Weak mitochondrial targeting sequence determines tissue-specific subcellular localization of glutamine synthetase in liver and brain cells

Gideon D. Matthews¹, Noa Gur¹, Werner J. H. Koopman², Ophry Pines³ and Lily Vardimon¹,*

¹Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, 69978 Tel Aviv, Israel
²Department of Biochemistry, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
³Department of Microbiology and Molecular Genetics3, IMRIC, Faculty of Medicine, Hebrew University of Jerusalem, 91120 Jerusalem, Israel

*Author for correspondence (vardi@post.tau.ac.il)

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Summary
Evolution of the uricotelic system for ammonia detoxification required a mechanism for tissue-specific subcellular localization of glutamine synthetase (GS). In uricotelic vertebrates, GS is mitochondrial in liver cells and cytoplasmic in brain. Because these species contain a single copy of the GS gene, it is not clear how tissue-specific subcellular localization is achieved. Here we show that in chicken, which utilizes the uricotelic system, the GS transcripts of liver and brain cells are identical and, consistently, there is no difference in the amino acid sequence of the protein. The N-terminus of GS, which constitutes a ‘weak’ mitochondrial targeting signal (MTS), is sufficient to direct a chimeric protein to the mitochondria in hepatocytes and to the cytoplasm in astrocytes. Considering that a weak MTS is dependent on a highly negative mitochondrial membrane potential (ΔΨ) for import, we examined the magnitude of ΔΨ in hepatocytes and astrocytes. Our results unexpectedly revealed that ΔΨ in hepatocytes is considerably more negative than that of astrocytes and that converting the targeting signal into ‘strong’ MTS abolished the capability to confer tissue-specific subcellular localization. We suggest that evolutional selection of weak MTS provided a tool for differential targeting of an identical protein by taking advantage of tissue-specific differences in ΔΨ.

Key words: Glutamine synthetase, Mitochondrial targeting signal, MTS, Uricotelic vertebrates, Hepatocytes, Astrocytes, Mitochondrial membrane potential

Introduction
Glutamine synthetase [GS; L-glutamate-ammonia ligase (ADP forming); EC6.3.1.2] catalyzes the ATP-dependent formation of glutamine from glutamate and ammonia and therefore plays an important role in ammonia detoxification and in the recycling of the neurotransmitter glutamate. In neural tissues, GS expression is confined to astrocytes and is cytoplasmic in all examined vertebrates (Kennedy et al., 1974; Linser and Moscona, 1983; Smith and Campbell, 1983; Smith and Campbell, 1987). However, the subcellular localization of GS in liver cells is dependent on the ammonia detoxification system used. In higher vertebrates, such as mammals, which utilize the ureotelic system of ammonia detoxification, GS is cytoplasmic in cells of both liver and neural tissue (Smith and Campbell, 1988; Wu, 1963). By contrast, in the marine elasmobranchs, such as dogfish shark, which utilize the ureosmotic system of ammonia detoxification, GS is cytoplasmic in neural tissue but mitochondrial in liver cells (Smith et al., 1987). Mitochondrial localization of hepatic GS is also required for the uricotelic system of ammonia detoxification. This system has apparently evolved as a water-conserving mechanism in the dinosaurs and their kin, and is utilized today by several species including birds (Campbell et al., 1987). In this system, intramitochondrially created ammonia is converted to glutamine by the action of GS, and this is followed by synthesis of the excreted final product, uric acid (Vorhaben and Campbell, 1972). The hepatic GS in these species is confined to the mitochondrial matrix of all liver cells (Smith and Campbell, 1987; Vorhaben and Campbell, 1972; Vorhaben and Campbell, 1977). Thus, uricotelic and ureosmotic vertebrates share a common trait of targeting the GS enzyme to the mitochondria in liver cells and cytoplasm in neural tissue.

Proteins targeted to the mitochondrial matrix are often expressed as a pre-polypeptide with an N-terminal extension that contains a mitochondrial targeting signal (MTS). These targeting presequences are commonly between 10 and 80 amino acids long and are capable of forming an amphipathic α-helix that is rich in basic and hydroxyl residues and lacks acidic residues (von Heijne, 1986). Import into the matrix is dependant on two energy sources: the mitochondrial membrane potential (ΔΨ), which exerts an electrophoretic pulling force on the positively charged MTS, and ATP hydrolysis in the matrix (Mokranjac and Neupert, 2008). Once inside the matrix, the MTS sequences are proteolytically cleaved, although in a number of cases the signals remain uncleaved (Neupert, 1997).

In the dogfish shark, which utilizes the ureosmotic system for ammonia detoxification, we have recently found that the differential targeting of GS is achieved by tissue-specific alternative splicing (Matthews et al., 2005). This species contains a single GS gene, but express two isoforms of the GS protein: a large isoform that occurs mainly in the mitochondrial compartment of liver cells, and a smaller isoform in the cytoplasm of brain cells (Laud and Campbell, 1994). The formation of neural and liver isoforms and their differential subcellular localization is controlled by an alternative splicing process, which generates two different GS transcripts (Matthews et al., 2005). The liver transcript contains an
alternative exon that is not present in the neural one. This exon leads to acquisition of an upstream in-frame start codon (uAUG), the addition of 29 residues to the N-terminus of the molecule, and formation of a MTS. Therefore, the liver product is targeted to the mitochondria, whereas the neural one is retained in the cytoplasm.

