Phosphorylation Enhances Mitochondrial Targeting of GSTA4-4 through Increased Affinity for Binding to Cytoplasmic Hsp70* 

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Recently we showed that three different isoforms of cytosolic glutathione S-transferases (GST), including GSTA4-4, are also localized in the mitochondrial compartment. In this study, we have investigated the mechanism of mouse GSTA4-4 targeting to mitochondria, using a combination of in vitro mitochondrial import assay and in vivo targeting in COS cells transfected with cDNA. Our results show that the mitochondrial GSTA4-4 is more heavily phosphorylated compared with its cytosolic counterpart. Protein kinase activators (cAMP, forskolin, or phorbol-12-myristate-13-acetate) markedly increased GSTA4-4 targeting to mitochondria, whereas kinase inhibitors caused its retention in the cytosol. Immunoinhibition and immunodepletion studies showed that the Hsp70 chaperone is required for the efficient translation of GSTA4-4 as well as its translocation to mitochondria. Co-immunoprecipitation studies showed that kinase inhibitors attenuate the affinity of GSTA4-4 for cytoplasmic Hsp70 suggesting the importance of phosphorylation for binding to the chaperone. Mutational analysis show that the putative mitochondrial targeting signal resides within the C-terminal 20 amino acid residues of the protein and that the targeting signal requires activation by phosphorylation at the C-terminal most protein kinase A (PKA) site at Ser-189 or protein kinase C (PKC) site at Thr-193. We demonstrate for the first time that PKA and PKC modulate the cytoplasmic and mitochondrial pools of GSTA4-4.

* This work was supported by National Institutes of Health Grant GM-34883-18. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: GST, glutathione S-transferase; CCCP, carbonyl cyanide-m-chlorophenylhydrazone; CDNB, 1-chloro-2,4-dinitrobenzene; CYP, cytochrome P450; Bt2cAMP, dibutyryl cAMP; GSH, glutathione; HNE, 4-hydroxynonenal; MPI, myristoylated peptide inhibitor; PMA, phorbol-12-myristate-13-acetate; PKA, protein kinase A; PKC, protein kinase C; ROS, reactive oxygen species; RRL, rabbit reticulocyte lysate; TIM, translocase of the inner mitochondrial membrane; WGL, wheat germ lysate; DHFR, dihydrofolate reductase; Hsp70, heat-shock protein 70; CMV, cytomegalovirus; MES, 4-morpholineethanesulfonic acid; MPI, myristoylated peptide inhibitor; WT, wild-type; ER, endoplasmic reticulum.

GSTA4-4 belongs to a major group of enzymes involved in detoxification and protection of cells against chemical and oxidative stress. The compounds detoxified by GSTs include an array of carcinogens, anticancer drugs, and environmental chemicals (1). Some GSTs also catalyze the reduction of cellular peroxides and aldehydes generated endogenously during lipid peroxidation (2). These aldehydes, such as HNE, are known to cause various cell and tissue degenerations and associated pathologies (3). HNE is produced in large amounts (10 μM to 5 mM) in response to oxidative insults, and it exhibits wide range of biological activities, including oxidation and alkylation of cellular proteins and DNA (3, 4). As a result, HNE has been implicated in a number of diseases, including cancer (5) and hepatic (6), neuronal (7), and cardiovascular (8) diseases. GSTA4-4 catalyzes the detoxification of HNE and related lipid peroxides by conjugation to GSH (9). This enzyme also exhibits GSH peroxidase activity for the metabolic inactivation of phospholipid hydroperoxides (10).

Mitochondria play a crucial role in maintaining the metabolic homeostasis and are important regulators of cell death (11). The mitochondrial electron transport chain accounts for nearly 90% of the molecular O2 consumed by cells and tissues. ROS are generated as undesirable side products of the energy yielding oxidative metabolism, leading to mitochondrial and cellular damages (11). Detoxification of intracellular ROS is catalyzed by antioxidant molecules such as vitamin E and GSH as well as a number of enzyme systems, including superoxide dismutase, catalase, GSH peroxidase, and GST. Recently, it has been shown that transfection with GSTA4-4 cDNA protects cells against oxidative stress and HNE-induced apoptosis (12). Although mitochondria have enzyme systems to regulate ROS produced in the electron transfer chain (13), induced ROS production under various pathophysiological conditions can be overwhelming, leading to mitochondrial DNA and membrane damages. Regulation of ROS by intramitochondrial GSH and GSH-metabolizing enzymes would therefore be critical for maintaining the antioxidant potential and structural/functional integrity of the mitochondrial membrane.

A majority of GSTs in mammalian cells are localized in the cytosol, where they exist as homodimers or heterodimers of subunits with molecular masses from 24 to 29 kDa (14). However, GSTs have also been reported in microsomes (15, 16), nucleus (17), plasma membrane (18), and mitochondria (19–22). Various subcellular locations of GSTs are consistent with their role in protecting cell components against chemical and oxidative damage. Recently, we showed the presence of three different forms of GSTs belonging to the alpha and mu family, namely, GSTA1-1, A4-4 and M1-1 in mouse liver mitochondria (22). We have also provided evidence that the mitochondrial GSTA4-4 level markedly increases in COS cells under oxidative stress conditions, suggesting its critical role in maintaining the GSH homeostasis under conditions of chemical and oxidative stress (21, 22).
In the present study, we have investigated the mechanism of targeting of recombinant mouse GSTA4-4 to mitochondria. Using in vivo and in vitro systems, we show that the mitochondrial targeting is highly dependent on the intact C-terminal end as well as the phosphorylation status of the protein. Translocation of GSTA4-4 is vastly increased under increased intracellular PKA and PKC activities. Results suggest that phosphorylation increases the affinity of the protein for binding to cytoplasmic chaperone, Hsp70, as a possible basis for its enhanced mitochondrial targeting.

EXPERIMENTAL PROCEDURES

Materials—CAMP analog Bt.cAMP, PKA, 2,4-dinitrophenol; CCP; purified Hsp70 protein; monoclonal antibodies to Hsp70, to phosphoserine, and to phosphothreonine; and polyclonal antibody to β-actin were purchased from Sigma Chemical Co. (St. Louis, MO). PKA-specific inhibitor myristoylated peptide, H98, PKC, PKC-inhibitor GI109203X, H7, forskolin, and PMA were purchased from Calbiochem (San Diego, CA). H89 is a PKA-specific inhibitor, and H7 is a wide spectrum kinase inhibitor that inhibits both PKA and PKC at the level of 6 μM (23). Myristoylated peptide inhibitor is a highly specific PKA inhibitor targeted to its active site (24). Polyclonal antibody against β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal and monoclonal antibodies to Hsp70, to phosphothreonine, and polyclonal antibody to phosphoserine, and to phosphothreonine; and polyclonal antibody to purified Hsp70 protein; monoclonal antibodies to Hsp70, to phosphothreonine, and to phosphoserine; and polyclonal antibody to purified Hsp70 protein were provided by B. Mannevik (University of Upsala, Sweden) and D. Pain (University of Medicine and Dentistry of New Jersey, Newark, NJ), respectively.

