Physiological Role of the N-terminal Processed P4501A1 Targeted to Mitochondria in Erythromycin Metabolism and Reversal of Erythromycin-mediated Inhibition of Mitochondrial Protein Synthesis*

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Recently, we showed that the major species of β-naphthoflavone-inducible rat liver mitochondrial P450MT2 consists of N-terminal truncated microsomal P4501A1 (+33/1A1) and that the truncated enzyme exhibits different substrate specificity as compared with intact P4501A1. The results of the present study show that P450MT2 targeted to COS cell mitochondria by transient transfection of P4501A1 cDNA is localized inside the mitochondrial inner membrane in a membrane-extrinsic orientation. Co-expression with wild-type P4501A1 and adrenodoxin (Adx) cDNAs resulted in 5–7-fold higher erythromycin N-demethylation (ERND) in the mitochondrial fraction but minimal changes in the microsomal fraction of transfected cells. Erythromycin, a potent inhibitor of bacterial and mitochondrial protein synthesis, caused 8–12-fold higher accumulation of CYP1A1 mRNA, preferential accumulation of P450MT2, and 5–6-fold higher ERND activity in the mitochondrial compartment of rat C6 glioma cells. Consistent with the increased mitochondrial ERND activity, co-expression with P4501A1 and Adx in COS cells rendered complete protection against erythromycin-mediated mitochondrial translation inhibition. Mutations that specifically affect the mitochondrial targeting of P4501A1 also abolished protection against mitochondrial translation inhibition. These results for the first time suggest a physiological function for the xenobiotic inducible cytochrome P4501A1 against drug-mediated mitochondrial toxicity.

Mitochondria in mammalian organisms are important subcellular targets for a variety of xenobiotics including drugs, carcinogens, and environmental contaminants (1–6). Structurally diverse chemicals and pharmacologically important drugs, which alter mitochondrial membrane properties and affect mitochondrial enzyme complexes or gene expression, are thought to be contributing factors in a variety of human disorders (3, 5–8). Efforts in our laboratory have been focused on the characterization of mitochondrial class I and class II drug metabolizing enzymes to determine their roles in modulating the toxic effects of various xenobiotic chemicals on the mitochondrial genetic system. Our studies showed the presence of five different xenobiotic-inducible P4503 monocooxygenases in rat liver mitochondria, which cross-react with antibodies to similarly induced microsomal P450 isoforms (9–14). Enzyme reconstitution and immunochromic studies also showed the presence of several P450 isoforms, resembling the liver mitochondrial forms, in induced rat brain and human brain mitochondria (15–17). Additionally, reports from various groups have shown the occurrence of different isoforms of glutathione S-transferases in the mitochondrial membrane compartment of rat liver, brain, and lung (18–21), suggesting the existence of both class I and class II enzymes in mitochondria.

Recent studies in our laboratory showed that the two BNF-inducible hepatic mitochondrial P450s, designated as P450MT2A and MT2B, are derived by differential proteolytic processing of the similarly induced microsomal P4501A1 with cleavage sites past the 4th and 32nd amino acid residues, respectively (22). Transient transfection in COS cells and site-specific mutagenesis studies showed that the N terminus of P4501A1 contains a chimeric signal for targeting the protein to both the ER and mitochondrial compartments. About 20–25% of the nascent P4501A1 chains in both BNF-induced liver and COS cells transfected with expression cDNA constructs escape ER targeting by an unknown mechanism and are processed by an endoprotease to activate a cryptic mitochondrial targeting sequence (22, 23). The nature of the protease and also steps involved in redirecting this otherwise predominantly ER-targeted protein to mitochondria remain unclear. Enzyme reconstitution experiments with purified mitochondrial P450 from BNF-treated liver and bacterially expressed P450 forms showed that P450MT2 exhibits high ERND activity in an Adx + Adr-supported system. In light of the established evidence that P4501A1 in a NADPH P450 reductase-supported system has low ERND activity (23, 24), the observed high ERND activity of the mitochondrial P450 form suggested significant difference in the conformation of the two forms. This possibility was further supported by a high affinity domain-specific interaction of P450MT2 with Adx under in vitro conditions (24).

In the present study, the difference in the catalytic properties of P4501A1 targeted to the two subcellular compartments was used as a marker to further investigate the dual targeting of P4501A1 to the ER and mitochondria and also to elucidate the physiological significance of mitochondrial targeting.

