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Mistargeting of peroxisomal L-alanine:glyoxylate aminotransferase to mitochondria in primary hyperoxaluria patients depends upon activation of a cryptic mitochondrial targeting sequence by a point mutation

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ABSTRACT **In approximately one-third of primary hyperoxaluria type 1 patients, disease is associated with a unique protein sorting defect in which hepatic L-alanine:glyoxylate aminotransferase (AGT; EC 2.6.1.44), which is normally peroxisomal, is mistargeted to mitochondria. In all such patients analyzed to date, the gene encoding the aberrantly targeted AGT carries three point mutations, each of which specifies an amino acid substitution. In this paper we show that one of these substitutions, a proline-to-leucine at residue 11, is necessary and sufficient for the generation of a mitochondrial targeting sequence in the AGT protein. AGT with this substitution appears to interact specifically with the mitochondrial protein import machinery, via a discrete N-terminal domain of the AGT protein. The N-terminal 19 amino acids of AGT with this substitution are sufficient to direct mouse cytosolic dihydrofolate reductase to mitochondria, and a synthetic peptide corresponding to this same 19-amino acid region reversibly inhibits mitochondrial protein import, not only of AGT but also of ornithine transcarbamoylase, a genuine cytoplasmically synthesized mitochondrial protein. We have extended these studies to analyze a region of normal human AGT cDNA directly upstream of the coding region. This sequence appears to correspond to an ancestral mitochondrial targeting sequence deleted from the human coding region by point mutation at the initiation codon. We show that reestablishment of this initiation codon produces an active mitochondrial targeting sequence that is different to that found in the hyperoxaluria patients. These results are discussed with reference to the AGT targeting defect in primary hyperoxaluria and also in relation to the highly unusual species specificity of subcellular distribution of AGT among mammals.**

Primary hyperoxaluria type 1 (PH1) is a lethal autosomal recessive disease caused by a deficiency of the liver-specific peroxisomal enzyme L-alanine:glyoxylate aminotransferase (AGT; EC 2.6.1.44) (1). Whereas the majority of PH1 patients have a complete deficiency of AGT catalytic activity (2) and AGT immunoreactive protein (3), approximately one-third possess significant levels of residual AGT activity and protein. In all of these latter patients so far examined it appears that the disease is due, at least in part, to a unique protein sorting defect in which AGT is erroneously targeted to the mitochondrion instead of its normal intracellular location, the peroxisome (4). In an attempt to explain the molecular basis of this protein mistargeting phenomenon, we recently cloned and sequenced AGT cDNA from the liver of a PH1 patient with mitochondrial AGT (mAGT) and compared this with the sequence of normal human liver AGT cDNA (5, 6). This identified the presence of three point mutations, each specifying an amino acid substitution, which were subsequently shown to be common to all PH1 patients with mAGT analyzed to date (6). One of these substitutions, a proline-to-leucine at residue 11 of the AGT protein (Pro-11 → Leu), was predicted to greatly enhance the likelihood of the N-terminal region of AGT folding as an amphiphilic α-helix (6). In view of the fact that the vast majority of characterized mitochondrial targeting sequences (MTSs) are N-terminal domains with predicted ability to form amphiphilic α-helices, we have postulated that this substitution (Pro-11 → Leu) might be responsible for generation of a MTS in the mutant mAGT (6). In this paper we have investigated the roles of the three cosegregating mutations in PH1 patients with mAGT in the generation of an AGT MTS via the use of *in vitro* mutagenesis and an *in vitro* mitochondrial import system. Furthermore, we have extended these studies to analyze the mitochondrial targeting activity associated with a second, distinct, region of human AGT cDNA. This latter region shows high sequence identity to the corresponding portion of AGT cDNA from rat, which is one of several species that target AGT to mitochondria (7). However, whereas this region encodes the MTS of rat AGT (8), it is excluded from the open reading frame of the human gene due to evolutionary loss of the translation initiation codon by a single base change (ATG in the rat, ATA in the human) (5). We have investigated the consequences for mitochondrial targeting of AGT of reintroduction of the rat-equivalent translation initiation codon to normal human AGT cDNA.

