Dual targeting of cytochrome P4502B1 to endoplasmic reticulum and mitochondria involves a novel signal activation by cyclic AMP-dependent phosphorylation at Ser128

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We have investigated mechanisms of mitochondrial targeting of the phenobarbital-inducible hepatic mitochondrial P450MT4, which cross-reacts with antibody to microsomal P4502B1. Results show that P4502B1 and P450MT4 have identical primary sequence but different levels of phosphorylation and secondary structure. We demonstrate that P4502B1 contains a chimeric mitochondrial and endoplasmic reticulum (ER) targeting signal at its N-terminus. Inducers of cAMP and protein kinase A-mediated phosphorylation of P4502B1 at Ser128 activate the signal for mitochondrial targeting and modulate its mitochondrial or ER destination. S128A mutation inhibits *in vitro* mitochondrial transport and also *in vivo* mitochondrial targeting in COS cells. A fragment of P4502B1 containing the N-terminal signal and the phosphorylation site could drive the transport of dihydrofolate reductase (DHFR) into mitochondria. Ser128 phosphorylation reduced the affinity of 2B1 protein for binding to SRP, but increased the affinity of the 2B1–DHFR fusion protein for binding to yeast mitochondrial translocase proteins, TOM40 and TIM44, and matrix Hsp70. We describe a novel regulatory mechanism by which cAMP modulates the targeting of a protein to two distinct organelles.

Keywords: cAMP/chimeric signal/mitochondrial targeting/P4502B1/protein phosphorylation

Introduction

Protein targeting to the endoplasmic reticulum (ER) and mitochondria involves distinctly different pathways and signal recognition mechanisms. Proteins targeted to mitochondria are translated on membrane-free ribosomes and post-translationally transported to the organelle. Binding of N-terminal or internal signal to components of mitochondrial translocase complexes, TOM, TIM and matrix Hsp70, appears to be the initial critical step in mitochondrial protein targeting (Glick and Schatz, 1991; Schneider *et al.*, 1994; Ryan and Jensen, 1995; Neupert, 1997; Pfanner and Meijer, 1997). The ER targeted proteins, on the other hand, contain distinct N-terminal hydrophobic

signal for binding to signal recognition particle (SRP), which, in turn, targets the emerging nascent chains to the ER (Gilmore *et al.*, 1982). With some exceptions (Wickner and Lodish, 1985; Schatz and Dobberstein, 1996), ER targeting is thought to be co-translational. Current models of protein targeting imply that the ER or mitochondrial destination of a protein is determined at the pre-translation level by virtue of the signal sequence it carries. Most mitochondrial and ER targeted proteins are encoded by a distinct set of genes. In a limited number of cases, characteristic mitochondrial targeting sequences are generated by differential expression of the gene using alternate transcription start sites or differential splicing of the primary transcripts (Jaussi *et al.*, 1995; Neupert, 1997; Isenmann *et al.*, 1998). Thus, proteins targeted to different cytoplasmic organelles carry a specific signal, which directs the protein to a specific organelle.

In contrast to this prevailing dogma, a recent study from our laboratory showed that the major xenobiotic-inducible P4501A1 is targeted to both the ER and mitochondria (Addya *et al.*, 1997; Anandatheerthavarada *et al.*, 1999). In a series of studies, Birdsey and Danpure (1998) showed that mammalian alanine:glyoxylate aminotransferase protein, targeted to both mitochondria and peroxisomes in some species, contains N-terminal mitochondrial and C-terminal peroxisomal targeting signals, although the former is the more dominant signal. Our results with P4501A1 showed that proteolytic cleavage of an N-terminal segment of the protein by a cytosolic endoprotease activates a cryptic mitochondrial targeting signal (Addya *et al.*, 1997). Dual mitochondrial and microsomal association of P450 isoforms, including P4502B1/2, 2E1, 3A1/2, 2D6 and 2C12, has been reported by various investigators (references cited in Anandatheerthavarada *et al.*, 1997; Bhagwat *et al.*, 1999), although mechanisms of dual membrane targeting remain unclear. The major phenobarbital (PB)-inducible mitochondrial P450MT4 was shown to resemble the similarly induced microsomal P4502B1/2 with respect to N-terminal sequence and antibody cross-reactivity (Shayiq and Avadhani, 1990; Anandatheerthavarada *et al.*, 1997). Extensive protein sequencing in the present study showed that the PB-inducible mitochondrial and microsomal P450s have identical primary sequence. P450 enzymes belonging to family 2, including P4502B1, contain cAMP-dependent protein kinase A (PKA) phosphorylation sites; their activity and steady-state levels in the microsome are thought to be modulated by protein phosphorylation (Pyerin and Taniguchi, 1989; Eliasson *et al.*, 1992). In the present study, we have investigated the mechanism of dual targeting of P4502B1 to the ER and mitochondria and found that phosphorylation at Ser128 activates a cryptic mitochondrial targeting signal at amino acid residues 20–36 of the protein. Phosphorylation also reduced the stability

Fig. 1. Characteristics of the microsomal P4502B1 and microsomal P450MT4. **(A)** Coomassie-stained pattern of 1 μ g each of purified 2B1 and MT4 resolved on 14–16% gradient SDS–polyacrylamide gel (left); Western blot of a companion gel using monoclonal antibody to 2B1 (middle); and the migration pattern of 35 S-labeled 2B1 and +20/2B1 translation products (right). **(B)** Peptide fingerprint patterns of purified P4502B1 and P450MT4. Protein (0.5–0.7 mg) transblotted to polyvinylidene difluoride (PVDF) membrane was subjected to tryptic digestion and peptides were analyzed by HPLC. **(C)** CD patterns of purified P4502B1 (dotted line) and P450MT4 (solid line) using 0.3 mg/ml enzyme suspension. Values underneath the spectra (mean $\pm$ SE) were derived from four different spectral patterns. **(D)** Purified P450s (1.2 μ g of protein each) or microsomal and mitochondrial proteins (75 μ g of each) were immunoprecipitated with polyclonal antibody to 2B1; half of the precipitate was probed with monoclonal antibody to 2B1 (1:100 dilution) and the other half probed with phospho-Ser antibody (1:1000 dilution) by Western blotting. In the lane marked 2B1 Phospho., purified 2B1 (1.2 μ g) was incubated with 1 U of PKA and 0.1 mM ATP for 30 min at 28°C before immunoprecipitation with 2B1 antibody. Western blots were developed using the Super Signal Ultra kit from Pierce Chemical Co. and imaged through a Bio-Rad FluorS imager.

of the nascent chain-bound SRP complex, while increasing the affinity of 2B1 protein for binding to mitochondrial translocases. Thus, cAMP-dependent phosphorylation serves as a signal for redirecting this otherwise ER-predominant protein to mitochondria.

