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Dictyostelium discoideum Ax2 as an Assay System for Screening of Pharmacological Chaperones for Phenylketonuria Mutations

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In this study, we developed an assay system for missense mutations in human phenylalanine hydroxylases (hPAHs). To demonstrate the reliability of the system, eight mutant proteins (F39L, K42I, L48S, I65T, R252Q, L255V, S349L, and R408W) were expressed in a mutant strain (*pah*$^-$) of *Dictyostelium discoideum* Ax2 disrupted in the indigenous gene encoding PAH. The transformed *pah*$^-$ cells grown in FM minimal medium were measured for growth rate and PAH activity to reveal a positive correlation between them. The protein level of hPAH was also determined by western blotting to show the impact of each mutation on protein stability and catalytic activity. The result was highly compatible with the previous ones obtained from other expression systems, suggesting that *Dictyostelium* is a dependable alternative to other expression systems. Furthermore, we found that both the protein level and activity of S349L and R408W, which were impaired severely in protein stability, were rescued in HL5 nutrient medium. Although the responsible component(s) remains unidentified, this unexpected finding showed an important advantage of our expression system for studying unstable proteins. As an economic and stable cell-based expression system, our development will contribute to mass-screening of pharmacological chaperones for missense PAH mutations as well as to the in-depth characterization of individual mutations.

Keywords: Phenylalanine hydroxylase, phenylketonuria, phenotype analysis, protein misfolding disease, tetrahydropteridines, pharmacological chaperone

Introduction

Phenylalanine hydroxylase (PAH, E.C. 1.14.16.1) is an iron-dependent enzyme catalyzing the conversion of L-phenylalanine (Phe) to L-tyrosine (Tyr) in the presence of molecular oxygen and L-erythro-tetrahydrobiopterin (BH4) [6]. PAH is expressed highly in the human liver and is crucial for preventing the accumulation of dietary Phe. Mutations in the human PAH (hPAH) gene are notorious for causing phenylketonuria (PKU), which develops severe mental retardation if not treated early. Missense mutations in hPAH are now understood to produce folding defects causing reduced stability and accelerated proteolytic degradation *in vivo* [10]. Current treatment includes a low-Phe diet and BH4 supplementation with other options [10]. BH4 exerts a pharmacological chaperone effect, preventing protein misfolding and protecting from inactivation [16]. Because BH4 is not effective in all PKU genotypes, however, other pharmacological chaperones were developed [18, 19] and patient-tailored therapy according to the genotype was suggested [22].

The analysis of mutant hPAH genotypes has revealed a spectrum of over 850 variants (BioPKU; http://www.biopku.org). More than two thirds of them are missense mutations, and their genotype-phenotype correlations have been studied extensively based on expression analysis of variant hPAHs (PAHdb; http://www.pahdb.mcgill.ca/). *In vitro* protein analysis was carried out with the mutant proteins prepared from *Escherichia coli*, yeast, mammalian transfected cells, or cell-free protein expression system using TNT-T7 reticulocyte lysate. These studies developed a paradigm of protein misfolding disease for PKU [10]. Based on this principle, the energetic impact on the PAH native-state stability of PKU-associated missense mutations was also studied using

computational analysis [17, 20]. In spite of these efforts, there still remains a significant inconsistency between *in vitro* and *in vivo* phenotypes with similar PAH genotypes [11, 20].

PAH is also known from *Dictyostelium discoideum* Ax2, a well-known nonmammalian model organism but also a promising expression system for eukaryotic proteins [1]. *Dictyostelium* PAH carries three domains, where the catalytic and tetramerization domains share over 70% sequence homology with hPAH [12]. Interestingly, *Dictyostelium* synthesizes BH4 and a much higher level of D-threo-isomer (DH4) [3]. Both tetrahydropteridines were determined to be effective cofactors for human as well as *Dictyostelium* PAHs [21]. Recently, we found that a mutant strain (*pah*$^-$) disrupted in the indigenous PAH gene did not grow at all in a minimal medium, which is devoid of Tyr [12]. As the result implied that *pah*$^-$ rescued with variant hPAH would display PAH activity-dependent growth in the medium, *pah*$^-$ was presumed to be useful for the screening of pharmacological chaperones for PKU as well as for characterization of PKU-associated mutations. In order to prove the hypothesis, we expressed eight mutant hPAHs in *pah*$^-$ and measured the growth rate and both the protein amount and activity of PAH from the transformed cells. Eight missense mutations were selected in the regulatory domain (F39L, K42I, L48S, I65T) and the catalytic domain (R252Q, L255V, S349L, and R408W), which have been studied in at least more than one expression system [2, 7, 8, 15, 23].