MATERIALS AND METHODS

Plasmids. Plasmid SPOTC, which carries the cDNA encoding the precursor to human ornithine transcarbamoylase (pOTC) cloned 3′ to the SP6 RNA polymerase promoter, has been described (9).

The eight AGT cDNA expression plasmids covering all combinations of the three point mutations in PH1 patients with mAGT, as well as the normal sequence (see Fig. 1*a*), were constructed as follows. Full-length AGT cDNA from a PH1 patient with mAGT, which contains the three point mutations, and full-length normal AGT cDNA were cloned into pBluescript (Stratagene) to produce clones pLRM and pPGI, respectively. These clones differed only at the sites of the three point mutations. By exchanging appropriate restriction fragments between these two plasmids, further pBluescript constructs containing each mutation in isolation (pLGI, pPRI, pPGM) and each pairwise combination (pLRI, pLGM, pPRM) were synthesized.

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Abbreviations: AGT, L-alanine:glyoxylate aminotransferase; DHFR, dihydrofolate reductase; DNP, dinitrophenol; mAGT, mitochondrial AGT; MTS, mitochondrial targeting sequence; PH1, primary hyperoxaluria type 1.

Plasmid pAB105, a pGEM-1 (Promega) derivative containing the entire mouse dihydrofolate reductase (DHFR) open reading frame, was a gift of Alison Baker (University of Cambridge, U.K.). To construct the AGT–DHFR fusions, regions of pPGI and pLGI covering the cDNA sequence up to amino acid 19 and the polylinker 5′ to the cDNA were PCR amplified. The downstream primer (5′-TTGGGGCTC-GAGAGGGGCTT-3′, complementary to cDNA nucleotides 187–168) contained mismatches to introduce an *Xho* I site. The PCR product was digested with *Eco*RI (cuts within the amplified region of the pBluescript polylinker) and *Xho* I and cloned into *Eco*RI/*Xho* I-digested pAB105. The sequence at the junction of the fusions was . . . CTCTCGAGATCC-GGCATCATG . . . . The TCG codon within the *Xho* I site (underlined) corresponds to amino acid 19 of AGT, and the ATG codon (double underlined) specifies the first amino acid of DHFR. The intervening 12 base pairs encodes a 4-amino acid linker.

To construct the plasmid encoding AGT_{-22}, the region 5′ to the *Bal* I site of pPGI (underlined in Fig. 4*a*) was replaced by the corresponding region of marmoset AGT cDNA (P.E.P., unpublished observations). This introduces the rat-equivalent translation initiation site to the human cDNA but leaves otherwise unaltered the amino acid sequence potentially encoded by this (normally untranslated) region of the human cDNA.

All constructs were checked by plasmid sequencing with modified T7 DNA polymerase (Sequenase, United States Biochemical) in accordance with the manufacturer's instructions.

***In Vitro* Transcription/Translation.** Linearized plasmids were transcribed (37°C, 60 min) with T3, T7, or SP6 RNA polymerase (GIBCO/BRL) as appropriate, and the resultant transcripts were translated (30°C, 60 min) in a rabbit reticulocyte lysate (Amersham) in accordance with the manufacturers' instructions.

***In Vitro* Mitochondrial Protein Import.** Mitochondria were prepared from rat liver as described (10) and immediately used in import reactions. Four microliters of mitochondrial suspension [40 mg/ml of protein in HMS buffer (220 mM D-mannitol/70 mM sucrose/2 mM Hepes/KOH, pH 7.4)] was mixed with 6 μl of translation product in lysate and incubated at 28°C for 20 min. Where appropriate, mitochondria were preincubated with 200 μM dinitrophenol (DNP) in HMS buffer at 4°C for 10 min. Following import, mitochondria were reisolated by centrifugation (12,000 × *g*, 5 min), and 10% of each pellet and supernatant was analyzed alongside an equivalent amount (0.6 μl) of nascent translation product by SDS/PAGE and fluorography. Proteinase K digestion (final concentration, 17 μg/ml) was at 4°C for 30 min with or without a previous incubation (4°C, 10 min) in 1% (vol/vol) Triton X-100. Higher amounts of proteinase K (final concentration, 70 μg/ml) were required for the AGT–DHFR import reactions due to the intrinsically high resistance to proteinase K digestion of the DHFR moiety.