Results

Nature of the PB-inducible mitochondrial P450MT4

The microsomal P4502B1 and mitochondrial P450MT4 purified from PB-induced rat livers showed similar migration rates on high-resolution gradient polyacrylamide gel (Figure 1A, left panel). Consistent with previous results (Shayiq and Avadhani, 1990; Anandatheerthavarada *et al.*, 1997), both of these proteins reacted with monoclonal antibody against microsomal P4502B1/2 (Figure 1A, middle panel). Proteins differing by 19 amino acid residues (Figure 1A, right panel) migrated differently on the gel system, indicating its resolving power. Purified P450MT4 and P4502B1 exhibited identical peptide fingerprints on a microbore high-pressure liquid chromatography (HPLC) system (Figure 1B), and N-terminal sequencing of the purified proteins and internal peptides showed identical

sequence properties (latter results not shown). These results, revealed for the first time that the mitochondrial and microsomal counterparts represent the same primary translation product of 2B1 mRNA. The results also suggest that the mitochondrial targeting of P4502B1 may not involve endoproteolytic cleavage of primary translation product as shown previously for P4501A1 (Addya *et al.*, 1997; Anandatheerthavarada *et al.*, 1999).

Despite similar primary sequence, the microsomal and mitochondrial enzymes exhibited a significant difference in circular dichroism (CD) spectra (Figure 1C). A comparison of spectra in Figure 1C and analysis of secondary structure contents showed that mitochondrial P450MT4 contains 50% lower α -helical content but increased β -sheet structure compared with microsomal P4502B1, suggesting a possible difference in post-translational protein modification. Members of P450 family 2 contain a single PKA consensus phosphorylation site. Indeed, phosphorylation of P4502B1 resulted in a CD spectral shift resembling that of P450MT4 (not shown).

A possible difference in the level of Ser phosphorylation of mitochondrial and microsomal P450s was investigated by probing 2B1 and MT4 proteins with a monoclonal

Fig. 2. Signal properties of the N-terminus of P4502B1. (A) Chimeric nature of the N-terminal signal of P4502B1 showing various domains. (B) The sequence of the wild-type 2B1 protein, site-specific mutations (2B1/3Mut and 2B1/S128A) and also N-terminal deletion mutations (+20/2B1 and +36/2B1) are shown. (C) A cartoon of wild-type 1-160/2B1-DHFR fusion protein.

antibody against phospho-Ser. As expected, 2B1 antibody immunoprecipitated a 52 kDa protein from various sources (Figure 1D, lower panel, lanes 2–9), excepting purified P4501A1 (lane 1). Interestingly, P450MT4 (Figure 1D, upper panel, lane 2), but not the microsomal P4502B1 (upper panel, lane 3), cross-react with phospho-Ser antibody. However, 2B1 protein subjected to PKA-mediated phosphorylation interacted with the antibody (Figure 1D, upper panel, lane 9). Similarly, 2B1 protein from PB-induced rat liver microsomes (Figure 1D, upper panel, lanes 4 and 5) did not interact with the antibody, while mitochondria from the same source (upper panel, lane 6) did cross-react with the antibody. Similar results were obtained with the mitochondrial (Figure 1D, upper panel, lane 7) and microsomal (upper panel, lane 8) fractions from COS cells overexpressing P4502B1 protein. These results, together with the CD spectral analysis, suggest that the difference in secondary structure contents of P450MT4 and P4502B1 may be related to their phosphorylation states. The mitochondrial P450MT4 with a higher level of phosphorylation exhibited a more unfolded structure compared with the more compact conformation of the underphosphorylated P4502B1. These results also suggest that protein phosphorylation may serve as a signal for mitochondrial targeting of P4502B1.

Chimeric signal properties of the N-terminal sequence of P4502B1

The N-terminal 20-amino-acid region with a hydrophobic helical structure is thought to function as an ER-targeting and also a membrane anchor domain (Sakaguchi *et al.*, 1987; Monier *et al.*, 1988). This domain is followed by a region rich in positively charged residues at positions 21, 23, 25 and 27 (Figure 2A and B), mimicking the cryptic mitochondrial targeting signal of P4501A1. Typical of most microsomal P450 proteins, there is a Pro-rich region between residues 31 and 39, which probably acts as a hinge for the folding of the globular catalytic domains (Figure 2A and B). Finally, peptide mapping of P4502B1 by two different groups showed the presence of a single PKA target phosphorylation site at Ser128 (Koch and Waxman, 1989; Pyerin and Taniguchi, 1989) as shown in Figure 2A.

Effects of phosphorylation on mitochondrial import of P4502B1

To study the requirements for mitochondrial targeting, we constructed N-terminal deletions (+20/2B1 and +36/2B1)

and point mutations (2B1/3Mut) at the positively charged residues within the putative mitochondrial targeting signal region, as well as the S128 phosphorylation site (2B1/S128A) (see Figure 2B). A rat liver mitochondrial system (Addya *et al.*, 1997) was used to study the transport of protein constructs listed in Figure 2B. The Western blot in Figure 3B shows that the mitochondrial preparations used for the transport assays contained no detectable microsome-specific protein P450 reductase, but normal levels of mitochondria-specific P450c27 protein, indicating their purity.