Materials and Methods

Growth Conditions

D. discoideum Ax2 cells were grown at 22°C in HL5 medium (10 g glucose, 5 g yeast extract, 10 g proteose peptone, 0.35 g KH_2PO_4, 0.35 g $Na_2HPO_4 \cdot 12H_2O$, pH 6.4, per liter) with 100 μg/ml streptomycin sulfate and 100 U/ml benzylpenicillin potassium [24]. The PAH knockout mutant (*pah*$^-$) was grown in HL5 containing 10 μg/ml Blasticidine S [12]. The mutant cells transformed with hPAH cDNAs were maintained in HL5 medium containing 10 μg/ml each of Blasticidine S and G418. The mutant cells were also cultured in a chemically defined FM minimal medium (ForMedium, UK) [5].

Site-Directed Mutagenesis of hPAH

Single substitution of the amino acid residues at 39, 42, 48, 65, 252, 255, 349, and 408 of hPAH was performed by the overlap extension PCR method [9] using the following primer pairs: F39L, 5′-CCATATCACTGATCTT**G**TCACTCAAAGAAGAA-3′ and 5′-TTCTTCTTTGAGTGA**C**AAGATCAGTGATATGG-3′; K42I, 5′-CTGATCTTCTCACTCA**T**AGAAGAAGTTGGTGC-3′ and 5′-GCACCAACTTCTTCT**A**TGAGTGAGAAGATCAG-3′; L48S, 5′-GAAGTTGGTGCAT**C**GGCCAAAGTATT-3′ and 5′-AATACTTTGGCC**G**ATGCACCAACTTC-3′; I65T, 5′-GTAAACCTGACCCACA**C**TGAATCTAGACCTTC-3′ and 5′-GAAGGTCTAGATTCA**G**TGTGGGTCAGGTTTAC-3′; R252Q, 5′-TGCTTTCCTCTC**A**GGATTTCTTGGGTG-3′ and 5′-CACCCAAGAAATCC**T**GAGAGGAAAGCA-3′; L255V, 5′-CTCGGGATTTC**G**TGGGTGGCCTG-3′ and 5′-CAGGCCACCCA**C**GAAATCCCGAG-3′; S349L, 5′-CTGGGCTCCTGT**T**ATCCTTTGGTGAATT-3′ and 5′-AATTCACCAAAGGAT**A**ACAGGAGCCCAG-3′; R408W, 5′-GCCACAATACCT**T**GGCCCTTCTCAG-3′ and 5′-CTGAGAAGGGCC**A**AGGTATTGTGGC-3′. The incorporated nucleotide change is marked by underline. The PCR was performed with the wild-type hPAH cDNA cloned in pMAL vector (a gift of Prof. Aurora Martinez in University of Bergen, Norway) as a template [14]. The amplified DNAs were purified by agarose gel electrophoresis and then mixed in equal proportions in a PCR to amplify the full-length sequence using a primer pair of 5′-GGTACCATGTCCACTGCGGTCCTGGAAAAC-3′ and 5′-ATGCATTTACTTTATTTTCTGGAGGGCACTGCAAA-3′. PCR amplifications were performed with *pfu* polymerase in 1× reaction buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM each of primer pairs, and templates, under the following conditions: 5 min at 95°C, followed by 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, and a final polymerization at 72°C for 10 min. The amplified DNAs were confirmed by sequencing.

Extrachromosomal Expression of hPAH in *pah*$^-$ and *spr*$^-$ Strains

The mutant hPAH cDNAs were cloned as *Kpn*I/*Nsi*I restriction fragments into pDXA-3H plasmid [13]. Cells were harvested by centrifugation at 350 ×*g* for 3 min and washed twice with ice-cold H-50 buffer (20 mM HEPES, pH 7.0, 50 mM KCl, 1 mM $MgSO_4$, 5 mM $NaHCO_3$, 1 mM NaH_2PO_4). A 100 μl aliquot of the cell suspension (5 × 10^6 cells) was mixed with 10 μg of DNA and electroporated with Electrophorator 1000 (Stratagene) (0.85 kV, 2 times). After cooling on ice for 5 min, cells were transferred to a Petri dish containing 20 ml of HL5 medium and incubated at 22°C for 24 h. The medium was supplemented with G418 and changed every 3 days. Live clones in the selection medium were isolated between 7 and 10 days.

