Short Communication

Glutathione Transferase P1 Interacts Strongly with the Inner Leaflet of the Plasma Membrane

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ABSTRACT:

GSH transferases (GSTs) are a superfamily of proteins best known for detoxifying harmful electrophilic compounds by catalyzing their conjugation with GSH. GSTP1 is the most prevalent and widely distributed GST in human tissues, helping to detoxify a diverse array of carcinogens and drugs. In contrast with its protective role, overexpression of GSTP1 in a variety of malignancies is associated with a poor prognosis due to failure of chemotherapy. Although GSTP1 is classified as a cytosolic GST, we discovered previously that it is associated with the plasma membrane of the small cell lung cancer cell lines, H69 and H69AR. In the current study, endogenous and overexpressed GSTP1 in human embryonic kidney (HEK) 293 and MCF-7 cell lines, respectively, were found also to associate with the plasma membrane, indicating that this interaction is not unique to H69 and H69AR cells. GSTP1 immunostaining in HEK293 and MCF7-GSTP1 cells only occurred under permeabilized conditions, suggesting that GSTP1 is associated with the intracellular surface of the plasma membrane. Cell surface biotinylation studies confirmed this finding. Immunogold electron microscopy revealed the presence of GSTP1 in close proximity to the plasma membrane. GSTP1 was not dissociated from plasma membrane sheets by high salt [potassium iodide (KI; 1 M) or KI/EDTA (1 M/2 mM)] or alkaline Na2CO3 (100 mM, pH 11.4), conditions known to strip peripherally associated membrane proteins. Thus, we report for the first time that GSTP1 is associated with the inner leaflet of the plasma membrane through a remarkably strong interaction.

Introduction

GSH transferases (GSTs) are a superfamily of proteins best known for their role in detoxification. These enzymes catalyze the nucleophilic addition of reduced GSH to harmful electrophilic compounds, thus neutralizing reactivity. Human GSTs are classified into three major families: cytosolic, mitochondrial, and microsomal (Hayes et al., 2005). The cytosolic proteins are further divided, based on amino acid sequences, into seven classes: Alpha (A), Mu, Omega, Pi (P), Sigma, Theta, and Zeta. The human GSTP class contains a single member, GSTP1, the most prevalent and widely distributed GST in cells and tissues. GSTP1 is important in the detoxification of a diverse array of carcinogens, and variations in its genotype have been associated with differences in susceptibility to cancer (McIlwain et al., 2006). In contrast with this protective role, GSTP1 is overexpressed in a variety of malignancies, including lymphomas as well as ovarian, breast, colon, and pancreatic cancers; this overexpression has been associated with the failure of chemotherapy and poor prognosis of patients (McIlwain et al., 2006).

GSTP1 has multiple roles in addition to its GSH conjugation activity. These include the regulation of cell signaling pathways through S-glutathionylation, as well as the sequestration and negative regulation of the stress kinase c-jun N-terminal kinase (Tew and Townsend, 2011). Furthermore, GSTP1 can function nonenzymatically by binding nonsubstrate ligands and reactive compounds, leading to sequestration and intracellular transport (Howie et al., 1989; Hayes et al., 2005; Ralat and Colman, 2006). Monomers of GSTP1 are found in the media of cultured tumor cells and human platelets as well as in human plasma and bile (Howie et al., 1989; Kura et al., 1996; Ranganathan et al., 2005). The passage of GSTP1 across the cell membrane has been proposed as an excretory pathway for bound ligands, but the mechanism by which GSTP1 might traverse the membrane is not understood.

During the course of investigating the cellular metabolism and efflux of arsenic, a high-priority environmental carcinogen, we detected GSTP1 in plasma membrane fractions of the H69 and H69AR small-cell lung cancer cell lines (Leslie et al., 2004). Imaging experiments showed a colocalization of GSTP1 with the H69AR plasma membrane (Leslie et al., 2004). The association of GSTP1 with the plasma membrane was unexpected, because GSTP1 is classified as a cytosolic GST. The close association of GSTP1 with the plasma membrane could be important for numerous cell functions. For example, nonsubstrate ligands bound to GSTP1 could be brought closer to the plasma membrane for excretion. Alternatively, GSTP1 is known to confer resistance to the toxic effects of electrophilic drugs and carcinogens by acting synergistically with several ATP-binding cassette (ABC) transport proteins (Depeille et al., 2004; Leslie et al., 2004; Smitherman et al., 2004; Peklak-Scott et al., 2005), including plasma membrane efflux transporters such as the multidrug resistance

ABBREVIATIONS: GST, GSH transferase; MRP, multidrug resistance protein; ABC, ATP-binding cassette; HEK, human embryonic kidney; pAb, polyclonal antibody; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CT-B, cholera toxin subunit B; PAGE, polyacrylamide gel electrophoresis.
proteins MRP1 (ABCC1) and MRP2 (ABCC2). We and others have proposed that the conjugating enzymes and the plasma membrane must be in close proximity for efficient conjugation and efflux of either labile conjugates or hydrophobic toxicants (Singh et al., 2002; Leslie et al., 2004; Bauer et al., 2008).

