Nuclear translocation of glutathione S-transferase π is mediated by a non-classical localization signal

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Abbreviations: GSH, reduced form of glutathione; GST, glutathione S-transferase; NLS, nuclear localization signal; MTS, mitochondrial targeting signal; GFP, green fluorescent protein; PCR, polymerase chain reaction; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; RCC1, regulator of chromosome condensation 1; Cu,Zn-SOD, Cu,Zn-superoxide dismutase; GPx, glutathione peroxidase

Abstract

Glutathione S-transferase π (GSTπ), a member of the GST family of multifunctional enzymes, is highly expressed in human placenta and involved in the protection of cellular components against electrophilic compounds or oxidative stress. We have recently found that GSTπ is expressed in the cytoplasm, mitochondria, and nucleus in some cancer cells, and that the nuclear expression of GSTπ appears to correlate with resistance to anti-cancer drugs. Although the mitochondrial targeting signal of GSTπ was previously identified in the amino-terminal region, the mechanism of nuclear translocation remains completely unknown. In this study, we find that the region of GSTπ195-208 is critical for nuclear translocation, which is mediated by a novel and non-classical nuclear localization signal. In addition, using an *in vitro* transport assay, we demonstrate that the nuclear translocation of GSTπ depends on the cytosolic extract and ATP. Although further experiments are needed to understand in depth the precise mechanism of nuclear translocation of GSTπ, our results may help to establish more efficient anti-cancer therapy, especially with respect to resistance to anti-cancer drugs.

Key words: glutathione S-transferase π, nuclear localization signal

Introduction

Glutathione S-transferases (GSTs) are multifunctional enzymes involved in the protection of cellular components against electrophilic compounds or oxidative stress. Glutathione S-transferase π (EC 2.5.1.18) belongs to the family of GSTs involved in cellular detoxification [1, 2]. The expression of GSTπ increases in various pre-cancerous and cancer tissues [3-5]. We have recently found that the mature form of GSTπ is distributed in the cytoplasm and mitochondria, and we have further identified the mitochondrial targeting signal located in the amino-terminal (N-terminal) region of GSTπ [6]. In other studies, nuclear translocation of GSTπ was observed in some cancer cells, and nuclear expression of GSTπ seems to associate with resistance to anti-cancer drugs [7-9]. However, the precise mechanism of nuclear translocation of GSTπ remains completely unclear.

In general, the translocation of proteins from the cytoplasm to the nucleus is dependent upon a specific sequence, which is called a nuclear localization signal (NLS). The NLS occurs in both classical and non-classical types. The classical NLS is composed of positively charged amino-acid cluster(s) [10-12] that can be recognized by importin-α (karyopherin-α) and forms a transport complex with importin-β (karyopherin-β) [13, 14]. However, a non-classical NLS lacks a contiguous stretch of positively charged residues and bears no sequence similarity to the classical NLS [15]. Thus, a non-classical NLS is directly recognized by importin-β family members without the need for adapter proteins, such as importin-α [16-18]. Because GSTπ has no known NLS in the amino acid sequence, the precise mechanism of the nuclear translocation of GSTπ has not yet been determined.

Considering the potential roles of nuclear GSTπ in physiological and pathological functions, we have tried to elucidate the mechanism of the nuclear translocation of GSTπ in this study. We have found that the carboxyl-terminal

(C-terminal) region of GSTπ is critical for nuclear translocation, likely through a novel

mechanism that involves a non-classical NLS.

Materials and Methods

Materials

All materials for this study are listed in the supplementary file.

Cell culture

We used HCT8, COS-1, and HeLa cells for this study. The cells were maintained in DMEM (COS-1 and HeLa) or RPMI 1640 (HCT8) basic medium, supplemented with 10% FBS at 37°C under a humidified atmosphere of 5% CO_2.

Immunostaining to detect endogenous GSTπ

To identify the intracellular localization of endogenous GSTπ, the cells were maintained with medium containing 10% FBS on glass coverslips in a six-well culture plate (Nalge Nunc International, Naperville, IL). After 24 hrs of culture, the cells on the glass coverslips were fixed with 3% paraformaldehyde for 20 min, treated with 1% Triton-X 100 for 10 min, and blocked with 3% BSA for 30 min at room temperature. The cells were incubated with a polyclonal antibody against human GSTπ for 60 min and were then treated with FITC-conjugated anti-rabbit IgG secondary antibody for 30 min, as described previously [6]. The nuclei were labeled with 10 μM Hoechst 33342 for 15 min. The intracellular distribution of endogenous GSTπ was observed under a confocal laser scanning microscope (LSM5 pascal, Carl Zeiss, Jena, Germany).

Construction of vectors for protein expression

The GSTπ cDNA was prepared by a reverse transcription polymerase chain reaction (PCR) method using total RNA extracted from HCT8 cells as described previously [7]. The cDNA of full-length GSTπ was inserted into pcDNA3.1/NT-GFP or pcDNA3.1/CT-GFP, using *Eco*RI-*Eco*RV restriction sites, for the construction of an

expression vector for GFP fused at the N-terminal (GFP-GSTπ) or C-terminal (GSTπ-GFP) end of GSTπ.

To create GFP-GSTπ182-210, GFP-GSTπ182-210 (K209G), GFP-GSTπ182-208, GFP-GSTπ182-194, and GFP-GSTπ195-208, GSTπ fragments were prepared by PCR using GSTπ cDNA as a template and an appropriate set of primers (*Supplemental material*). Following digestion with *Eco*RI and *Eco*RV, the resulting PCR products were subcloned into the *Eco*RI-*Eco*RV restriction sites of pcDNA3.1/NT-GFP.

To obtain FLAG-HA-tags at the N-terminal end of full-length or deletion mutants of GSTπ (FLAG-GSTπ1-210, FLAG-GSTπ1-181, and FLAG-GSTπ1-194), GSTπ fragments were prepared by PCR using an appropriate set of primers (*Supplemental material*). The PCR products were digested with *Not*I and *Apa*I and subcloned into pcDNA3/FLAG-HA.

Evaluation of nuclear translocation of GSTπ by fusion proteins

To observe the nuclear translocation of GSTπ, the expression vectors (2 μg) for various GFP-fused proteins were transfected into COS-1 cells or HCT8 cells with Lipofectamine reagent or Lipofectamine 2000 according to the manufacturer's instructions. The fusion proteins were expressed by cultivating the transfectants for 24 hrs at 37°C and were then fixed with 3% paraformaldehyde in PBS for 20 min. After three washes with PBS, the nuclei were stained with Hoechst 33342. The intracellular distribution of GFP-fused proteins was directly observed as green fluorescence under a confocal laser scanning microscope.