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† The abbreviations used are: P450, cytochrome P450; P450 reductase, NADPH cytochrome P450 reductase; BNF, β-naphthoflavone; Adx, adrenodoxin; Adr, adrenodoxin reductase; P450MT2, N-terminal truncated P4501A1 targeted to mitochondria; ERND, erythromycin N-demethylase, EROD, ethoxyresorufin O-deethylase; ER, endoplasmic reticulum.

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Erythromycin is a potent antibiotic with known inhibitory effects on the bacterial ribosome system and minimal effects on the eukaryotic cytoplasmic ribosomes. Because of the reported prokaryotic nature of the mitochondrial ribosomes with respect to sensitivity to various antibiotics (25–30), we tested the effects of P450MT2 overexpression in COS cells on the mitochondrial erythromycin metabolism and erythromycin-mediated inhibition of mitochondrial translation. Our results provide further supporting evidence for the dual ER and mitochondrial targeting of P450MT2 in addition to suggesting the role of the mitochondrial targeted P450MT2 in erythromycin detoxification and protection against the drug-induced inhibition of mitochondrial protein synthesis. Additionally, we demonstrate that erythromycin treatment not only induces CYP1A1 mRNA accumulation but also causes an enhanced accumulation of P450MT2 in the mitochondrial compartment and an associated increase in ERND activity.

MATERIALS AND METHODS

Subcellular Fractionation and Isolation of Mitochondria—Mitochondria and microsomes from BNF-induced rat livers were isolated as described before (9, 13). Mitochondria and microsomes from transfected COS cells and C6 glioma cells were isolated by sucrose density banding and digitonin treatment as described recently (22). Cells from about 10 plates (100 mm) were pooled and homogenized in a sucrose-mannitol buffer (13) with 10–12 strokes (about 5,000 revolutions/min) of a Teflon fitted glass homogenizer, and subcellular membrane fractions were isolated by differential centrifugation. The crude mitochondrial fraction was suspended in the sucrose-mannitol buffer by gentle homogenization and further purified by sedimentation through a discontinuous sucrose gradient. Mitochondrial particles banding at the interface of 1.35 and 1.6 m sucrose were recovered, washed twice with the sucrose-mannitol buffer, and used for further analysis. As shown recently, this method yields intact mitochondrial free of detectable microsomal marker proteins (23). In some experiments, mitochondria were subjected to digitonin treatment (75 µg/mg protein for 5 min on ice) as described (13).

cDNA Constructs and Expression in COS Cells—The cDNA constructs expressing full-length P4501A1 cDNA, N-terminal deletions, and digitonin treatment as described recently (22). The pCD mammalian expression cDNA construct for bovine Adx was generously provided by Dr. Michael Waterman (31, 32). Transfection of COS cells with Adx transfected DNA was carried out using 0.1 M Na2CO3 buffer (pH 11) as described before (23, 39). Mitochondria and microsomal fractions were resuspended in a reaction mixture (150–200 µl) were removed and used for determining the extent of the mitochondrial targeted P450MT2 in erythromycin detoxification and protection against the drug-induced inhibition of mitochondrial protein synthesis. Additionally, we demonstrate that erythromycin treatment not only induces CYP1A1 mRNA accumulation but also causes an enhanced accumulation of P450MT2 in the mitochondrial compartment and an associated increase in ERND activity.

Reconstitution of Enzyme Activities—Enzyme activities were reconstituted in a reaction mixture containing the appropriate buffer system, 100–200 µg of microsomal or sonic disrupted mitochondrial protein, either using 400 pmol/mg purified rat microsomal P450 reductase or 180 pmol/ml bovine Adx reductase essentially as described before (9, 23, 24). ERND and EROD activities were measured according to published methods (33, 34).

In Vitro Protein Import Into Isolated Mitochondria—cDNAs encoding +5/1A1 and +33/1A1 were cloned in pcDNA3 plasmid and used as templates to program the synthesis of [35S]labeled in vitro translation products in a Promega TNT coupled system essentially as described (22). In vitro mitochondrial import was carried out in 200-µl reaction volumes consisting of 2–4 µl of reticulocyte lysate translation products (80,000 cpm), 500 µg of freshly isolated rat liver mitochondria, 60 µl of buffer, and 70 µl of transport buffer as described previously (35, 36). The final mitochondrial pellet was dissolved in Laemmli sample buffer (37) and analyzed by SDS-polyacrylamide gel electrophoresis. The proteins were subjected to fluorography. Digitonin fractionation of mitochondria before or after in vitro incubation was carried out essentially as described before using 75 µg of digitonin/mg of protein (13, 38), which selectively eliminates the outer membrane without any measurable loss of the inner membrane matrix components (38). Fractionation of mitochondrial proteins into alkaline-soluble and -insoluble fractions was carried out using 0.1 M Na2CO3 buffer (pH 11) as described before (23, 39).