Intact WT/2B1 protein translated in rabbit reticulocyte lysate (RRL) in the absence of added PKA bound poorly (7%) to mitochondria and imported at <0.5% (Figure 3B and table at the bottom of the figure). Addition of PKA to the translation mix, however, vastly increased mitochondrial binding as well as import to ~35–38% of input protein. The N-terminal deletion product, +20/2B1, was imported at ~35% with or without PKA-mediated phosphorylation. The intramitochondrial location of the labeled protein was ascertained by adding 0.1% Triton X-100, which made the protein fully accessible to added protease (Figure 3B). Progressive deletion to +36/2B1 reduced protein transport to ~0.4% even when the protein was fully phosphorylated (see Figure 3C), suggesting that the sequence 21–36 is critical for mitochondrial import. Furthermore, full-length 2B1 protein with S128A substitution was imported only at a very low level (0.2%) even when translated in the presence of added PKA (Figure 3C), suggesting that phosphorylation may have a role in the activation of mitochondrial targeting signal in intact 2B1 protein. Likewise, 2B1/3Mut protein was not imported significantly even when it was translated in the presence of added PKA (Figure 3D, left panel), suggesting the critical importance of positively charged residues at 21, 25 and 27 for mitochondrial transport.

As shown in Figure 3D, purified P4502B1 protein with bound heme group, and phosphorylated in the presence of [γ - 32 P]ATP and PKA, bound to mitochondria at 45% but was not imported, suggesting that fully folded proteins or proteins with bound substrates or prosthetic groups are import-incompetent (Chen and Douglas, 1987). In contrast, 32 P-labeled translation product synthesized in RRL in the presence of [γ - 32 P]ATP and PKA (Figure 3E) was imported efficiently and the imported protein became sensitive to protease digestion after treatment with Triton X-100 (Figure 3E). Although not shown, S128A mutant protein was not labeled when incubated with [γ - 32 P]ATP, confirming previous reports (Koch and Waxman, 1989; Pyerin and Tanaguchi, 1989) that S128 is the only cAMP-dependent phosphorylation site on the P4502B1 protein. Results (Figure 3E) showed that the *in vitro* import of 32 P-labeled 2B1 was inhibited by agents like 2,4-dinitrophenol (DNP) and carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP), which disrupt mitochondrial transmembrane potential, and also by oligomycin, which depletes the mitochondrial ATP pool. For unknown reasons, these treatments caused a 40–50% reduced mitochondrial binding. These experiments indicate that the present *in vitro* system shows requirements similar to the established *in vitro* mitochondrial transport systems from different cells (Gasser *et al.*, 1982; Schwartz and Neupert, 1994).

Fig. 3. Sequence requirements for the *in vitro* transport of P4502B1 into mitochondria. (A) Immunoblot of rat liver mitochondrial and microsomal fractions (100 μ g of protein each) co-developed with antibodies to microsome-specific P450 reductase and mitochondria-specific P450c27. Purified enzymes (0.2 μ g of each) were loaded as controls. (B–E) *In vitro* transport of labeled proteins in isolated rat liver mitochondria resolved on a 12–16% gradient gel. (B and C; D, left) *In vitro* translation products were generated in the RRL system in the absence (–) or presence (+) of PKA (10 U/50 μ l). (D, right) Purified P4502B1 (100 ng) labeled with 50 μ Ci of [γ - 32 P]ATP as described in Figure 1D and 10 μ l of RRL as the source of essential factors were used. (E) 2B1 translation product labeled with [γ - 32 P]ATP and PKA were used. Digestion of mitochondria with trypsin (300 μ g/mg protein) was for 30 min on ice. In (E), mitochondria were pre-incubated on ice for 10 min with 2,4-DNP (25 μ M), oligomycin (25 μ M), CCCP (25 μ M) or without inhibitor (none) before adding the translation mix containing labeled protein. Triton X-100 (0.1%) was added at the end of the transport reaction, before the addition of trypsin. 0.4 to 1 $\times$ STD represents fractions of input counts used for transport.

Effects of phosphorylation on *in vivo* mitochondrial targeting of P4502B1

The mitochondrial targeting of 2B1 protein and the effects of PKA-mediated phosphorylation were investigated using COS cells transfected with cDNA constructs. We tested the effects of the cAMP analog dibutyryl cAMP, the cAMP inducer forskolin and PKA inhibitor H89 on the levels of mitochondrial and microsomal associated proteins. After 62 h of transfection, subcellular fractions were isolated by sucrose density banding (Addya *et al.*, 1997) and the proteins were analyzed by Western blot analysis using affinity-purified polyclonal antibody to microsome P4502B1. As described before (Addya *et al.*, 1997), marker enzyme assays and Western blot analysis for organelle-specific proteins showed that the mitochondrial isolates from transfected COS cells contained <1% contaminating microsomal fragments.