Synthesis and Use in Mitochondrial Import Reactions of Synthetic Peptides. The sequences of peptides PL and PP were as follows:

PL NH_2-MASHKLLVTPLKALLKPLSC-COOH;

PP NH_2-MASHKLLVTPPKALLKPLSC-COOH.

The cysteine residues at the C termini are not present in AGT. Peptides were synthesized using fluoren-9-ylmethoxycarbonyl chemistry on a model 431A peptide synthesizer (Applied Biosystems). Purification was by reversed-phase HPLC and the peptides were characterized by amino acid analysis and mass spectrometry. Mitochondria (40 mg/ml of protein) in HMS buffer were preincubated (4°C, 5 min) in the absence or presence of various concentrations of peptide prior to import. For the reimport assays, mitochondria were reisolated by centrifugation (12,000 × *g*, 5 min) following import, washed, resuspended in 4 μl of HMS buffer plus 6 μl of translation product in lysate, and reincubated as for normal import.

RESULTS

Mitochondrial Import of Mutant AGT. AGT cDNA from PH1 patients with mAGT contains three point mutations (C-154 → T, G-630 → A, and A-1142 → G) that specify amino acid substitutions [Pro-11 → Leu, Gly-170 → Arg, and Ile-340 → Met, respectively (where Gly-170 → Arg indicates a glycine-to-arginine substitution at residue 170 of the AGT protein and Ile-340 → Met indicates an isoleucine-to-methionine substitution at residue 340 of the AGT protein)] (Fig. 1*a*). *In vitro* transcription/translation of each of the

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Mutation	$C_{154}T$	$G_{630}A$	$A_{1142}G$
αα substitution	$Pro_{11}Leu$	$Gly_{170}Arg$	$Ile_{340}Met$

Plasmid	Trans product			
pPGI	AGT_{PGI}	-	-	-
pLGI	AGT_{LGI}	+	-	-
pPRI	AGT_{PRI}	-	+	-
pPGM	AGT_{PGM}	-	-	+
pLRI	AGT_{LRI}	+	+	-
pLGM	AGT_{LGM}	+	-	+
pPRM	AGT_{PRM}	-	+	+
pLRM	AGT_{LRM}	+	+	+

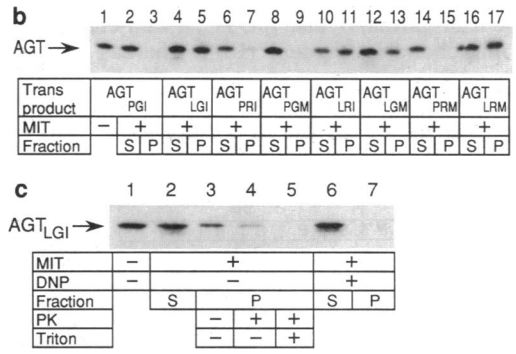

FIG. 1. A proline-to-leucine substitution at residue 11 is necessary and sufficient to allow membrane potential-dependent mitochondrial import of AGT. (*a*) Description of the eight AGT expression plasmids incorporating various combinations of the three co-segregating point mutations ($C_{154}T$, $G_{630}A$, $A_{1142}G$) in AGT cDNA of PH1 patients with the peroxisome-to-mitochondrion targeting defect and the various forms of AGT encoded by these plasmids. For example, AGT_{LGI} is encoded by plasmid pLGI, which contains a C → T mutation at base 154 of the cDNA ($C_{154}T$) specifying a proline-to-leucine substitution at residue 11 ($Pro_{11}Leu$). AGT_{PGI} is the normal protein, and AGT_{LRM} is the mutant protein found in PH1 patients with mAGT (see ref. 6). αα substitution, amino acid substitution. (*b*) *In vitro* mitochondrial import with these various forms of AGT (lanes 2–17). S, supernatant; P, pellet (all figures) [following reisolation of mitochondria (MIT, all figures) after import]. Lane 1, nascent translation product of normal AGT (AGT_{PGI}). (*c*) Triton X-100/proteinase K (PK) protection assay (lanes 4 and 5) and the effect of DNP (lanes 6 and 7) on import of AGT with leucine at residue 11 (AGT_{LGI}). Lane 1, nascent AGT_{LGI} translation product.