Chicken, which utilize the uricotelic system for ammonia detoxification, also contain a single copy of the GS gene (Pateujunas and Young, 1987) and target the GS enzyme to the mitochondria in liver cells and to cytoplasm in neural tissue. Although regulation of GS expression in chicken has been extensively studied in the past (for a review, see Vardimon et al., 1999), the mechanism for differential targeting of GS remained largely unknown. Here we show that in chicken, unlike dogfish shark, tissue-specific subcellular localization is not achieved by alternative splicing, but rather by a novel mechanism that can differentially target an identical protein to the mitochondria in hepatocytes and to cytoplasm in astrocytes. This mechanism relies on the evolutional selection of a ‘weak’ MTS, which is known to depend on a highly negative $\Delta\Psi$ for import. We show that the $\Delta\Psi$ in hepatocytes is considerably more negative than in astrocytes and that converting the targeting signal of GS into ‘strong’ MTS abolished the capability to confer tissue-specific subcellular localization. Thus, although the GS gene has been highly conserved during evolution, the mechanism for tissue-specific subcellular localization has evolved independently twice.

**Results**

**Differential targeting of chicken GS in liver and brain cells is not achieved by alternative splicing**

Differential targeting of chicken GS was microscopically observed following immunostaining of monolayer cultures of primary hepatocytes and astrocytes with anti-GS antibody and with MitoTracker (a mitochondrial marker). In agreement with previous studies (Smith and Campbell, 1983), GS was mitochondrial in hepatocytes and cytoplasmic in astrocytes (Fig. 1A). Similar results were also obtained by western blot analysis of mitochondrial and cytoplasmic fractions of liver and brain tissues, using antibodies against the mitochondrial protein mHsp60 and the cytoplasmic protein tubulin as controls. Here too, accumulation of GS was mitochondrial in liver cells and cytoplasmic in the brain (Fig. 1B).

As described above, differential targeting of GS in liver and neural tissue in marine elasmobranchs such as dogfish shark is achieved by an alternative splicing: the liver cell splice product encodes a MTS, whereas the neural tissue splice product lacks this sequence (Matthews et al., 2005). To determine whether this GS targeting mechanism has been evolutionary conserved, we examined the possible presence of differentially spliced GS transcripts in chicken liver and brain. Northern blot analysis revealed no size difference between GS transcripts from the two tissues (Fig. 2A). Similarly, RT-PCR products obtained by using primers for exon 1 and 7, the two end exons of the GS gene, displayed no difference in size (Fig. 2B). This is consistent with a previous report that demonstrated that sequences of two cloned GS cDNAs from chicken liver and brain were identical (Campbell and Smith, 1992). Yet, considering that a cDNA clone represents a single transcript, this finding does not exclude the possibility that liver cells contain other GS transcripts that differ from those of brain. Signals that target proteins

![Fig. 1. Avian GS is mitochondrial in hepatocytes and cytoplasmic in astrocytes. (A) Chicken hepatocytes and astrocytes were stained with antibodies against GS and mitochondria were stained using MitoTracker. Confocal images show GS staining, mitochondria staining and overlay of GS and mitochondria staining, as indicated. When GS localizes to mitochondria, the green and red colors merge and yellow appears in overlay images. (B) Western blot analysis of chicken liver and brain subcellular fractions. Equivalent portions from the total (T), cytoplasmic (C) and mitochondrial (M) fractions were analyzed using the indicated antibodies.](image)

![Fig. 2. The GS transcript of liver cells is not different from that of brain. (A) Northern blot analysis of chicken brain and liver GS mRNA using a specific $^{32}$P probe. (B) RT-PCR analysis of chicken brain and liver mRNA using primers for exon 1 and exon 7 of the GS gene. (C) Schematic representation of the first two GS exons and their intermediary intron. Labeled open reading frames (ORFs) found to encode potential MTSs are indicated. Numbers below give location of the exons and ORFs on the gene relative to the transcription initiation site. The primers used for RT-PCR and PCR analysis are shown as numbered arrows with arrowhead indicating the 3’ end. (D) Results of RT-PCR on liver (lanes 7-12) and brain (lanes 13-18) RNA and PCR on the genomic clone pGS-116 (lanes 1-6) as a control, using the indicated primers. The bands representing the GS transcript in liver and brain are indicated by an arrow.](image)
Tissue-specific subcellular localization

