Membrane Association of Glutathione S-Transferase mgSTAA4-4, an Enzyme That Metabolizes Lipid Peroxidation Products*

Received for publication, October 5, 2001, and in revised form, November 14, 2001
Published, JBC Papers in Press, November 19, 2001, DOI 10.1074/jbc.M109678200

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Lipid peroxidation products have signaling functions and at higher concentrations are toxic and may trigger cell death. The compounds are metabolized predominantly by glutathione S-transferases exemplified by mgSTAA4-4, an enzyme highly efficient in glutathione conjugation of 4-hydroxyalkenals, and possessing glutathione peroxidase activity toward phospholipid hydroperoxides. mgSTAA4-4 belongs to the predominant group of “canonical” glutathione S-transferases that are soluble and generally localized in the cytoplasm. The intracellular localization of mgSTAA4-4 was examined in hepatocytes of normal mouse liver and in transfected HepG2 cells by fluorescence microscopy and digital deconvolution. mgSTAA4-4 was found to be predominantly localized at or near the plasma membrane in transfected HepG2 cells, as well as in hepatocytes endogenously expressing the protein. In vitro, mgSTAA4-4 associated with liposomes, and this interaction was potentiated when the liposomes contained negatively charged phospholipids. Mutating lysine 115 to glutamic acid resulted in a loss of the plasma membrane targeting of mgSTAA4-4 as well as in a significant reduction of its binding to liposomes in vitro. These data suggest preferential targeting of mgSTAA4-4 to the plasma membrane that may contain the major substrate(s) for this enzyme. Lysine 115 is critically important for the membrane association of mgSTAA4-4, most likely by entering into an electrostatic interaction with negatively charged phospholipid headgroups.

Lipid peroxidation is a detrimental outcome of oxidative stress. However, in the course of evolution products of lipid peroxidation have also acquired a physiological function: they signal the presence of an oxidative insult and trigger an appropriate cellular response. Although lipid hydroperoxides can act directly as messengers (1), the signaling function appears to be associated primarily with downstream products derived from hydroperoxides of polyunsaturated fatty acids. These products, the highly electrophilic and diffusible 4-hydroxyalkenals, including the predominant 4-hydroxynonenal (4-HNE)1 (2), were thought to be formed in a spontaneous, non-catalyzed reaction, although recent results indicate a possible involvement of enzymes in this process (3). Because of their chemical properties as Michael acceptors, 4-HNE and similar a,b-unsaturated aldehydes have the ability to modify proteins (4, 5), often with considerable selectivity (6). Such modifications of a number of key regulatory proteins result in specific physiological outcomes, including effects on the cell cycle, cell differentiation, and cell death (reviewed in Ref. 7).

Given the signaling functions, and, at higher concentrations, the toxicity of lipid peroxidation products, their metabolism is of dual importance in detoxification and in signal termination. Phospholipid hydroperoxides are substrates for selenium-containing glutathione peroxidases (1) and for the glutathione peroxidase activity of Alpha-class glutathione S-transferases (GSTs) (8–11). Of these, the selenoenzyme Gpx4 (12, 13) and GSTs do not require prior phospholipase action. It has been recently shown that Alpha-class GSTs contribute the majority of activity for phospholipid hydroperoxides, at least in some cells (10, 11). 4-Hydroxyalkenals are metabolized predominantly by conjugation to glutathione by a subclass of Alpha-class GSTs exemplified by mgSTAA4-4, which also possesses glutathione peroxidase activity toward phospholipid hydroperoxides (reviewed in Ref. 14). Early stress response is characterized by increased lipid peroxidation and by induction of an Alpha-class GST that conjugates 4-HNE (15). Thus, Alpha-class GSTs play a key role in the catabolism of lipid peroxidation products.

Lipid peroxidation products either reside in membranes (phospholipid hydroperoxides) or partition into membranes to a significant degree (4-HNE). Strikingly, the Alpha-class GSTs responsible for the majority of the metabolism of these compounds were not thought to be associated with membranes. GSTs are ubiquitous enzymes with diverse functions (16–18). At least three structurally unrelated groups of these enzymes arose through convergent evolution: the canonical soluble GSTs, microsomal GSTs, and the bacterial fosfomycin resistance protein (19–21). The subcellular localization of the canonical GSTs, which include the major mammalian Alpha, Mu, and Pi classes, has been studied in several tissues (22–28). The
enzymes are present mainly in the cytosol. For several GSTs, especially of the Pi class, nuclear localization has also been reported (22, 24–26), which is consistent with a role in protecting DNA from damage by electrophiles and oxidants. Mitochondrial localization has been reported for hGSTA4-4 (28) and for rGSTA4-4 (27), enzymes involved in the metabolism of lipid peroxidation products (29, 30).