To further confirm the nuclear translocation of GSTπ obtained by the direct detection of GFP-fused GSTπ proteins, expression vectors (2 μg) of FLAG-GSTπ1-210, FLAG-GSTπ1-181 and FLAG-GSTπ1-194 were also transfected into COS-1 cells or

HCT8 cells. After 24 hrs, the cells were stained by anti-FLAG primary antibody and FITC-labeled anti-mouse IgG secondary antibody, as described above. The nuclei were visualized by staining the cells with Hoechst 33342. The nuclear translocation of GSTπ was observed as positive staining of FLAG under a confocal laser scanning microscope.

Preparation of cytosolic and nuclear proteins

The cytosolic and nuclear proteins were prepared as described previously [6]. Briefly, the cell pellets (1×10^6 cells) were treated with 100 μl of hypotonic buffer (10 mM HEPES at pH 7.8, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride [PMSF], 2 μg/ml pepstatin, and 2 μg/ml leupeptin). After centrifugation of the sample (1,800 x g, 4°C, 1 min), the supernatant and debris were collected as rough cytosolic and nuclear fractions, respectively. The rough cytosolic fractions were centrifuged at 15,000 x g for 20 min and 100,000 x g for 30 min at 4°C. The final supernatant was collected as the cytosolic fraction for use in the following study. The rough nuclear fractions were washed three times with hypotonic buffer and treated with 100 μl of 50 mM HEPES (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 mM PMSF, 2 μg/ml pepstatin, and 2 μg/ml leupeptin and were then gently rotated with a rotator at 4°C for 30 min. The supernatant was collected as the nuclear fraction.

Immunoblot analysis

Expression levels of GSTπ, RCC1, Cu,Zn-SOD, GFP and FLAG-tagged proteins in the cells were estimated by immunoblotting. Lysate (30 μg of total protein) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% or 15% gel, transferred to a nitrocellulose membrane, and

immunologically stained first with each appropriate primary antibody, then with HRP-labeled anti-rabbit, anti-mouse or anti-goat IgG as the secondary antibody. Blots were developed by enhanced chemiluminescence using an ECL kit. The protein concentration was determined according to Redinbaugh and Turley [19], with bovine serum albumin (BSA) as the standard.

Preparation of recombinant protein for *in vitro* nuclear transport assay

The cDNA of GFP or GFP-GSTπ195-208 was inserted into the pGEX-6P-1vector using *Sma*I-*Not*I restriction sites, resulting in an expression vector for recombinant GFP or GFP-GSTπ195-208, in which GFP is fused at the C-terminal end of GST from *Schistosoma japonicum*. To create cDNA of GFP and GFP-GSTπ195-208, each fragment was prepared by PCR using pcDNA3.1/NT-GFP or pcDNA3.1/NT-GFP-GSTπ195-208 as a template and an appropriate set of primers (*Supplemental material*). Following digestion with *Sma*I and *Not*I, the resulting PCR products were subcloned into *Sma*I-*Not*I restriction sites of pGEX-6P-1. Expression in the BL21 *Escherichia coli* host and purification of recombinant protein were performed according to the manufacturer's instructions.

The nucleotide sequences of vectors

All newly constructed vectors were transformed into a JM109 *Escherichia coli* host and purified using a GeneElute Hp plasmid midiprep kit (Sigma Aldrich). The nucleotide sequences of all constructs were determined using the CEQ DTCS-Quick Start kit on a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA).

In vitro nuclear transport assay

In vitro transport assays were performed according to the method described by Adam et al. [20]. Briefly, HeLa cells were grown on glass coverslips in a 6-well plate. The cells were washed twice on the glass coverslips with ice-cold transport buffer (20 mM Hepes-KOH, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM DTT, 1.0 mM EGTA, and 1 μg/ml each aprotinin, leupeptin, and pepstatin) and permeabilized for 5 min in ice-cold transport buffer containing 40 μg/ml digitonin. After washing with cold transport buffer, the permeabilized cells were incubated with 150 μl of import assay mixture containing recombinant GST-GFP or GST-GFP-GSTπ 195-208 protein with or without cytosol extract or the ATP regeneration system (0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 50 μg/ml creatine kinase), for 30 min at either 30°C or 4°C. After three washes with import buffer and PBS, the cells were fixed with 3% formaldehyde for 20 min, and then nuclei were labeled with 10 μM Hoechst 33342 for 10 min. Fluorescence was observed under a confocal laser scanning microscope.

Results

The C-terminal region of GSTπ is critical for nuclear translocation

Immunostaining with anti-GSTπ antibody clearly showed the expression of endogenous GSTπ in the nuclei of HCT8 and COS-1 cells under steady-state conditions (**Fig.1A**), although the expression of GSTπ in the cytoplasm was stronger in HCT8 than in COS-1 cells.

We constructed vectors encoding GFP at the N-terminal end (GFP-GSTπ) or the C-terminal end (GSTπ-GFP) of GSTπ, which were then transfected into HCT8 and COS-1cells, respectively. The intracellular distribution of GFP-fused GSTπ proteins (M.W. 52,000) was detected as GFP fluorescence under a confocal laser scanning microscope 24 hrs after transfection. GFP fluorescence was observed in both the cytoplasm and the nuclei of cells transfected with GFP-GSTπ vectors (**Fig. 1B**), indicating that the N-terminally GFP-fused GSTπ translocated into the nuclei. However, GFP fluorescence was observed only within the cytoplasm, not within the nuclei, in cells transfected with GSTπ-GFP vectors (**Fig. 1C**), which indicated a lack of C-terminally GFP-fused GSTπ in the nuclei. The treatment of cells with Leptomycin B, a specific inhibitor of nuclear export of protein, did not change the nuclear distribution of fusion proteins (data not shown), which eliminated the possibility that the fusion proteins were exported from the nuclei in cells transfected with GSTπ-GFP vectors. These microscopy findings were corroborated by immunoblot analysis (**Fig. 1D**). Our results suggest that artificial modification of GSTπ by fusion with GFP may cause a conformational change of the protein and that the C-terminal region, but not the N-terminal region, of GSTπ is critical for nuclear translocation.