Measurement of Growth Rate and PAH Activity

Growth rate was determined by the increase in cell number after 2 day culture in FM medium. The transformed cells with hPAH cDNAs were proliferated first in HL5 medium, washed with FM medium, and then transferred to FM medium (1 × 10^6 cells/ml). After 2 days, the cells were counted using a hemacytometer and harvested for PAH assay.

Crude extract was prepared fresh from the cells dissolved in 50 mM Tris–HCl (pH 7.5), 1 mM DTT, and 1 mM PMSF. Cells were disrupted by three cycles of freeze-thawing and centrifuged to remove precipitates. The supernatant was desalted using a

Sephadex G-25 spin column. The PAH assay was carried out at 37°C for 10 min in a volume of 50 μl containing 100 mM Tris–HCl (pH 7.5), 2 mM Phe, 100 units catalase, 5 mM DTT, 0.4 mM BH4, and 10 μg of crude extract [12]. The reaction mixture was mixed with an equal volume of 5% (v/v) TCA solution and centrifuged for 10 min to discard proteins. The supernatant was analyzed by HPLC using an Inertsil ODS-3 C18 column eluted with 40 mM sodium acetate (pH 3.5) at a flow rate of 1 ml/min. The Tyr peak was detected by fluorescence at 290 nm/340 nm (ex/em).

Miscellaneous

Western blot analysis was performed with the crude extracts prepared fresh from the harvested cells. Samples were boiled in Laemmli buffer and loaded (equivalent to 50 μg of protein per well) on a 12.5% denaturing polyacrylamide gel, and then transferred to a nitrocellulose membrane in Tris-glycine buffer, pH 8.3. For immunoblot of hPAH, the desired primary antibodies (Abcam) were used at a dilution of 1:2,000, followed by horseradish peroxidase-conjugated secondary antibodies, and developed by enhanced chemiluminescence reaction (Millipore). The digital chemiluminescence images were taken by Fusion-SL4 Spectra (Vilber, Germany). Protein was measured by the Bradford method using bovine serum albumin as a standard.

All the data were collected from three independent experiments and expressed as the mean ± SD.

Results and Discussion

Growth Rate and PAH Activity

The *pah*⁻ transformed with each of the mutant hPAH cDNAs was cultured in FM medium. The growth rate determined by cell counting was compared with the PAH activity measured from the crude extract (Fig. 1A). Whereas *pah*⁻ cells were unable to proliferate in the medium, all the transformed cells were not, indicating successful expression of mutant hPAHs in *Dictyostelium*. As if representing PKU pathogenesis, the growth rate and PAH activity were worst for S349L, which is known as one of the most severe PKU-associated mutations. Overall, the growth rate of each transformed cell seemed parallel with the PAH activity in the cell lysate. The putative relationship between growth rate and PAH activity was examined by linear regression analysis (Fig. 1B). The positive linear correlation between them was statically significant to support PAH activity-dependent growth of *pah*⁻ cells in FM medium. The result suggests that by using *pah*⁻, cells with missense mutations in hPAH can be evaluated quantitatively by growth rate, which is more convenient to measure and more *in vivo* like than PAH activity assay. Thus, the expression system may be valuable for the mass screening of pharmacological chaperones for PKU.