...to the mitochondrial matrix are usually encoded by the 5' region of the transcript. This is the case in dogfish shark, where GS contains the initiator AUG codon in exon 2 and an alternative exon of only 95 bases, which leads to acquisition of a mitochondrial targeting signal in intron 1 (Matthews et al., 2005). Because the initiator AUG codon of chicken GS is also located in exon 2, we examined the possible presence of an upstream alternative exon. To this end, we sequenced intron 1 of the GS gene (GenBank accession no. EU369427) using the chicken genomic GS clone pGS116 (Vardimon et al., 1986), and subjected the sequence to analysis by MTS prediction programs (PsortII, MitoprotII, Predotar, Mitopred and TargetP). Several potential MTS regions were identified within intron 1 (Fig. 2C). PCR or RT-PCR analysis, using primers for sequences upstream or inside the potential MTS regions, generated products of anticipated sizes on the genomic GS clone (Fig. 2D, lanes 2-6), but not on liver or brain RNA (Fig. 2D, lanes 8-12 and 14-18). Consistently, RT-PCR analysis using primers for exon 1 and 2 resulted in a single fragment in both liver and brain (Fig. 2D, lanes 7 and 13, indicated by an arrow) that corresponded to the size predicted from splicing out of the whole intron 1 region. Taken together, these findings suggest that chicken liver and brain cells contain an identical GS transcript and that tissue-specific subcellular localization of chicken GS is not achieved by a mechanism of alternative splicing as found in elasmobranchs.

The N-terminus of chicken GS is sufficient to confer tissue-specific subcellular localization

Differential targeting of a protein product might also be achieved by alternative initiation of translation, which leads to the production of proteins that differ in their N-terminal domain and molecular mass. However, Western blot analysis revealed no size difference between liver and brain GS (Fig. 3A). Considering that the N-terminus of mitochondrial proteins is often cleaved on entry into the mitochondrial matrix, the difference between liver and brain GS might be diminished and not detectable by western blot analysis. Therefore, we compared sequences of the GS protein from brain and liver using mass spectrometry. Liver and brain GS were isolated by immunoprecipitation (Fig. 3B), cleaved with trypsin, AspN or GluC and subjected to analysis by mass spectrometry (Fig. 3C). We obtained coverage of 68% of the primary GS sequence (Fig. 3C, underlined) including the first 85 residues at the N-terminal of the protein. The results revealed that the N-terminus of liver GS is not cleaved on mitochondrial translocation and that the detected amino acid sequences of liver and brain GS are identical. In both liver and brain, the GS protein contains an identical sequence that is identical to the mitochondrial matrix Localization signal.

Fig. 3. The GS protein in liver cells is identical to that in brain. (A) Cellular protein samples were prepared from liver and brain and analyzed by western blot using anti-GS antibodies. (B) Protein samples from brain (lanes 1,3) and liver (lanes 2,4) were immunoprecipitated with anti GS antibodies (lanes 3,4) or non-specific antibodies (NS, lanes 1,2) and resolved on SDS-PAGE. The GS bands (marked by arrows) were excised and used for analysis by mass spectrometry. Lane M shows a mixture of proteins used as standards. (C) The sequence of chicken GS protein. In bold and underlined are the sections identified by mass spectrometry sequencing.

Fig. 4. The N-terminus of chicken GS confers differential localization in hepatocytes and astrocytes. Hepatocytes and astrocytes were transfected with the chimeric constructs pCh-50N-EGFP, pEGFP-Ch-50C, pdf-MTS-EGFP or with pEGFP-N1, as indicated. Mitochondria were stained using MitoTracker. Confocal images show mitochondrial staining, EGFP localization and overlay of EGFP and mitochondrial staining, as indicated. When EGFP localizes to mitochondria, the green and yellow colors merge and yellow appears in overlay images.
tissues, the initial methionine was removed and, apart from acetylation of alanine at position 2, no other post-translational modifications in the sequenced fragments were detected.

Most mitochondrial matrix proteins carry N-terminal targeting sequences, termed MTS, although some might contain MTS sequences in the C-terminal domain (Folsch et al., 1998; Lee et al., 1999). To probe the existence of an N- or C-terminal mitochondrial targeting signal in chicken GS, we fused the first 50 N-terminal or the last 50 C-terminal residues of the protein upstream (pCh-50N-EGFP) or downstream (pEGFP-Ch-50C) to the reading frame of the reporter EGFP, respectively. As controls, we used the reporter construct that contains the MTS sequence of dogfish shark GS fused in frame to EGFP [pDF1-MTS-EGFP (Matthews et al., 2005)], and the EGFP reporter alone (pEGFP-N1). Monolayer cultures of hepatocytes and astrocytes were transfected with the different constructs, and the subcellular localization of the chimeric proteins was assayed. The cells were stained with MitoTracker to identify mitochondrial localization. It should be noted that not all cells are transfected and consequently more cells are seen stained with MitoTracker than express EGFP. In cells transfected with the pEGFP-N1 construct, EGFP was homogenously distributed in the cytoplasm and nucleus of both hepatocytes and astrocytes (Fig. 4C).

A similar pattern was observed in cells transfected with the chimeric C-terminal construct, p-EGFP-Ch-50C (Fig. 4A). There too, the chimeric EGFP was homogenously distributed in hepatocytes and astrocytes, indicating that the attached GS residues did not contain a localization signal. By contrast, transfection of the N-terminal pCh-50N-EGFP construct resulted in a cell-type-specific distribution of EGFP (Fig. 4D): similarly to the endogenous GS, this Ch-50N-EGFP was mitochondrial in hepatocyte cells, but mainly cytoplasmic in astrocytes. This cell-type-specific distribution was not due to an intrinsic failure of astrocytes to transport EGFP to the mitochondrion because pDF1-MTS-EGFP (harboring the dogfish MTS) was mitochondrial in both hepatocytes and astrocytes (Fig. 4B).