The goal of the present study was to examine the subcellular localization of mGSTA4-4, a GST with high activity for both 4-HNE and phospholipid hydroperoxides, and thus to address the apparent paradox of an incongruent localization of an enzyme and its substrate(s). Our results demonstrate that mGSTA4-4 is enriched at or near the plasma membrane of hepatocytes and transfected hepatoma cells, a localization likely to facilitate its access to substrates and thus its biological function. Furthermore, through site-directed mutagenesis followed by transfection and in vitro binding experiments, we elucidated the mechanism by which mGSTA4-4 associates with membranes and identified the amino acid residues (predominantly lysine 115) responsible for the binding.

**Experimental Procedures**

**Plasmid Constructs and Transfection of Cells**—Vectors for constitutive mammalian cell expression of the following proteins were used in the course of the present work. (i) Wild-type mGSTA4-4: the coding sequence of murine mGSTA4-4 (31, 32) was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) as described by this group previously (33, 34), resulting in plasmid pcR-CMV/mGSTA4. (ii) mGSTA4-4(K115E): the AAG codon encoding K115 in vector pcR-CMV/mGSTA4 was mutated to GAG using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). (iii) Mitochondrially targeted mGSTA4-4: DNA encoding the 24-aa amino mitochondrial targeting sequence of human ornithine transcarbamoylase (Ref. 35, the clone was a generous gift of Dr. Wayne A. Penton, Yale University) was amplified by PCR introducing the following two modifications. 1) To create a downstream NdeI site needed for subsequent in-frame ligation, the last codon of the leader signal was changed from AAT (Asn) to CAT (His). 2) To convert the suboptimal Kozak consensus sequence of the last codon of the leader signal was changed from AAT (Asn) to CAT (His). 2) To convert the suboptimal Kozak consensus sequence of the

**Bacterial Expression and Purification of GSTs for in Vitro Studies**—The bacterial expression vector pET-Theta/mGSTA4 was described previously (33). The plasmid was subjected to site-directed mutagenesis to obtain the desired variants of codons 115 and/or 112. Wild-type

**Enzyme Assays**—All bacterially expressed GSTs, including the mutants of mGSTA4-4, were assayed for activity with a model substrate (CDNB) and with the electrophilic lipid peroxidation product 4-HNE. GST activities were measured spectrophotometrically in a microtiter plate reader (SPECTRAMax Plus, Molecular Devices, Sunnyvale, CA). Activity with CDNB was measured at 25 °C according to Ref. 39. 4-HNE activity was determined at 30 °C as described in Ref. 40.

**Anti-GST Antibodies**—Polyclonal antibodies against affinity-purified mGSTA4-4 and hGSTA4-4 were raised in chicken. The IgY fraction (corresponding to mammalian IgG) was obtained from egg yolks by polyethylene glycol precipitation (41). Control IgY was purified by the same protocol from preimmune eggs. Anti-hGSTA1-1 (cross-reacting with mGSTA1-1) was raised in rabbits as described previously (42), and the IgG fraction was purified by protein A-Sepharose chromatography. Identity of all antibodies was established by Western blotting using authentic purified GSTs.