As shown in Figure 4A and quantitation of the gel pattern at the bottom of the figure, ~15% of the wild-type P4502B1 was associated with mitochondria and 85% with the microsome. Treatment with forskolin and dibutyryl cAMP resulted in a marked increase in the mitochondrial content of 2B1 to ~62% of the total cellular pool, and a parallel decline in the microsomal content to 35%. Treatment with H89, on the other hand, resulted in a marked decrease (5%) in the mitochondrial content, but a sharp increase (95%) in the microsomal 2B1 content. Interestingly, mutations targeted to the phosphorylation site at Ser128 showed a distribution similar to H89 treatment. The mitochondrial level of S128A mutant protein was neither induced nor inhibited by cAMP inducers or PKA inhibitor, further confirming that phosphorylation is important for mitochondrial targeting and that S128 is the critical PKA target site. Additionally, mutations targeted to the positively charged residues at 21, 25 and 27 (3Mut/2B1) also reduced mitochondrial targeting by >85% (see Figure 4B). Expression of N-terminal truncated protein, +20/2B1, resulted in a 4-fold higher level of mitochondrial accumulation compared with intact 2B1 protein. Surprisingly, this protein lacking the transmembrane anchor domain was also detected in the microsomal fraction, although at a reduced level compared with intact 2B1 protein. As indicated by a previous study, the ER targeting of +20/2B1 may be due to the occurrence of secondary ER targeting and membrane association domains (Monier *et al.*, 1988). Transfection with cDNA for +36/2B1 protein lacking the positively charged region, on the other hand, did not result in significant accumulation in either the mitochondrial or microsomal fractions. However, a significant amount of the immunoreactive protein is detected in total cell homogenate (Figure 4B, bottom panel, last lane), suggesting its efficient translation. Results also showed that both PKA inhibitor and cAMP inducers did not affect the mitochondrial content of truncated +20/2B1 protein (Figure 4B). Similarly, treatment with cAMP inducers failed to increase the mitochondrial content of the 2B1/3Mut protein. These results support the view that the N-terminal 20–30 sequence region of the protein, containing positively charged residues, functions as a cryptic mitochondrial targeting signal, which can be activated either by removal of the N-terminal 20 hydrophobic region, or by cAMP-mediated phosphorylation at Ser128 of the intact protein. Interestingly, results of gel quantita-

Fig. 4. Role of phosphorylation in *in vivo* mitochondrial targeting of P4502B1. COS cells were co-transfected with the cDNA constructs indicated and used for isolating mitochondrial and microsomal fractions. Membrane fractions (30 µg of protein each) or total cell homogenates (100 µg protein) were resolved on 12% gel and subjected to Western blot analysis. The blots with mitochondrial proteins in both (A) and (B) were co-developed with polyclonal antibodies to 2B1 and mtTFA (1:3000 dilutions each). The blots with microsomal protein in both (A) and (B) were sequentially probed with 2B1 antibody and calreticulin antibody (1:1500 dilution). Forskolin (10 µM) and dibutyryl cAMP (5 µM) were added 6 h after transfection and H89 (48 nM) was added 3 h before transfection. Purified P4502B1 (0.5 µg) was run as a positive control.

tion also suggested that cAMP inducers and PKA inhibitor may affect the subcellular distribution of the 2B1 pool in transfected cells without significantly affecting the total cellular contents.

The role of cAMP in *in vivo* mitochondrial targeting of P4502B1 was further verified by immunohistochemical analysis. COS cells were transfected with wild-type 2B1 cDNA and the effects of added dibutyryl cAMP on the subcellular distribution of immunoreactive protein were studied. The cells were double stained with antibody to 2B1 and antibody to mitochondrial genome-coded protein COX I. In some experiments, the cells were also co-stained with antibody to β -COP to visualize the Golgi network. As seen from the histological staining patterns in Figure 5A (a, b and c), expression of WT/2B1 protein resulted in a widespread staining of various cytoplasmic organelles in addition to the plasma membrane. A significant amount of the stain co-localized with COX I antibody-stained punctate structures, reminiscent of mitochondria (Figure 5A, b and c). Addition of dibutyryl cAMP resulted in a preferential clustering of 2B1 antibody-staining protein in particulate punctate structures (Figure 5A, d) that co-localized with organelles stained with COX I antibody (e and f). Some studies indicate that low levels of 2B1 are also targeted to plasma membranes of barbiturate-induced rats and human patients (Robin *et al.*, 1997). To distinguish these extensively stained membrane structures in cAMP-treated cells from the Golgi transport vesicles, we also co-stained with β -COP antibody. It is seen that a small part of the 2B1 antibody stain (Figure 5B, i) co-localized with Golgi-specific β -COP antibody stain (j and k), which was not increased under induced cAMP levels (not shown). These results confirm and extend the view that induced cAMP levels cause an increased mitochondrial accumulation of P4502B1.

Effects of S128 phosphorylation on ER membrane insertion and SRP binding

The effects of Ser128 phosphorylation on ER membrane insertion of 2B1 protein were studied under *in vitro* conditions. Wild-type or S128A mutant proteins were translated in the wheat germ lysate (WGL), supplemented with 0–10 U of PKA and 5 U of unwashed rough endoplasmic reticular membranes (REM) or KCl-washed REM (K-RM) membrane. After 30 min of incubation, the alkaline Na_2CO_3 -soluble and -insoluble fractions were analyzed by polyacrylamide gel electrophoresis. Results (Figure 6A) showed that in the presence of unwashed REM and the absence of added PKA, both the wild-type and S128A mutant proteins partitioned quantitatively in the membrane (pellet) fraction, indicating efficient membrane insertion. With added PKA from 2.5 to 10 U in the presence of unwashed REM, increasing levels of labeled 2B1 protein partitioned in the soluble fraction and correspondingly reduced levels in the membrane fraction. The S128A mutant protein, on the other hand, inserted nearly quantitatively into the membrane even in reactions with the highest level (10 U) of PKA. Membrane insertion of 2B1 was SRP-dependent, since addition of salt-washed K-RM (and no PKA) resulted in negligible membrane insertion (Figure 6A, right panel). These results show that phosphorylated 2B1 is not efficiently inserted into the ER, and instead, remains in the soluble fraction.

Fig. 5. Mitochondrial localization of P4502B1. COS cells were transfected with WT/2B1 cDNA and grown for 60 h with or without added dibutyl cAMP (5 μ M) as indicated. Cells were subjected to double immunostaining with polyclonal antibody against P4502B1/monoclonal antibody to COX I protein (A) or monoclonal antibody to 2B1/polyclonal antibody to β -COP (B). (A) Histological (a and d) 2B1 antibody staining patterns (developed with Alexa dye 488, anti-rabbit); (b and e) COX I antibody staining patterns (developed with Alexa dye 594, anti-mouse); (c and f) the digital overlay of the images. (B) (g) Staining pattern with 2B1 antibody (developed with Alexa dye 594, anti-mouse); (h) staining with Golgi-specific β -COP antibody (developed with Alexa dye 488, anti-rabbit); (i) digital overlay of (g) and (h).