Construction of Wild Type and Mutant cDNA Constructs and Fusions with DHFR—Full-length mouse GSTA4-4 cDNA was generated by reverse transcriptase-based PCR, using RNA extracted from mouse liver (25). N- and C-terminal deletions containing a start codon preceding by a Kozak consensus sequence were constructed essentially as described before (26). Mutant GSTA4-4 cDNA constructs with substitutions at the phosphorylation site and C-terminal-positively charged residues were constructed using a QuikChange site-directed mutagenesis kit supplied by Stratagene. These included T113A (Mut 113), T130A (Mut 130), S189A-T130A (Mut 189/130), and mutations at the C-terminal signal domain R217A/K221A (Mut RK/AA). The 172–222 sequence of GSTA4-4 was fused to the C terminus of mouse DHFR by in-frame ligation of DNA through an EcoRI linker as described before (27). The cDNAs were engineered to contain 5’ Xbal and 3’ Hind III restriction sites and cloned in pCMV4 and pGEM7zf (Promega Biotech, Madison, WI) vectors, for cell transfection and in vitro translation studies, respectively.

In Vitro Translation and Phosphorylation of GSTA4-4—In vitro translation of mouse GSTA4-4 cDNA was performed in WGL or RRL systems (Promega Biotech, Madison, WI) in the presence of added [35S]Met (40 μCi/50 μl reaction, PerkinElmer Life Sciences, Boston, MA) as described before (26). In some experiments, GSTA4-4 translation product was phosphorylated either by PKA or PKC. PKA-dependent phosphorylation was performed for 45 min at 30 °C in MgCH3COO COOH acetate buffer (pH 6.5) by adding 10 units of PKA and 100 μM ATP to the translation mix as described (28). PKC-dependent phosphorylation of GSTA4-4 was carried out for 45 min at 30 °C in 50 mM MES buffer (pH 6) containing 1 mM EGTA, 10 mM MgCl2, 10 units of PKC, and 100 μM ATP as recommended by the manufacturer (Calbiochem, San Diego, CA). In some cases, translation products were generated in the WGL in the absence of [35S]Met, and phosphorylation was carried out in the presence of [32P]ATP.

In Vitro Import of GSTA4-4 into Mitochondria—Mitochondria were isolated from rat liver and used for in vitro import using a system described before (26). The import assays (containing approx. 9,000 cpn of [35S]-labeled GSTA4-4) were carried out in a 200-μl final volume and treated with 0–300 μg/ml trypsin for 20 min on ice, as described before (26). Trypsin-treated samples were mixed with a 10-times excess of trypsin inhibitor, and mitochondria were recovered by sedimentation through 1 ml sucrose. Untreated samples were diluted with mitochondrial isolation buffer (1 ml) and similarly treated through sucrose cushion. Resulting mitochondrial pellets were washed twice with mitochondrial isolation buffer, and mitochondrial proteins were solubilized in 2× Laemmli buffer for 10 min at 75 °C and analyzed by SDS-PAGE (29) and fluorography.

Expression of GSTA4-4 cDNA in COS Cells and Isolation of Subcellular Fractions—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium to 50% confluency and GSTA4-4 cDNA cloned in pCMV4 vector was transfected using FuGENE, a non-liposomal transfection reagent (Roche Applied Science, Molecular Biochemicals, Indianapolis, IN) as described before (30). After 48 h of transfection, cells from six to eight plates (100 mm) were homogenized in sucrose-mannitol buffer containing protease inhibitor mixture with a Teflon–fitted glass homogenizer (about 25 strokes). Mitochondria were isolated by differential centrifugation and sucrose density banding as described before (26). The postmitochondrial supernatant was centrifuged for 1 h at 100,000 × g, and the cytosolic fraction was recovered in the supernatant.

Immunoprecipitation—Mitochondria and cytosol (100 μg each) from GSTA4-4-transfected COS cells were solubilized in 250 μl of phosphate-buffered saline containing 0.1% Triton X-100 and protease inhibitors. The 15,000 × g supernatant fraction was incubated with rabbit polyclonal GSTA4-4 antibody (5 μg of IgG) overnight at 4 °C. 30 μl of protein A-agarose beads was added to the sample and shaken for 3 h at room temperature. Beads were pelleted by centrifugation and washed three times with phosphate-buffered saline containing 0.1% Triton X-100 and protease inhibitors. Immunoprecipitated proteins were eluted from the protein A-agarose beads by heating at 95 °C for 5 min in 30 μl of 2× Laemmli buffer without added β-mercaptoethanol and analyzed by immunoblot using phosphoserine, phosphothreonine, and GSTA4-4 antibodies.

SDS-PAGE and Immunoblot Analysis—Proteins were resolved by SDS-PAGE (29) and transferred to a nitrocellulose membrane for immunoblot analysis as described previously (31). Immunoblots were developed with a chemiluminescence Super Signal Ultra kit (Pierce Chemical Co., Rockford, IL). Imaging and quantitation were performed using a Fluor-S imaging system (Bio-Rad, Hercules, CA).

GST Activity—GST activity in the COS cell mitochondrial and cytosolic extracts was assayed using CDNB and HNE as substrates following the procedures of Habig et al. (32) and Alin et al. (33), respectively.

Depletion of Hsp70 from WGL—GSTA4-4 was translated in the WGL as described above, and 25 μl of the translation mix was incubated for 30 min at room temperature with 25 μl of 5 μm urea in 50 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA. The mixture was dialyzed for 1 h against two changes of 20 mM Tris-HCl (pH 7.5) containing 1 mM MgCl2, 50 mM KCl, and 0.5 mM dithiorethiol. Monoclonal Hsp70 antibody (10 μg of IgG) was added and incubated overnight at 4 °C. The mixture was incubated for 3 h at room temperature with protein A-agarose beads under agitation, and the beads were removed by centrifugation. The supernatant was discarded, and the Hsp70 antibody was added and used as a GSTA4-4 translation product devoid of Hsp70.