The purpose of this study was to determine whether the GSTP1 plasma membrane association was an anomaly of the H69 and H69AR cell lines and to biochemically characterize the plasma membrane association. Endogenous and overexpressed GSTP1 in HEK293 and MCF7 cell lines, respectively, were found to associate with the plasma membrane. Cell surface biotinylation assays and immunoelectron microscopy suggested that GSTP1 is localized to the inner leaflet of the plasma membrane. Lastly, the association of GSTP1 with the plasma membrane was found to be remarkably strong and was not dissociated under harsh membrane stripping conditions.

Materials and Methods

Materials. The GSTP1 GS72 rabbit polyclonal antibody (pAb) was obtained from Oxford Biomedical Research (Oxford, MI). The GAPDH (6C5) mouse monoclonal and Na⁺/K⁺ ATPase (H-300) rabbit polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The MRPl rat monoclonal antibody MRPr1 was from Novus Biologicals (Littleton, CO). The anti-calnexin and anti-Mn superoxide dismutase rabbit pAbs were from Stressgen (Ann Arbor, MI), and the anti-histone H1 mouse monoclonal antibody was from US Biological (Swampscott, MA). Alexa Fluor 555-conjugated cholera toxin subunit B (CT-B), and 4′,6-diamidino-2-phenylindole were obtained from Invitrogen (Carlsbad, CA). G418 (Geneticin) was from Invitrogen. Mini Complete protease inhibitor tablets and FuGENE6 were from Roche Diagnostics (Indianapolis, IN). All other reagents were purchased from Sigma-Aldrich.

Construction of Vector Encoding GSTP1 Protein. pBluescriptSK(−) GSTP1 was used as a template in polymerase chain reaction using a 5′-primer with an engineered BamHI site (indicated in italics) (5′-AGAGATCCGGCCATGTCGCCGTA TACCCGTTGTCGTTGTCGTT-3′) and a 3′-primer with an engineered XhoI site (indicated in italics) (5′-GAGAGATCCGGCCATGTCGCCGTA TACCCGTTGTCGTTGTCGTT-3′). The polymerase chain reaction product was digested with BamHI and XhoI and ligated into pcDNA3.1(+).

Cell Lines. The SV40-transformed HEK293 cell line was maintained in Dulbecco’s modified Eagle’s medium supplemented with 4 mM l-glutamine and 10% fetal bovine serum. The MCF7-GSTP1 and MCF7-vector stable cell lines were created as described previously (Leslie et al., 2004), except that levels of GSTP1 expression in G418-selected populations were determined by immunoblot analysis with the GSTP1-specific antibody GS72, as described previously (Leslie et al., 2004). GSTP1-expressing populations were cloned by limiting dilution to obtain populations of ~90% as assessed by GSTP1 immunostaining and fluorescence microscopy. Cells were maintained in Dulbecco’s modified Eagle’s medium with 4 mM l-glutamine, 110 mg/ml sodium pyruvate, 10% fetal bovine serum, and 1000 µg/ml G418.

Confocal Microscopy. Triple staining experiments for nuclei, plasma membrane, and GSTP1 were performed by using the HEK293 and MCF7-GSTP1 cell lines, as described previously (Leslie et al., 2004), with minor modifications. HEK293 cells were transiently transfected with pcDNA3.1(−)MRP1 (as a plasma membrane marker) (Ito et al., 2001) using FuGENE6, according to the manufacturer’s instructions. Alexa Fluor 555-conjugated CT-B was used as a plasma membrane stain for the MCF7-GSTP1 cell line. Seventy-two hours after seeding, cells were fixed with paraformaldehyde for 10 min and MCF7-GSTP1 cells were incubated with CT-B (1:1000) for 10 min. The rest of the staining procedure was identical to that described in Leslie et al., 2004 except that the anti-GSTP1 antibody GS72 was used. Cells were also stained under nonpermeabilizing conditions by using the conditions described above, but in the absence of Triton X-100. Cells were viewed with a Zeiss LSM510 confocal microscope and photographed.