eight expression plasmids (see Fig. 1*a*) that cover all combinations of these mutations as well as the normal sequence produced a single major product of the predicted size (43 kDa, as estimated by SDS/PAGE) that was identified as AGT by immunoblotting (not shown). These eight different forms of AGT translation product could be divided into two classes on the basis of their behavior in *in vitro* mitochondrial import assays (Fig. 1*b*). All four variant forms of AGT that included Pro-11 → Leu showed significant association with the mitochondria during the assay. In contrast, wild-type AGT and the three variant forms lacking Pro-11 → Leu failed to associate with the mitochondria. The influence of Pro-11 → Leu was exemplified by the observation that this substitution alone resulted in ≈50% of the translation product associating with mitochondria (Fig. 1*b*). We concluded from this that Pro-11 → Leu was necessary and sufficient to mediate the events involving association between the mutant AGT and mitochondria *in vitro*. In view of this, AGT with this substitution alone (AGT_{LGI}, Fig. 1*a*) was used for the further characterization of this association. Mitochondrial targeting of AGT_{LGI} appeared to involve specific interaction with the mitochondrial protein import machinery, in that it was accompanied by translocation of at least part of the AGT to a proteinase K-inaccessible/Triton X-100-labile location, and was abolished by DNP-induced collapse of the membrane potential (Fig. 1*c*). That none of the AGT in the mitochondrial pellet appeared to have undergone any proteolytic processing (Fig. 1*b*) was in agreement with the observation that mAGT in PH1 patients is of identical size to normal peroxisomal AGT (4). Moreover, AGT with either proline or leucine at residue 11 could not be cleaved *in vitro* by purified rat liver mitochondrial matrix processing peptidases (11) (not shown).

Effects of Synthetic Peptides on Import of AGT. Synthetic peptides corresponding to the first 19 amino acids of normal AGT (peptide PP, proline at residue 11) and mutant AGT (peptide PL, leucine at residue 11) were chemically synthesized and analyzed for their ability to inhibit import. As shown in Fig. 2, increasing concentrations of peptide PL progressively inhibited import of AGT_{LGI} and the precursor to ornithine transcarbamoylase [pOTC, a well-characterized imported mitochondrial protein (10)], whereas equivalent concentrations of peptide PP had no apparent effect. The inhibitory effect of PL was reversible, in that mitochondria reisolated following incubation in the presence of PL were still as capable of import as mitochondria isolated following incubation in the absence of peptide (Fig. 2*b*), suggesting that the effect of PL was not due to indiscriminate damage to membrane integrity. Significant levels of AGT_{LGI} and pOTC still associated with mitochondria in the presence of PL but were readily digested by proteinase K (not shown). These results are in agreement with other similar recent studies using synthetic peptides corresponding to known MTSs, which concluded that such peptides inhibit import of a wide range of precursors at a level subsequent to surface binding of precursors (12, 13).

Mitochondrial Import of AGT–DHFR Fusion Proteins. Fusion proteins consisting of mouse DHFR attached C-terminal to the first 19 amino acids of AGT with proline (AGT_{P19}–DHFR) and leucine (AGT_{L19}–DHFR) at residue 11 were synthesized *in vitro* and tested in import assays (Fig. 3). AGT_{L19}–DHFR associated with mitochondria, albeit with low efficiency, whereas DHFR and AGT_{P19}–DHFR did not. This association was sensitive to DNP and was accompanied by translocation of AGT_{L19}–DHFR to a proteinase K-inaccessible/Triton X-100-labile location.

