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Quantitative analysis by flow cytometry of green fluorescent protein-tagged human phenylalanine hydroxylase expressed in *Dictyostelium*

https://doi.org/10.1515/pterid-2017-0015
Received August 19, 2017; accepted October 2, 2017; previously published online November 8, 2017

Abstract: We have developed a fluorescence assay system to monitor the protein levels of human phenylalanine hydroxylase (hPAH). Wild-type (WT) and three mutant hPAHs (I65T, L255V, and S349L) were expressed as green fluorescent protein (GFP)-tagged forms in a PAH knockout mutant (*pah*$^-$) of *Dictyostelium discoideum* Ax2. The fluorescence-activated cell sorting (FACS) analysis showed that the GFP positive cells were the most frequent in WT but were rare in *pah*$^-$, demonstrating the successful expression of GFP-tagged hPAHs in *Dictyostelium*. The fluorescence levels of mutants relative to WT were higher than expected from the protein amounts determined from the non-tagged forms, probably due to the presence of the N-terminal GFP. However, treatment of the cells with cumene hydroperoxide, which is known to accelerate protein degradation, decreased fluorescence levels, suggesting that protein stability changes in individual mutations can be monitored by FACS analysis. For an evaluation study, a putative pharmacological chaperone effect of yeast extract on S349L was examined by Western blot and FACS analysis. Both the protein amount and the fluorescence levels were increased by yeast extract, supporting that the FACS analysis could replace the time- and labor-consuming procedures such as the Western blot and cell culture. The fluorescence-based cell assay system may be valuable for the high-throughput screening of pharmacological chaperones for phenylketonuria mutations.

Keywords: *Dictyostelium*; FACS analysis; pharmacological chaperone; phenylalanine hydroxylase; phenylketonuria.

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Introduction

Phenylalanine hydroxylase (PAH, EC 1.14.16.1) catalyzes the conversion of L-phenylalanine (Phe) to L-tyrosine (Tyr) in the presence of molecular oxygen and L-erythro-tetrahydrobiopterin (BH4) [1]. Human PAH (hPAH) is expressed highly in the liver, preventing the accumulation of dietary Phe in the body. Hereditary mutations in the hPAH gene are notorious for causing phenylketonuria (PKU), which results in a broad range of neuropsychiatric problems including severe mental retardation, if not treated early. Currently, there are 970 hPAH variants (BioPKU, http://www.biopku.org) and more than two-thirds of them are missense mutations. Missense mutations in the hPAH gene are now understood to produce folding defects in the proteins causing accelerated proteolytic degradation *in vivo* [2]. The current treatment includes a low Phe diet and BH4 supplementation [2]. BH4 exerts pharmacological chaperone effect, preventing protein misfolding and protecting from inactivation [3]. However, BH4 is not effective in all PKU genotypes, stimulating development of a number of potential pharmacological chaperones [4–6]. It is expected that more pharmacological chaperones and their synergistic combinations could provide patient-tailored therapeutic options for PKU treatment [6, 7].

Dictyostelium discoideum Ax2 is a cellular slime mold that serves as a well-known nonmammalian model organism for biomedical research. We previously developed an assay system for the evaluation of missense mutations in hPAH using a PAH knockout strain (*pah*$^-$) of *D. discoideum* Ax2 [8, 9]. The *pah*$^-$ mutant transformed with expression vectors for hPAH variants demonstrated that hPAH mutations can be evaluated quantitatively by the growth rate and characterized further for both protein stability and catalytic activity. The assay system, however, has several limitations. Because *Dictyostelium* cells have a doubling time of 8–12 h in axenic medium [10], it takes at least 2 days to observe a significant level of difference in cell numbers. Furthermore, to determine protein stability the assay system needs chemiluminescence imaging analysis following a Western blot of the crude extract from a large number of cells. In order to replace the time-consuming procedures, we speculated about using the green fluorescence protein (GFP) for direct measurement of the hPAH protein stability changes. To accomplish this idea, we transformed *pah*⁻ cells to express GFP-tagged hPAHs and evaluated the fluorescence-based cell assay system for the screening of potential pharmacological chaperones.