Co-immunoprecipitation of Hsp70 with GSTA4-4—GSTA4-4 protein was synthesized in the WGL in the absence or the presence of PKC inhibitors (6 μM H7 or 50 mM GF109203X) or PKA inhibitors (50 nM H89 or 50 mM H8). Interaction of GSTA4-4 with Hsp70 from the WGL under these conditions was analyzed by co-immunoprecipitation. Reaction mixtures were immunoprecipitated with a polyclonal antibody to GSTA4-4 as described above and resolved by SDS-PAGE on a 12% gel. The proteins were transferred to a nitrocellulose membrane, and co-immunoprecipitated proteins were analyzed by fluorography and immunoblot analysis using mouse monoclonal antibody for Hsp70.

RESULTS

Mitochondrial Translocation of GSTA4-4—A recent study from our laboratory showed that the mitochondrial GSTA4-4 exhibits size and sequence properties similar to the cytosolic counterpart (22). In the present study, we first investigated the mitochondrial transport of GSTA4-4 using an in vitro mitochondrial import system in which resistance to limited protease digestion is used as a criterion for mitochondrial import (26, 30). Fig. 1 shows the in vitro import of 35S-labeled GSTA4-4 into isolated rat liver mitochondria. For comparison, in vitro translation was carried out either in RRL or in the WGL system. The in vitro translated GSTA4-4 protein showed two very closely migrating bands indicating alternative translation at immediately adjacent AUG codons. In both RRL and WGL systems, we obtained comparable translation efficiency (lanes 1 and 4), although the RRL products showed a surprisingly low (less than 2%) mitochondrial binding as well as import (lanes 2 and 3). The in vitro product from the WGL system, on the other hand, showed high level of binding (about 75% of the input counts, lane 5) and a high level of import (15% of input, lane 6). The reason for the poor import of translation products programmed in RRL remains unclear, although it is likely due to either a lack of essential protein factors for mitochondrial import of GSTA4-4 or rapid folding of the nascent...
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Fig. 1. Translocation of GSTA4-4 into mitochondria. A, in vitro transport of GSTA4-4 in rat liver mitochondria. 35S-Labeled GSTA4-4 translated either in RRL (lanes 1-3) or in WGL (lanes 4-14) was incubated with mitochondria. The extent of import was determined by resistance to treatment with trypsin (300 μg/ml), and mitochondria from treated and untreated samples were pelleted through 1 M sucrose. Solubilized proteins were resolved by SDS-PAGE on a 14% gel and subjected to fluorography. Std represents 50% of the input counts. Mitochondria were preincubated for 20 min on ice without (lanes 2, 3, 5, and 6) or with added 2,4-dinitrophenol (2,4-DNP, 25 μM, lanes 9 and 10), carbonyl cyanide-m-chlorophenylhydrazone (CCCP, 25 μM, lanes 11 and 12), or in the absence of added energy mix (EMIX, lanes 13 and 14) before initiating import reaction. Triton X-100 (0.1%) was added at the end of the transport reaction, before addition of trypsin (lanes 7 and 8). B, in vivo transport of GSTA4-4 in COS cell mitochondria. Mitochondria (80 μg) and cytosol (40 μg) from cells transfected with vector alone (mock) or GSTA4-4 were resolved by SDS-PAGE on 14% gels. When indicated, both fractions were subjected to trypsin treatment (5 μg of trypsin/100 μg of mitochondria for 20 min on ice). Proteins were transferred to nitrocellulose and probed with GSTA4-4 antibody. TIM44 and β-actin were used as loading controls and markers for determining cross-contamination of cytoplasmic and mitochondrial fractions. C, GST activities in mitochondria and cytosol. Mitochondrial and cytosolic fractions were prepared as in B. GST activity was measured as described under “Experimental Procedures” using CDNB or HNE as substrates.

Mitochondrial GSTA4-4 Is Hyperphosphorylated—Recent studies in our laboratory showed that CYP enzymes belonging to family 2, which are predominantly microsomal, are also targeted to mitochondria and that the bimodal targeting of these proteins is modulated by PKA-dependent phosphorylation (30, 34). GSTA4-4 contains a number of consensus sites for both PKA- and PKC-mediated phosphorylation (see the schematic at the top of Fig. 2). As shown in Fig. 2A, nascent GSTA4-4 translated in WGL was efficiently phosphorylated by adding PKA and PKC. Phosphorylation was apparent both in the presence of added PKC along with PKA inhibitor H89, or PKA along with PKC inhibitor GF109203X. PKA and PKC inhibitors together markedly reduced the level of phosphorylation. We also investigated the steady-state levels of Ser and Thr phosphorylation of cytosolic and mitochondrial GSTA4-4. As shown in Fig. 2B, GSTA4-4 was immunoprecipitated from both cell fractions, and the immunoprecipitates were subjected to immunoblot analysis with monoclonal antibodies to Ser-phosphate and Thr-phosphate. Fig. 2B shows that nearly the same amounts of immunoprecipitated GSTA4-4 from mitochondria and cytosolic fractions (left-most panel) show vastly varying levels of phosphorylation. As seen from the middle and right-most panels, mitochondrial GSTA4-4 was more heavily phosphorylated than its cytosolic counterpart.
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Role of Phosphorylation on Mitochondrial Targeting of GSTA4-4—The role of phosphorylation on mitochondrial targeting of GSTA4-4 was studied using in vitro and in vivo approaches. In the first approach, GSTA4-4 translated in WGL in the presence or absence of added PKA and PKC and, with or without added kinase inhibitors, was used for import in isolated rat liver mitochondria. Although not shown, none of the protein kinase inhibitors affected the translational efficiency of GSTA4-4 at the concentrations used. Results of in vitro import in Fig. 3A (lanes 1 and 2) show that translation products formed in the absence of added kinases or inhibitors bind to mitochondria efficiently (about 70% of input protein), although they were imported at a very low level (~6% of input cpm). Addition of PKA (10 units) during translation caused a marked increase of in vitro import to about 40% of input protein as indicated by resistance to trypsin treatment (Fig. 3A, lanes 3 and 4). Addition of PKC also caused a marked increase in import to about 60% of input (Fig. 3A, lanes 5 and 6). Inhibitors of PKA, H89 (50 nM), and MPI (a myristoylated peptide inhibitor of PKC (50 nM), PKC inhibitor GF109203X (50 nM), and PKA/PKC inhibitor H7 (6 µM) were added during translation of GSTA4-4. G, 35S-labeled translation products were generated in WGL in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of added PKA or PKC (lanes 5 and 6 without and lanes 7 and 8 without inhibitors). Unlike in A–F, inhibitors were added post-translationally and used for the import. H, 35S-labeled translation products were generated in WGL in the absence or presence of added PKA or PKC as in A–F. Mitochondria were incubated for 20 min with 50-nm H89 (lanes 3 and 4) or 50-nm GF109203X (lanes 5 and 6) without added inhibitors (lanes 1 and 2) and used for the import. In A–H, mitochondrial import, treatment with trypsin and recovery of mitochondria were as described under “Experimental Procedures” and Fig. 1. In all cases proteins were resolved by SDS-PAGE on a 14% gel and subjected to fluorography.
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Fig. 4. Effect of phosphorylation on in vivo targeting of GSTA4-4 in COS cells. A, the effect of PKA-dependent phosphorylation; B, the effect of PKC-dependent phosphorylation. COS cells were transfected with empty vector (mock) or with GSTA4-4, and mitochondria and cytosolic fractions were isolated as described under "Experimental Procedures." Mitochondrial (80 μg) and cytosolic proteins (40 μg) were resolved by SDS-PAGE on a 14% gel and subjected to Western blot analysis. Blots were sequentially probed with anti-GSTA4-4 antibody (1:2000 dilution) and with anti-TIM44 (1:1000 dilution) antibody for mitochondrial proteins or with anti-β-actin antibody (1:2000 dilution) for cytosolic proteins as loading controls. Bt2cAMP (10 μM) and forskolin (10 μM) were added 3 h after transfection. H89 (50 nM), MPI (50 nM), H7 (6 μM), and GF109203X (50 nM) were added 1 h before transfection. PMA (100 nM) was added 1, 3, or 24 h before harvesting the cells.