Total and Cell Surface Expression of GSTP1. HEK293 and MCF7-GSTP1 cells were seeded at 1 × 10⁶ cells in a T-75 flask and 72 h later washed with ice-cold borate buffer (0.62 mg/ml boric acid, pH 9.0). Biotinylation reactions and sample preparation for SDS-polyacrylamide gel electrophoresis (PAGE) were performed as described previously (Leslie et al., 2007). GSTP1 protein was detected by using the anti-GSTP1 antibody GS72 (1:1000). To ensure that the cell surface biotinylation worked correctly, blots were stripped and probed for the Na⁺/K⁺ ATPase (integral plasma membrane protein) using the rabbit anti-Na⁺/K⁺ ATPase pAb (1:500). To ensure that the biotinylation was specific to cell surface proteins, the blots were also probed for the cytosolic protein GAPDH using the rabbit anti-GAPDH pAb (1:1000).

Immunoelectron Microscopy. The subcellular localization of GSTP1 in MCF7-GSTP1 cells was examined by using immunogold electron microscopy. Cells were seeded at 1 × 10⁶ cells in a T-75 flask and 72 h later pelleted and washed with phosphate-buffered saline. Cells were fixed with 0.1% glutaraldehyde, 3% paraformaldehyde, 3% sucrose, and 1.5 mM CaCl₂ in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 h at 4°C. Sample preparation, immunogold labeling [using primary antibody anti-GSTP1 GS72 (1:50) for 2 h and a 12-nm colloidal gold-donkey anti-rabbit IgG secondary antibody (1:10) for 1 h], and viewing followed previously described procedures (Ickowitz et al., 2010).

Plasma Membrane Sheet Preparation, Stripping, and Immunoblot Analysis. Plasma membrane sheets were prepared from MCF7-GSTP1 cells collected from 10 150-mm² Petri plates, as described previously (Hubbard et al., 1983). The purity (of the plasma membrane sheets) was assessed by immunoblot using antibodies against calnexin (endoplasmic reticulum marker), nuclear histone H1 (nuclear protein marker), GAPDH (cytosolic marker), and manganese superoxide dismutase (mitochondrial marker). Plasma membrane sheets were then subjected to various chemical treatments known to strip peripherally associated proteins. Thus, plasma membrane sheets (10 µg) were incubated with 10 volumes of 1 M Tris HCl (pH 7.4), 1 M potassium iodide (KI), 1 M KI with 2 mM EDTA, or 100 mM Na₂CO₃ (pH 11.4) for 30 min at 4°C. To increase the stringency of stripping, the Na₂CO₃ condition was also done using 50 and 100 volumes. Treated plasma membrane sheets were then pelleted by centrifugation at 100,000 g for 1 h, resuspended in Laemmli buffer, resolved by 11% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with the anti-GSTP1 GS72 antibody (1:1000) as described previously (Leslie et al., 2004). The blot was then stripped and probed with the anti-Na⁺/K⁺ ATPase pAb and finally stripped and probed with the anti-GAPDH antibody.

Results and Discussion

The purpose of the current study was to extend our investigations of the plasma membrane association of GSTP1 (Leslie et al., 2004). Thus, in an investigation of MRP1-mediated transport of arsenic tri-GSH, GSTP1 was found to be in close proximity with the plasma membrane of H69 and H69AR cells (Leslie et al., 2004). To determine whether the GSTP1 plasma membrane association was a characteristic unique to the H69 and H69AR cell lines, the localization of GSTP1 was investigated in HEK293 and MCF7-GSTP1 cells. HEK293 cells contain endogenous GSTP1, whereas the MCF7-GSTP1 cell lines are stable transfectants. Cell lines were immunostained with the anti-GSTP1 GS72 antibody, and a significant fraction of GSTP1 was localized to a region resembling the plasma membrane of HEK293 and MCF7-GSTP1 cells (Fig. 1A, first column). To define the plasma membrane region of the HEK293 cells, they were immunostained with the anti-MRP1 antibody MRPr1 after transient transfection with the plasma membrane protein MRP1 (Fig. 1A, column 2, top). To define the plasma membrane region of the MCF7-GSTP1 cells, the plasma membrane marker CT-B was used (Fig. 1A, column 2, bottom). Merged images of GSTP1 and the respective plasma membrane marker are shown (Fig. 1A, column 3), and for cellular orientation the nuclei were stained (Fig. 1A, column 4).