In Vitro Protein Synthesis with Isolated Mitochondria—Protein synthesis with isolated mitochondria was carried out essentially as described before (40). Digitonin-treated mitochondria from transfected COS cells and erythromycin-treated C6 glioma cells were suspended in a medium containing 0.25 M sucrose, 10 mM Tris HEPES (pH 7.4), 100 mM KCl, 10 mM MgCl2, 10 mM potassium phosphate (pH 7.4), and 5 mM b-mercaptoethanol at 2 mg of protein/ml. The medium was supplemented with 2 mM ADP, 2 mM GTP, 5 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 2 mM sucinate, 1 mM isocitrate, 200 µM cycloheximide, and 100 µM each of t-9-minois acids except methionine. After loading, ATP was added at a final concentration of 2 mM. The mixture was preincubated by gentle shaking for 5 min at 32 °C with added erythromycin or chloramphenicol as indicated in the legend to Fig. 3. At the end of preincubation, protein synthesis was initiated by adding 100 µCi/ml [35S]Met (1175 Ci/mmol; NEN Life Science Products), and the incubation was continued for 45 min. At intervals, aliquots (2 µl) were removed and used for determining the extent of [35S]Met incorporation by the hot trichloroacetic acid method (40). The labeled proteins (10 µg) were dissociated in Laemmli (37) sample buffer at 37 °C for 60 min and electrophoresed on 15–20% exponential gradient SDS-polyacrylamide gel electrophoresis. The translation products were quantitated by using a Bio-Rad GS-525 molecular imager.

In Vivo Mitochondrial Protein Synthesis in 1A1-transfected COS Cells—COS cells were transfected with Adx and P4501A1 cDNAs as described before (22). After 62 h of transfection, cells were washed with Met-free medium and incubated for 10 min with cycloheximide (200 µg/ml). This was followed by a 1-h incubation with varying concentrations of erythromycin, and labeling was carried out with [35S]Met (100 µCi/plate) for 4 h. Cells were washed and homogenized gently in mitochondrial isolation buffer, and mitochondria were isolated by differential centrifugation as described above. 50 µg of mitochondrial protein was subjected to electrophoresis on a 15–30% exponential gradient SDS-polyacrylamide gel (41, 42). The translation products were quantitated by using a Bio-Rad GS-525 molecular imager.

Northern and Western Blot Analysis—Northern hybridization was carried out using 30 µg of total RNA from treated or untreated C6 glioma cells under stringent conditions as described before (19). Western immunoblots were developed with polyclonal antibody against P4501A1 (23) using the Pierce Super Signal ULTRA chemiluminescent substrate kit and imaged, and the blots were quantitated in a Bio-Rad Fluor-s imaging system.

RESULTS

Membrane Topology and Intramitochondrial Location of P450MT2—As reported recently, P450MT2 purified from the similarly induced liver mitochondria resolves as two closely migrating proteins of about 51–54 kDa, both of which cross-react with antibody to P4501A1 (22, 23, 24). Northern and internal peptide sequencing (22) showed that the two mitochondrial P450 species have primary sequence similar to the microsomal P4501A1. The slower migrating P450MT2A lacks the terminal 4 amino acid residues (+5/1A1), while the faster migrating P450MT2B lacks the first 32 residues (+33/1A1).