The effects of phosphorylation of 2B1 on SRP binding were studied by two different approaches. In the absence of ER, SRP binds to nascent chains, causing a translational pause under *in vitro* conditions (Belin *et al.*, 1996). In the first approach, we used SRP-mediated inhibition of *in vitro* translation (in WGL) as an indicator of its affinity for phosphorylated or unphosphorylated nascent chains. Cytosolic protein, dihydrofolate reductase (DHFR), was used as an internal control. As seen from Figure 6B (left panel), in the absence of added PKA, SRP inhibited translation rates of both wild-type and S128A mutant proteins in a concentration-dependent manner to reach >95% inhibition at 2 U of SRP. In the presence of added PKA (Figure 6B, right panel), the rate of translation inhibition of the S128A mutant protein remained nearly the same, while the inhibitory effects on the wild-type protein were less pronounced even with 5 U of added SRP. The rate of translation of DHFR in both the panels was unaffected by SRP addition. In the second approach, we tested the effects of phosphorylation on the association of 2B1 nascent chains with SRP by direct chemical cross-linking. The emerging wild-type or S128A mutant 2B1 chains translated in the WGL in the presence of [35 S]Met were allowed to interact with an added unit of SRP, and subjected to cross-linking with *m*-maleimidobenzoyl-

N-hydroxysulfosuccinimide ester (*S*-MBS) after the complete synthesis and dissociation of the protein from the ribosome. The cross-linked products were immunoprecipitated in parallel with antibody to P4502B1 and also antibody against the 54 kDa subunit of SRP (SRP Ab). As seen in Figure 6C (upper panel), immunoprecipitates with 2B1 antibody showed faster migrating 2B1 protein in all the reactions. Reactions with added cross-linker yielded a slower migrating component of ~105 kDa, possibly representing the cross-linked product. Phosphorylation of wild-type protein reduced the efficiency of cross-linking by 60–70%, while translation of S128A mutant protein, in the presence of PKA, had no effect on the rate of cross-linking with SRP. Immunoprecipitation of reaction mixture with SRP Ab (Figure 6C, bottom panel) showed that the 105 kDa putative cross-linked product indeed contains SRP protein. Together, these results show that phosphorylation of 2B1 at Ser128 drastically reduced the affinity of the protein for binding to SRP and hence its insertion into the ER membrane.

It is known that SRP-mediated translation arrest results in the accumulation of short peptide fragments (Belin *et al.*, 1996). The nature of 2B1 arrest fragments was tested using well characterized preprolactin (PPL) as a control. Figure 6D shows that *in vitro* translation of

Fig. 6. Effects of Ser128 phosphorylation on SRP binding and ER membrane targeting. (A) Extent of ER integration of proteins translated in the RRL system with [35 S]Met, 5 U of unwashed REM and 0–10 U/50 μ l of PKA (first 16 lanes). In the last two lanes, WT/2B1 translated in the WGL system in the absence of PKA was used. P (insoluble) and S (soluble) represent 0.1 M Na_2CO_3 fractions equivalent to 20 μ l of reaction volumes. (B) WT/2B1, S128A and DHFR were translated in the absence (left) or presence (right) of PKA with added [35 S]Met in WGL and the indicated amounts of SRP. (C) WT/2B1 and S128A protein chains were initiated in the presence (+phos) or absence (–phos) of PKA, with added 1 U SRP in WGL. Cross-linking was carried out using S-MBS as described in Materials and methods. Reactions without added S-MBS were run in parallel. Half of the reaction was immunoprecipitated with polyclonal antibody to 2B1 and the other half with antibody to the 54 kDa subunit of SRP (SRP Ab). (D, first four lanes) gel profile of control translation products (no SRP) and translation-arrested (+ 1 U of SRP) products of PPL and WT/2B1 proteins in the WGL system. (D, last four lanes) Na_2CO_3 -soluble (S) and -insoluble (P) fractions showing rates of membrane insertion of SRP-arrested 2B1. After 20 min translation in WGL with 1 U of added SRP, 4 mM $^7\text{mGMP}$ was added to block chain initiation, and incubated for a further 10 min with added K-RM (5 U) and with or without 5 U of added PKA. (E) Cross-linking with SRP was carried out as in (C), except that DHFR and PPL proteins were co-translated as competitors. Template ratios of 1:2.5 in the case of DHFR and 1:2 in the case of PPL were used to yield a competitor:2B1 protein ratio of 3:1. The cross-linked products were immunoprecipitated with the antibodies indicated. In (A) to (C) and (D), 10% polyacrylamide gels were used, and in (E), a 16% gel was used.

PPL in the presence of 1 U of SRP (in WGL) resulted in a vastly reduced level of 30 kDa intact protein but an increased level of the <7 kDa component. In support of studies with P4502B4 (Sakaguchi *et al.*, 1984) and our unpublished results (H.K.Anandatheerthavarada and N.G.Avadhani) with P4501A1, SRP-dependent translation arrest of 2B1 protein, however, did not yield any short fragments. Instead, we observed near-full-length arrest product. Although not shown, the SRP-arrested 2B1 protein readily associated with K-RM, and addition of PKA following SRP arrest inhibited membrane insertion. As seen from the cross-linking experiment in Figure 6E,

co-translation of 2B1 mRNA (in WGL with 1 U of SRP), with DHFR as a non-specific competitor, yielded normal levels of 105 kDa cross-linked products that could be immunoprecipitated with both 2B1- and SRP-specific antibodies. Co-translation with PPL, on the other hand, resulted in vastly reduced 2B1-specific 105 kDa cross-linked product and the appearance of a 62 kDa, SRP reactive product. The latter probably represents the <7 kDa translation arrest product of PPL cross-linked to the 54 kDa subunit of SRP. These results show the specificity of 2B1 nascent chain binding to SRP in addition to providing further support for the unusual mode of SRP-mediated