**Immunolocalization of mGSTA4-4 in Transfected HepG2 Cells**—Confluent monolayers of HepG2 cells on glass coverslips were rinsed with Dulbecco’s PBS and fixed with 3% paraformaldehyde (Sigma) in PBS, pH 7.4, for 30 min. The permeabilization and blocking of non-specific binding sites were performed in one step by incubating the monolayers with PBS containing 0.3% Triton X-100, 0.3% bovine serum albumin (Goldmark Biologicals,Phillipsburg,NJ),and5%normalgoatserum(JacksonImmunoresearch,WestGrove,PA)beforeincubationfor45 min. Monolayers were then incubated with anti-mGSTA4-4 chicken IgY (1:100 in PBS supplemented with 0.2% bovine serum albumin) overnight at room temperature, followed by extensive washing with PBS. For control of nonspecific binding, preimmune chicken IgY at 1:100 dilution was substituted for the anti-mGSTA4-4 antibody. Monolayers were subsequently incubated with Alexa 488-conjugated goat anti-chicken IgY (1:200 dilution; Molecular Probes, Eugene, OR) for 45 min, postfixed with 3% paraformaldehyde, and mounted in 50% glycerol in PBS containing 0.2% p-phenylenediamine as an anti-photobleaching agent. The monolayers were examined using an Olympus microscope equipped with DeltaVision PC 4.08 g deconvolution system (Applied Precision, Issaquah, WA). Images were reconstructed in XY and XZ planes using MetaMorph software (version 3.5, Universal Images, West Chester, PA).

The mitochondrial localization of mGSTA4-4 fused to an ornithine transcarbamoylase mitochondrial-targeting signal was confirmed by co-localization studies with the mitochondria-specific fluorophore Mitotracker Orange (Molecular Probes, Eugene, OR). Stably transfected HepG2 cells cultured on glass coverslips were rinsed with PBS twice, and incubated with standard culture medium containing 0.2 μM Mitotracker Orange for 15 min at 37 °C. The monolayers were then rinsed twice with PBS and fixed and immunolabeled for mGSTA4-4 as described above. Co-localization was examined using Olympus IX 70 equipped with a DeltaVision deconvolution system. The signal from Alexa 488-conjugated secondary antibody (for mGSTA4-4) was collected using 490/20 nm and 528/36 nm excitation/emission band pass filters, and the signal from Mitotracker Orange was collected using the 555/28 nm and 617/73 nm band pass filter set. Neutral density filters were applied to reduce the leak of the fluorescence signal in either direction to undetectable levels.

**Immunolocalization of mGSTA4-4 in Mouse Hepatocytes**—Swiss-Webster mice were sacrificed by cervical dislocation, and small cubes of liver were excised and immediately placed in ice-cold fixative (3% paraformaldehyde, pH 7.4). Following fixation for 4 h, tissue blocks were rinsed with PBS, immersed in Optimum Cutting Temperature (OCT) compound (Tissue-Tek, Sakura, Torrance, CA) overnight at 4 °C, and then cryoprotected in liquid N2 and sectioned. Cryosections were immunolabeled for mGSTA4-4 using essentially the same approach as described above for HepG2 cells with the exception that the permeabilization/blocking step was performed for 2 h.

**Binding of GSTs to Liposomes in Vitro**—Liposomes (small unilamellar vesicles, SUV) from asolectin (crude soybean phospholipids; Associated Concentrates, Woodside, NY, or Sigma, cat. no. P9844) were prepared by swelling 50 mg of unpurified asolectin in 1 ml of water under nitrogen for 1 h with intermittent shaking at room temperature followed by exhaustive sonication in a cylindrical bath sonicator (Laboratory Supplies, Hicksville, NY) under nitrogen to reduce the leakage of the fluorescence signal in either direction to undetectable levels.

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RESULTS

mGSTA4-4 Is Targeted to the Plasma Membrane in Mouse Hepatocytes and in Transfected HepG2 Cells—Immunostaining of frozen sections of mouse liver with an anti-mGSTA4-4 antibody revealed a localization of the enzyme predominantly at the plasma membrane of the hepatocytes (Fig. 1A). Some labeling was also present within discrete areas of the cytoplasm, but no significant diffuse cytoplasmic labeling was observed in any of the examined preparations. Interestingly, the plasma membrane was labeled to a similar degree regardless of the membrane domain, i.e. apical (canalicular) versus basolateral. No significant labeling was observed in liver sections incubated with preimmune chicken IgY (Fig. 1B).

The microscopic analysis of the mGSTA4-4-transfected HepG2 cells revealed that ~40% of all cells exhibited a strong and specific fluorescent signal from mGSTA4-4. The majority of mGSTA4-4 was localized to the plasma membrane and, to a lesser degree, to the narrow zone of adjacent cytoplasm (Fig. 2A). Analysis of images reconstructed in the XZ plane (Fig. 2B) confirmed the above-mentioned localization of mGSTA4-4 in transfected HepG2 cells. The specificity of the immunostaining was demonstrated by the absence of fluorescent signal in monolayers in which the anti-mGSTA4-4 antibody was replaced with preimmune chicken IgY (Fig. 2C).