Next, we examined whether GS can be imported into liver mitochondria in a standard in vitro import reaction. Purified chicken liver mitochondria were isolated and incubated with in vitro translated GS. Import into mitochondria was determined by resistance to externally added proteinase K. We detected essentially no import of GS or of GS-DHFR (GS N-terminal 50 amino acid residues fused to DHFR) (Fig. 5A, bottom and top left panels, respectively). By contrast, Su9-DHFR, a known control of mitochondrial matrix targeting that is cleaved upon entry (Karniely et al., 2006) (Fig. 5A, top right panel), or Su9-GS (Fig. 5A, bottom right panel) were imported efficiently. Similar results were obtained when import was assayed under coupled translation and import conditions (Knox et al., 1998), in the presence of elevated concentrations of NADH (8 mM), ATP (4 mM) or succinate (4 mM) or following denaturation with urea (not shown). These findings suggest that the N-terminal residues of chicken GS establish a non-conventional MTS that requires for import mitochondrial properties and/or cellular components not available in an in vitro system.

Mitochondrial membrane potential in hepatocytes is more negative than in astrocytes

Tissue-specific modification of MTS residues represents an attractive mechanism for differential targeting of GS. Considering that phosphorylation of MTS residues has been shown to affect mitochondrial import (Amutha and Pain, 2003; Anandasatheerthavarada et al., 1999; Robin et al., 2003), we decided to examine the possible involvement of tissue-specific phosphorylation in differential targeting of GS. Inspection of the GS N-terminal 50 amino acid sequence, using a program that predicts Ser/Thr/Tyr phosphorylation sites (NetPhos), revealed two main candidates for phosphorylation: Tyr17 and Ser7. Substitution of Tyr17 with phosphomimetic Glu residue (pCh-50N-EGFP_{Y17E}) was indeed sufficient to impair mitochondrial import: the chimeric protein was cytoplasmic not only in astrocytes, but in hepatocytes as well (Fig. 5B). By contrast, substitution of Tyr17 or Ser7 with Ala (pCh-50N-EGFP_{S17A} or pCh-50N-EGFP_{Y17A}, respectively), an amino acid residue that cannot accept a phosphor group, had no effect on the subcellular localization of the chimeric protein: in both cases EGFP was mitochondrial in hepatocytes and cytoplasmic in astrocytes (Fig. 5B). These finding suggested that tissue specificity is not facilitated by differential phosphorylation of these residues. In agreement, MS/MS analysis did not detect phosphorylated residues in brain or liver GS and no phosphorylation was observed by western blot analysis of immunoprecipitated GS molecules using anti phospho-tyrosine Abs (not shown).

Another possible mechanism for differential targeting of GS is based on the fact that translocation of mitochondrial proteins across the inner mitochondrial membrane is dependent on the magnitude of ∆Ψ (Huang et al., 2002; Martin et al., 1991). ∆Ψ is created by pumping protons from the matrix to the inter-membrane space in conjunction with electron transport through the respiratory chain,
and facilitates import by exerting an electrophoretic effect on the positively charged MTS. We examined whether the magnitude of $\Delta \Psi$ of hepatocytes is different from that of astrocytes. We performed live-cell imaging of hepatocytes and astrocytes stained with tetramethyl rhodamine methyl ester (TMRM), a mitochondria-specific fluorescent cation that accumulates in the mitochondrial matrix according to $\Delta \Psi$. The acquired images were analyzed by a computer-assisted method for automated quantification of $\Delta \Psi$ (Koopman et al., 2008). Remarkably, the results showed that TMRM intensity of hepatocytes is about 40% higher than that of astrocytes and, therefore, that the $\Delta \Psi$ in astrocytes is substantially less negative than that in hepatocytes (Fig. 6A,B). Further quantification of the TMRM images also revealed that hepatocyte mitochondria are more branched, larger and longer than those of astrocytes (Fig. 6C–E). Analysis by electron microscopy showed that hepatocyte mitochondria are internally more complex, containing intricate folding of the mitochondrial inner membrane (Fig. 6F). Given that the inner membranes contain the respiratory chain components, it is possible that the magnitude of $\Delta \Psi$ is a reflection of mitochondrial morphology (Benard and Rossignol, 2008).