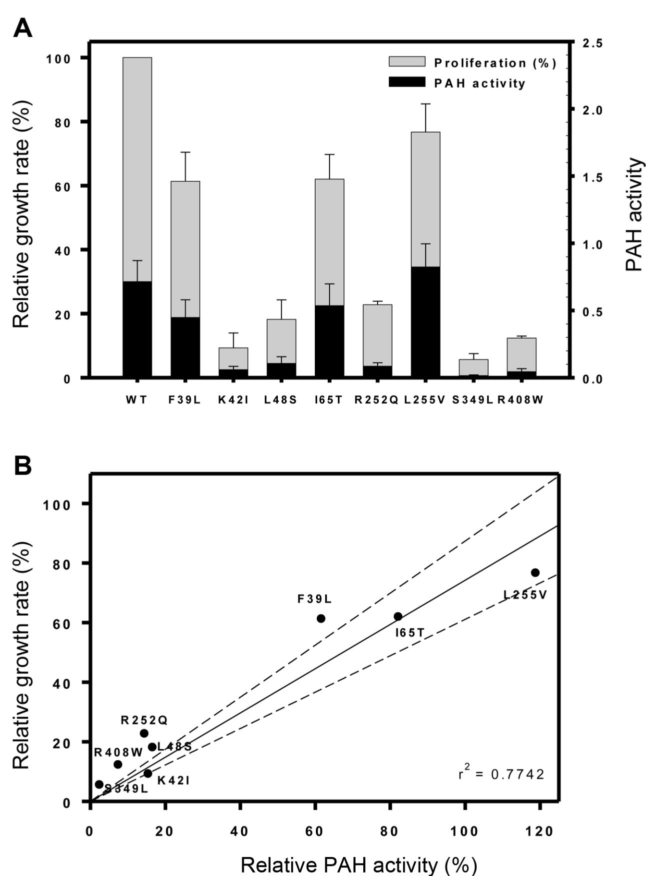

Fig. 1. Comparative analysis of growth rate and PAH activity of *pah*⁻ cells transformed with wild-type and mutant hPAH cDNAs.

(**A**) Relative levels of growth rate and PAH activity. The growth rate was plotted by percentage of wild type (WT). The graph represents data (mean ± SD) from three independent experiments. (**B**) Quantitative relationship between growth rate and PAH activity. The mean values shown in Fig. 1A were plotted by percentage of WT. The correlation coefficient (r^2) is provided with 95% confidence intervals (dotted lines).

Protein Level and Specific Activity

The protein level of hPAH was also determined from the crude extracts of *pah*⁻ cells cultured in FM medium by western blot analysis (Fig. 2A) and the quantified data were used for calculating the specific activity relative to WT (Table 1). Since the relative specific activity represents catalytic activity in this study, we were able to assess the impact of mutations on protein structure and function. A protein level and a specific activity of less than 1.0 indicate protein instability and catalytic inactivity, respectively.

The relative specific activity was more than 1.0 for F39L, K42I, L48S, I65T, and L255V (Table 1). It was not expected to find mutant enzymes having a higher catalytic activity

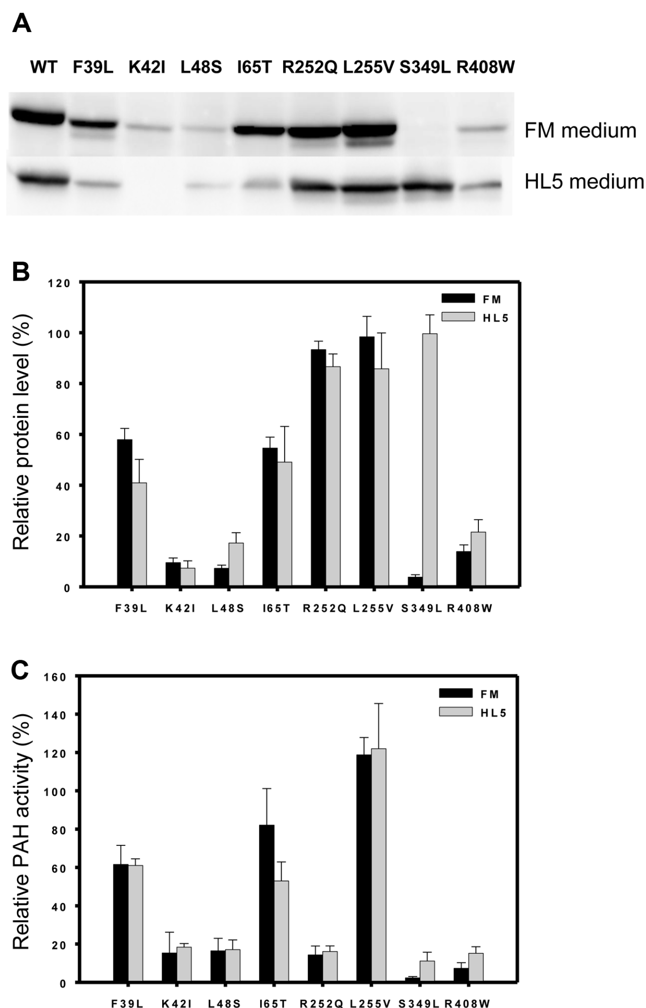

Fig. 2. Medium effect on hPAHs expressed in *pah*⁻ cells.
(**A**) Representative western blots developed by chemiluminescense reaction. Crude extracts equivalent to 50 μg of total protein were analyzed by 12.5% SDS-PAGE and western blotting. (**B**) Relative levels of protein amount from the cells cultured in FM medium (black bars) and HL5 medium (gray bars). The images obtained from western blots were quantified and plotted by percentage of wild type (WT). (**C**) Relative levels of PAH activity plotted by percentage of WT. The original data of FM medium are shown in Fig. 1A.