Materials and methods

Dictyostelium discoideum Ax2 cells were cultured at 22°C in HL5 medium (10 g glucose, 5 g yeast extract, 10 g protease 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 [11]. The *pah*⁻ strain was cultured in HL5 containing 10 μg/mL Blasticidin S [9]. The *pah*⁻ transformed with hPAH cDNAs were maintained in HL5 medium containing 100 μg/mL streptomycin sulfate and 10 μg/mL G418.

For the construction of GFP-tagged hPAH, the GFP sequence obtained from the pTX-GFP plasmid [12] was amplified by PCR using the primers (forward; catcatgatatcggtaccagtaaa, reverse; ggtaccagctctgaattctcttt) harboring KpnI sequences. The amplified GFP DNA was inserted into the corresponding sites of the pDXA-3H plasmid containing hPAH cDNAs, which were prepared beforehand [8, 13], and then used for the transformation of *pah*⁻ strain. The cells harvested by centrifugation at 350 × *g* for 3 min were 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 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, the 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. The viable clones in the selection medium were isolated between 7 and 10 days. The expression of GFP-hPAH in the transformed cells was also confirmed by fluorescence microscopy.

Western blot analysis of S349L was performed as described previously [8]. Briefly, the crude extract from *pah*⁻[S349L] cells (equivalent to 50 μg of protein per a well) was separated by the SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblot was performed with anti-PAH antibody (ab88740 from Abcam, England) and horseradish peroxidase-conjugated anti-mouse IgG (#7076 from Cell Signaling Technology, USA), followed by an enhanced chemiluminescense reaction (Millipore). The membrane was incubated with the diluted antibodies (1:2000) for 1 h at room temperature. The digital chemiluminescence images were taken using Fusion-SL4 Spectra (Vilber, Germany).

For the fluorescence-activated cell sorting (FACS) analysis, the cells were washed twice and cultured in a chemically defined FM minimal medium (ForMedium™, UK) [14]. Aliquots of 2 mL (1×10^6 cells/mL) were cultured in 12-well plates for 2 days and then supplemented with either cumene hydroperoxide or yeast extract. Fluorescent intensity was determined on a BD FACSCalibur (BD Biosciences) with 10,000 cells per sample. The cells were gated for GFP signals based on the background signal from the non-transformed cells. Data acquisition and analysis were carried out with BD FACSDiva software.

Results and discussion

We previously determined the protein stability of eight mutant hPAHs expressed in *pah*⁻ *Dictyostelium* as non-tagged native forms by Western blot analysis [8]. Among them, three mutant proteins, I65T, L255V and S349L, representing the different levels of protein stability [0.546, 0.984, and 0.038, respectively, relative to wild-type (WT) protein] were selected for expression in the same *pah*⁻ strain as GFP-tagged forms. Including non-transformed (*pah*⁻), the transformed cells were analyzed for GFP intensity by flow cytometry (Figure 1A). Expectedly, the GFP positive cells were most frequent (71.3%) in WT cells but were rare (4.74%) in *pah*⁻, demonstrating the successful expression of GFP-tagged hPAHs in *Dictyostelium*. The GFP positive cells of three mutations were plotted as the percentage of WT (Figure 1B). Given that there is a strong correlation between the intracellular fluorescence intensity and the GFP-tagged protein level [15, 16], we expected to see a result consistent with the relative protein stabilities determined from the native forms. Excluding GFP-tagged L255V, however, the others showed much higher fluorescence intensities than expected from the native forms. Because it is known that the N-terminal domain is flexible as a regulatory domain [1], we assume that the N-terminal tagged GFP might have increased the conformational stability of I65T and S349L. Otherwise, N-terminal tagging might have altered the quaternary structure equilibrium of hPAH rather than the tertiary or quaternary structure itself [17]. As these speculations are still insufficient to properly explain the observed fluorescence intensity of L255V, which was less than expected, the combined structural impacts of N-terminal GFP-tagging and point mutation on the protein dynamics of hPAH need further study.