Further confirmation of the specificity of immunolabeling was obtained from cells expressing mGSTA4-4 fused to a mitochondrial-targeting peptide. As expected, in these cells the majority of mGSTA4-4 was localized to discrete intracyttoplasmic structures relatively homogeneously distributed within the perinuclear cytoplasm (Fig. 3A). The fluorescent signal from the majority of these particles co-localized with mitochondria labeled with MitoTracker Orange (Fig. 3, B and C). Importantly, neither labeling of the plasma membrane nor diffuse cytoplasmic staining was observed in these cells, which confirmed the high specificity of the anti-GST antibody used in these studies. Some cells expressed very little of the transfected mGSTA4-4. In these cells, no immunolabeling with mGSTA4-4 antibody was detected, whereas the typical mitochondrial staining pattern was still observed (Fig. 3C, asterisks).

In contrast to the transfected cells described above, no detectable immunostaining was observed in control HepG2 cells transfected with insert-free vector (data not shown).

Intracellular Localization of mGSTA4-4(K115E)—The intracellular distribution of the mGSTA4-4(K115E) mutant was distinctively different from that observed in HepG2 cells expressing the wild-type protein. The mutated protein was diffusely distributed within the cytoplasm, and no preferential targeting to intracellular organelles or plasma membrane was observed (Fig. 4). This diffuse cytoplasmic distribution was observed in the XY as well as in the XZ planes of the HepG2 cells (Fig. 4, A and B, respectively). This observation suggested that lysine 115 is critically important for the plasma membrane targeting of the protein.

Binding of mGSTA4-4 to Asolectin Liposomes in Vitro—In agreement with the membrane association of mGSTA4-4 observed microscopically in intact cells, the enzyme had the abil-
ity to bind to phospholipid (asolectin) liposomes (Fig. 5). Under the conditions used, approximately half of mGSTA4-4 associated with the vesicles. Although true binding constants were not measured, the ratio of bound to free protein (used as the binding parameter in Fig. 5) constitutes an apparent binding constant. This is justified because the vesicle surface is in large excess compared with the protein. Thus, the concentration of free liposomal binding sites will remain constant for all GSTs tested, and can be subsumed into the apparent (composite) binding constant. The conclusion that liposomal binding surface in excess over GST protein was reached as follows. Under the experimental conditions used, the protein concentration was 0.6 μM, and the approximate concentration of liposomes was 0.4–0.5 μM (as determined from an average radius of SUVs of 14.9 nm (43), a membrane thickness of 3 nm, and an average surface requirement of 0.71 nm² per phospholipid molecule (44), or from the experimentally obtained internal volume of SUVs of ~1 μl/mg lipid). Given that the cross-section of a mGSTA4-4 molecule is less than 40 nm² (on the basis of crystal structures; Refs. 45, 46) and the surface of a SUV is ~3000 nm², less than 2% of the total surface of liposomes is occupied by protein. It should also be noted that the dissociation of mGSTA4-4 from its complex with liposomes is slow because discrete peaks of bound and free protein (as opposed to a smear) are obtained on Sephadex gel filtration, which requires several hours to complete.

Binding of mGSTA4-4 Mutants and of Other GSTs to Asolec- tin Liposomes in Vitro—Loss of plasma membrane association of the mGSTA4-4(K115E) mutant in transfected cells suggested that lysine 115 may play a role in the mechanism involved in the targeting and/or binding of the enzyme to the plasma membrane. To verify this hypothesis we examined the binding of mGSTA4-4(K115E) to liposomes in vitro. As shown in Fig. 5, membrane association was essentially abolished in the mGSTA4-4(K115E) mutant expressed in HepG2 cells. Fifteen micrograms of the respective protein were incubated with 1 mg of sonicated liposomes composed of asolectin. Binding to liposomes was evaluated as described under “Experimental Procedures.” Results are presented as the ratio of vesicle-bound to free protein.

