. The lack of effect seen for Mu and Pi class enzymes is consistent with an electrostatic interaction, because the isoelectric points of GSTM2-2 and GSTP1-1 are 5.4 and 4.5, respectively (total calculated charges at pH 7.4 are −4 and −6, for GSTM2-2 and GSTP1-1, respectively).

This conclusion is further supported by the pH and ionic strength dependence of the liposome aggregation process (Fig. 5*B*). By lowering the pH to 5.4, a lower concentration of GSTA1-1 is needed to induce vesicle aggregation, in agreement with the higher total charge of the enzyme under these conditions (+12). Interestingly, at pH 5.4 liposome aggregation is caused also by GSTM2-2, which is close to its isoelectric point at this pH. Finally, the addition of 10 mM NaCl completely inhibits vesicle aggregation at pH 7.4 in the presence of GSTA1-1 (Fig. 5*A*). Because a relatively high ionic strength reduces the intensity of electrostatic interactions, this result cannot be due to a direct effect of the ionic strength on the aggregation process (which would be favored by the reduced intervesicle repulsion in the presence of salts), but only to the lack of protein-membrane association under these conditions. This finding nicely paralleled the observation that dissociation of nuclear GSTs can be induced by the addition of 10 mM potassium phosphate.

Direct visualization of GSTA1-1 association to anionic lipid membranes was obtained by employing the so-called GUVs, *i.e.* liposomes with a diameter of several micrometers, which can be easily observed by optical microscopy. To allow direct visualization by confocal fluorescence microscopy, GSTA1-1 and GSTM2-2 were labeled with NBD-Cl, a fluorescent probe (λ_{em} = 520 nm) that binds to proteins by reacting with lysines and cysteines. We labeled the proteins at pH 6.0 to achieve selective alkylation of cysteines (35) and to minimize the perturbation of the electrostatic potential of the protein surface. As shown in Fig. 4*F* accumulation of GSTA1-1 on the surface of anionic GUVs is demonstrated by strong NBD fluorescence. This phenomenon is absent with GSTM2-2 (Fig. 4*G*) or in the presence of salts (not shown).

Molecular Modeling of GST Electrostatics—A more detailed analysis of the electrostatic properties of the GST isoenzymes provides further information regarding the mode of association. In addition to a net positive charge, GSTA1-1 also exhibits an asymmetric distribution of electric charges, which endows this protein with a strong electric dipole (1000 Debye, as calculated by the Dipole server), as shown in Fig. 4. Interestingly, this was not the case for the other isoenzymes (the calculated electric dipoles of GSTM2-2 and P1-1 are 240 and 40 Debye, respectively). A further illustration of this point is provided by a calculation of the electrostatic potential generated by GSTs, performed by solving the Poisson-Boltzmann equation with the APBS software (27). Fig. 4 shows the isopotential surfaces corresponding to +3 kT/e (in *blue*) and −3 kT/e (in *red*), demonstrating a significant positive potential on the surface of the dimer located on the protein side opposite to the active sites. Very likely this is the protein region interacting electrostatically with the membrane. In agreement with this conclusion, a positive potential region was not present in the other isoenzymes, which did not interact with the nuclear membrane or with lipo-

somes. It is worth noting that very similar results were obtained for both human and rat enzymes (data not shown).

DISCUSSION

Data reported in this paper clearly show that specific GSTs are involved in subcellular compartmentalization in rat hepatocytes. About 20% of cytosolic Alpha class GSTs are electrostatically associated with the outer nuclear membrane, and an equivalent amount is present in the nuclear interior. The use of 0.25 M sucrose without exogenous salts for nuclei isolation, and the particular assay for GST activity of intact nuclei, made it possible to discover this double modality of association that, to a lesser extent, may also be present in other subcellular components (see Table 1). Previous observations contrary to the nuclear compartmentalization of GST were probably because of the use of standard subcellular fractionation procedures that caused the loss of the electrostatically bound GST, and to the extensive inactivation that occurs during sonication of nuclei or extraction with detergents. Immunostaining and chromatographic data indicate that GSTA1-1. GSTA2-2, and GSTA3-3 are the enzymes mainly involved in the nuclear association. Confocal microscopy and the use of NBDHEX, a specific fluorescent probe for Alpha GSTs, give a direct visualization of the presence of these isoenzymes both at the outer nuclear membrane and in the nuclear interior. The electrostatic binding of Alpha GSTs observed in isolated nuclei is well reproduced with liposomes, and the results also confirm that Mu and Pi class GSTs do not interact at physiological pH values. Although our experiments with liposomes have been done with GSTA1-1, a similar behavior is expected for GSTA2-2 and GSTA3-3 that display very similar electrostatic properties. Finally, electrostatic potential calculations, performed on Alpha, Mu, and Pi GSTs indicate that only Alpha GSTs display a proper electrostatic potential at the protein surface to interact with the negatively charged membrane. In this context, another peculiar feature of the Alpha dimer is the strong dipolar character that is not found in the other GST isoenzymes.