In order to determine if the fluorescence analysis can monitor changes in protein level, we induced accelerated protein degradation in the transformed cells using cumene hydroperoxide, which was known to increase the rate of protein degradation in human red blood cells via free radical generation [18]. Following treatment with various concentrations of cumene hydroperoxide for 4 h, the cells were subjected to the FACS analysis. The GFP positive cells relative to those of the corresponding non-treated cells are shown in Figure 2. Although the overall declining patterns were similar in every kind of hPAH, it was clear that the least stable S349L was the most severely affected. The result supports that the fluorescence-based assay system might be useful for quantifying the protein levels in individual mutant hPAHs.

For the evaluation of the assay system, a putative pharmacological chaperone effect of yeast extract on

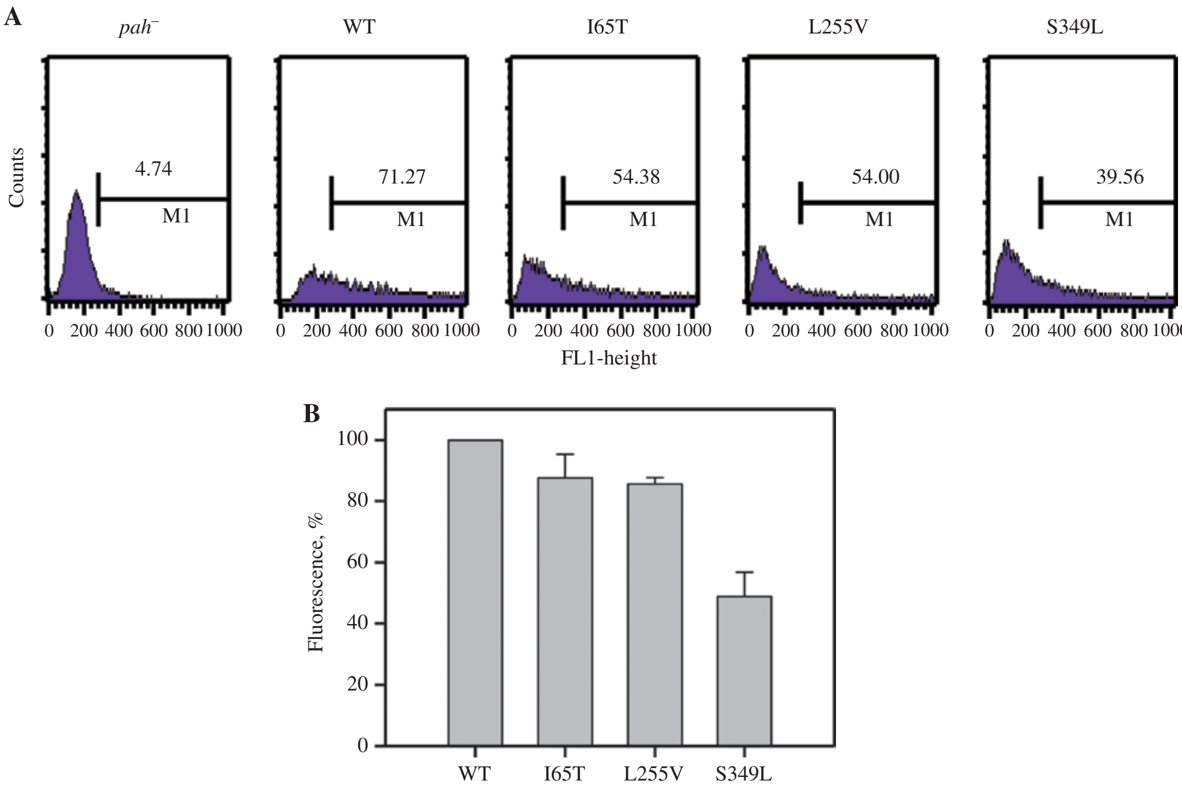

Figure 1: FACS analysis of *pah*⁻ cells expressing GFP-hPAHs.
(A) Representative FACS histograms. The peak pattern represents fluorescent profile of the cells. The gated region (M1) for positive GFP cells is indicated by the bar. The number reflects positive cell percentage. (B) The positive cell percentages relative to WT values were plotted as mean ± SD of the data from three independent experiments.