(Fig. 3, E and F, lanes 3–6). The PKA and PKC inhibitors as well as the general kinase inhibitor H7 inhibited both mitochondrial binding and import of GSTA4-4 synthesized in the control WGL system, suggesting that the low level of import seen in Fig. 3A (lane 2) may be due to a low level of endogenous kinases (Fig. 3, B–F, lanes 1 and 2).

The use of translation mix containing PKA or PKC in the in vitro import experiments in Fig. 3A above leaves open the possibility that phosphorylation of mitochondrial membrane proteins also contribute to the increased import. We carried out two types of experiments to test this possibility. In the first approach, the residual PKC and PKA activities at the end of post-translational phosphorylation were quenched by adding specific inhibitors, and the translation mix in the presence of inhibitors was used for in vitro import. As seen from Fig. 3G, control translation products without added kinase and kinase inhibitor (lanes 1 and 2) yielded a low level of transport. Translation products phosphorylated with PKA and its specific inhibitor H89 (lanes 3 and 4), PKC and its inhibitor GF109203X (lanes 5 and 6), or with both kinases and both inhibitors yielded nearly 50–60% import. This same level of import was obtained when kinase reactions were not terminated (see Fig. 3A). In the second approach, control untreated mitochondria and those preincubated with H89 or GF109203X were used for protein import. As seen from Fig. 3H, the levels of import of control translation product or those phosphorylated with PKA or PKC had imported levels correspondingly similar in untreated mitochondria (Fig. 3, A and G). Additionally, PKA and PKC inhibitors did not affect the import of mitochondrial cytochrome oxidase subunit Vb, which contains a cleavable N-terminal targeting signal (results not shown). These results suggest that the increased import in post-translationally phosphorylated translation products is not due to indirect effects of kinases on mitochondrial translocase proteins.

The role of PKA- and PKC-dependent phosphorylation on in vivo targeting of GSTA4-4 to mitochondria was investigated using COS cells transfected with GSTA4-4 cDNA (Fig. 4). Cells were treated with PKA inducers or inhibitors (Fig. 4A) and harvested 48 h post-transfection, and the levels of GSTA4-4 in the subcellular fractions were determined by immunoblot analysis (Fig. 4A). As described before, the cell fractionation procedure yielded mitochondrial preparations with less than 1% extramitochondrial contamination as tested by marker enzyme assays (22, 30, 35). Addition of the cAMP analog, Bt2cAMP (10 μM), or cAMP inducer, forskolin (10 μM), increased the mitochondrial targeting of GSTA4-4 by 40 and 70%, respectively (Fig. 4A, left panel). The cytosolic GSTA4-4 pool under these conditions was reduced by 50–60% (Fig. 4A, right panel). Addition of H89 (50 nM) or MPI (50 nM) decreased the mitochondrial GSTA4-4 level to that of control cells, whereas the level of cytosolic GSTA4-4 was increased markedly.

We used the PKC inducer PMA to assess the effects of PKC on in vivo mitochondrial targeting of GSTA4-4. In all cases, cells were harvested 48 h post-transfection, and PMA (100 nM) was added 1, 3, or 24 h before harvesting. As shown in Fig. 4B, addition of PMA for 1 h caused a rapid increase of intramitochondrial GSTA4-4 with a corresponding decrease in the cytosolic fraction. At longer durations of 3 and 24 h, the mitochondrial GSTA4-4 content steadily declined with corresponding increases in the cytosolic GSTA4-4. These results suggest that PMA-mediated phosphorylation causes a rapid redistribution of cytosolic GSTA4-4 by channeling it to mitochondrial compartment. Consistent with this possibility, PKC-specific inhibitor GF109203X as well as general kinase inhibitor H7 markedly retarded the PMA-mediated increase in mitochondrial GSTA4-4 (Fig. 4B).

Localization of Mitochondrial Targeting Signal of GSTA4-4 at the C Terminus—With the goal of localizing the mitochondrial targeting signal of GSTA4-4, we generated deletion cDNA constructs, which express proteins lacking the first 19 amino acids at the N terminus (GSTA4NT) and 19 amino acids at the C terminus (GSTA4CT) of GSTA4-4. Both of these segments contain positively charged residues that are characteristics of mitochondrial-targeting signals (Fig. 5A). Wild type GSTA4-4 and the two deletion constructs were transfected in COS cells (Fig. 5B), and translation products were used for in vitro import
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assay (Fig. 5, B and C). Our results show that the N-terminal deletion had no effect on the mitochondrial targeting of the protein, whereas the C-terminal deletions inhibited it in vitro (Fig. 5B) as well as in vitro (Fig. 5C) transport, indicating that the targeting signal might reside at the C terminus of GSTA4-4. Interestingly, the N-terminal deletion was not recovered in the cytosolic compartment, suggesting that it may be unstable and/or degraded in the cytosol. Because positively charged residues at 217 and 221 are within the putative mitochondrial targeting signal region, we tested the double mutant of R217A/K221A (GSTRA/AA) protein in the in vitro transport assay. Fig. 5D shows that the GSTRA/AA mutant protein synthesized in the absence of kinases was imported very poorly. The proteins synthesized in the presence of PKA and PKC were imported at a slightly higher level of about 8%. This rate, however, is markedly lower than the 40–50% import with WT protein (Fig. 3). The residual activity with the GSTRA/AA mutant may be a result of two additional positive residues at positions 204 and 205. The results of protein transport with deletion and point mutations show that the C-terminal 20-amino acid stretch functions as a cryptic mitochondrial targeting signal.