An examination of the present data discloses a surprising scenario. The amount of the cytosolic Alpha GSTs is about 2.2 mg/g of liver (reaching a concentration of 0.3 mM in the cytosol, 43% of the total GSTs), whereas about 0.5 mg is electrostatically associated with the nuclear membrane, and an equivalent amount is probably compartmentalized inside the nucleus. As the nuclear volume is about 10% of the cytosolic volume, the results are that the intranuclear GST concentration is ~0.7 mM, a value comparable with that found in cytosol. This concentration may reflect a free diffusional in/out traffic of Alpha GSTs between the cytosolic and nuclear compartments. Notably, the nuclear access seems to be denied for Mu GSTs. In contrast, the local concentration of Alpha GSTs at the outer nuclear membrane will be much higher. An estimation of the minimum surface occupied by all electrostatically bound GSTA1-1 assembled in a layer (calculated on the basis of an area of 19.6 nm^2 for each GSTA1-1 dimer, based on the x-ray structure) paradoxically results about five times larger than the surface of the entire nuclear membrane (Table 2). In addition, the specific activity of the Alpha GSTs, extracted from the nuclear membrane after salt treatment, is about 3 units/mg, five times lower than that of the purified enzymes (16 units/mg). Thus, other proteins must be electrostatically associated with the nuclear envelope together with GSTs; this appears to be an additional paradox, given the absence of free membrane area for further electrostatic interactions. One possible explanation for this excessive amount of bound proteins is that Alpha GSTs could be assembled in a multilayer disposition near the nuclear membrane in an alternate sequence with negatively charged proteins. This peculiar onion layer-like assembly of proteins is not a complete novelty. It has been demonstrated *in vitro* that positively charged proteins easily aggregate in such a way to negatively charged colloidal particles in the presence of anionic polyelectrolytes (36, 37), and our preliminary results also indicate a prevalent anionic nature of the accompanying proteins. To our knowledge, the present data represent the first indication for the existence *in vivo* of a similar protein assembly, which obviously needs further confirmation. The possibility that a few cytosolic enzymes are not in a completely disordered distribution but electrostatically ordered near specific intracellular membranes opens a new and fascinating scenario in cell physiology. Is it possible that this hypothetical protein shell escaped visualization by advanced spectroscopy and microscopy techniques? The answer may be affirmative. We must remember that the protein concentration in the cytosol is above 300 mg/ml, a borderline value for protein crystallization. Thus all cytosolic proteins are extremely crowded, but no spectroscopic or microscopic evidence has been reported for this almost crystal-like condition. Obviously in the absence of a detectable selective marker no trace can be expected if this crowding is formed by selected proteins near specific subcellular components. Whatever the true assembly of Alpha GSTs at the nuclear envelope, the presence of large amounts of these specific GSTs near the nucleus demonstrated here is a novelty and probably has a physiological finality. In fact, all GSTs represent a very efficient defense system against many toxic alkylating compounds, but Alpha class GSTs display an additional and peculiar peroxidase activity, not shown by Mu and Pi GSTs, and eliminate efficiently dangerous organic peroxides (2). Alpha class GSTs also have a 10 times higher affinity than Pi and Mu GSTs for DNDGIC, a natural carrier of NO that displays strong oxidizing properties and inactivates irreversibly key enzymes like glutathione reductase (38, 39). Thus, the present results seem to indicate that the nucleus and its precious genetic content have a further mechanism of protection, not considered until now. Quite a few questions remain. For example, given the similar lipid composition of most intracellular membranes, it is not clear why the Alpha GST displays such an impressive accumulation on the nuclear membrane (see Table 2). Interestingly,

TABLE 2
Quantitative analysis of GSTs bound electrostatically to subcellular membranes

Cell components	Membrane area[a]	GST/membrane area	Area of GST/membrane area
	m^2/g	mg/m^2	
Nuclei	0.022	22.7	5
Mitochondria	0.77	0.06	0.01
Lysosomes	0.044	2.4	0.5
Microsomes	5.5	0.02	0.01

[a] Data were derived from Ref. 41.

it was shown many years ago by Virtanen (40) that the outside of the nucleus is strongly negatively charged, but the inner membrane is practically neutral. However, it is also possible that specific nuclear membrane proteins recognize Alpha GSTs and favor their accumulation. We did not explore in this study the status of the tightly bound GSTs that probably reside in the nuclear interior. The curiously similar amount of the "external" and "internal" Alpha GSTs could be merely a coincidence, or it could reflect a specular modality of interaction of Alpha GSTs with the outer and inner membrane. Work is in progress to answer these questions.

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Enzyme Catalysis and Regulation:

Electrostatic Association of Glutathione Transferase to the Nuclear Membrane: EVIDENCE OF AN ENZYME DEFENSE BARRIER AT THE NUCLEAR ENVELOPE

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