**Fig. 3.** Immunolocalization of mGSTA4-4 in HepG2 cells transfected with mGSTA4 cDNA fused to a mitochondrial-targeting sequence. Cells were incubated with MitoTracker Orange, fixed, and immunolabeled for mGSTA4-4 as described under “Experimental Procedures.” Note that most areas positive for mGSTA4-4 (Panel A) colocalize with mitochondria labeled with MitoTracker (Panel B). Panel C is an overlay showing co-localization of both fluorescent signals (arrows; orange to yellow). Asterisks mark cells with no significant mGSTA4-4 labeling.

**Fig. 4.** Immunolocalization of mGSTA4-4(K115E) mutant protein expressed in HepG2 cells. Cells were fixed at 80–90% confluency and immunolabeled using anti-mGSTA4-4 chicken IgY. The immunolabeling is diffusely distributed throughout the cytoplasm, which is well visible in both XY and XZ planes (Panels A and B, respectively). No plasma membrane staining could be seen. Note the absence of a fluorescent signal in cells incubated with preimmune chicken IgY (Panel C). The line in Panel A indicates the XZ plane of reconstruction shown in Panel B. The broken line in Panel B indicates culture support. The bar represents 5 μm.

**Fig. 5.** Binding of mGSTA4-4, its mutants, and unrelated GSTs to asolectin liposomes. Fifteen micrograms of the respective protein were incubated with 1 mg of sonicated liposomes composed of asolectin. Binding to liposomes was evaluated as described under “Experimental Procedures.” Results are presented as the ratio of vesicle-bound to free protein.
and hGSTA4-4, enzymes that contain a negatively charged amino acid in position 115 (Fig. 6), exhibited liposome association that was moderate but higher than that of the mGSTA4-4 mutant carrying a negative charge on the side chain in position 115 (Fig. 5).

mGSTA4-4 Mutants Are Enzymatically Active—To rule out the possibility that the loss of membrane association of certain mGSTA4-4 mutants is due to a gross change of conformation and/or loss of protein stability, the catalytic activity of all enzymes was determined. As shown in Table I, all enzymes were fully active with the model substrate CDNB as well as with the lipid peroxidation end product 4-HNE (with the exception of mGSTA1-1, which is known to have low activity for 4-HNE). Thus, the proteins fold normally upon bacterial expression and are able to retain a native conformation, indicating that the effects of mutations of residues 115 and/or 112 are local rather than global.

Binding of mGSTA4-4 to Liposomes of Defined Lipid Composition—Asolectin is a crude mixture of lipids that may contain residual proteins. To ascertain that membrane association of mGSTA4-4 does not require additional proteins, binding assays were carried out with liposomes made from chromatographically purified lipids. A lipid composition was chosen that mimics that of the plasma membrane. As shown in Fig. 7, mGSTA4-4 was able to bind to liposomes composed of phosphatidylcholine, phosphatidylethanolamine, and cholesterol, although not as well as to asolectin liposomes. The inner leaflet of the plasma membrane is enriched in acidic phospholipids, particularly phosphatidylethanolamine and phosphatidylinositol (47, 48). The incorporation of acidic phospholipids into liposomes increased the binding of mGSTA4-4 to the level seen with asolectin. Although the effects of the individual lipids differed somewhat from each other, all tested acidic lipids supported increased binding of mGSTA4-4 to liposomes. The mGSTA4-4(K115E) mutant associated poorly with either defined or asolectin liposomes, and in vesicles of defined lipid composition that residual binding was not dependent on the presence of acidic phospholipids.

**DISCUSSION**

Among canonical GSTs, mGSTA4-4 and its orthologs in other species are distinguished by their high catalytic efficiency for glutathione conjugation of 4-hydroxyalkenals (14, 40). At elevated concentrations the latter are highly toxic electrophiles (49), whereas at physiological levels they appear to serve as messenger molecules (reviewed in Refs. 7, 50). Because 4-hydroxyalkenals are derived from membrane-bound phospholipid hydroperoxides (51) but are diffusible, they would be most efficiently metabolized by enzymes located at or near the site of their formation, i.e. the membrane. Such localization would also augment the glutathione peroxidase activity of GSTs toward phospholipid hydroperoxides. Contrary to older reports (52), there is now ample evidence that GSTs, including mGSTA4-4, can act directly on phospholipid hydroperoxides without the need for prior release of the oxidized fatty acid by a phospholipase (9, 10). Because phospholipid hydroperoxides are likely to be associated with membranes, their metabolism would be facilitated by a membrane localization of the GST.