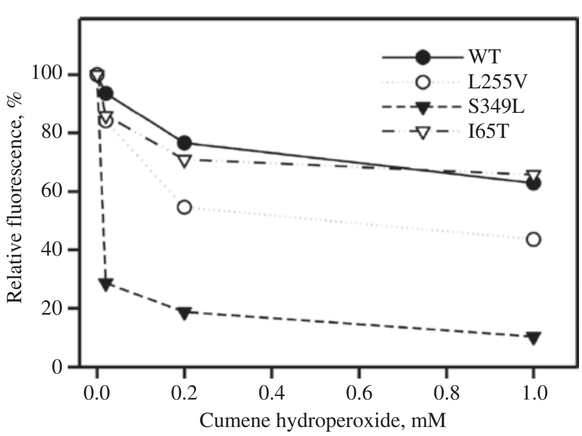

Figure 2: FACS analysis of cumene hydroperoxide effect.
The transformed cells cultured in FM medium were treated with the indicated amounts of cumene hydroperoxide for 4 h and subjected to FACS analysis. The fluorescence levels were relative to those of the corresponding non-treated cells. The data shown is representative of two independent experiments.

S349L was examined (Figure 3). In the previous report, we showed that S349L protein was rescued to WT level when the strain was cultured in HL5 medium for 2 days [8]. The activity of S349L in the crude extract was also increased about five-fold, amounting to 11% of WT. Although the discrepancy between the protein amount and the activity was not fully understood, we hypothesized that the effect of the HL5 medium might have come from some component(s) in the yeast extract and therefore determined the putative chaperone effect of yeast extract on S349L by Western blot analysis (Figure 3A). The S349L protein level was increased about two-fold with 1× yeast extract. We repeated the experiment using the FACS analysis system and found a four-fold increase in fluorescence intensity with 1× yeast extract (Figure 3B). Furthermore, the chaperone effect of 1× yeast extract began to be seen clearly after 8 h (Figure 3B inset). Although the putative component(s) of yeast extract and its underlying mechanism remain unidentified, the results show that the newly developed assay system using FACS analysis could replace the time- and labor-consuming procedures such as Western blot and cell culture.

In conclusion, the fluorescence-based cell assay system appears to be feasible for monitoring changes n the protein level in individual hPAH mutations, despite an insufficiency in evaluating the intrinsic structural impact of each hPAH mutation on the native protein stability. As an assay system having a great advantage of saving time and

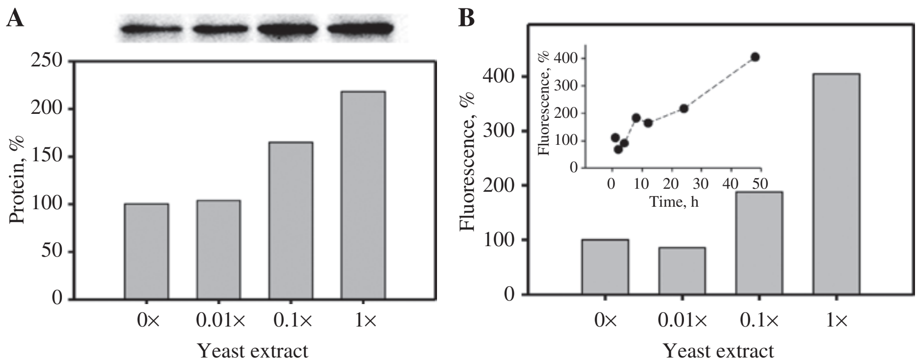

Figure 3: Effect of yeast extract on S349L.
(A) Protein levels determined by Western blot assay. The images obtained from chemiluminescense reaction (shown above) were quantified and plotted by percentage of the control (0× yeast extract). Crude extracts equivalent to 50 μg of total protein were used for each well. (B) Relative levels of fluorescence. Time-course of changes in fluorescence with 1× yeast extract (inset). The transformed cells (*pah*⁻[S349L] for Western blot and *pah*⁻ [GFP-349L] for FACS analysis) were cultured in FM medium with the indicated amounts of yeast extract. 1× yeast extract corresponds to 5 g per liter. The FACS data is representative of two independent experiments.

labor, it would be valuable for high-throughput screening of pharmacological chaperones for hPAH variants.