Phosphorylation Sites of GSTA4-4 That Are Critical for Mitochondrial Targeting—As shown in Fig. 6A, the mouse GSTA4-4 contains five PKA and PKC target sites. Deletion studies shown in Fig. 5 (B and C) showed that the N-terminal 19-amino acid stretch of GSTA4-4 does not play a significant role in mitochondrial import suggesting that the PKA/PKC site at Ser-18 may not be important for mitochondrial transport. We therefore generated T113A and T193A (Mut113 and Mut130) and a double mutant of S189A and T193A (Mut189/193) constructs as shown in Fig. 6A. It is seen that the in vitro transport of both Mut113 and Mut130 proteins (Fig. 6B) exhibit PKA- and PKC-mediated increase in transport similar to the WT protein. Furthermore, PKA inhibitor, H89, and PKC inhibitor, GF190203X, inhibited the specific kinase-mediated increase of in vitro import. The double mutant Mut189/193, on the other hand, showed a vastly reduced mitochondrial binding and mitochondrial import. Post-translational incubation with PKA and PKC had no significant effect on the import of this protein. In support of our studies with CYP2B1 and CYP2E1 (30, 34), these results suggest that the PKA and PKC sites closest to the C-terminal targeting signal are important for mitochondrial targeting and that phosphorylation at these positions may have a role in the activation of cryptic signal.

Phosphorylation Enables the Cryptic Mitochondrial Signal to Target Heterologous Proteins to Mitochondria—To further ascertain the mitochondrial targeting property of the C-terminal end of GSTA4-4 and the role of phosphorylation at the Ser-189 and Thr-193 sites, we generated fusion constructs with DHFR. The construct contained sequence 172–222 of GSTA4-4, which includes the PKA and PKC phosphorylation sites, fused to DHFR (Fig. 7A). The second construct consisted of sequence 172–222 of GSTA4-4 with mutated Ser-189 and Thr-193 residues fused to DHFR (Fig. 7A). Results of in vitro import in Fig. 7B show that the fusion construct DHFR-C50GST was im-

![Image](https://example.com/image1.png)

**Fig. 5.** Mutational analysis for localizing the mitochondrial targeting signal. A, schemes of N- and C-terminal deletions and point mutations at the positively charged residues at the C terminus. B, COS cells were transfected with either empty vector (mock) or with cDNA for GST WT, GST½NT, or GST½CT, and subcellular fractions were isolated as described under “Experimental Procedures.” Mitochondrial (80 μg) and cytosolic proteins (40 μg) were resolved by SDS-PAGE on a 14% gel and subjected to Western blot analysis. The blot was probed with anti-GSTA4-4 antibody (1:2000 dilution). C, GST WT, GST½NT, and GST½CT were translated in the WGL in the presence of [35S]Met with or without PKA and used for in vitro import into rat liver mitochondria. D, mutant protein GSTMutRK/AA with substituted Arg-217 and Lys-221 was translated in the presence of [35S]Met in the absence (lanes 1 and 2) or the presence of added PKA (lanes 3 and 4) or PKC (lanes 5 and 6) and used for import in isolated rat liver mitochondria. The proteins synthesized in the absence of kinases was imported very poorly. This rate, however, is markedly lower than the 40–50% import with WT protein (Fig. 3). The residual activity with the GSTMutRK/AA mutant may be a result of two additional positive residues at positions 204 and 205. The results of protein transport with deletion and point mutations show that the C-terminal 20-amino acid stretch functions as a cryptic mitochondrial targeting signal.

**Fig. 6.** PKA and PKC target sites of GSTA4-4 critical for mitochondrial targeting. A, schemes depicting the mutations at consensus PKA and PKC sites. B, [35S]-labeled translation products were generated in WGL in the absence (lanes 1 and 2), presence of added PKA (lanes 3–6), or PKC (lanes 7–10). Inhibitors H89 (50 nm) (lanes 3 and 4) and GF190203X (lanes 7 and 8) were also added during translation and used for import in rat liver mitochondria. Mitochondria from companion sets of reactions were treated with trypsin (300 μg/ml), both treated and untreated mitochondria were recovered by sedimentation through 1 μ sucrose as described in Fig. 1, and the proteins were resolved by SDS-PAGE on a 14% gel and subjected to fluorography.
ported at a high level of 50–60% when phosphorylated with PKA or PKC. The fusion construct (DHFR-C50GST/Mut189, 193), carrying mutations at the phosphorylation sites, was imported poorly even when the protein was subjected to post-translational phosphorylation with PKA or PKC.

The mitochondrial targeting of the fusion proteins under in vivo conditions was tested by expressing the proteins in COS cells by cDNA transfection and by probing the cell fractions with antibody to DHFR. Fig. 7 shows the immunoblot analysis of cytosolic and mitochondrial fractions with DHFR antibody from cells transfected with the DHFR-C50GST construct and treated with either inducers or inhibitors of cAMP. It is seen (upper panels) that the mitochondrial fusion protein content was increased 2- to 3-fold by Bt2cAMP and forskolin as compared with control cells and was reduced to near control cell level by H89 and MPI, known inhibitors of PKA. The cytoplasmic fraction (Fig. 7A, upper panel), on the other hand, showed a relatively high DHFR fusion protein level but markedly reduced levels in cells treated with Bt2cAMP and forskolin. Additionally, treatment with H89 and MPI reversed the PKA-mediated reduction of fusion protein in the cytoplasm.

These results suggest that, similar to that observed with WT GSTA4-4, PKA and PKC treatment cause a reduction in the mitochondrial targeting of the fusion protein by PKA- and PKC-mediated phosphorylation. However, there was no significant change in the level of mitochondrial TIM44 and cytoplasmic β-actin used as loading controls. The fusion construct DHFR-C50GST/Mut189/193, on the other hand, was not targeted to mitochondria in control cells as well as in cells treated with cAMP inducers and inhibitors. The level of this protein in the cytoplasm remained at relatively high and constant level under all the treatment conditions, suggesting that the protein does not respond to cellular PKA levels.