The intramitochondrial location of the two P450MT2 components and their topological organization in the mitochondrial membrane compartment was studied by using a combination of digitonin treatment, trypsin digestion, and extraction with alkaline buffers. It is seen from Fig. 1A that both [35S]labeled +5/1A1 and +33/1A1 are imported into isolated rat liver mitochondria and rendered relatively insensitive to trypsin digestion. It is also seen that both +5/1A1 and +33/1A1 proteins are resistant to trypsin even after digitonin treatment (Fig. 1A), which removes over 80% of the mitochondrial outer membrane, leaving relatively intact inner membrane, suggesting that they are localized inside the intramitochondrial compartment. Nearly 80% of the labeled proteins are extracted in the alkaline-soluble fraction, suggesting that both proteins may be organized in a membrane-extrinsic topology. Fig. 1B represents immunoblot analysis of mitochondrial protein from +5/1A1- and +33/1A1-expressing COS cells using CYP1A1-specific antibody. It is seen that both with +5/1A1 and +33/1A1, over 80% of the

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antibody-reactive protein associated with the mitochondrial membrane fraction was resistant to trypsin digestion and was soluble in alkaline Na₂CO₃ buffer. In support of our observation with BNF-induced rat liver mitochondria (23), the results of the present study with the isolated mitochondrial import system (Fig. 1A) and also transient transfection in COS cells (Fig. 2B) show that both of the in vitro expressed MT2 components (+5/1A1 and +33/1A1) are localized within the mitochondrial inner membrane, probably as membrane-extrinsic proteins. These results provide further confirmation for the mitochondrial targeting of P4501A1 and also possible differences in the topological properties of the P450 protein targeted to the two membrane compartments.

Catalytic Profiles of P450 Targeted to the Mitochondrial and ER Compartments in 1A1-expressing COS Cells—Previous results from our laboratory showed that whole mitochondrial isolates and also partially purified P450MT2 from BNF-induced liver exhibit high ERND activity in an Adx + Adr-supported system. The bacterially expressed +33/1A1, resembling P450MT2, also showed high ERND activity, while the nearly intact +5/1A1 showed low activity (24). In the present study, direct evidence for the differential catalytic properties of P450 targeted to the two subcellular membrane compartments was sought by using the COS cell expression system. COS cells contain relatively low endogenous levels of Adx, Adr, and P450 reductase and no detectable P450 (43), thus providing a valuable cell system to study the catalytic properties of the transiently expressed P450s and their electron transport protein requirements. COS cells were transfected with the WT/1A1 cDNA, or various mutant constructs with or without co-transfection with the bovine Adx cDNA construct. Mitochondria and microsomes were isolated by a combination of differential cen-

trifugation and density banding methods (13, 22), which was shown to yield a nearly qualitative separation of the two membrane fractions with no detectable cross-contaminating marker proteins. The effects of overexpression of the mitochondrial targeted P450MT2 alone or in combination with Adx were examined by reconstituting the activity either with Adr or P450 reductase. We have assayed ERND activity and compared it with the EROD activity of P4501A1 targeted to mitochondria (44). In the present study, direct evidence for the differential catalytic properties of P450 targeted to the two subcellular membrane compartments was sought by using the COS cell expression system. COS cells contain relatively low endogenous levels of Adx, Adr, and P450 reductase and no detectable P450 (43), thus providing a valuable cell system to study the catalytic properties of the transiently expressed P450s and their electron transport protein requirements. COS cells were transfected with the WT/1A1 cDNA, or various mutant constructs with or without co-transfection with the bovine Adx cDNA construct. Mitochondria and microsomes were isolated by a combination of differential cen-

- **Table I**

<table>
<thead>
<tr>
<th>cDNA construct</th>
<th>Co-transfected with Adx</th>
<th>Reductase used for reconstitution</th>
<th>ERND</th>
</tr>
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<tr>
<td>Adx</td>
<td>None</td>
<td>Adr</td>
<td>0.166 ± 0.01 0.04 ± 0.01</td>
</tr>
<tr>
<td>WT/1A1</td>
<td>None</td>
<td>Adr</td>
<td>0.208 ± 0.01 0.087 ± 0.07</td>
</tr>
<tr>
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<td>Adr</td>
<td>0.17 ± 0.04 0.07 ± 0.04</td>
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<tr>
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<td>Adr</td>
<td>0.191 ± 0.03 0.103 ± 0.01</td>
</tr>
<tr>
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<td>Adr</td>
<td>0.114 ± 0.01 0.09 ± 0.02</td>
</tr>
<tr>
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<td>Adr</td>
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</tr>
<tr>
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<td>Adr</td>
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<tr>
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<td>P450 reductase</td>
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</tr>
<tr>
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<td>Adx</td>
<td>P450 reductase</td>
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</tr>
<tr>
<td>Adx</td>
<td>None</td>
<td>P450 reductase</td>
<td>0.085 ± 0.01 0.065 ± 0.01</td>
</tr>
</tbody>
</table>

- Transfections were carried out with 10 μg of reporter plasmid.
- For co-transfection, 5 μg of Adx expression cDNA was used.
- In vitro reconstitution of enzyme activities was carried out using 180 pmol of Adr/ml or 400 pmol of P450 reductase/ml of reaction mixture.