The pattern of distribution of immunofluorescence in mouse liver sections indicates that mGSTA4-4 is indeed enriched at or near the plasma membrane of normal mouse hepatocytes (Fig. 1). We extended this initial observation, and determined the mechanism governing the localization by two distinct but complementary approaches: transfection of cultured cells, and direct in vitro binding experiments.

HepG2 cells do not express endogenous enzymes cross-reacting with anti-mGSTA4-4 antibodies or exhibiting 4-HNE conjugating activity (33), and they were therefore chosen for transfection studies. Fluorescent microscopy on stable HepG2 transfectants expressing mGSTA4-4 clearly demonstrated a localization of the enzyme to the plasma membrane and/or a narrow adjacent zone of the cytosol. Image reconstruction in the XZ plane shows that staining that could be interpreted as diffuse or cytosolic in standard epifluorescent whole-cell images is actually because of enzyme associated with plasma membranes oriented parallel to the culture substrate. These observations confirmed our original hypothesis that the transfected HepG2 cell model closely resembles normal mouse hepatocytes with respect to the intracellular targeting of mGSTA4-4.

The plasma membrane association of mGSTA4-4 was lost...
when the protein was fused to a mitochondrial import signal peptide that targets it to the mitochondrial matrix. Technically, the observed shift of the intracellular distribution of the fluorescence validates the overall methodology used in these studies and, in particular, the specificity of the antibody. Conceptually, the result indicates that the plasma membrane association of wild-type mGSTA4-4 is of relatively low-affinity as it cannot effectively compete with the mitochondrial protein import machinery. It should be noted that mitochondrial localization has been reported for the human hGSTA4-4 (28), a GST whose function but not sequence is similar to that of mGSTA4-4 (38). Interestingly, at least a partial localization to the mitochondrial matrix has also been described for rGSTA4-4 (27), the rat ortholog of mGSTA4-4. Our present results do not indicate a mitochondrial localization of native mGSTA4-4; such localization was observed only for an engineered protein that contained a heterologous mitochondrial-targeting peptide. The difference in localization could be because of the species (rat versus mouse) or perhaps to a modification of the rat enzyme leading to an alternative form with an increased molecular weight in the rat (27) but not in the mouse.

Tissue-purified (53) or bacterially expressed (31) mGSTA4-4 is soluble in aqueous buffers, in which it resembles other canonical GSTs. The structural basis of the ability of mGSTA4-4 to act as a peripheral membrane protein is thus of obvious mechanistic interest. Inspection of the recently available crystal structure of the enzyme (46) revealed the presence of a positively charged residue, lysine 115, located in each subunit of the dimeric enzyme in the surface loop connecting α helices α4 and α5 and thus highly exposed to solvent. The lysine 115 residues of both subunits flank the intersubunit cleft that harbors the active sites of the enzyme (Fig. 8). In contrast, the predominant Alpha-class GSTs, which are devoid of 4-HNE-conjugating activity, e.g. mGSTA1-1 or human hGSTA1-1, contain a negatively charged residue (aspartic or glutamic acid, respectively) in position 115. This raised the possibility that the lysine 115 “fingers” of mGSTA4-4 interact with negative charges on the surface of membranes. To test this hypothesis, we mutated lysine 115 of mGSTA4-4 to glutamic acid. The mutation did not affect the catalytic properties of the enzyme (Table I), but caused a dramatic shift of its intracellular localization from the plasma membrane to a diffuse cytosolic distribution (compare Figs. 2 and 4).