Fig. 8A shows the effects of PKC inducers and inhibitors on the mitochondrial and cytoplasmic levels of the fusion proteins as well as DHFR-C50GST and its 189/193 mutant. The relative distribution under different treatment conditions is nearly similar to that with PKA inducers and inhibitors shown above. In confirmation with the results of in vitro transport in Fig. 7, these results show that the C-terminal 50-amino acid sequence of GSTA4-4, when fused in the same orientation as the parent GSTA4-4, not only directs the mitochondrial targeting of a heterologous protein DHFR but also imparts responsiveness to PKA and PKC.

Phosphorylated GSTA4-4 Exhibits a Higher Affinity for Binding to Cytosolic Hsp70—Binding of newly synthesized proteins to the cytoplasmic Hsp70 family chaperones is an important first step of mitochondrial targeting, which ensures efficient presentation of properly unfolded polypeptide to the mitochondrial translocase proteins (36). With the goal of understanding the role of phosphorylation on mitochondrial targeting, we investigated its role on GSTA4-4 binding to cytoplasmic Hsp70 proteins. We examined the role of Hsp70 at two different levels: 1) during the translation of GSTA4-4 protein and 2) post-translationally, during the import process. When GSTA4-4 was translated in WGL in the presence of a monoclonal antibody to Hsp70, the translation efficiency was reduced by 2- to 3-fold (Fig. 9A, lanes 1 and 2). In lanes 3–10 (Fig. 9A), we compared the rate of in vitro import of GSTA4-4 synthesized in the presence and absence of added antibody (80,000 cpm each). It is seen that addition of Hsp70 antibody to the translation mix strongly inhibited both binding and import of the protein, even when the protein was phosphorylated (Fig. 9A, lanes 9 and 10). Addition of mouse IgG affected neither the binding nor the import into mitochondria (not shown). The GSTA4-4 synthesized in the absence of added Hsp70 antibody
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35S-Labeled GSTA4-4 translation products were generated in the absence (lanes 1–4) as well as presence of PKA (lanes 5–10) inhibited the in vitro import in a concentration-dependent manner (Fig. 9B), whereas mouse IgG had no effect (not shown). Although not shown, the import competence of GSTA4-4 was not restored by the addition of pure Hsp70 protein, probably due to excess of antibody present in the translation mixture. In the second series of experiments, therefore, the translation mixture was depleted of Hsp70 as well as the residual antibody by adsorption to protein A-Sepharose beads. To reduce the loss of GSTA4-4 bound to Hsp70 during immunodepletion, the translation mixture was subjected to urea treatment followed by dialysis before immunodepletion. Fig. 9C shows the immunoblot analysis of translation products in the presence of PKA at different steps of immunodepletion (left panel) and autoradiography of the same gel (right panel) to determine the levels of 35S-labeled GSTA4-4. As seen from Fig. 9C, immunodepleted lysate lacks detectable Hsp70 (left panel, lane 2) as compared with control lysate (left panel, lane 1). As expected, the protein adsorbed to the protein A-Sepharose beads contains Hsp70 (left panel, lane 3) and a number of unidentifiable proteins. The autoradiogram (Fig. 9C, right panel) shows that nearly 50% of GSTA4-4 was lost even when the lysate was subjected to urea treatment (lane 2), which was nearly quantitatively recovered in the protein A-Sepharose-bound fraction (lane 3). Fig. 9D shows the mitochondrial import of PKA-phosphorylated GSTA4-4 from control and Hsp70-depleted lysates. It is seen that control WGL supported both mitochondrial binding and import of GSTA4-4 efficiently (lanes 1 and 2). However, both mitochondrial binding and import of GSTA4-4 were drastically reduced with Hsp70-depleted lysates (lanes 3 and 4). Furthermore, addition of purified Hsp70 restored both binding and import, suggesting its direct role in the mitochondrial import of GSTA4-4 (lanes 5 and 6).

In the second series of experiments, we investigated the effect of phosphorylation on the affinity of GSTA4-4 for binding to Hsp70 protein. GSTA4-4 was translated and phosphorylated (PKA and PKC) in WGL in the absence or presence of specific inhibitors, and the in vitro translation products were immunoprecipitated with GSTA4-4 antibody. In some cases, the immunoprecipitation was carried out with pre-immune serum or antibody to β-actin as negative controls. The immunoprecipitated proteins were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membrane. The blot was successively probed with anti-Hsp70 antibody (left panel) and subjected to fluorography (right panel). D, requirement of Hsp70 for the in vitro import of GSTA4-4 into rat liver mitochondria. 35S-Labeled GSTA4-4 translation products were generated in the presence of PKA. Untreated translation products (lanes 1 and 2) or Hsp70-depleted translation products (lanes 3–6) were used for mitochondrial import. Pure Hsp70 (2 μg) was re-added just before the import reaction (lanes 5 and 6). Details of mitochondrial transport, treatment with trypsin, and recovery of treated and untreated mitochondria in A, B, and D were as described in Fig. 1.

was imported in a significant amount, and the import was further enhanced by PKA (Fig. 9A, lanes 3–6). Although not shown, complete depletion of Hsp70 from the WGL by immunoadsorption resulted in a vastly reduced GSTA4-4 translation, suggesting that Hsp70 is required for the synthesis and/or stabilization of GSTA4-4 protein.

To understand the role of Hsp70 in the mitochondrial import of GSTA4-4, we carried out two types of experiments. In the first series, Hsp70 antibody was added to the WGL following complete synthesis of GSTA4-4 and just prior to the start of in vitro import reaction. Addition of antibody to translation products programmed in the absence (lanes 1–4) as well as presence of PKA (lanes 5–10) inhibit the in vitro import in a concentration-dependent manner (Fig. 9B), whereas mouse IgG had no effect (not shown). Although not shown, the import competence of GSTA4-4 was not restored by the addition of pure Hsp70 protein, probably due to excess of antibody present in the translation mixture. In the second series of experiments, therefore, the translation mixture was depleted of Hsp70 as well as the residual antibody by adsorption to protein A-Sepharose beads. To reduce the loss of GSTA4-4 bound to Hsp70 during immunodepletion, the translation mixture was subjected to urea treatment followed by dialysis before immunodepletion. Fig. 9C shows the immunoblot analysis of translation products in the presence of PKA at different steps of immunodepletion (left panel) and autoradiography of the same gel (right panel) to determine the levels of 35S-labeled GSTA4-4. As seen from Fig. 9C, immunodepleted lysate lacks detectable Hsp70 (left panel, lane 2) as compared with control lysate (left panel, lane 1). As expected, the protein adsorbed to the protein A-Sepharose beads contains Hsp70 (left panel, lane 3) and a number of unidentifiable proteins. The autoradiogram (Fig. 9C, right panel) shows that nearly 50% of GSTA4-4 was lost even when the lysate was subjected to urea treatment (lane 2), which was nearly quantitatively recovered in the protein A-Sepharose-bound fraction (lane 3). Fig. 9D shows the mitochondrial import of PKA-phosphorylated GSTA4-4 from control and Hsp70-depleted lysates. It is seen that control WGL supported both mitochondrial binding and import of GSTA4-4 efficiently (lanes 1 and 2). However, both mitochondrial binding and import of GSTA4-4 were drastically reduced with Hsp70-depleted lysates (lanes 3 and 4). Furthermore, addition of purified Hsp70 restored both binding and import, suggesting its direct role in the mitochondrial import of GSTA4-4 (lanes 5 and 6).