Results in Table I show that expression of intact 1A1 or +33/1A1 proteins, and reconstitution with Adr yielded marginal ERND activity (0.1–0.2 nmol) in both mitochondria and microsomes. Coexpression with Adx cDNA in both cases...
yielded about 5–7-fold higher Adr-dependent activity with the mitochondrial but not the microsomal fractions. These results are consistent with the ERND activity profiles of purified microsomal P4501A1 in a P450 reductase-supported system and purified P450MT2 or bacterially expressed 1A1 in Adx-supported systems, respectively (23, 24). These results are also in accordance with the observations that cells transfected with wild type 1A1 yield the two forms of mitochondrial P450s, including the putative +33/1A1 (MT2b) with high ERND activity in an Adx + Adr-supported system. Following the entry into mitochondria, the latter species interacts efficiently with mitochondrially targeted Adx to metabolize erythromycin, while the +5/1A1 has very low ERND activity (24). Expression of mutant forms, Mut/1A1 and +33/Mut, yielded activities similar to those of cells transfected with the Adx cDNA alone. The mitochondrially targeted P450MT2 also showed a significant but reduced ERND activity when reconstituted with the P450 reductase electron donor system. As expected, the P450 reductase-supported activity was not affected by co-expression with Adx cDNA. The microsomal fraction in both Adx + Adr-supported and NADPH P450 reductase-supported systems showed a marginal activity. These latter results are consistent with the reported low ERND activity of the microsomal P4501A1 (23, 24).
micromosomal EROD activity. Reconstitution with P450 reductase protein, however, yielded a low, but significant activity (2.5 pmol/min/mg protein), while the micromosomal fraction showed a high activity in the range of 70–80 pmol/min/mg protein. It should be noted that P450 reductase-supported EROD activity with both the mitochondrial and micromosomal enzymes was not affected by Adx cDNA co-expression. These results show that the mitochondrial localized P450 can receive electrons from both P450 reductase and Adx electron transfer systems. Our results also show that in an Adx + Adr-supported system, the truncated mitochondrial form, +33/1AI exhibits substrate specificity and catalytic activity somewhat different from its more intact microsomal counterpart, reconstituted with the P450 reductase system.

Reversal of Erythromycin-induced Mitochondrial Translation by P450MT2—The inhibitory effects of chloramphenicol, streptomycin, and related macrolide antibiotics, including erythromycin, on the mitochondrial translation systems from various cell sources have been well established (25–30). Because of the observed ERND activity of P450MT2, we investigated its ability to provide protection against erythromycin-induced inhibition of mitochondrial translation. Fig. 3A shows the effects of 120 μg/ml erythromycin on protein synthesis by mitochondria from COS cells expressing Adx alone or Adx plus wild type or Mut/1A1 proteins. The pattern of total [35S]Met incorporation in isolated mitochondria shows nearly complete reversal of mitochondrial translation inhibition by co-expression with WT/1A1 and Adx cDNAs, but co-expression with Mut/1A1 cDNA had no protective effect. As shown in the autoradiogram in Fig. 3A, mitochondria from Adx-expressing cells incorporated [35S]Met into characteristic mitochondrial translation products. The identification and designation of 13 individual polypeptide species is based in part on immunoprecipitation with specific antibodies (45, 46) and follows the nomenclature and electrophoretic patterns reported by Chomyn et al. (41). It is seen that in mitochondria from Adx-expressing cells, erythromycin (120 μg/ml) inhibited the mitochondrial translation by 70–80% as seen by the reduced band intensities and also the total trichloroacetic acid-insoluble radioactivity presented in Fig. 3B. The gel pattern in Fig. 3A also shows that ND3 and ATPase 8 proteins appear to be major translation products, suggesting a relatively higher translation efficiency of these subunits under the in vitro conditions. Chloramphenicol (200 μg/ml), which is a well known inhibitor of mitochondrial specific translation also inhibited the activity of mitochondria from control (Adx-expressing) cells (see Fig. 3A, last lane). Co-expression with Mut/1A1 cDNA with altered mitochondrial targeting ability had no effect on the erythromycin-induced inhibition of mitochondrial translation (see lanes 3 and 4). Co-expression with WT/1A1 cDNA, however, resulted in a nearly complete reversal of translation inhibition. As shown in Fig. 4, A–F, autoradiograms from four independent cell transfection and mitochondrial translation experiments, as in Fig. 3A, were used to quantitate the levels of [35S]Met incorporation into individual mitochondrial translation products, namely CO II + CO III (Fig. 4A), ND1 (B), ND2 + cytochrome b5 (C), ND4 + CO I (D), ND3 (E), and ATPase 8 (F). Results show that all of these subunits were inhibited by 120 μg/ml of erythromycin by about 70–80%, and their inhibitory effects were fully reversed by co-expression with WT/1A1 but not Mut/1A1 cDNA.