**Altering the charge or length of the GS targeting signal facilitates mitochondrial import in astrocytes**

The magnitude of $\Delta \Psi$ required for mitochondrial import has been shown to depend on the length and net positive charge of the targeting sequence: proteins with a short or less positively charged MTS require a higher electrical potential for import than do proteins with a longer or a more positively charged MTS (Huang et al., 2002; Martin et al., 1991). To identify more precisely the targeting signal of GS, we inspected the N-terminal 50 residues by secondary structure prediction programs (PSIPRED, HNN, Jpred, SCRATCH and PredictProtein). The results revealed that, similarly to human GS, whose crystal structure has recently been resolved (Krajewski et al., 2008), the N-terminal residues of avian GS comprise three structural elements: an $\alpha$-helix at the most N-terminal region and two extended strands downstream to the $\alpha$-helix. Helical wheel projection of the predicted $\alpha$-helical region (residues 2-19) showed that, similarly to known MTSs, this region can form a positively charged amphipathic $\alpha$-helix (Fig. 7A). We examined whether GS N-terminal residues, which encompass the $\alpha$-helix structure, constitute a functional MTS. Analysis of chimeric EGFP constructs that contain the first 37 (pCh-N37-EGFP), 23 (pCh-N23-EGFP) or 14 (pCh-N14-EGFP) residues of GS or a 50 amino acid segment spanning between residues 35 and 84 (pCh-35-84-EGFP), revealed that the first 23 residues, which include the complete $\alpha$-helix structure, are indeed sufficient to confer mitochondrial localization in hepatocytes and cytoplasmic localization in astrocytes (Fig. 7B, see also supplementary material Fig. S1). This short targeting sequence constitutes a weak MTS: it has particularly low scores from MTS prediction programs (MitoProtII, PSORTII, TargetP) (e.g. with MitoProtII it scores 0.136 compared to the MTS of dogfish GS that scores 0.692). It contains only three positive residues, all of which are lysine, and two histidines that have a pKa of approximately 6.0 and probably do not contribute positive charges under physiological conditions.

Considering that the $\Delta \Psi$ in hepatocytes is more negative than in astrocytes it is possible that the weak MTS provides a tool for differential targeting of GS: In hepatocytes, the highly negative $\Delta \Psi$ might exert a sufficiently strong pulling force to drive translocation of GS, whereas in astrocytes, $\Delta \Psi$ might be below the threshold required for GS import. We examined whether converting the

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**Fig. 6. Mitochondria of hepatocytes and astrocytes differ in morphology and TMRM intensity.** (A) Typical confocal images of mitochondria from hepatocytes and astrocytes, stained with TMRM and used for statistical analysis of mitochondrial morphology and fluorescence intensity. Image processing of the background corrected image (COR) yielded a binary image (BIN). Masking of the COR image with the BIN image was used to create an intensity-coded image of the mitochondrial objects (MSK). Color coded scale bar on the right indicates the intensity of TMRM fluorescence from blue (low intensity) to red (high intensity). Quantification of the MSK image for hepatocytes (black columns) and astrocytes (white columns) was used to calculate (B) area and (E) aspect ratio. The individual values for hepatocyte mitochondria are expressed as % of mean value for these cells in each graph. Data for astrocytes is expressed as % of the mean observed in hepatocytes. Error bars indicate s.e. ***$P<0.001$. For hepatocytes and astrocytes, a total of 12498 and 22884 objects (mitochondria) and 420 and 490 cells were analyzed, respectively. (F) Electron microscope (EM) images of typical mitochondria in hepatocytes (left panels) or astrocytes (right panels). Scale bars: 1 $\mu$m (upper panels), 200 nm (lower panels).
targeting signal of GS into a strong MTS, by elevating the net positive charge or extending the size of the targeting sequence, would compensate for a less-negative $\Delta \Psi$ and facilitate mitochondrial targeting also in astrocytes. Indeed, substitution of the histidine residues with arginine (pCh-50N-EGFP_H11003 and pCh-50N-EGFP_H11003), a positively charged residue under physiological pH, resulted in mitochondrial localization in both hepatocytes and astrocytes (Fig. 7C, see also supplementary material Fig. S1). Similarly, extending the size of the targeting sequence by including two copies of the targeting sequence (pCh-N23×2-EGFP) facilitated mitochondrial targeting also in astrocytes (Fig. 7D, see also supplementary material Fig. S1). These findings suggest that avian GS has evolved to include a weak MTS, which allows tissue-specific targeting of GS by taking advantage of the differential magnitude of $\Delta \Psi$.

**Discussion**

The most common means to target a protein to two different subcellular localizations is to maintain two copies of the gene. This is the case in *Drosophila melanogaster*, which contains two distinct GS genes (Caizzi et al., 1990): one encodes a GS isoform that contains a MTS and is targeted to the mitochondria, whereas the other encodes a protein that lacks a MTS and is retained in the cytoplasm (Caizzi et al., 1990). Marine elasmobranchs and birds, which utilize the ureosmatic and uricotelic systems for ammonia detoxification, respectively, contain a single GS gene (Laud and Campbell, 1994; Patejunas and Young, 1987). This gene apparently arose from a gene sharing common ancestry with the *Drosophila melanogaster* cytoplasmic GS (Pesole et al., 1991). Nevertheless, in these two species, the GS enzyme is targeted to the mitochondria in liver cells and cytoplasm in neural tissue. Analysis of the molecular mechanism that underlies the differential targeting of GS in these two species revealed that although the GS gene has been highly conserved during evolution (Kumada et al., 1993), two distinct mechanisms for differential targeting of GS have evolved independently.