The C-terminal region of GSTπ182-210 is required for nuclear translocation

Positively charged residues are known to be critical components of the classical-NLS. As the C-terminal region of GSTπ182-210 is rich in positively charged arginine and lysine (bold-typed, **Fig. 2A**), this C-terminal region may mediate the nuclear translocation of GSTπ as a NLS. To test this possibility, we constructed expression vectors encoding N-terminally FLAG-tagged full-length GSTπ (FLAG-GSTπ1-210) or a C-terminal deletion mutant GSTπ (FLAG-GSTπ1-181) (**Fig. 2A**). These vectors were introduced into COS-1 cells, and the intracellular distribution of FLAG-tagged proteins was detected by immunostaining with anti-FLAG antibody. As expected, the nuclear localization of FLAG-tagged proteins was observed in cells transfected with the FLAG-GSTπ1-210 vector but was not observed in cells transfected with the FLAG-GSTπ1-181 vector (**Fig. 2B**), which suggests that the C-terminal region of GSTπ (residues 182-210) is required for nuclear translocation.

Non-classical NLS mediates the nuclear translocation of GSTπ

To further define the required C-terminal region of GSTπ and understand the relevant mechanism of nuclear translocation of this protein, expression vectors encoding various N-terminally GFP-fused C-terminal fragments of GSTπ were introduced into the COS-1 cells (**Supplementary Fig. 1A**). Immunoblot analysis using anti-GFP antibody showed the expression of GFP under all conditions (**S. Fig. 1B**). A clear band corresponding to endogenous GSTπ was also detected by the anti-GSTπ antibody under all conditions, but a band for GFP-fused GSTπ was not detected in cells transfected with GFP-GSTπ182-194 vector. It is likely that the anti-GSTπ antibody did not recognize the GFP-fused GSTπ protein that was expressed from GFP-GSTπ182-194 vector, because a clear band corresponding to the fusion protein was detected by anti-GFP antibody in these cells.

The intracellular distribution of GFP-fused GSTπ was detected as GFP fluorescence under confocal laser scanning microscopy (**S. Fig. 1C**). Nuclear translocation of GSTπ was detected in cells transfected with vectors of GFP-GSTπ182-208, GFP-GSTπ195-208, GFP-GSTπ182-210, and GFP-GSTπ182-210 (K209G), in which lysine 209 is replaced with glycine. However, nuclear translocation of GSTπ was not detected in cells transfected with the GFP-GSTπ182-194 vector. To confirm the critical nature of the 195-208 sequence, COS-1 and HCT8 cells were transfected with FLAG-GSTπ1-210 and FLAG-GSTπ1-194 vectors. Immunostaining by anti-FLAG antibody clearly showed nuclear translocation of GSTπ in cells transfected with the FLAG-GSTπ1-210 vector, but not with the FLAG-GSTπ1-194 vector (**Fig. 3**). Our results suggest that the 195-208 region of GSTπ is critical for nuclear translocation. Considering the lack of a contiguous stretch of positively charged amino acid residues within this region, nuclear translocation of GSTπ is likely mediated by a non-classical rather than a classical NLS.

Nuclear translocation of GSTπ is dependent on cytosolic factor(s) and ATP

Using the nuclear transport assay, we tried to further clarify how GSTπ enters the nucleus. We subjected recombinant GST-GFP or GST-GFP-GSTπ195-208 proteins to permeabilized HeLa cells under various conditions (**Fig. 4**). GFP fluorescence was not observed in the nuclei following the addition of GST-GFP-GSTπ195-208 proteins alone to the permeabilized HeLa cells at 30°C or 4°C (data not shown). However, GFP fluorescence was clearly observed in the nuclei of permeabilized HeLa cells following the addition of GST-GFP-GSTπ195-208 protein along with both cytosolic extract and the ATP regeneration system at 30°C (but not with either of these components alone). In contrast, GFP fluorescence was not observed in the nuclei of permeabilized HeLa cells following the addition of GST-GFP protein, even in the presence of both cytosolic

extract and ATP regeneration system at 30°C. These results indicate that the nuclear translocation of GSTπ is dependent on cytosolic factor(s) and ATP. Although further experiments are needed to understand the mechanism in depth, our results suggest that different signals mediate the import of GSTπ into the nucleus and mitochondria (**S. Fig. 2**).

Discussion

Proteins smaller than 40 kDa are believed to enter the nucleus by passive diffusion through the nuclear pore complex. Although GSTπ is a homodimer consisting of two subunits each with a molecular mass of 24 kD in the cytosol, it is possible that GSTπ may form a monomer prior to passing through the nuclear pore complex by passive diffusion. However, the present study and earlier results suggest that nuclear translocation of GSTπ likely does not result from passive diffusion from the cytoplasm, as 1) nuclear translocation is selectively observed in N-terminal GFP-fused GSTπ, the molecular weight of which is about 52 kD (too large to diffuse through the nuclear pore complex), but not in C-terminally GFP-fused GSTπ (Fig. 1B, C); and 2) a previous study demonstrated nuclear translocation of GSTπ in some but not all cancer cell lines [7]. These observations suggest the existence of a specific mechanism for the nuclear translocation of GSTπ.

Various mechanisms have been proposed for the nuclear translocation of proteins from the cytoplasm [10-18]. In the present study, we found that amino acid residues 195-208 at the C-terminus were necessary for the nuclear localization of GSTπ. The GSTπ NLS lacks the contiguous stretch of positively charged amino acid residues characteristic of a classical NLS. In addition, we found that the GSTπ NLS bears no sequence similarity to other proteins, except for the same isoform of GST from other species, using the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (USA). We also found no registered motifs in the C-terminal region of GSTπ in PROSITE, a database of protein families and domains curated by the Swiss Institute of Bioinformatics (Switzerland). Therefore, it is most likely that a non-classical rather than a classical NLS in the C-terminal region mediates the nuclear translocation of GSTπ. As the nuclear translocation of GSTπ was found to depend on cytosolic factor(s) and ATP, it is possible that GSTπ forms complex

with other cytosolic protein(s) that recognize the NLS and facilitate nuclear translocation.