Mitochondrial Import and Processing of AGT_{-22}. By the use of *in vitro* mutagenesis, the open reading frame of human AGT cDNA was extended to encode an additional 22 amino acids, homologous to the MTS of rat AGT, at the N terminus (see *Materials and Methods*). The resultant extended form of AGT (AGT_{-22}) was imported and processed by mitochondria in a DNP-sensitive fashion (Fig. 4). By analogy with the rat (8), the processing site is probably between the threonine and methionine (residues 22 and 23) of AGT_{-22}, such that the mature form is identical to normal AGT. A minor second product of the *in vitro* transcription/translation of the clone encoding AGT_{-22} (Fig. 4*b*, lane 1) probably corresponds to normal human AGT, arising via internal initiation of translation. This product presumably fails to associate with mitochondria (Fig. 1*b*) and accounts for the material of this size in the postimport supernatant. The possibility that any of this material corresponded to processed AGT_{-22} that had been

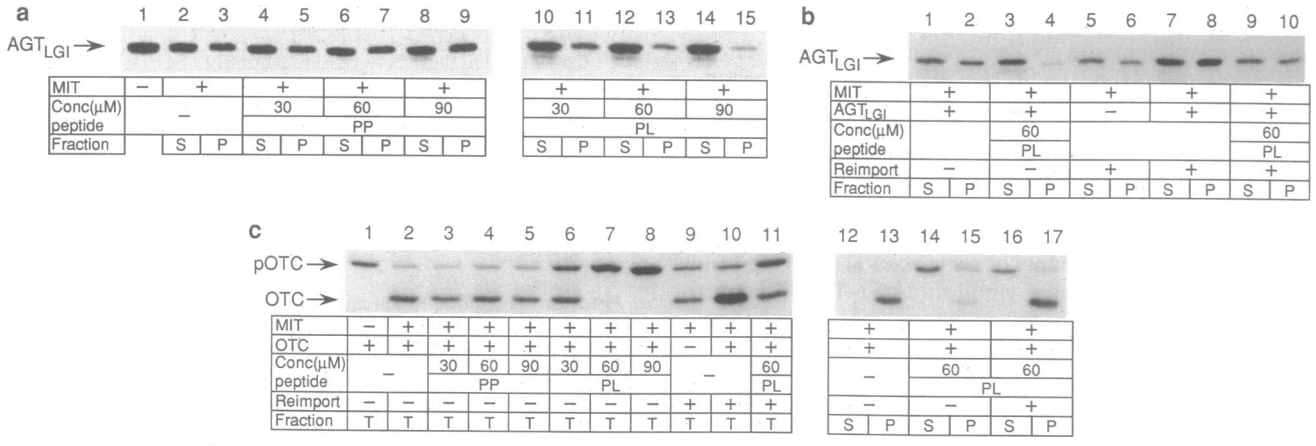

FIG. 2. Mitochondrial import of AGT_{LGI} and the precursor to mitochondrial ornithine transcarbamoylase (pOTC) is reversibly inhibited by a peptide corresponding to the N-terminal 19 amino acids of mAGT (peptide PL) but not by the equivalent peptide of normal AGT (peptide PP). (*a*) Increasing concentrations of peptide PL progressively inhibit import of AGT_{LGI} (lanes 10–15, compared with lanes 2 and 3), whereas equivalent concentrations of peptide PP do not (lanes 4–9, compared with lanes 2 and 3). The final concentrations of peptide are shown in μmol/liter. Lane 1, nascent AGT_{LGI} translation product. (*b*) Inhibition of AGT_{LGI} import by PL is reversible. Mitochondria from import with AGT_{LGI} plus PL (lanes 9 and 10), AGT_{LGI} minus PL (lanes 7 and 8), and straight lysate (no AGT, lanes 5 and 6) were washed and reincubated (Reimport) with AGT_{LGI} (no peptide) and compared to standard import with (lanes 3 and 4) and without (lanes 1 and 2) peptide. (*c*) Import and processing of pOTC are inhibited by peptide PL (lanes 6–8, compared with lane 2) but not by peptide PP (lanes 3–5, compared with lane 2). Inhibition is reversible (reimport assays, lanes 9–11). As expected, all of the mature ornithine transcarbamoylase (OTC) is associated with mitochondria, even after reimport (lanes 12–17). However, inhibition of import with peptide PL leads to a significant increase in the amount of pOTC that associates with mitochondria (compare lanes 13 and 15). Lane 1, nascent pOTC translation product. T, total (also Fig. 4) (i.e., mitochondria not reisolated prior to SDS/PAGE analysis).