In marine elasmobranchs, such as dogfish shark, previous studies have shown that differential localization is achieved by tissue-specific alternative splicing that generates two different GS transcripts (Matthews et al., 2005). The liver transcript contains an upstream alternative exon that is not present in the neural one and leads to the formation of MTS. Here, extensive RT-PCR analysis of the chicken GS transcript excluded the presence of an upstream alternative exon, and sequence analysis of the GS protein showed that the amino acid sequence of liver and brain GS is the same. Furthermore, analysis of chimeric constructs that contain various regions of the GS protein revealed that the capability to confer tissue-specific subcellular localization is confined to the first 23 N-terminal residues of chicken GS, which are sufficient to target a chimeric EGFP construct to the mitochondria in hepatocytes and to the cytoplasm in astrocytes. These findings indicate that tissue-specific subcellular localization of avian GS is achieved by a novel mechanism that can differentially localize an identical protein in liver and brain cells.

Differential targeting of a single translation product might be achieved by several possible mechanisms, one of which is post-translational modification. Protein modification, such as phosphorylation, might affect the accessibility of a targeting sequence by altering its folding or ability to interact with another protein or by directly modulating its targeting properties (Karnieli and Pines, 2005). Protein phosphorylation activates, for example, the cryptic MTS of the cytochrome P450 family member CYP2B1 (Anandatheerthavarada et al., 1999), enhances the mitochondrial import of the glutathione S-transferase protein (Robin et al., 2003), but inhibits the mitochondrial import of the yeast protein YNK1 (nucleotide diphosphate kinase 1) (Amutha and Pain, 2003). The possibility that differential targeting of GS involves the function of a tissue-specific kinase was examined by amino acid substitutions. Our results showed that although substitution of Tyr17 with the phosphor-mimetic Glu residue was sufficient to impair mitochondrial import in hepatocytes, substitution of Tyr17 and Ser7 with Ala, did not facilitate mitochondrial import in astrocytes. In addition, MS/MS analysis revealed that apart from acetylation of Ala at position 2 in GS from both liver and brain, there are no other post-translational modifications in the sequenced fragments. These findings suggest that tissue-specific subcellular localization is not achieved by post-translational modification of the GS protein.

Translocation of proteins into the mitochondrial matrix is ultimately dependent on a sufficiently large electrochemical proton gradient across the mitochondrial inner membrane. It has been suggested that the electrophoretic effect produced by $\Delta \Psi$ on MTS leads to an active pulling mechanism that includes catalyzed unfolding of protein domains (Huang et al., 2002; Shariff et al., 2004). The magnitude of $\Delta \Psi$ required for import is dependent on the length and/or net positive charge of the mitochondrial targeting signal. Proteins with a ‘strong’ signal, which is characterized by the presence of basic amino acids and the absence of acidic ones, can be imported at a moderately negative $\Delta \Psi$, whereas proteins with a ‘weak’ signal, characterized by a low content of positive charged residues, require higher $\Delta \Psi$ (Martin et al., 1991). In addition, proteins with a long targeting signal (i.e. 40-50 residues) are not dependant on a highly negative $\Delta \Psi$ for import, possibly...
because they reach the matrix at the initial interaction with the import machinery and become unfolded by the mitochondrial Hsp70 (Huang et al., 2002; Shariff et al., 2004). Recently, we have shown that dual-targeted mitochondrial proteins tend to have a weaker MTS than exclusive mitochondrial proteins (Dinur-Mills et al., 2008). GS is an example in which the weak MTS has functional significance. Analysis of the GS N-terminal residues revealed that the targeting signal is relatively short and is encompassed within the first 23 residues. This region forms, according to prediction programs and to the crystal structure of the highly homologous human GS, an α-helical structure. Imposition of this region onto an α-helical wheel projection shows that it has the properties of an amphipatic α-helical MTS with a hydrophilic face that contains some positive and polar residues and lacks negative ones. This short targeting sequence established a weak MTS, as judged by the particularly low scores given by MTS prediction programs and by the low number of positively charged residues. In contrast to the canonical MTS of Su9, this weak MTS was incapable of directing mitochondrial import in a standard in vitro import reaction. Considering that a weak MTS is dependent on a highly negative ΔΨ for import, we decided to examine the magnitude of ΔΨ of liver and brain cells. Remarkably, our results revealed that ΔΨ in hepatocytes is considerably more negative than in astrocytes. This finding represents, to the best of our knowledge, the first example of cell-type-specific differences in ΔΨ. Changes in ΔΨ have been previously observed in neurological disorders (Abou-Sleiman et al., 2006; Mortiboys et al., 2008), aging cells (Sugrue and Tatton, 2001) and tumors. A more negative ΔΨ has been detected in a variety of carcinomas (Chen, 1988; Fantin et al., 2002; Fantin et al., 2006) and in chemically induced and oncogene-induced malignant transformation in various cell types (Liang et al., 1999; Zarbl et al., 1987). The more negative ΔΨ in tumor cells has been attributed to the shift in glucose metabolism: normal cells produce most of the ATP from glucose through oxidative phosphorylation whereas many cancer cells exhibit lower oxidative phosphorylation activity and produce ATP by conversion of glucose to lactate. This change in glucose metabolism is causatively related to the more negative ΔΨ in tumor cells (Fantin et al., 2006). Liver is the major organ involved in glucose homeostasis by means of gluconeogenesis (i.e., glucose production from precursor compounds such as lactate) and glycolysis. A highly negative ΔΨ in liver cells might be functionally related to the complex metabolic functions exerted by the cells, but might also reflect liver mitochondria ultrastructure, which exhibits a larger surface area (Benard and Rossignol, 2008).