The ability of P450MT2 to reverse the erythromycin inhibition of mitochondrial translation in whole cells was investigated using the COS cell expression system. As shown in Fig. 5, cells transfected with various cDNA constructs for 62 h were labeled with [35S]Met in the presence of 200 μg/ml cycloheximide to suppress cytosolic translation or in combination with cycloheximide and the indicated amounts of erythromycin. The results show that in cycloheximide-treated cells, the mitochondrial translation continues as seen by the characteristic electrophoretic pattern (lane 1) and size distribution of proteins, similar to the gel profile of in vitro translation products in Fig. 3A. A notable difference from the in vitro translation pattern (Fig. 3A) was that ND4L and ATPase 8 are translated as minor products under the in vivo translation conditions (see Fig. 5A). Currently, reasons for this difference remain unclear. The gel pattern in Fig. 5A and quantitation in Fig. 5B also show that mitochondrial translation was inhibited by 85–90% by the addition of 120 μg/ml chloramphenicol or thiamphenicol (lanes 8 and 9). Erythromycin inhibited mitochondrial protein synthesis in a dose-dependent manner (lanes 2 and 3). Co-expression with the WT/1A1 construct (lanes 6 and 7) but not the Mut/1A1 cDNA (lanes 4 and 5) reversed the erythromycin effect in these cells. These results collectively show that in both intact cells and isolated mitochondria, P450MT2 can provide protection against erythromycin-induced mitochondrial translation possibly by inducing the metabolic inactivation of erythromycin into its demethylated form.

Induction of mRNA and Mitochondrial P450MT2 Levels by Erythromycin Treatment—A hallmark of class I family drug-metabolizing monoxygenases is that the steady state levels of a given P450 enzyme are induced severalfold by its cognate xenobiotic substrate (47). Induction of 1A family enzymes by diverse polycyclic aromatic hydrocarbons, of the 2B family by phenobarbital, and of the 3A family by dexamethasone and related glucocorticoids (48) are some examples. We therefore decided to see if erythromycin had any effect on CYP1A1 mRNA and its steady state levels in the mitochondrial fraction. We have used rat glioma C6 cells for this study because of the
known response of these cells to CYP1A1-specific inducers (49, 50). Results in Fig. 6 show that erythromycin doses that are shown to inhibit mitochondrial translation in COS cells (see Figs. 3 and 5) also induced the steady state levels of P4501A1 mRNA (lane 3) compared with untreated control cells, which contain a low level of the mRNA (lane 1). The extent of induction with erythromycin was similar to that obtained with the well known P4501A1 inducer, BNF. The 18 S rRNA hybridization pattern in Fig. 6 indicate a similar RNA loading in all three cases. Neither erythromycin nor BNF had any inducible effect on the P4503A1/2 mRNA level (Fig. 6, middle). This was surprising in view of the fact that P4503A1 is a marker enzyme for the microsomal ERND activity (47, 48). In this paper, we demonstrate that overexpression of P4501A1 either by its cognate inducer or by transfection with expression cDNA constructs effectively rendered protection against erythromycin-mediated inhibition of mitochondrial translation (Fig. 6D). These results together demonstrate that in susceptible cells, erythromycin induced both the CYP1A1 mRNA level and also preferentially elevated the level of mitochondrial P450MT2.