Several previous studies have identified the relevant mechanisms of the nuclear and mitochondrial localizations of proteins. For example, apurinic/apyrimidinic endonuclease 1 (APE1), a key enzyme of DNA base excision repair, localizes in both the nucleus and mitochondria. The nuclear form of APE1 is intact, whereas the mitochondrial form lacks the N-terminal region (including the NLS), suggesting that the cleavage of the intact form is a prerequisite for the mitochondrial import of APE1 [21]. Alternatively, phospholipid hydroperoxide glutathione peroxidase (PHGPx) is encoded by a single gene, *gpx-4*, that has two distinct promoter regions. The upstream region transcribes cytosolic PHGPx and mitochondrial PHGPx, while the downstream region yields nuclear PHGPx [22]. However, we found that endogenous GSTπ in the cytoplasm, the mitochondria, and the nucleus has a similar molecular size [6]. This means that the localization of GSTπ in the nucleus and the mitochondria depends on internal peptide signals without the need for alternative splicing or post-translational modifications, such as proteolysis (S. Fig. 2). Although our data suggest the existence of specific mechanisms for the regulation of the intracellular distribution of GSTπ, how cells allow the translocation of GSTπ to subcellular compartments will need to be the subject of further studies. Our recent studies have shown that positive expression of GSTπ in the nucleus seems to correlate with resistance to anti-cancer drugs [7-9]. Understanding the biochemical and physiological significance of the nuclear translocation of GSTπ may therefore help to establish more efficient anti-cancer therapies.

Acknowledgements

References

[1] L. F. Chasseaud, The role of glutathione and glutathione S-transferases in the metabolism of chemical carcinogens and other electrophilic agents, Adv. Cancer Res. 29 (1979) 175-274.

[2] D. J. Meyer, D. Beale, K. H. Tan, B. Coles and B. Ketterer, Glutathione transferases in primary rat hepatomas: the isolation of a form with GSH peroxidase activity, FEBS Lett. 184 (1985) 139-143.

[3] K. Satoh, A. Kitahara, Y. Soma, Y. Inaba, Y. I. Hatayama and K. Sato, Purification, induction, and distribution of placental glutathione transferase: a new marker enzyme for preneoplastic cells in the rat chemical hepatocarcinogenesis, Proc. Natl. Acad. Sci. USA.82 (1985) 3964-3968.

[4] B. Mannervik, V. M. Castro, U. H. Danielson, M. K. Tahir, J. Hansson and U. Ringborg, Expression of class Pi glutathione transferase in human malignant melanoma cells, Carcinogenesis. 8 (1987) 1929-1932.

[5] A. F. Howie, L. M. Forrester, M. J. Glancey, J. J. Schlager, G. Powis, G. J. Beckett, J. D. Hayes and C. R. Wolf, Glutathione S-transferase and glutathione peroxidase expression in normal and tumour human tissues, Carcinogenesis. 11 (1990) 451-458.

[6] S. Goto, M. Kawakatsu, S. Izumi, Y. Urata., K. Kageyama, Y. Ihara, T. Koji, T. Kondo, Glutathione S-transferase π localizes in mitochondria and protects against oxidative stress, FRBM. 46 (2009) 1392-403.

[7] S. Goto, Y. I hara, Y. Urata, S. Izumi, K. Abe, T. Koji and T. Kondo, Doxorubicin-induced DNA intercalation and scavenging by nuclear glutathione

S-transferase π, FASEB J. 15 (2001) 2702–2714.

[8] S. Goto, K. Kamada, Y. Soh, Y. Ihara and T. Kondo, Significance of nuclear glutathione S-transferase π in resistance to anti-cancer drugs, Jpn. J. Cancer Res. 93 (2002) 1047-1056.

[9] Y. Soh, S. Goto, M. Kitajima, S. Moriyama, K. Kotera, T. Nakayama, H. Nakajima, T. Kondo and T. Ishimaru, Nuclear Localization of Glutathione S-Transferase π is an Evaluation Factor for Drug Resistance in Gynecological Cancers, Clinical Oncology 17(4) (2005) 264-270.

[10] C. Dingwall and R. A. Laskey, Nuclear targeting sequences--a consensus? Trends Biochem. Sci.16 (1991) 478–481.

[11] K. Nakai, M. Kanehisa, A knowledge base for predicting protein localization sites in eukaryotic cells, Genomics. 14 (1992) 897–911.

[12] F. Melchior and L. Gerace, Mechanisms of nuclear protein import, Curr. Opin. Cell Biol. 7 (1995) 310–318.

[13] D. Görlich and I.W. Mattaj, Nucleocytoplasmic transport, Science .271 (1996) 1513–1518.

[14] E. A. Nigg, Nucleocytoplasmic transport: signals, mechanisms and regulation, Nature. 386 (1997) 779–787.

[15] H. Siomi, G. Dreyfuss, A nuclear localization domain in the hnRNP A1 protein, J. Cell .Biol. 129 (1995) 551-560.

[16] E. Nagoshi, N. Imamoto, R. Sato and Y. Yoneda, Nuclear import of sterol regulatory element-binding protein-2, a basic helix-loop-helix-leucine zipper

(bHLH-Zip)-containing transcription factor, occurs through the direct interaction of importin beta with HLH-Zip, Mol. Biol. Cell. 10 (1999) 2221–2233.

[17] Y. Yoneda, Nucleocytoplasmic protein traffic and its significance to cell function, Genes Cell. 5 (2000) 777–787.

[18] S. J. Lee, T. Sekimoto, E. Yamashita, E. Nagoshi, A. Nakagawa, N. Imamoto, M. Yoshimura, H. Sakai, K. T. Chong, T. Tsukihara and Y. Yoneda, The structure of importin-beta bound to SREBP-2: nuclear import of a transcription factor, Science. 302 (2003) 1571–1575.

[19] M. G. Redinbaugh, R. B. Turley, Adaptation of the bicinchoninic acid protein assay for use with microtiter plates and sucrose gradient fractions, Anal. Biochem. 153 (1986) 267-271.

[20] S. A. Adam, L. Gerace, Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. Cell. 166(5) (1991) 837–847.

[21] R. Chattopadhyay, L. Wiederhold, B. Szczesny, I. Boldogh, T. K. Hazra, T. Izumi and S. Mitra, Identification and characterization of mitochondrial abasic (AP)-endonuclease in mammalian cells, Nucleic Acids Res. 34 (2006) 2067-2076.