The possibility that a weak MTS provides a crucial tool for differential targeting of GS was assayed by converting the targeting signal of GS into a strong MTS. Our results clearly showed that elevation of the net positive charge of the targeting sequence, by substitution of one or two histidine residues with arginine, or by extending the size of the MTS through including two copies of the targeting sequence, abolished the capability to confer tissue-specific subcellular localization. Under these conditions, the chimeric protein was mitochondrial in both liver and brain cells. Our results suggest that uricotelic species have evolved by the selection of a weak MTS to the otherwise highly conserved GS enzyme. This targeting sequence allows taking advantage of the tissue-specific differences in ΔΨ and directing the GS protein to the mitochondria in liver cells and to cytoplasm in brain. The functional link between MTS properties and the magnitude of ΔΨ might provide a mechanistic basis for the redirection of cellular protein under physiological conditions in which ΔΨ is altered.

Materials and Methods

Plasmid construction

Chimeric EGFP plasmids were constructed by using first strand cDNA generated from RNA of chicken liver cells. pEGFP-Ch-50C was constructed by PCR amplification of first strand cDNA from chicken liver using primers pGS, which contains the entire GS coding sequence under the control of Sp6, was used as the template to generate a PCR fragment obtained by amplification using pSu9-DHFR as template with the primers 5′-ATGGATCCCGTGGCGGAGGTC-3′ and 5′-CGGTCCCGTCGCCGTGGCC-3′. The resulting plasmid was used as template to generate a PCR fragment with the primers 5′-ATGGATCCCGTGGCGGAGGTC-3′ and 5′-CGGTCCCGTCGCCGTGGCC-3′. This PCR fragment was cloned into plasmid pEGFP-Cs (Clontech). All other chimeric EGFP plasmids were constructed by cloning of PCR products into pEGFP-N1 (Clontech). The following primers were used to amplify chicken cDNA: For pch-50N-EGFP 5′-ATGGAAGCTGCGAGCCATGCGGAGG-3′ (primer A) and 5′-ATGGATCCTCGGCGGAGGTC-3′ (primer B); for pch-37N-EGFP primer A 5′-ATGGATCCTCGGCGGAGGTC-3′ and cloning into pEGFP and for pch-14N-EGFP primer A 5′-ATGGATCCTCGGCGGAGGTC-3′ and cloning into pEGFP and B 5′-ATGGAAGCTGCGAGCCATGCGGAGG-3′ as well as primer B and 5′-ATGGATCCTCGGCGGAGGTC-3′ as well as primer B and 5′-ATGGATCCTCGGCGGAGGTC-3′ as well as primer B and 5′-ATGGATCCTCGGCGGAGGTC-3′ as well as primer B and 5′-ATGGATCCTCGGCGGAGGTC-3′ as well as primer B and 5′-ATGGATCCTCGGCGGAGGTC-3′. For pch-50N-EGFP(S7A) primer A 5′-ATGGATCCTCGGCGGAGGTC-3′ as well as primer B and 5′-ATGGATCCTCGGCGGAGGTC-3′ as well as primer B and 5′-ATGGATCCTCGGCGGAGGTC-3′ as well as primer B and 5′-ATGGATCCTCGGCGGAGGTC-3′ as well as primer B and 5′-ATGGATCCTCGGCGGAGGTC-3′ as well as primer B and 5′-ATGGATCCTCGGCGGAGGTC-3′ as well as primer B and 5′-ATGGATCCTCGGCGGAGGTC-3′ as well as primer B and 5′-ATGGATCCTCGGCGGAGGTC-3′ as well as primer B and 5′-ATGGATCCTCGGCGGAGGTC-3′. Plasmid pch-32N-EGFP is described elsewhere [pL-GS-EGFP (Matthews et al., 2005)]. Plasmid pEGFP, which contains the entire GS coding sequence under the control of Sp6, was used in the creation of PCR amplified first strand cDNA from chicken liver using primers A 5′-ATGGATCCCGTGGCGGAGGTC-3′ and B 5′-ATGGATCCTCGGCGGAGGTC-3′ and cloning into pEGFP as template with primers A 5′-ATGGATCCTCGGCGGAGGTC-3′ and B 5′-ATGGATCCTCGGCGGAGGTC-3′ and cloning into pEGFP as template with primers A 5′-ATGGATCCTCGGCGGAGGTC-3′ and B 5′-ATGGATCCTCGGCGGAGGTC-3′. Plasmid pEGFP(Δ7A) was generated by a Ncol/BfgI fragment in plasmid pGS with a PCR fragment obtained by amplification using pEGFP-DHFR as template with the primers 5′-TACATGACGTTCAATGGCTTGGCC-3′ and 5′-TCACATCCCTAATGGCTTGGCC-3′. Plasmid pEGFP(Δ9B) was generated by a Ncol/BfgI fragment in plasmid pGS with a PCR fragment obtained by amplification using pEGFP-DHFR as template with the primers 5′-TACATGACGTTCAATGGCTTGGCC-3′ and 5′-TCACATCCCTAATGGCTTGGCC-3′.