**DISCUSSION**

Erythromycin, a 14-membered ring macrolide, is well known for its antibacterial and anti-inflammatory effects in different target tissues. Its pharmacological potency is known to reside in its ability to selectively inhibit bacterial protein synthesis (27, 28). In keeping with the hypothesis on the endosymbiotic origin of mitochondria (51, 52), erythromycin also inhibits mitochondrial translation (26–29). It is also suggested that the anti-inflammatory action of this antibiotic may reside in its ability to induce NO production in endothelial cells and also its ability to modulate interleukin-8 gene expression (53). In this paper, we demonstrate that overexpression of P4501A1 either by its cognate inducer or by transfection with expression cDNA constructs effectively rendered protection against erythromycin-mediated inhibition of mitochondrial protein synthesis. The
protective effects of 1A1 specifically stem from the N-terminal truncated P450 targeted to mitochondria, which exhibits a remarkably high ability to metabolize erythromycin into inactive demethylated product.

Studies reported over the past 10 years showed that hepatic mitochondria from BNF- and PB-treated rats contain P450 proteins cross-reacting with the antibody to similarly induced major microsomal P450 forms (9, 14, 23). Although the putative mitochondrial forms exhibited electrophoretic migration similar to the microsomal counterparts, the former species showed preference for mitochondrial specific electron transfer proteins, Adx and Adr, for enzyme reconstitution, suggesting that they may be different molecular forms. A recent study (22) on P4501A1 in our laboratory showed that N-terminal truncation of the protein by a cytosolic endoprotease activates the cryptic mitochondrial targeting signal at the N-terminal 32–44 sequence region of the protein. Our results also showed that the bacterially expressed +33/1A1 not only had a higher affinity for Adx binding but also high ERND activity in an Adx- and Adr-supported system, suggesting a possible conformational change in the P450 protein devoid of the N-terminal transmembrane domain. Results presented in this study confirm and extend our previous observations on the mitochondrial targeting of P4501A1 and also its altered activity in the mitochondrial compartment. Our results show that expression of wild type 1A1 or +33/1A1 by transient transfection in COS cells results in the accumulation of antibody-reactive protein in mitochondria and increased ERND activity in the mitochondrial compartment. Interestingly, coexpression with mitochondrial targeted Adx protein resulted in a severalfold higher activity, further confirming the intramitochondrial location of the P450 and hence the ERND activity. Furthermore, mutations targeted to the putative mitochondrial targeting sequence abolished both mitochondrial accumulation of antibody-reactive protein and the ERND activity, further confirming the mitochondrial targeting of the N-terminal modified version of P4501A1.

Consistent with the ERND activity of the mitochondrial tar-

FIG. 6. Induction of P4501A1 mRNA and mitochondrial P450MT2 by erythromycin in C6 glioma cells. C6 glioma cells were cultured with added erythromycin (30 μM) or BNF (12 μM) for 92–96 h, and the cells were used for total RNA isolation or isolation of mitochondria using the sucrose density gradient method. A, Northern blot analysis using 30 μg of total RNA from each of the untreated or treated cells. The same blot was sequentially hybridized with 32P-labeled rat CYP1A1 cDNA probe, followed by stripping and hybridization with 32P-labeled rat 3A1 cDNA and 18 S rDNA probes. B, mitochondrial and microsomal proteins (50 μg each) from Me2SO (DMSO) control cells or cells treated with BNF or erythromycin (ERM) were subjected to Western blot analysis using polyclonal antibody to P4501A1. The blot was developed with the Super Signal Ultra chemiluminescence kit as described under “Materials and Methods” and imaged and quantitated in a Bio-Rad Fluor-S imager. The relative levels of antibody-reactive P450MT2 in the mitochondrial fractions of treated and untreated cells are presented at the bottom of the immunoblot. The values are averages of two separate immunoblots. C, the ERND activities of mitochondrial isolates from control Me2SO-treated cells, and cells treated with BNF and erythromycin. The results represent the average of two independent experiments. D shows the effects of 120 and 240 μg of erythromycin on the [35S]Met incorporation by mitochondrial isolates from control Me2SO-treated cells and cells treated with 30 μM erythromycin for 92 h as described for A above. Total [35S]Met incorporation was measured by extraction with hot trichloroacetic acid as described under “Materials and Methods.”
targeted P4501A1 in an Adx + Adr-supported system, P450 targeted to the COS cell mitochondrial compartment under the transient transfection conditions is detected as membrane-extrinsic protein (see Fig. 2B). Similar is the case with both +5/1A1 and +33/1A1 proteins imported into isolated mitochondria. It is not clear why +5/1A1 with nearly complete transmembrane domain assumes a membrane extrinsic topological arrangement within the mitochondrial membrane compartment, although the mitochondrial matrix environment may favor this organization. In any case, this mode of organization is highly conducive for interaction with soluble electron transfer proteins Adx and Adr. These results support and extend previous observations of our own and others that mitochondrial monoxygenase activities with either total mitochondrial membrane fragments or purified proteins (9–17, 23, 24) are preferentially supported by soluble electron transfer proteins Adx + Adr but very poorly by P450 reductase.