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Fig. 10. Effect of protein kinases inhibitors on the affinity of GSTA4-4 for binding to Hsp70. A, WT GSTA4-4 protein was translated and phosphorylated in WGL in the absence or presence of GF109203X (50 nM), H7 (50 nM), MPI (50 nM), H89 (50 nM), or a combination of GF109203X and MPI. Translation products (25 μl) were immunoprecipitated either with pre-immune serum, non-related antibody or with antibody against GSTA4-4 and separated by SDS-PAGE on 12% gel. The upper part of the gel (containing Hsp70) was transferred to nitrocellulose and immunoblotted with Hsp70 monoclonal antibody. The bottom part of the gel (containing GSTA4-4) was subjected to autoradiography. B, reduced binding of phosphorylation site mutant of GSTA4-4, Mut189/193 to Hsp70. 2S-Labeled translation products of WT GSTA4-4 (lane 1), Mut189/193 (lane 2), and Mut130 (lane 3) were immunoprecipitated with mouse monoclonal Hsp70 antibody, resolved by SDS-PAGE on 12% gel, transferred to nitrocellulose membrane, and analyzed by immunoblot using GSTA4-4 antibody or mouse monoclonal antibody for Hsp70. The levels of input WT and mutant GSTA4-4 proteins are presented in the bottom panel.

system and phosphorylated post-translationally when both PKA and PKC were added. The translation mix was immunoprecipitated with Hsp70 antibody, and the immunoprecipitates were probed with Hsp70 antibody and GSTA4-4 antibody by immunoblot analysis. It is seen from Fig. 10B that in all three cases nearly similar amounts of Hsp70 protein were immunoprecipitated. However, the levels of GSTA4-4 protein varied markedly. Wild type and Mut130 proteins were co-immunoprecipitated at comparably high levels, whereas the Mut189/193 protein was co-immunoprecipitated at very low levels. These results provide confirmatory evidence that phosphorylation at Ser-189/Thr-193 is important for binding to Hsp70.

DISCUSSION

Proteins destined to the mitochondrial compartment carry a specific targeting signal that is selectively recognized by the mitochondrial protein translocation machinery. A majority of the mitochondrial targeting signals are N-terminal extensions that are cleaved by mitochondrial matrix protease. Nevertheless, uncleaved signals at the N terminus, internal sites, or C terminus of the protein chain have also been reported (34). In a limited number of cases where the same gene product is targeted to the mitochondrial and cytoplasmic compartments, mRNA encoding the mitochondrial protein is thought to be transcribed from an upstream transcription start site so as to include an N-terminal mitochondrial-targeting signal. In extension of the concept that a given protein is targeted to a specific subcellular membrane site based on its specific targeting signal, we showed that several members of the CYP family 1 and 2 heme proteins are targeted to both the ER and mitochondrial compartments (26, 30, 34). Dimodal targeting of CYP apoproteins to the ER and mitochondria was modulated by the N-terminal chimeric signal sequence, which requires activation either by endoproteolytic processing, as in the case of CYP1A1 (26), or PKA-mediated phosphorylation at an internal site as in the case of CYP2B1 and 2E1 (30, 34). In the present study we show that GSTA4-4, an important member of cytosolic phase II drug-metabolizing enzymes, is also targeted to mitochondria and that its mitochondrial localization is modulated by both PKA- and PKC-dependent phosphorylation.

Using in vitro and in vivo transport methods (Figs. 1 and 3), we have presented compelling evidence that GSTA4-4 localizes inside the mitochondrial membrane system particularly when cellular PKA or PKC activities are induced. We have used a series of approaches to dispel the possibility that GSTA4-4 associated with the mitochondrial fraction represents a cross-contaminant from the cytosolic fraction: 1) mitochondrial preparations were devoid of β-actin, a cytosolic-specific marker but contained mitochondrial-specific membrane protein TIM44 and soluble matrix protein mtTFA, 2) cytosolic GSTA4-4 from transfected COS cells was readily degraded by limited digestion with trypsin, whereas mitochondrial associated GSTA4-4 was resistant to this treatment, and 3) immunohistochemical studies with transfected COS cells showed that a significant amount of immunostained GSTA4-4 was co-localized with punctate membrane structures that were also stained with antibody to mitochondrial genome encoded CytoX I protein (22). Furthermore, treatment with PMA, an inducer of PKC, or forskolin, an inducer of PKA, intensified the staining of punctate structures with GSTA4-4 antibody suggesting increased mitochondrial localization (not shown). Results of this study also suggest that full-length GSTA4-4 translocated to mitochondria is catalytically active as indicated by its ability to conjugate CDNB and HNE.

Mitochondrial compartment is an important subcellular site for ROS production as part of the electron transport chain coupled oxidative metabolism (38). We, as well as others, have shown the occurrence of GST isofoms in the hepatic and brain mitochondria. Our recent study showed that three of the mitochondrial GSTs exhibit size and sequence similar to those of the cytosolic GST counterparts, suggesting that the same primary translation product is translocated to mitochondria (22). In this study we investigated mechanisms of mitochondrial targeting of GSTs using GSTA4-4 as a model system. The choice of this form was based on its known physiological function in the metabolic detoxification of HNE and related end product of lipid peroxidation in human (39), rat (21), and mouse (22, 40). A recent study showed that cytosolic and mitochondrial isoenzymes of fumarase in yeast exhibit identical size and N-terminal sequence and are encoded by the same gene (41). Interestingly, the primary translation product of fumarase with N-terminal extension is first targeted to mitochondria, and following N-terminal processing by the matrix metalloprotease, part of the protein pool is shuttled back to the cytosol (41). However, currently there is no evidence for the expression of GSTs with N-terminal extensions (22). We therefore investigated the possibility of protein phosphorylation playing a role in mitochondrial targeting of GSTA4-4, by a mechanism simi-
lar to that shown for CYP2B1 and 2E1 (30, 34).