The key role of lysine 115 in membrane association of mGSTA4-4 could be confirmed by in vitro binding experiments to liposomes. The ability of wild-type mGSTA4-4 to bind to lipid vesicles was largely abrogated by the K115E mutation. The requirement is for a positive charge rather than for the specific structure of lysine because arginine could substitute for lysine 115 without a loss of membrane association. A mutant with an uncharged amino acid in position 115 had intermediate binding properties. On the membrane side the binding is in part dependent on the presence of acidic phospholipids. Unlike certain cytoskeletal proteins that bind specific lipids, particularly phosphatidylinositol phosphates, via complex binding sites involving multiple amino acids (reviewed in Ref. 48), mGSTA4-4 appears to interact with any acidic phospholipid. This may explain the association of mGSTA4-4 with the plasma membrane but not, or to a lesser degree, with intracellular membranes (Figs. 1 and 2). Approximately 20% of plasma membrane lipids carry a net negative charge, and the majority of these lipids (80–100%) are localized in the cytoplasmic leaflet of the membrane (47, 54). In contrast, with the exception of the inner mitochondrial membrane, which is normally inaccessible to cytosolic mGSTA4-4, the percentage of acidic lipids in intracellular membranes is lower than in the plasma membrane. Moreover, in membranes such as the endoplasmic reticulum the distribution of lipids between membrane leaflets is thought to be essentially symmetrical (55), further decreasing the effective membrane surface charge available to cytosolic proteins. Although it is currently unknown whether mGSTA4-4 binding to the plasma membrane is modulated by structures such as lipid rafts (56), data on lipid composition

![Model of membrane association of mGSTA4-4](image117x565 to 229x729)

**Fig. 8. Model of membrane association of mGSTA4-4.** The crystal structure of mGSTA4-4 (46) was docked to a model (in scale) of a phospholipid bilayer in an orientation allowing electrostatic interaction of lysine 115 side chains with negatively charged phosphates or phosphatidylserine head groups on the surface of the membrane. The possible channeling of membrane-derived substrates such as 4-HNE to the active sites of the enzyme under exclusion of the bulk solution is schematically depicted by arrows.

![Relationship between binding of GSTs to liposomes and the presence of charged amino acids in positions 110–120](image338x645 to 524x729)

**Fig. 9. Relationship between binding of GSTs to liposomes and the presence of charged amino acids in positions 110–120.** Panel A, the ratio of liposome bound to free GST for mGSTA4-4 and its mutants (●), in order of increasing ordinate value: mGSTA4-4(K112C/K115E), mGSTA4-4(K115E), mGSTA4-4(K115Q), mGSTA4-4, mGSTA4-4(K112C), and mGSTA4-4(K115R), for mGSTA1-1 (○), and for hGSTA4-4 (□). Panel B, the same data (●) as in Panel A was assumed except that only the amino acid in position 115 was taken into account in calculating the effective electrostatic interaction of the proteins with the membrane.
and asymmetry clearly indicate that the plasma membrane should be the primary target for such binding. Diffuse staining in a zone immediately adjacent to the plasma membrane (Fig. 2) could be because of endocytic vesicles that, until they fuse with intracellular structures, retain the lipid composition of the plasma membrane.

In addition to pinpointing the amino acid residue in mGSTA4-4 that is involved in membrane binding, the in vitro experiments have also demonstrated that the membrane association can occur in a simple, well-defined system consisting of pure mGSTA4-4 and liposomes of defined composition. Thus, the binding is not mediated by, nor does it require, additional proteins, at least in the system studied.

Unexpectedly, two GSTs with a negative charge in position 115 had the ability to associate with liposomes, albeit relatively poorly. These were mGSTA1-1, by analogy with human hGSTA1-1 likely to play an important role in the metabolism of phospholipid hydroperoxides (10), and hGSTA4-4, one of two human enzymes with high 4-HNE-conjugating activity (38). It should be noted, however, that mGSTA4-4 and hGSTA4-4 are human enzymes with high 4-HNE-conjugating activity (38). It should be noted, however, that mGSTA4-4 and hGSTA4-4 are human enzymes with high 4-HNE-conjugating activity (38).

Thus, consideration of additional, nearby residues could be close enough to the membrane to facilitate the metabolism of substrates generated within or at the membrane could serve as an example. Such localization may be transient and can be mediated by weak interactions. Such localization may be transient and can be mediated by weak interactions. Such localization may be transient and can be mediated by weak interactions. Such localization may be transient and can be mediated by weak interactions.

Acknowledgments—We thank Dr. Wayne A. Fenton, Yale University, for the cDNA encoding human ornithine transcarbamoylase, and Dr. Slawomir Pikula, Department of Cellular Biochemistry, Nencki Institute of Experimental Biology, Warsaw, Poland, for helpful discussions.

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J. Biol. Chem. 2002, 277:4232-4239.
doi: 10.1074/jbc.M109678200 originally published online November 19, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109678200

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