Subcellular fractionation and western blot analysis

Liver or brain tissues of chicken embryos (E18) were excised, suspended in isotonic HIM buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES-KOH pH 7.4) including 0.1% BSA and homogenized in a Dounce homogenizer by five strokes with pestle A and two strokes with pestle B. The homogenate was centrifuged at 1000 g for 10 minutes. The supernatant was centrifuged at 12,000 g for 15 minutes to obtain the mitochondrial pellet and the cytoplasmatic supernatant. The mitochondrial pellet was washed twice at 12,000 g and resuspended in HIM. The cytoplasmatic supernatant was centrifuged at 120,000 g for 60 minutes and the pellet was discarded. Total protein extract was obtained by homogenization in Passive Lysis Buffer (Promega) by five strokes with pestle A and 20 strokes with pestle B, sonication for 5 minutes and centrifugation at 20,000 g for 15 minutes. All steps were carried out at 4°C. Equal portions of total, cytoplasmatic and mitochondrial fractions were resolved on 10% SDS-PAGE gel. For western blot analysis, antibodies against tubulin (DM 1A; Sigma), mHsp60 (LK-2 Sigma), GS (Gorovitz et al., 1997) or phosphotyrosine (PY20; Santa Cruz Biotechnology) were used. The corresponding horseradish-peroxidase-conjugated secondary antibodies were used, and the cross-reactivity was visualized by the enhanced chemoluminescence (ECL) procedure (Pierce).

Immunoprecipitation and mass spectrometry sequencing

For immunoprecipitation, protein extracts (0.5 mg) were precleared by incubation for 16 hours at 4°C with pre-immunus serum bound to protein-A-Sepharose (Amersham). Cleared extracts were immunoprecipitated with protein-A-Sepharose bound to anti-GS antibodies overnight at 4°C. The immunoprecipitated proteins were separated on 10% SDS-PAGE and analyzed by western blotting or stained with Coomassie blue. The Coomassie-blue-stained gel slices, containing the GS protein band, were incubated with either trypsin, AspN or GluC, and MS carried out with Qtof2 (Micromass, England) using a nanospray attachment. Data analysis was done using the bio lynx package (Micromass, England) and database searches were performed
Mitochondrial morphology and membrane potential analysis

For electron microscopy, hepatocytes and astrocytes were harvested and fixed with 2% glutaraldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM NaH2PO4, 2 mM KH2PO4, pH 7.4) and post-fixed in 1% OsO4 in PBS. After dehydration in graded ethanol solutions, the samples were embedded in glycid ether (Serva). Ultrathin sections (~0.1 μm) were stained with 4% aqueous uranyl acetate in MilliQ water for 10 min and then with 2% aqueous aqueous potassium permanganate in MilliQ water for 10 min. The sections were then washed in MilliQ water and dried on formvar-coated nickel grids. For cryoelectron microscopy, hepatocytes and astrocytes were harvested and fixed with 2.5% glutaraldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM NaH2PO4, 2 mM KH2PO4, pH 7.4) and post-fixed in 1% OsO4 in PBS. After dehydration in graded ethanol solutions, the samples were embedded in a glycid ether-based resin (Epon) and polymerized at 60°C for 24 h. Ultrathin sections (~0.1 μm) were stained with 4% aqueous uranyl acetate in MilliQ water for 10 min and then with 2% aqueous aqueous potassium permanganate in MilliQ water for 10 min. The sections were then washed in MilliQ water and dried on formvar-coated nickel grids.

In vitro import into isolated mitochondria

The pGEM4 constructs were used as templates for in vitro SP6 transcription/translation carried out with a TNT Coupled Reticulocyte Lysate System (Promega) in the presence of [35S]methionine. In some reactions, labeled proteins were denatured on coverslips in 8 M urea and 20 mM HEPES-KOH pH 7.4, at room temperature for 2 hours before the import reaction. Coupled transcription and import was performed as described before (Knos et al., 1998) using GS mRNA transcribed from pGS. Import reactions into isolated mitochondria (100 μg per reaction) were carried out in 200 μl SI buffer (0.6 M mannitol, 50 mM HEPES-KOH pH 7.4, 80 mM KCl, 10 mM MgAc2, 2 mM KH2PO4, 2 mM EDTA, 2 mM MnCl2 and 3% BSA) for 60 minutes at 30°C. The reaction was started by the addition of buffer B (2 mM ATP, 4 mM NADH, 100 μg/ml creatine kinase and 4.5 mM creatine phosphate) and 2 μl of labeled protein. Import was stopped by diluting the reaction fivefold in ice-cold SHKCL medium (0.6 M sorbitol, 80 mM KCl and 20 mM HEPES-KOH pH 7.4). The samples were washed in TFM (10 mM Tris, pH 7.4, 150 mM KCl, 250 mM NaCl, 0.1 mM EDTA) before being centrifuged at 10,000 g for 20 minutes. The mitochondrial pellet was resuspended in sample buffer, boiled for 5 minutes at 95°C and subjected to SDS-PAGE. Signals of radiolabeled proteins were detected by autoradiography.

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References


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