There is no well documented evidence either for the phosphorylation of any of the soluble GST isoforms, regulation of their expression, or modulation of activity by phosphorylation. However, GSTA4-4 (and other cytosolic GSTs) contains a number of consensus sites for both PKA and PKC (see Fig. 2). Results presented in this study show for the first time that GSTA4-4 is the target for both PKA- and PKC-mediated phosphorylation. Additionally, in both transfected COS cells and in mouse liver, the mitochondrial GSTA4-4 contains a high level of both Ser and Thr phosphorylation, whereas the cytosolic counterparts contained no detectable phosphorylation. These results suggest that phosphorylation plays a direct role in the distribution of soluble GST pool between the cytosolic and mitochondrial compartments. These results are consistent with our previous study showing an increase of mitochondrial GSTA4-4 and parallel decrease in the cytosolic compartment of COS cells subjected to oxidative stress by treatment with HNE — 44). Preliminary results on enzyme activity with different substrates show no detectable difference in the specific activities of enzymes from the mitochondrial and cytosolic GSTs, suggesting that phosphorylation may not be a major factor in their catalytic function. However, results of both in vitro import and in vivo transport in transfected COS cells clearly show that PKA- and PKC-mediated phosphorylation plays a direct role in mitochondrial translocation of the protein. Because mitochondria are the direct targets of chemical and oxidative stress conditions, which also induce cellular kinase activities (42–44), we postulate that phosphorylation induced mitochondrial translocation of GSTA4-4, and possibly other GSTs represent an important cellular defense system.

The cryptic mitochondrial targeting signals of CYP1A1, 2B1, and 2E1 have one common feature in that they precede the hydrophobic ER targeting/transmembrane anchor domains (26, 30, 34). The cryptic signals, however, vary markedly in terms of overall secondary structure and number as well as relative positions of positively charged residues that are critical for mitochondrial targeting. Both the N- and C-terminal 20- to 25-amino acid regions of GSTA4-4 show sequence properties reminiscent of the cryptic mitochondrial targeting sequences of CYPs with variable distribution of positive residues. Deletion studies, however, showed that the N-terminal 19 residues had no effect on the mitochondrial targeting under both in vitro and in vivo conditions, although deletion of the C-terminal 19 residues vastly reduced its mitochondrial entry, suggesting its role in mitochondrial targeting. Consistent with other cleaved and uncleaved mitochondrial targeting signals, the positively charged residues Arg-217 and Lys-221 of the cryptic C-terminal signal of GSTA4-4 are important for mitochondrial targeting of protein. Direct support for the mitochondrial targeting property of the C-terminal end of GSTA4-4 comes from experiments showing that the 50-amino acid sequence from the C terminus can drive the mitochondrial targeting of a cytosolic protein, DHFR.

Mutational analysis of putative phosphorylation sites (Fig. 5) showed that two closely located PKA and PKC target sites at Ser-189 and Thr-193 are critical for signal-mediated shuttling of cytosolic GSTA4-4 to the mitochondrial compartment. The concept of activation of cryptic signals for protein targeting is relatively new and emerged mainly from our studies on CYP2B1 and 2E1 targeting to mitochondria. The present results support and extend this concept, because both PKA- and PKC-dependent phosphorylation appears to activate the putative signal. Additionally, similar to that shown for CYPs, the overlapping PKA/PKC sites closest to the cryptic signal at the C-terminal most 20-amino acid region are important for activation and subsequent mitochondrial targeting of the protein. The precise mechanism by which phosphorylation activates the cryptic targeting signal remains unclear, although it may be associated with conformational changes in the protein as shown for CYP2B1 and 2E1 (30, 34).

The cytoplasmic Hsp70 and related chaperones are thought to play important roles in mitochondrial protein targeting, particularly in unfolding the newly synthesized protein in the cytosol, maintaining it in an import-competent state and presenting the protein to mitochondrial import. The cytoplasmic Hsp70 and related chaperones are thought to play important roles in mitochondrial protein targeting, particularly in unfolding the newly synthesized protein in the cytosol, maintaining it in an import-competent state and presenting the protein to mitochondrial import. The precise mechanism by which phosphorylation activates the cryptic targeting signal remains unclear, although it may be associated with conformational changes in the protein as shown for CYP2B1 and 2E1 (30, 34). An important observation of the present study is that cytoplasmic Hsp70 is critical for both efficient translation of GSTA4-4 under in vitro conditions and for the mitochondrial import. Selective immunodepletion of WGL resulted in vastly reduced translation rates and in vitro mitochondrial import of both phosphorylated and non-phosphorylated GSTA4-4. Addition of purified Hsp70 fully restored the in vitro import competence of the protein further establishing its role in mitochondrial targeting of GSTA4-4. Our results are in general agreement with previous studies showing the requirement for Hsp70 for efficient translation of precursor protein and/or mitochondrial import under in vitro conditions (36, 37, 45, 48). We hypothesize that phosphorylation-induced conformational changes in GSTA4-4 increase its affinity for binding to Hsp70, which in turn helps present the protein to mitochondrial translocase complexes in an import-competent conformation. We also observed a major difference in the import competence of GSTA4-4 translated in the RRL and WGL, the former being an unsupportive system for in vitro transport of GSTA4-4. Interestingly, addition of 4 M urea to RRL-translated protein vastly increased the import competence (not shown). It is known that urea treatment and associated protein unfolding improve the import competence of some proteins (48, 49). Based on this we postulate that reticulocyte Hsp70 either binds to GSTA4-4 inefficiently or is unable to properly unfold the nascent chains, thus...
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contributing to inefficient import of this protein. In summary, we provide direct experimental evidence for the mitochondrial transport of GSTA4-4 and its organization in the mitochondrial matrix compartment in a catalytically active form. Our results demonstrate that phosphorylation of GSTA4-4 in the cytoplasmic compartment increases its affinity for binding to cytosolic Hsp70, which might be the basis for increased mitochondrial localization under increased cellular PKA and PKC activities. Based on these in vivo and in vitro results we present a model for GSTA4-4 targeting to mitochondria under different physiological conditions (Fig. 11). This study also sheds light on mechanisms by which mitochondrial GSTA4-4 is modulated under chemical or oxidative stress conditions.

Acknowledgments—We thank Drs. D. Pain and B. Mannervik for providing antibodies to TIM44 and GSTA4-4, respectively. We also thank members of the Avadhani laboratory for valuable suggestions and criticisms.

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Phosphorylation Enhances Mitochondrial Targeting of GSTA4-4 through Increased Affinity for Binding to Cytoplasmic Hsp70
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J. Biol. Chem. 2003, 278:18960-18970.
doi: 10.1074/jbc.M301807200 originally published online March 19, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M301807200

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