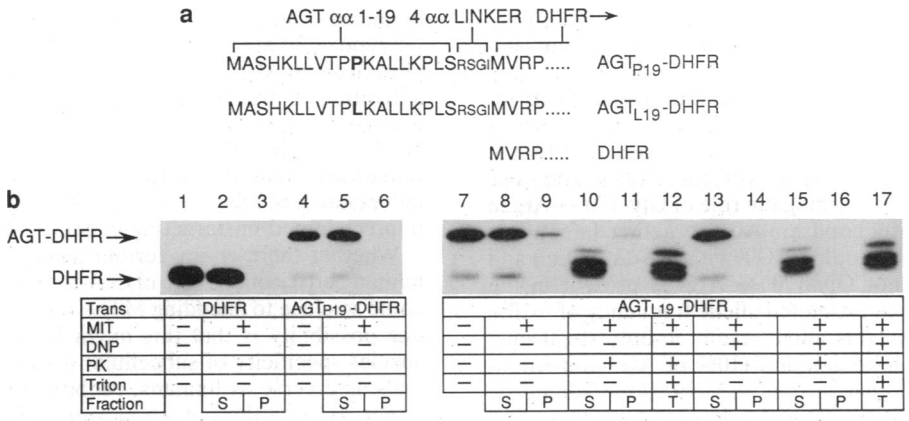

FIG. 3. The first 19 amino acids (αα) of mAGT, but not the corresponding region of normal AGT, can direct DHFR to mitochondria. (*a*) Predicted N-terminal amino acid sequences of fusion proteins AGT$_{P19}$–DHFR and AGT$_{L19}$–DHFR compared with normal DHFR. The proline/leucine at residue 11 of the fusion proteins is in boldface type, and the 4-amino acid linker is shown in small capital letters. (*b*) Neither DHFR (lanes 2 and 3) nor AGT$_{P19}$–DHFR (lanes 5 and 6) associates with mitochondria, whereas AGT$_{L19}$–DHFR does (lanes 8 and 9). A proportion of the AGT$_{L19}$–DHFR is translocated to a Triton X-100-labile/proteinase K (PK)-inaccessible location (lanes 10–12). AGT$_{L19}$–DHFR import was sensitive to DNP (lanes 13–17). Lanes 1, 4, and 7, nascent DHFR, AGT$_{P19}$–DHFR, and AGT$_{L19}$–DHFR translation products, respectively. The lower bands in the AGT$_{P19}$–DHFR and AGT$_{L19}$–DHFR translations probably represent internal initiation of translation at the start of the DHFR open reading frame. The lower bands in lanes 10, 12, 15, and 17 are presumed to be proteinase K digestion products of AGT$_{L19}$–DHFR.

released by mitochondria was eliminated by demonstrating that mitochondria isolated from import reactions with AGT$_{-22}$ did not release any material during reincubation with fresh lysate (not shown).