[22] A. Borchert, N.E. Savaskan and H. Kuhn, Regulation of expression of the phospholipid hydroperoxide/sperm nucleus glutathione peroxidase gene. Tissue-specific expression pattern and identification of functional cis- and trans-regulatory elements, J. Biol. Chem. 278 (2003) 2571-2580.
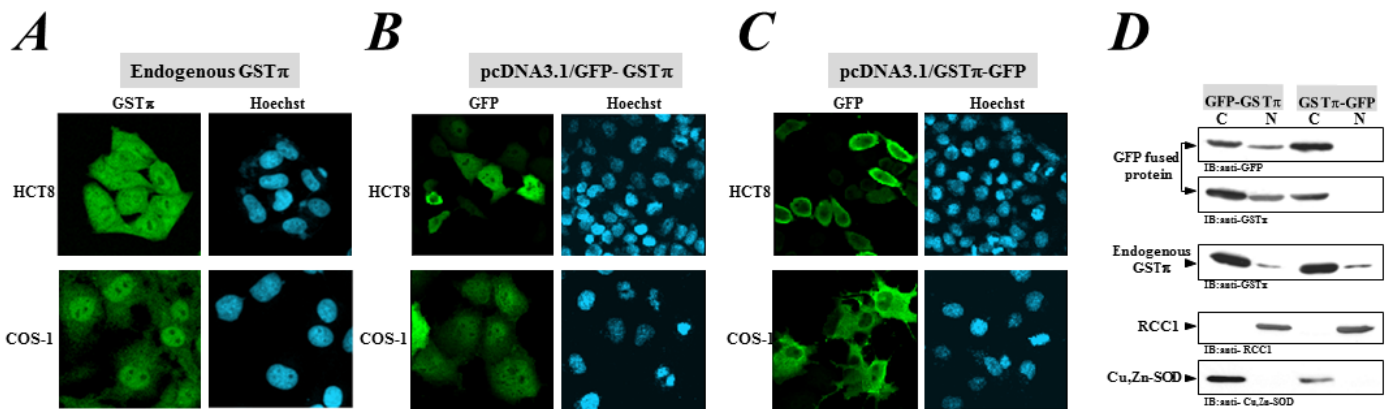
Figure legends

Figure 1. The C-terminal region of GSTπ is critical for nuclear translocation.

A) Immunostaining with anti-GSTπ antibody shows positive expression of endogenous GSTπ in the nuclei of HCT8 or COS-1 cells. The location of the nuclei was determined by staining the cells with Hoechst 33342.

B) After 24 hrs of transfection with 2 μg of vector encoding N-terminally GFP-fused GSTπ (GFP-GSTπ), the fusion protein was clearly observed (as GFP fluorescence) in the nuclei of HCT8 or COS-1 cells under a confocal laser scanning microscope.

C) Fusion protein was not detectable in the nucleus of HCT8 or COS-1 cells after 24 hrs of transfection with 2 μg of vector encoding C-terminally GFP-fused GSTπ (GSTπ-GFP).

D) Immunoblot analysis using anti-GFP and anti-GSTπ antibodies clearly shows the presence of fusion proteins in both the cytoplasm (C) and the nucleus (N) in COS-1 cells after 24 hrs of transfection with vector encoding N-terminally GFP-fused GSTπ (GFP-GSTπ), but fusion protein was detected only in the cytoplasm in COS-1 cells transfected with vector encoding C-terminally GFP-fused GSTπ (GSTπ-GFP). Possible contamination of cytosolic and nuclear protein fractions was checked by immunoblotting for Cu,Zn-SOD and RCC-1, respectively.
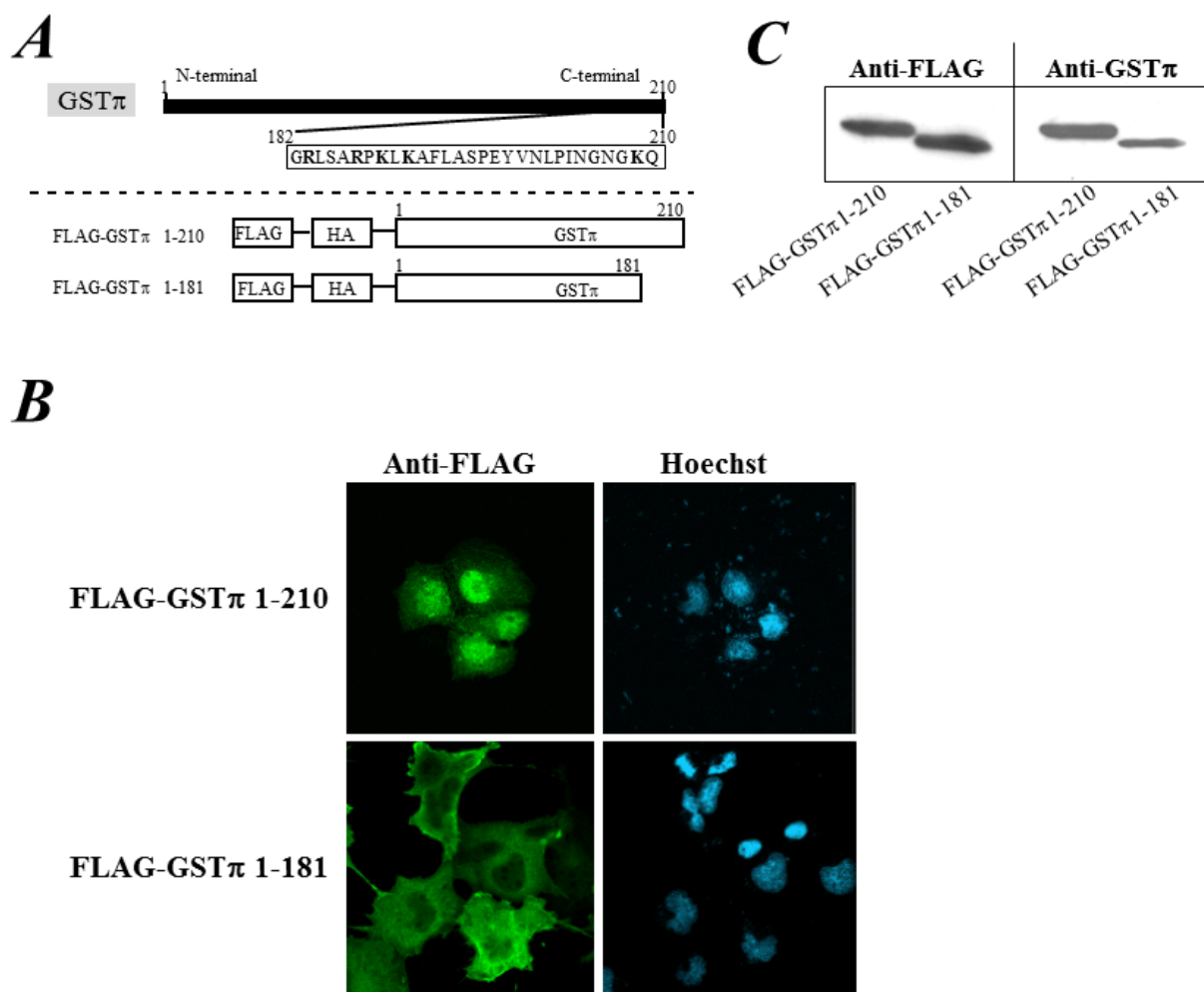
Figure 2. Identification of the C-terminal GSTπ sequence required for nuclear translocation.