Fig. 7. Association of 2B1 signal sequence with mitochondrial translocase proteins. (A) Effects of translocation arrest on *in vitro* transport of 1-160WT/2B1-DHFR fusion protein, run on 14% gel. (B) Chemical cross-linking of translocation-arrested 1-160WT/2B1-DHFR fusion protein with yeast mitochondrial translocase complexes, run on 10% gel. Yeast mitochondria (250 μ g in 100 μ l reaction volume) were incubated with *in vitro* translation products (Murakami *et al.*, 1988). Translocation arrest was caused by pre-treating mitochondria with 1 μ M MTX or 0.25 μ g/100 μ l Efrapreptin (Sigma Chemical Co.) for 20 min at room temperature before initiating the import. A synthetic Put2p signal peptide (300 nM) was added as a competitor. In (A), protease digestion was carried out with 150 μ g/mg (+) or 300 μ g/mg (++) trypsin. In (B), cross-linking with MBS and immunoprecipitation were carried out as described in Materials and methods. In both (A) and (B), 35 S-labeled fusion protein (50 000 c.p.m.) translated in RRL in the presence of PKA was used, except in the last lane of (B), where translation was carried out in the presence of 5 U of PKA inhibitor, PKI (New England Biolabs).

inhibition of 2B1 translation, which yields a near-full-length SRP arrested product.

Association of 2B1 protein with mitochondrial translocase complexes

We tested the ability of 2B1 signal sequence to interact with yeast mitochondrial translocase complexes using an experimental system similar to that described by Schneider *et al.* (1994). We used the 1-160WT/2B1-DHFR fusion protein shown in Figure 2C to allow the methotrexate (MTX), a competitive inhibitor of DHFR, to mediate translocation arrest of protein in association with translocase complexes. *In vitro* import in yeast mitochondria (Figure 7A) showed that the fusion protein translated in RRL in the presence of added PKA was imported efficiently into mitochondria. Both the mitochondrial binding and transport were inhibited by 300 nM synthetic mitochondrial targeting sequence of Put2p protein. Addition of MTX yielded protease-resistant fragments of ~14–17 kDa, which probably represent a pre-translocation

intermediate(s) in contact with different translocase complexes. Depletion of matrix ATP by the addition of Efrapreptins also yielded similar protected fragments, demonstrating that the 2B1 N-terminal signal forms a pre-translocation intermediate as established previously for other mitochondrial targeted proteins (Schneider *et al.*, 1994; Neupert, 1997). These results also show that the 1–160 sequence of P4502B1 can target a heterologous reporter protein to mitochondria.

In Figure 7B, cross-linking of transport-arrested 1-160WT/2B1-DHFR fusion protein in intact yeast mitochondria was carried out using the membrane-permeable chemical cross-linking agent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS). Immunoprecipitation of reaction mixture without added cross-linker with 2B1 antibody yielded 36 kDa fusion protein, while the incubation mixture with added cross-linker yielded two major and a number of minor slow-migrating components. Immunoprecipitation with translocase-specific antibodies shows that the 106 kDa species may be the cross-linked product of the fusion protein with Hsp70, and the 75 kDa band may consist of similarly migrating cross-linked products with TIM40 and TOM44 proteins. It should be noted that mitochondrial membrane-associated TOM40 and TIM44 exhibit comparable electrophoretic mobility. Furthermore, translocation arrest with added MTX or Efrapreptins yielded essentially similar putative cross-linked products. Addition of 300 nM synthetic Put2p signal peptide inhibited both the mitochondrial binding and cross-linking of the fusion protein, suggesting specificity. Use of fusion protein, translated in the RRL in presence of added PKA inhibitor (PKI), resulted in a 10-fold reduction of mitochondrial binding and no detectable cross-linked products, further illustrating the importance of phosphorylation in the activation of mitochondrial targeting signal. Although not shown, S128A/2B1-DHFR fusion protein behaved similarly to the unphosphorylated protein. These results show that phosphorylation markedly increases the affinity of 2B1 protein to bind to yeast mitochondrial translocases, TOM40 and TIM44, as well as matrix Hsp70 protein.

Discussion

We have elucidated mechanisms of bimodal targeting of the barbiturate-inducible drug metabolizing enzyme, cytochrome P4502B1, to the ER and mitochondria. Interestingly, the extent of ER and mitochondrial targeting of 2B1 protein is modulated by the cellular cAMP level through phosphorylation at the PKA signature sequence RRFSL at 125–129. cAMP is known to regulate the activity of an array of cellular processes including rate-limiting steps of metabolic pathways, metabolite flux, receptor function, various ionic fluxes across membranes and regulation of gene expression through activation and inactivation of transcription factors, etc. (Francis and Corbin, 1994). However, this is probably the first demonstration of cAMP tightly regulating the subcellular destination of a protein by activation/inactivation of its N-terminal chimeric signal components. In view of the known role of cAMP in the regulation of intramitochondrial enzyme activities (Linn *et al.*, 1969), our results suggest a novel and

Fig. 8. A model for the cAMP-regulated biogenesis of P450MT4 *in vivo*.

direct role for extramitochondrial cAMP in mitochondrial protein targeting and organelle biogenesis.

Using a combination of *in vitro* transport in isolated mitochondria and *in vivo* protein expression in COS cells, we show that wild-type underphosphorylated or S128A mutant 2B1 protein is poorly targeted to mitochondria. *In vitro* phosphorylated wild-type protein, or that expressed under elevated cAMP, preferentially localizes in the mitochondrial compartment but shows reduced ER localization. Studies on the mutational analysis of proteins using both *in vivo* and *in vitro* transport systems demonstrate a critical requirement for the positively charged residues (sequence 20–30) of the protein for mitochondrial targeting. Equally important for the mitochondrial targeting of P4502B1 apoprotein is the consensus sequence for PKA-dependent phosphorylation at Ser128. Although not shown, these two domains of P4502B1 were also essential for targeting a C-terminal fused non-mitochondrial reporter protein, DHFR, to mitochondria, further confirming their critical role in mitochondrial targeting.