DISCUSSION

In this paper we have shown that one of the three amino acid substitutions in the AGT protein of PH1 patients with mAGT, Pro-11 → Leu, is necessary and sufficient to allow *in vitro* association between AGT and mitochondria. Wild-type AGT failed to associate with mitochondria, as did mutant forms of AGT with either or both of the other two substitutions (Gly-170 → Arg and Ile-340 → Met) but not Pro-11 → Leu. Association between mitochondria and mutant forms of AGT bearing Pro-11 → Leu was accompanied by translocation of at least part of the AGT to a proteinase K-inaccessible/Triton X-100-labile location and was sensitive to collapse of the mitochondrial membrane potential with DNP. This indicates that the AGT is interacting specifically with the mitochondrial protein import machinery and is subsequently being translocated across one or both of the mitochondrial membranes. This *in vitro* effect of Pro-11 → Leu appears to be exerted independently of Gly-170 → Arg and Ile-340 → Met and to involve local changes restricted to the N-terminal region of the AGT protein. This is evidenced by the observation that the first 19 amino acids of mAGT (AGT$_{L19}$) can direct DHFR to the mitochondrion (Fig. 3). Additionally, import of AGT can be inhibited by a synthetic peptide corresponding to the first 19 amino acids of mAGT (peptide PL) but not by the corresponding peptide of normal AGT (peptide PP) (Fig. 2). These results are consistent with our previous theoretical arguments regarding possible consequences of Pro-11 → Leu (6). The vast majority of characterized MTSs exists as discrete N-terminal domains with predicted ability to fold as amphiphilic α-helices, and Pro-11

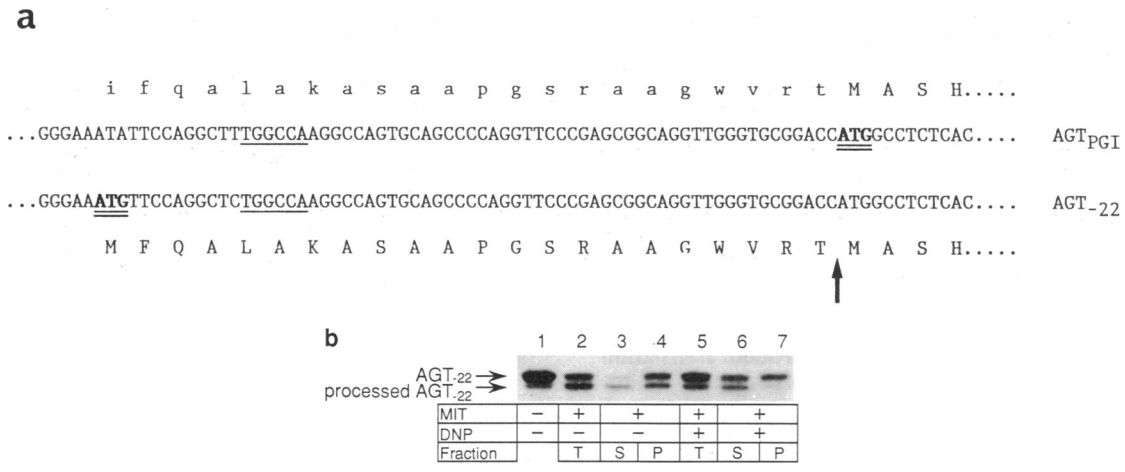

FIG. 4. A second, "ancestral," cryptic MTS in AGT cDNA can also be activated by a point mutation. (*a*) Nucleotide sequences and predicted amino acid sequences of the N-terminal regions of AGT$_{PGI}$ (normal AGT) and AGT$_{-22}$ (normal AGT with an N-terminal 22-amino acid extension). Translation initiation codons are doubly underlined, and translated amino acids are in capital letters. The *Bal* I site used in cloning is singly underlined, and the putative processing site of AGT$_{-22}$ is marked by an arrow. (*b*) Mitochondrial import and processing of AGT$_{-22}$. Import reactions with or without DNP were analyzed directly (lanes 2 and 5) or after reisolation of mitochondria (lanes 3, 4, 6, and 7). Lane 1, nascent AGT$_{-22}$ translation product. The lower band in the translation product presumably corresponds to normal AGT, arising via internal initiation of translation. This product presumably fails to associate with mitochondria (see Fig. 1*b*, lane 3). The processed form of AGT$_{-22}$, which is also probably identical to normal AGT (see text), was not released by mitochondria during a reimport assay with fresh lysate (not shown).

→ Leu is predicted to greatly enhance the likelihood of the N-terminal region of AGT assuming such a discrete amphiphilic α-helical structure.