A) The sequence of the C-terminal region of GSTπ and diagrams of FLAG-tagged GSTπ are indicated.

B) Immunostaining using anti-FLAG antibody shows nuclear expression of FLAG-tagged GSTπ protein in COS-1 cells after 24hrs of transfection with pcDNA3/FLAG-GSTπ1-210 vector but not in cells transfected with pcDNA3/FLAG-GSTπ1-181 vector.

C) Intracellular expression of FLAG-tagged GSTπ was verified by immunoblot analysis using anti-FLAG and anti-GSTπ antibodies.
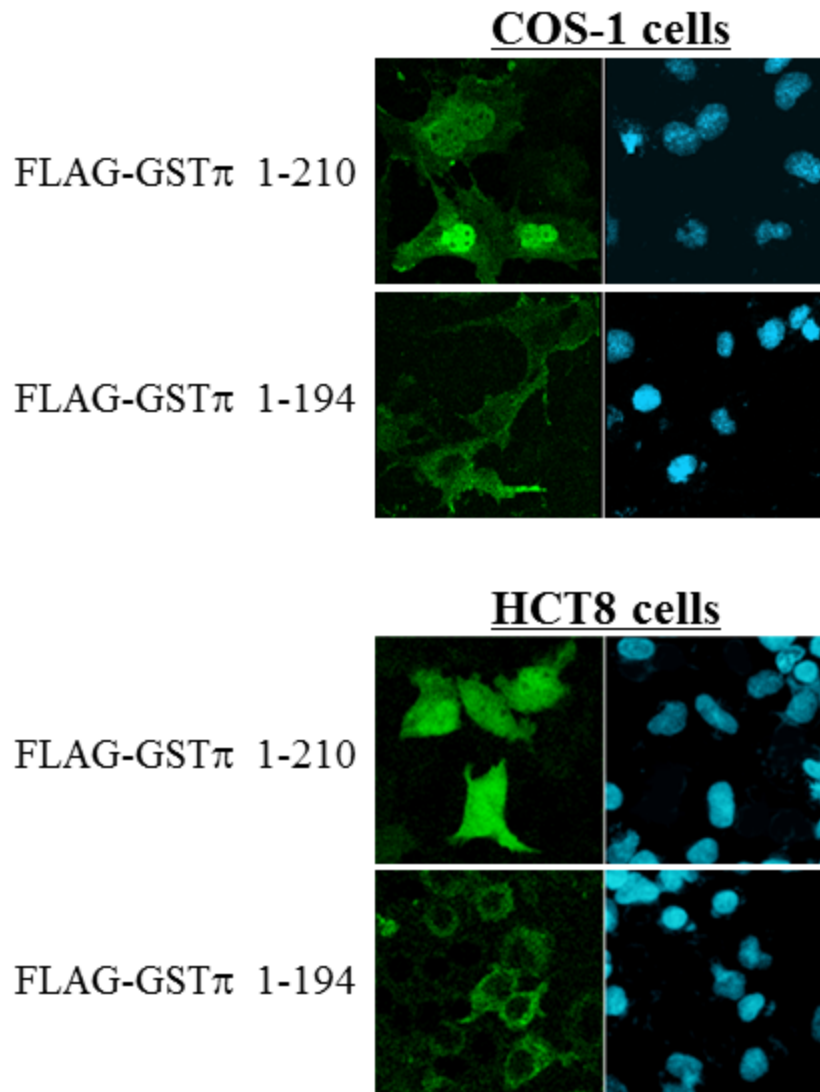
Figure 3. Nuclear translocation of N-terminally GFP-fused fragments of the C-terminal region of GSTπ.

Nuclear translocation of FLAG-tagged GSTπ was detected by immunostaining with anti-FLAG antibody. Nuclear translocation of FLAG-tagged GSTπ proteins in HCT8 or COS-1 cells was observed after 24 hrs of transfection with pcDNA3/FLAG-GSTπ 1-210 vector but was not observed after 24 hrs of transfection with pcDNA3/FLAG-GSTπ 1-194 vector.
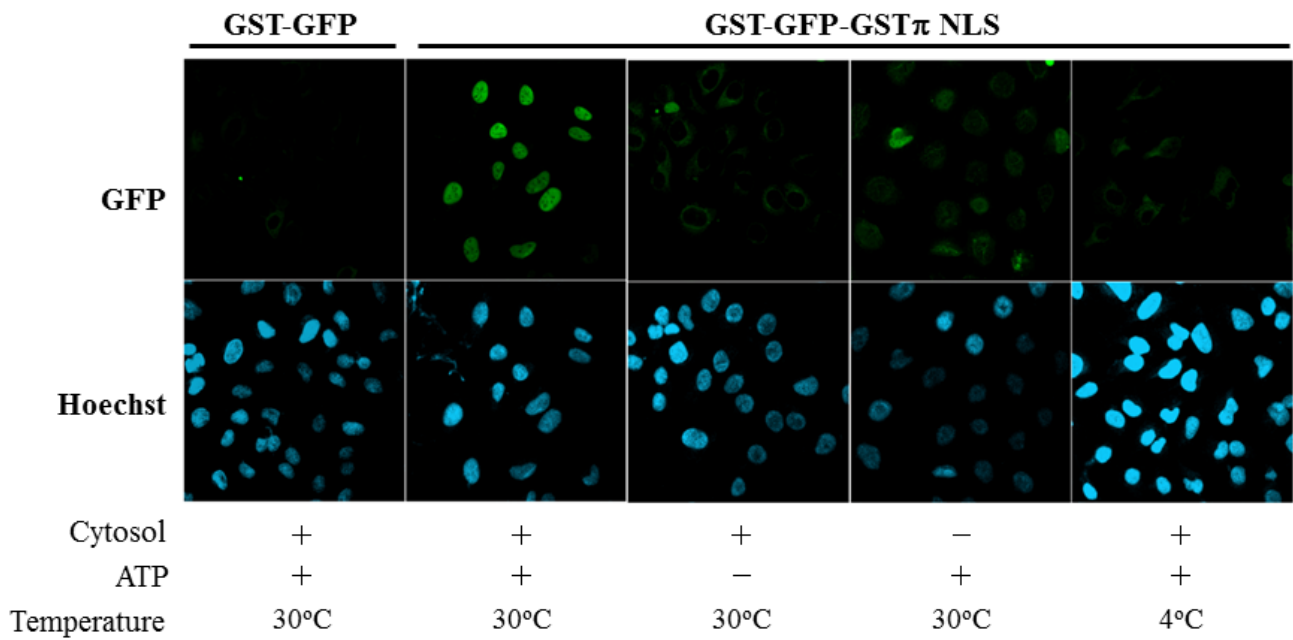
Figure 4. Nuclear translocation of GSTπ is dependent on cytosolic factor(s) and ATP.