We reasoned that the observed effects of PKA-dependent phosphorylation on the targeting of P4502B1 apoprotein to two membrane compartments may somehow be related to the functional activation of a cryptic mitochondrial signal at the 20–30 region of the protein. In support of such a possibility, results show that underphosphorylated 2B1 protein binds to SRP more efficiently and also preferentially associates with the ER membrane in a transmembrane orientation. On the other hand, underphosphorylated wild-type protein and the S128A mutant proteins are imported into mitochondria poorly and also interact with mitochondrial translocase proteins, TOM40 and TIM44, and matrix Hsp70 at a reduced rate. The phosphorylated 2B1 protein, however, shows quite opposite affinity for SRP and mitochondrial translocases. These results suggest a switch-like mechanism for the activation or inactivation of the chimeric signal elements of P4502B1, which in turn appears to regulate the bimodal transport of the protein to mitochondria or the ER (see the model in Figure 8). The model implies that in an underphosphorylated state, the ER targeting signal is more active, binds more efficiently with SRP, and the nascent chain is targeted to the ER. Under high cellular cAMP levels, phosphorylation at an internal site may destabilize the bound SRP by an unknown mechanism. Internal phosphorylation, therefore, results in inactivation of the ER targeting signal but activation of the mitochondrial targeting signal. In the latter state, the polypeptide is translated in a membrane-free form and is eventually targeted to mitochondria. Chain lengths from 72 amino

acids for PPL (Wolin and Walter, 1993) to 137 amino acids for opsin (Laird and High, 1997) have been reported for SRP binding to the nascent proteins. Surprisingly, our results with 2B1 show that the SRP-arrested product is a near-full-length translation product. Furthermore, the observation that phosphorylation of arrested product drastically reduces the rate of ER insertion of 2B1 protein directly supports our model of the inactivation of ER targeting signal by phosphorylation. To our knowledge, this is probably the first demonstration of regulation of protein targeting by post-translational activation or inactivation of signal sequence by hormonal factors.

Currently, structural details of how phosphorylation of an internal site activates a cryptic mitochondrial signal at a distant N-terminal region remain unclear. It is likely that phosphorylation-induced conformational change, as indicated in the CD spectral shift, may be part of the structural change associated with the activation of the cryptic mitochondrial signal domain. A highly interesting part of our observation is that cAMP appears to functionally regulate the signal property of 2B1 protein in an on-off mode, presenting a novel mode of regulation of protein targeting either to mitochondria or to the ER.

The concept of an N-terminal chimeric signal sequence consisting of signal elements for targeting the same protein to two different organelles was first suggested in our studies on the targeting of P4501A1 to the ER and mitochondria (Addya *et al.*, 1997; Anandatheerthavarada *et al.*, 1999). The present study confirms and extends the existence of a chimeric signal sequence in other P450 isoforms. Despite differences in the *in vivo* mechanisms of activation of mitochondrial targeting signals of the two P450 proteins, the chimeric signal of P4502B1 is functionally and structurally reminiscent of that of P4501A1. First, in both cases a stretch of positively charged amino acid region, immediately C-terminal to the transmembrane domain, appears to function as a cryptic mitochondrial targeting signal. Secondly, removal of the N-terminal transmembrane domain of 2B1 protein appears to constitutively activate the mitochondrial targeting signal, thus overriding the need for internal phosphorylation at Ser128. It is likely that in both cases the N-terminal hydrophobic domain, consisting of the ER targeting and transmembrane helical region, might impose steric restrictions for the basic region to bind to mitochondrial translocase proteins. The conformational shift induced by N-terminal deletion or Ser128 phosphorylation is also important for the catalytic function of the P450 protein inside mitochondria (not shown). Modified 2B1 protein targeted to mitochondria exists as a membrane-extrinsic matrix protein and interacts with the mitochondrial-specific soluble electron carrier protein, adrenodoxin, at a higher affinity compared with intact and underphosphorylated protein (H.K.Anandatheerthavarada and N.G.Avadhani, unpublished results). Unpublished results from our laboratory suggest that mitochondrial targeting of a number of other P450s, including P4502E1, 3A1/2, 2D6, 2C12, etc., is also regulated by a similar PKA-dependent phosphorylation mechanism. We believe that the cAMP-regulated preferential targeting of various P450 monooxygenase enzymes to the mitochondrial compartment has an important physiological role in providing protection against chemical/oxidative damage to mitochondria.

Materials and methods

Subcellular isolation and purification of P450MT4 and P4502B1

Mitochondria and microsomes were isolated from livers of PB-treated Sprague-Dawley rats as described before (Niranjan *et al.*, 1984). The final mitochondrial pellets were suspended in 50 mM phosphate buffer (pH 7.4) containing 20% glycerol, 1 mM dithiothreitol (DTT), 1 mM EDTA, and phosphatase inhibitor and protease inhibitor mix as indicated above. Mitochondria from the yeast *Saccharomyces cerevisiae* were prepared as described previously (Murakami *et al.*, 1988). P450MT4 and P4502B1 were purified from PB-induced rat liver mitoplasts and microsomes, respectively, as described (Shayiq and Avadhani, 1990).

Circular dichroism spectroscopy

CD spectra of P450MT4 and P4502B1 were taken on a Jasco J720 instrument at room temperature in a 0.2-mm-pathlength water-jacketed cell. The proteins were diluted in 50 mM phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM DTT at a concentration of 0.3 mg/ml, determined by amino acid analysis. Background spectra were obtained under identical conditions and were digitally subtracted from the test spectra. Mean residue ellipticity ([θ]MRW) is expressed in degrees cm²/dmol using a mean residue weight of 110.

Peptide mapping and amino acid sequencing

Peptide fingerprint analysis of purified P450MT4 and the microsomal P4502B1 by microbore HPLC and sequencing of intact proteins and internal peptides in a Model 475A sequencer (Applied Biosystems, Inc., Foster City, CA) were carried out as described before (Addya *et al.*, 1997).