Table 1. Relative specific activity of mutant hPAHs.

Protein	PAH activity[a]	Protein amount[b]	Specific activity
F39L	0.615 ± 0.100	0.579 ± 0.044	1.062
K42I	0.153 ± 0.108	0.095 ± 0.019	1.611
L48S	0.165 ± 0.065	0.073 ± 0.012	2.260
I65T	0.821 ± 0.191	0.546 ± 0.043	1.504
R252Q	0.143 ± 0.046	0.933 ± 0.033	0.153
L255V	1.187 ± 0.090	0.984 ± 0.080	1.206
S349L	0.023 ± 0.007	0.038 ± 0.010	0.605
R408W	0.073 ± 0.029	0.139 ± 0.026	0.525

All the data are relative to WT.
[a]The original data are shown in Fig. 1A.
[b]The protein amount as a percentage of WT is also presented in Fig. 2B.

than WT. Except for L255V, however, the others were found in the regulatory domain, which covers the active site and plays an autoregulatory role in hPAH [6]. According to the *in vitro* expression data, F39L, K42I, L48S, and I65T mutant enzymes expressed from *E. coli* as MBP-fusion proteins were 114%, 133%, 84%, and 92% of wild-type activity [23]. Furthermore, their proteolytic degradation rates in the TNT-T7 rabbit reticulocyte system were in the order of L48S, K42I, F39L, I65T, and WT, which was also confirmed by quantitative analysis of the proteins expressed in human kidney cells [23]. L255V needs further characterization, however, because it showed much higher protein stability and PAH activity than previously known in other expression systems [2]. A possible reason of the high protein stability of L255V might be the low culture temperature (22°C) with *Dictyostelium*. On the other hand, the relative specific activity was lower than 1.0 for the other mutations R252Q, S349L, and R408W (Table 1). Considering the high protein level of R252Q, the mutation may be a typical example of catalytic inactivity, not protein misfolding. R252 is positioned in the catalytic domain of hPAH and thus any substitution at the residue was expected to result in a disruption of catalytic activity [4]. The recombinant proteins of R252Q obtained from *in vitro* expression systems or *E. coli* were shown to have 3~11.4% of wild-type activity [2]. In contrast to R252Q, S349L and R408W were also low in protein level. Supporting this, the mutations were known to severely affect both the catalytic function and protein stability [7, 15]. Given the differences in host cells and purification levels of the expressed hPAHs, some inconsistencies might be inevitable between our data and the previous ones. Taken together, however, our data on mutant hPAHs are highly comparable with those obtained from other expression systems, thereby supporting strongly *Dictyostelium* as an expression system for the characterization of missense mutant hPAHs.

Effect of Nutrient Medium

Since the above results demonstrated that our expression system is convenient and reliable for the characterization of mutations in hPAH, we were encouraged further to test a possible application of the assay system. We cultured the transformed *pah*⁻ cells in HL5 medium, which contains

yeast extract, a rich blend of natural components. Instead of growth rate, which was independent of PAH activity in HL5 medium, the protein level was measured from the cells by western blot analysis (Fig. 2A). To our surprise, S349L protein was rescued to WT level (Fig. 2B). The activity of S349L was also increased, but about 5-fold, amounting to 11% of WT (Fig. 2C). The discrepancy between the protein amount and activity of S349L may be due to impaired catalytic activity, as shown in Table 1. R408W was partially rescued in protein amount with the concomitant increase in PAH activity (Figs. 2B and 2C). Although the result of S349L and R408W needs validation through further study involving identification of the responsible component(s) in yeast extract and the underlying mechanisms, this finding demonstrated clearly the merit of our expression system as a cell-based assay system. Because protein stability is severely impaired by S349L and R408W mutations (Table 1), they may not be available for protein-level study *in vitro*. Therefore, our expression system may be extremely valuable for studying mutant hPAHs affecting severely on protein stability and for screening pharmacological chaperones for them.

In summary, we demonstrated that through complementary expression in *pah*$^{-}$, *Dictyostelium* strain missense mutant hPAHs can be evaluated quantitatively by the growth rate and characterized further for both protein stability and catalytic activity in a more *in-vivo*-like conditions. As an economic and stable expression system, it may be useful for cell-based mass-screening of pharmacological chaperones as well as for in-depth characterization of individual mutant hPAHs towards the personalized therapy of individual PKU genotypes [22]. Finally, as demonstrated with S349L and R408W, our expression system may be particularly valuable for the study of mutations severely affecting protein stability.

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