In vitro transport assays show the presence of recombinant GST-GFP-GST195-208 protein (GFP fluorescence) in the nuclei of digitonin-permeabilized HeLa cells supplemented with cytosolic extract and the ATP regeneration system for 30 min at 30°C.

Supplementary file 1

Materials

RPMI 1640 medium and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma Aldrich (St. Louis, MO); fetal bovine serum (FBS) was from Invitrogen Corp. (Carlsbad, CA); horseradish peroxidase (HRP)-labeled anti-mouse IgG, HRP-labeled anti-rabbit IgG, and HRP-labeled anti-goat IgG were from DAKO A/S (Glostrup, Denmark); fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG were from ICN Pharmaceuticals (Aurora, OH); anti-green fluorescent protein (GFP) antibody was from Invitrogen Corp.; anti-FLAG antibody was from Sigma Aldrich; anti-human regulator of chromosome condensation (RCC1) antibody was from BD Biosciences Pharmingen (San Jose, CA); and anti-human Cu,Zn-superoxide dismutase (Cu,Zn-SOD) antibody was a gift from Dr. K. Suzuki (Hyogo College of Medicine, Nishinomiya, Japan). The Slow Fade Light Antifade Kit, Lipofectamine reagent, Lipofectamine 2000, pcDNA3.1/NT-GFP vector, and pcDNA3.1/CT-GFP vector were purchased from Invitrogen Corp. The QuickChange Site-Directed Mutagenesis Kit was purchased from STRATAGENE (La Jolla, CA). The FLAG-HA-tagged protein expression vector (pcDNA3/FLAG-HA vector), constructed by introducing a *Bgl*II-Kozack-ATG-FLAG-HA-*Eco*RI fragment into the *Bam*HI-*Eco*RI restriction sites of pcDNA3 (Invitrogen), was a gift from Dr. J. Yanagisawa (Institute of Applied Biochemistry, University of Tsukuba, Ibaraki, Japan). The Enhanced Chemiluminescence (ECL) Kit, pGEX-6P-1 vector and Glutathione

Sepharose 4 Fast Flow were purchased from GE Healthcare Bio-Sciences (Little Chalfont, UK). Hoechst 33342 and digitonin were from CALBIOCHEM (San Diego, CA). The other chemicals and reagents were purchased from Sigma Aldrich.

PCR primers

To create GFP-GSTπ182-210, GFP-GSTπ182-210 (K209G), GFP-GSTπ182-208, GFP-GSTπ182-194 and GFP-GSTπ195-208, GSTπ fragments were prepared by PCR using GSTπ cDNA as a template and an appropriate set of primers. The primers used were as follows: GSTπ182-210 sense, 5'-ACC GAA TTC TGG GGC GCC TCA GCG CCC GGC CC; GSTπ182-210 antisense, 5'-GCG GAT ATC TCA CTG TTT CCC GTT GCC ATT GAT; GSTπ182-210 (K209G) sense, 5'- ACC GAA TTC TGG GGC GCC TCA GCG CCC GGC CC; GSTπ182-210 (K209G) antisense, 5'- GCG GAT ATC TCA CTG GGC CCC GTT GCC ATT GAT; GSTπ182-208 sense, 5'-ACC GAA TTC TGG GGC GCC TCA GCG CCC GGC CC; GSTπ182-208 antisense, 5'- GCG GAT ATC TCA CCC GTT GCC ATT GAT GGG GAG; GSTπ182-194 sense, 5'- ACC GAA TTC TGG GGC GCC TCA GCG CCC GGC CC; GSTπ182-194 antisense, 5'- GCG GAT ATC TCA CAG GAA GGC CTT GAG CTT GGG; GSTπ195-208 sense, 5'- ACC GAA TTC TGG CCT CCC CTG AGT ACG TGA AC; GSTπ195-208 antisense, 5'- GCG GAT ATC TCA CCC GTT GCC ATT GAT GGG GAG; GSTπ1-194 sense, 5'-ACC GAA TTC GCC ACC ATG CCG CCC TAC ACC GTG GTC; and GSTπ1-194 antisense, 5'- GCG GAT ATC TCA CAG GAA GGC CTT GAG CTT GGG.

To obtain FLAG-HA-tagged at the N-terminal end of full-length or deletion mutants of GSTπ (FLAG-GSTπ1-210, FLAG-GSTπ1-181 and FLAG-GSTπ1-194), GSTπ fragments were prepared by PCR using an appropriate set of primers. The following primers were used: GSTπ1-210 sense, 5'-CTC GCG GCC GCA AAT GCC GCC CTA

CAC CGT GGT C; GSTπ1-210 antisense, 5'- ATA GGG CCC TCA CTG TTT CCC

GTT GCC ATT GAT; GSTπ1-181 sense, 5'-CTC GCG GCC GCA AAT GCC GCC CTA

CAC CGT GGT C; GSTπ1-181 antisense, 5'- ATA GGG CCC TCA CAC ATA TGC

TGA GAG CAG GGG; GSTπ1-194 sense, 5'- CTC GCG GCC GCA AAT GCC GCC

CTA CAC CGT GGT C; and GSTπ1-194 antisense, 5'-ATA GGG CCC TCA CAG GAA

GGC CTT GAG CTT GGG.