In contrast to Pro-11 → Leu, there are no evident theoretical arguments linking either Gly-170 → Arg or Ile-340 → Met with generation of a MTS, and this is borne out by the *in vitro* import data (Fig. 1). However, these results do raise interesting questions concerning the role of Gly-170 → Arg in the peroxisome-to-mitochondrion AGT targeting defect. It is known that the allele encoding AGT with Pro-11 → Leu and Ile-340 → Met (but not Gly-170 → Arg) is present in the normal population at an estimated allelic frequency of ≈10% and that expression of this allele results in only slight mistargeting of AGT to mitochondria (10% of AGT mitochondrial and 90% peroxisomal, as opposed to 95% mitochondrial and 5% peroxisomal if Gly-170 → Arg is also present) (6). The evidence presented here that Gly-170 → Arg has no direct role in the generation of a MTS (see Fig. 1*b*) is in agreement with our previous hypothesis that this substitution may contribute to the AGT mistargeting by inhibiting peroxisomal import of AGT. According to this hypothesis, AGT with Pro-11 → Leu but not Gly-170 → Arg retains its natural (as yet undefined) peroxisomal targeting activity that efficiently competes with the Pro-11 → Leu MTS. However, in the additional presence of Gly-170 → Arg, the MTS becomes the dominant sorting signal, resulting in the pattern of distribution of AGT found in PH1 patients with mAGT. One prediction of this model is that the Pro-11 → Leu MTS is rather weak, at least when compared with the putative AGT peroxisomal targeting signal. This would not be too surprising, bearing in mind that the Pro-11 → Leu MTS arose due to a single amino acid substitution in a peroxisomal protein, and the reduced import efficiency of AGT_{LGI} and AGT_{L19}–DHFR compared to pOTC, a genuine mitochondrial precursor (Figs. 1–3), tends to support this as does our previous observation that the levels of mAGT in PH1 patients tend to be rather low, despite apparently normal levels of AGT mRNA (4, 5). Also, the rather high concentrations of peptide PL required to inhibit import (Fig. 2) may reflect the inefficiency of this MTS.

With reference to the mistargeting of AGT to mitochondria in PH1, it is of interest to note that among mammals the subcellular distribution of AGT is species specific. The rat is one of a number of species in which AGT is a naturally occurring mitochondrial protein (7). Surprisingly, the MTS of rat mAGT (8) appears to be entirely different to that generated by Pro-11 → Leu in PH1 patients. In human AGT cDNA, the region homologous to that encoding the rat AGT MTS shows high sequence identity to its rat counterpart but lies within the 5′ untranslated region, being excluded from the open reading frame due to a coding difference (ATG in rat, ATA in human) at the rat-equivalent translation initiation site (5). We have postulated that evolutionary loss of this ATG codon explains the exclusive peroxisomal localization of human AGT and that reestablishment of this codon could represent another mechanism for mitochondrial mistargeting of AGT in humans (5). As shown in Fig. 4, the protein produced by reintroduction of the rat-equivalent translation initiation site (AGT_{-22}) is imported and processed by mitochondria. Thus, the phenotype of PH1 patients with mAGT [enzymically active mAGT of the same size as normal peroxisomal AGT (4)] would be entirely compatible with activation of this second, rat-equivalent, cryptic MTS by appropriate point mutation. Although no patients using this second cryptic MTS have yet been identified, it seems remarkable that two different point mutations, activating independent MTSs, are consistent with such an unusual and unprecedented mistargeting phenomenon.

Whether there is any further significance to the fact that human peroxisomal AGT cDNA contains two sequences that come so close to encoding MTSs remains unclear. However, one possibility is that this might be related to the unusual species specificity of subcellular distribution of AGT. Mammals as diverse as humans, rabbits, and guinea pigs do not target AGT to the mitochondrion, whereas rats, cats, and marmosets are among those species that do (7, 14–18). Loss (or gain) of mitochondrial targeting activity must therefore have occurred at more than one point during mammalian evolution. The intriguing possibility that some species with mAGT might share the human MTS activated in PH1 patients remains to be resolved.

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