Construction of cDNA for wild-type and mutant proteins

P4502B1 cDNA (Monier *et al.*, 1988) was used as a template to generate N-terminal deletions by polymerase chain reaction (PCR) using the appropriate sense primer. The 3Mut/2B1 cDNAs with internal mutations of R21G, K25T, R27L and S128A were generated by overlap PCR. cDNA for 2B1-DHFR fusion proteins was generated by ligating the DNA sequence encoding the wild-type 1–160 sequence of 2B1 protein in-frame with the mouse DHFR coding sequence through an *EcoRI* linker. All constructs were engineered to contain 5' *XbaI* and 3' *SmaI* sites. cDNA constructs were cloned in PCR TM II (Invitrogen), pGEM7zf and mammalian expression vector pCI (Promega).

In vitro transport of proteins into isolated mitochondria

cDNA constructs in pGEM7zf and PCR TM II vectors were used as templates in Sp6 or T7 polymerase-coupled RRL or WGL transcription-translation systems as described before (Addya *et al.*, 1997). The translation products were phosphorylated in two ways according to Koch and Waxman (1989). The translation mix was supplemented with 10 U of the catalytic subunit of PKA/50 μ l translation mix (Sigma Chemical Co., St Louis, MO). The translation was carried out in the presence of either 0.1 mM unlabeled ATP and [³⁵S]Met, or 3 μ Ci/50 μ l (6000 Ci/mmol) of [γ -³²P]ATP and no [³⁵S]Met. Import of ³⁵S- or ³²P-labeled translation products in RRL was carried out using the system initially described by Gasser *et al.* (1982) and recently modified by Addya *et al.* (1997) using freshly isolated rat liver mitochondria. Mitochondria from both trypsin-treated and untreated samples were re-isolated by pelleting through 1.35 M sucrose, and the proteins were subjected to SDS-PAGE followed by radiometric scanning through a Bio-Rad GS525 molecular imager.

Source of antibodies

The sources of polyclonal antibody to P4502B1, P450 reductase and P450c27 were described before (Addya *et al.*, 1997). Antibodies were raised against whole TIM44 protein and a chemically synthesized peptide (residues 1–19) of TOM40 protein. Monoclonal antibody (H8) to P4502B1, polyclonal antibody to the 54 kDa subunit of SRP and human MrTFA were provided by Drs M.Adesnik, D.Andrews and D.Clayton, respectively. Monoclonal antibody to phospho-Ser was from Sigma Chemical Co., human COX I subunit was from Molecular Probes, Inc., and polyclonal antibodies to β -COP and calreticulin were from Affinity Bio Reagents. KCl-washed and unwashed canine pancreatic ER and SRP were gifts from Dr R.Gilmore.

Expression of 2B1 proteins in COS cells by transient transfection

COS-M6 cells were transfected with plasmid DNAs (10 μ g/100 mm plate) using the liposomal agent Superfect (Qiagen). About 62 h after

transfection, cells from 10 plates were pooled, homogenized in a Teflon fitted glass homogenizer (10 strokes at 5000 revolutions) and used for the isolation of mitochondrial and microsomal fractions as described previously (Addya *et al.*, 1997).

Immunofluorescence microscopy

COS cells grown on poly L-lysine-coated coverslips were transfected with 2B1 cDNA. After 62 h of transfection, cells were fixed and double immunostained with antibodies to P4502B1 and with either monoclonal antibody against mitochondrial genome-coded COX I subunit, or polyclonal antibody to Golgi-specific protein β -COP. Details of transfection, antibody staining and confocal microscopy were as described (Addya *et al.*, 1997).

Chemical cross-linking of P4502B1 with SRP

Chemical cross-linking of 2B1 with SRP was carried out co-translationally as described (Chuck and Lingappa, 1992; Laird and High, 1997). Briefly, proteins were translated in WGL in the presence of 1 U of SRP, [³⁵S]Met, and with or without added PKA (10 U/50 μ l of reaction) to allow co-translational association of SRP with nascent chains. After 20 min at 30°C, the reaction was stopped by adding 1 mM cycloheximide and the incubation was continued for 10 min to allow chain completion. In some cases, chain initiation was inhibited with 4 mM ⁷mGMP. Cross-linking was carried out (50 μ l volumes) in reaction mixture containing 220 mM sucrose, 0.05% Triton X-100, 1 mM EDTA, 10 mM MOPS buffer pH 7.2, 20 mM KCl, 25 μ l of ³⁵S-labeled translation products with bound SRP and 500 μ M hetero-bifunctional cross-linker S-MBS (Pierce Chemicals Co.). The reaction was carried out at room temperature for 20 min and terminated by the addition of 80 mM Gly and 5 mM β -mercaptoethanol.

Cross-linking with isolated intact yeast mitochondria was carried out using 1–160WT2B1-DHFR fusion protein as described (Schneider *et al.*, 1994). Mitochondria (250 μ g protein) were incubated with *in vitro* translation products (80 000 c.p.m.) and MTX- or Efrapentin-arrested intermediates were cross-linked using the membrane-permeable cross-linker MBS (Pierce Chemical Co.) as described by Schneider *et al.* (1994). Immunoprecipitation of Triton X-100-solubilized proteins was carried out as described (Laird and High, 1997).

Membrane integration assay

Proteins were translated in the presence of 5 U/50 μ l of unwashed or KCl-washed canine ER in the RRL system, supplemented with varying amounts of PKA from 0 to 10 U essentially as described by Laird and High (1997). Translation was stopped by the addition of 1 mM cycloheximide and the reaction mixtures were extracted with 0.1 M Na₂CO₃ (pH 11.0) as described (Chuck and Lingappa, 1992). The supernatant and membrane pellet fractions were recovered by trichloroacetic acid precipitation and analyzed as described (Anandatheerthavarada *et al.*, 1999). The gels were scanned and quantitated using a Bio-Rad GS525 molecular imager.

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