Several reports have documented that GSTP1 is found in the culture media of human tumor cells and human platelets as well as in human plasma and bile (Howie et al., 1989; Kura et al., 1996; Ranganathan et al., 2005). In addition, GSTP1 has been found to associate with the external surface of cells (Hemachand et al., 2002). To determine whether GSTP1 was interacting with the internal surface of the plasma membrane or binding to the external surface after exiting the cell.
GSTP1 could be associated with organelles or other intracellular distributed throughout the cytoplasm but clustered, suggesting that GSTP1 cells (Fig. 2) (Goto et al., 2009). GSTP1 was not evenly throughout the cytoplasm, mitochondria, and nucleus of the MCF7-sustained with previous reports of other cell lines, GSTP1 was distributed were analyzed using electron microscopy immunogold labeling. Con-

To confirm the cellular localization of GSTP1, MCF7-GSTP1 cells were analyzed using electron microscopy immunogold labeling. Consistent with previous reports of other cell lines, GSTP1 was distributed throughout the cytoplasm, mitochondria, and nucleus of the MCF7-GSTP1 cells (Fig. 2) (Goto et al., 2009). GSTP1 was not evenly distributed throughout the cytoplasm but clustered, suggesting that GSTP1 could be associated with organelles or other intracellular structures. In addition, plasma membrane staining for GSTP1 was observed.

To determine the biochemical nature of the association between GSTP1 and the plasma membrane, plasma membrane sheets were prepared from MCF7-GSTP1 cells and treated under stringent membrane stripping conditions known to remove peripherally associated membrane proteins. Immunoblot analysis of plasma membrane sheets revealed that they were very pure with little contamination by endoplasmic reticular, mitochondrial, or nuclear proteins (data not shown).
Plasma membrane sheets treated with 10 volumes of Na₂CO₃ (100 mM, pH 11.4) or KI (1 M) ± EDTA (2 mM) retained virtually all of the GSTP1 and the integral membrane protein Na⁺/K⁺ ATPase (Fig. 3). A substantial amount of GSTP1 was retained in the membrane after treatment with 50 and 100 volumes of Na₂CO₃. It is noteworthy that even the integral membrane protein Na⁺/K⁺ ATPase was beginning to be stripped from the membrane at 100 volumes of Na₂CO₃, emphasizing how harsh this stripping condition was. In contrast, the cytosolic protein GAPDH was almost completely removed from the membrane by a wash with Tris (1 M, pH 7.4) as well as all of the harsh stripping conditions. Thus, these results unexpectedly showed that the association between the plasma membrane and GSTP1 was very strong and comparable with the integral membrane protein Na⁺/K⁺ ATPase.

A peripherally associated P and Mu GST isoforms with the plasma membrane of goat sperm has been described previously (Hemachand et al., 2002). Furthermore, murine GSTA4-4 and several human GSTA isoforms were found to associate with the plasma membrane and outer nuclear membrane, respectively, through electrostatic interactions (Singh et al., 2002; Stella et al., 2007). We have found that GSTP1 is still associated with the plasma membrane after high salt treatment and alkaline carbonate extraction, ruling out the possibilities of electrostatic and/or peripheral interaction of GSTP1 with the plasma membrane. This represents the first report of a strong, nonperipheral association of a “cytosolic” GST with the plasma membrane. Although the strength of the GSTP1 plasma-membrane interaction is characteristic of an integral membrane protein, nothing about the reported structure of GSTP1 suggests that this would be the case (Oakley et al., 1997). Intriguingly, members of the chloride intracellular channel family, which are also GST fold superfamily members, exist primarily as soluble proteins, but can also autoinsert into cellular membranes to form ion channels, through a poorly understood mechanism (Littler et al., 2010). Whether GSTP1 can interact with the plasma membrane through a similar mechanism is currently unknown. The observation that GSTP1 is simultaneously present in the cytosol and the plasma membrane of individual cells (Fig. 1A) also raises the possibility that GSTP1 associates with the plasma membrane after a reversible posttranslational modification. Investigations are currently underway to explore this possibility.

In conclusion, GSTP1 is associated with the inner leaflet of the plasma membrane through a remarkably strong interaction. The localization of GSTP1 to the plasma membrane could have important implications for the role of GSTP1 in detoxification, anticancer drug resistance as well as many of the other functions of GSTP1.

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Authorship Contributions

Participated in research design: Qazi, Osoria Pérez, and Leslie.
Conducted experiments: Qazi, Osoria Pérez, and Sam.
Performed data analysis: Qazi, Osoria Pérez, and Leslie.
Wrote or contributed to the writing of the manuscript: Qazi and Leslie.
Other: Leslie acquired funding for the research.

Department of Physiology,
Sohail S. Qazi¹
University of Alberta,
Aida Osoria Pérez
E EDMONTON, ALBERTA, CANADA
Medica Sam
Elaine M. Leslie

¹ Current affiliation: Molecular Entomology/Molecular Virology Laboratory, Great Lakes Forestry Centre, Natural Resources Canada, Sault Ste. Marie, Ontario, Canada.

References


**Address correspondence to:** Dr. Elaine M. Leslie, Department of Physiology, 7-10A Medical Sciences Building, University of Alberta, Edmonton, AB, Canada, T6G 2H7. E-mail: eleslie@ualberta.ca