Until recently, it was widely believed that the microsomal P450s require microsomal cytochrome P450 reductase for transfer of electrons from NADPH, while the mitochondrial P450s require mitochondrial specific Adx + Adr for substrate oxidation. A number of exceptions to this generality have recently been reported. Bacterially expressed microsomal P450c17 was fully active with bacterial flavodoxin and flavodoxin reductase, and also flavodoxin induced a change in the spin state of P450 indicating productive physical association (54). Similarly, Dong et al. (55) showed that N-terminal truncated bacterially expressed human P4501A2 is active with bacterial flavodoxin as well as mitochondrial Adx and Adr electron transfer proteins. In extension of these observations, we showed that wild type Adx but not mutant forms physically interact with the bacterially expressed, N-terminal truncated P4501A1 as well as that purified from BNF-induced mitochondria as shown by chemical cross-linking, a change in the spin state of P450, and reconstitution of enzyme activity (24). Parallel results from various laboratories also show that N-terminal modified mitochondrial P450c27 targeted to the ER can be efficiently reconstituted with microsomal P450 reductase (56).2 Similarly, removal of N-terminal transmembrane domain of microsomal P4501A2 and -2E1 resulted in a 40–50% reduced activity in a P450 reductase-supported system, suggesting inefficient interaction (55, 57). Furthermore (57, 58), N-terminal truncation of P450 reductase significantly reduced its ability to support the activity of microsomal P450s (57–59), suggesting that membrane anchoring of both the P450 and the reductase proteins is necessary for optimal activity. Results emerging from various studies, including ours, therefore suggest that some of the microsomal P450 forms can effectively interact with Adx + Adr electron transfer proteins when they are organized in a membrane-extrinsic mode. Some of the mitochondrial P450s attached to a transmembrane anchor domain and organized in a transmembrane topology, on the other hand, can interact with membrane-anchored P450 reductase.

Although some of the xenobiotic-inducible hepatic microsomal P450 forms have been shown to exhibit varied levels of steroid hydroxylase activities (60), the precise physiological roles in most cases remain unclear. A number of studies suggest that some of the xenobiotic-inducible P450s may have roles in the manifestation of oxidative stress, while other studies imply a protective role against oxidative stress (61, 62). We therefore decided to determine if the altered activity of the mitochondrial targeted P4501A1 has any physiological significance. An exciting new observation of this study is that erythromycin treatment not only induced the mRNA level but also caused a preferential accumulation of antibody-reactive protein in the mitochondrial compartment (Fig. 6). Erythromycin is known to modulate or potentiate the pharmacological effectiveness of various drugs, including astemizole, carbamazepine, corticosteroids, warfarin, etc., by yet unknown mechanisms (63–65). The finding of this study that erythromycin acts as a classical P450 inducer provides a rational basis for its reported modulatory effects on other drugs.

Many hepatic P450 forms such as P4501D1 family members play a central role in modulating the pharmacological potencies and efficacies of many drugs and antibiotics. Thus, evaluating interpatient differences in P450 levels and genotyping specific P450 forms are becoming major developments in human medicine. In fact, Watkins et al. (66) have recently developed a breath test to measure the rate of metabolism of 14C-erythromycin as a measure of glucocorticoid-inducible hepatic P4501A1/2 level. In view of our present results showing high ERND activity of P4501A1 targeted to mitochondria, mitochondrial targeting of various P450 forms, and their altered substrate specificity (24), the catalytic properties of the mitochondrial targeted forms should also be taken into account both in the development of tests and in the interpretation of test results. In summary, this study provides the first evidence for the physiological role of mitochondrial targeted P450MT2 in drug detoxification and protection against erythromycin-induced inhibition of mitochondrial protein synthesis.

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Physiological Role of the N-terminal Processed P4501A1 Targeted to Mitochondria in Erythromycin Metabolism and Reversal of Erythromycin-mediated Inhibition of Mitochondrial Protein Synthesis

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