Funding: This work was supported by a 2016 Inje University research grant.

References

1. Fitzpatrick PF. Allosteric regulation of phenylalanine hydroxylase. Arch Biochem Biophys 2012;519:194–201.
2. Heintz C, Cotton RG, Blau N. Tetrahydrobiopterin, its mode of action on phenylalanine hydroxylase, and importance of genotypes for pharmacological therapy of phenylketonuria. Hum Mutat 2013;34:927–36.
3. Pey AL, Pérez B, Desviat LR, Martínez MA, Aguado C, Erlandsen H, et al. Mechanisms underlying responsiveness to tetrahydrobiopterin in mild phenylketonuria mutations. Hum Mutat 2004;24:388–99.
4. Pey AL, Ying M, Cremades N, Velazquez-Campoy A, Scherer T, Thöny B, et al. Identification of pharmacological chaperones as potential therapeutic agents to treat phenylketonuria. J Clin Invest 2008;118:2858–67.
5. Santos-Sierra S, Kirchmair J, Perna AM, Reiss D, Kemter K, Röschinger W, et al. Novel pharmacological chaperones that correct phenylketonuria in mice. Hum Mol Genet 2012;21:1877–87.
6. Underhaug J, Aubi O, Martinez A. Phenylalanine hydroxylase misfolding and pharmacological chaperones. Curr Top Med Chem 2012;12:2534–45.
7. Hole M, Jorge-Finnigan A, Underhaug J, Teigen K, Martinez A. Pharmacological chaperones that protect tetrahydrobiopterin dependent aromatic amino acid hydroxylases through different mechanisms. Curr Drug Targets 2016;17:1515–26.
8. Kim YM, Yang YG, Kim HL, Park YS. *Dictyostelium discoideum* Ax2 as an assay system for screening of pharmacological chaperones for phenylketonuria mutations. J Microbiol Biotechnol 2015;25:782–7.
9. Kim HL, Park MB, Kim Y, Yang YG, Lee SW, Zhuang N, et al. *Dictyostelium* phenylalanine hydroxylase is activated by its substrate phenylalanine. FEBS Lett 2012;586:3596–600.
10. Ashworth JM, Watts DJ. Metabolism of the cellular slime mould *Dictyostelium discoideum* grown in axenic culture. Biochem J 1970;119:175–82.
11. Watts DJ, Ashworth JM. Growth of myxameobae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. Biochem J 1970;119:171–4.
12. Levi S, Polyakov M, Egelhoff TT. Green fluorescent protein and epitope tag fusion vectors for *Dictyostelium discoideum*. Plasmid 2000;44:231–8.
13. Manstein DJ, Schuster H-P, Morandini P, Hunt DM. Cloning vectors for the production of proteins in *Dictyostelium discoideum*. Gene 1995;162:129–34.
14. Franke J, Kessin R. A defined minimal medium for axenic strains of *D. discoideum*. Proc Natl Acad Sci USA 1977;74:2157–61.
15. Li X, Fang Y, Zhao X, Jiang X, Duong T, Kain SR. Characterization of NFkappaB activation by detection of green fluorescent protein-tagged IkappaB degradation in living cells. J Biol Chem 1999;274:21244–50.
16. Li X, Zhao X, Fang Y, Jiang X, Duong T, Fan C, et al. Generation of destabilized green fluorescent protein as a transcription reporter. J Biol Chem 1998;273:34970–5.
17. Jaffe EK, Stith L, Lawrence SH, Andrake M, Dunbrack RL Jr. A new model for allosteric regulation of phenylalanine hydroxylase: implications for disease and therapeutics. Arch Biochem Biophys 2013;530:73–82.
18. Runge-Morris M, Frank P, Novak RF. Differential effects of organic hydroperoxides and hydrogen peroxide on proteolysis in human erythrocytes. Chem Res Toxicol 1989;2:76–83.