The cDNA of GFP or GFP-GSTπ195-208 was inserted into the pGEX-6P-1vector

using the *Sma*I-*Not*I restriction sites to construct an expression vector for GFP or

GFP-GSTπ195-208 (in which GFP is fused at the C-terminal end of GST from

Schistosoma japonicum). To create cDNA of GFP and GFP-GSTπ195-208, each

fragment was prepared by PCR using pcDNA3.1/NT-GFP or

pcDNA3.1/NT-GFP-GSTπ195-208 as a template and an appropriate set of primers. The

primers used were as follows: GFP sense, 5'-ATA CCC GGG TAT GGC CAG CAA

AGG AGA AGA ACC T; GFP antisense, 5'-CTC GCG GCC GCT CAA CCA CAC

TGG ACT AGT GGA TC; GFP-GSTπ195-208 sense, 5'-ATA CCC GGG TAT GGC

CAG CAA AGG AGA AGA ACC T; and GFP-GSTπ195-208 antisense, 5'-CTC GCG

GCC GCT CAC TGT TTC CCG TTG CCA TTG AT.
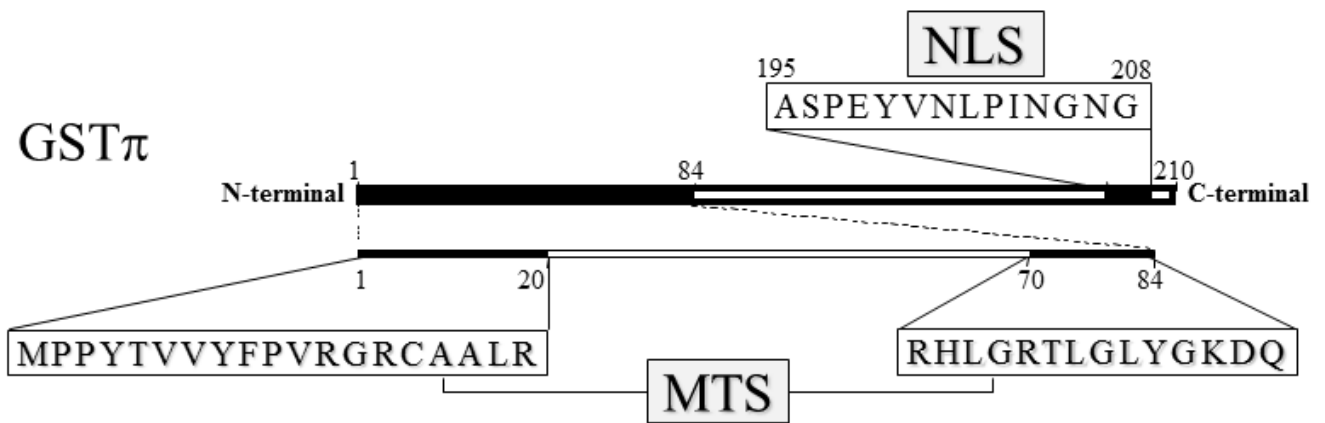
Supplementary Figure legends
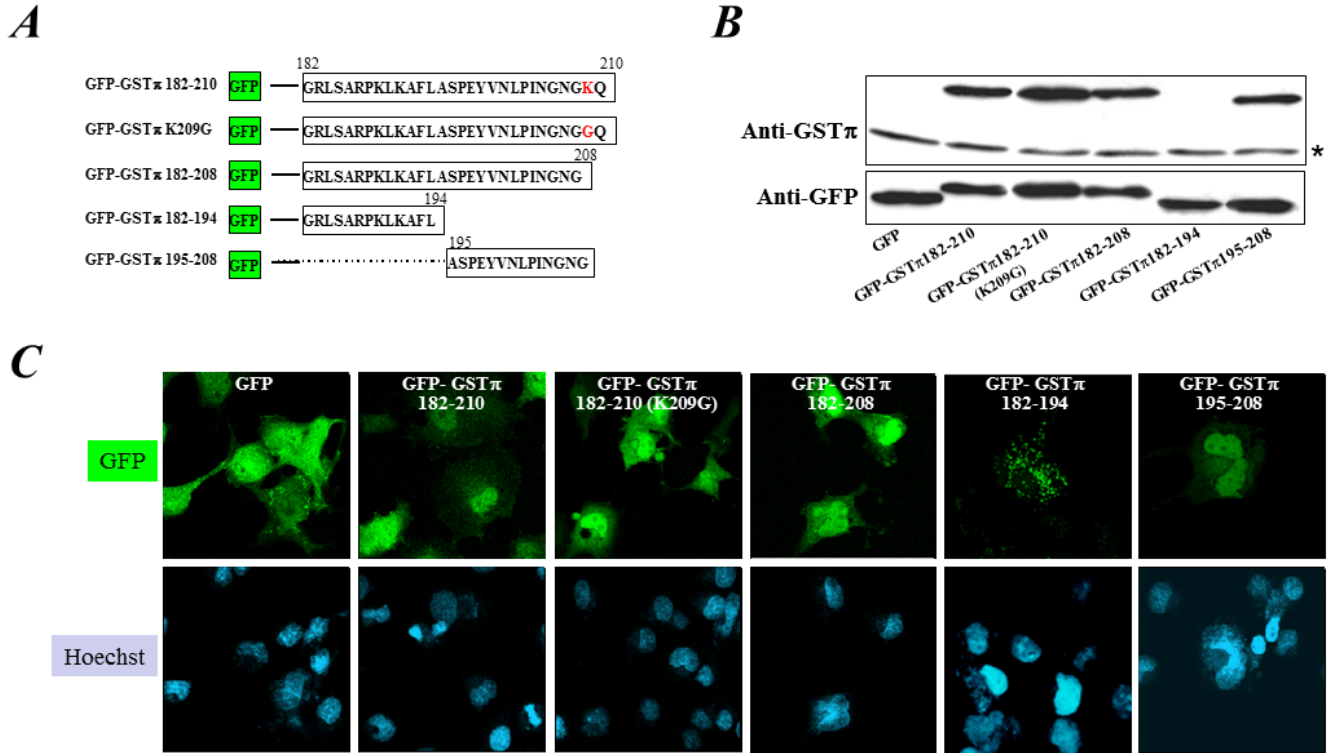
Supplementary Figure 1.

A) Diagrams of N-terminally GFP-fused fragments of GSTπ.

B) After 24 hrs of transfection with various vectors, intracellular expression of GFP-fused proteins was estimated by immunoblot analysis using anti-GFP and anti-GSTπ antibody.   The asterisk indicates endogenous GSTπ.

C) Nuclear translocation of GFP-fused GSTπ was detected by GFP fluorescence under a confocal laser scanning microscope after 24 hrs of transfection with various vectors. No fusion protein was detected in the cells transfected with vector encoding N-terminally GFP-fused fragments of the GSTπ182-194 (GFP-GSTπ182-194).

Supplementary Figure 2.

Diagram summarizing the mitochondrial targeting signal (MTS) and the nuclear localization signals for GSTπ.