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Melanosomal pH, Pink Locus Protein and their Roles in Melanogenesis

To the editor:

We read with interest the article by Puri *et al* (2000) that reports aberrant pH of melanosomes in pink-eyed dilution (p) mutant mouse melanocytes. Although it is recognized that the product of pink-eyed dilution locus (P protein) is important for melanogenesis its specific function has not yet been identified. In their paper Puri *et al* propose that the P-protein is involved in acidification of melanosomal pH and that this acidic pH is essential for melanogenesis. This proposal is based on the finding of a reduced localization of the melanogenic enzyme TRP-1 in acidic organelles. Whilst this could suggest that p-mutant mouse melanocytes have fewer acidic melanosomes there are other interpretations. For instance, it could be argued that in these melanocytes there is a misrouting of TRP-1 protein.

It would indeed be a unique finding if, as Puri *et al* suggest, the p-locus product is an ionic transporter involved in acidification of melanosomes. If this was the case then it is difficult to explain how the P-protein, which does not utilize energy from ATP, could function against a proton gradient of up to 1000-fold (pH 7.2 in cytosol *versus* pH < 4 in melanosomes); however, this hypothesis can be easily tested. If, as postulated by the authors, melanogenesis only occurs in acidic melanosomes then it follows that neutralization of intracellular acidic organelles should lead to a reduction of melanogenesis in wild-type cells. The same change in p null mouse melanocytes should result in little if any decrease in melanogenesis as most melanosomes are already at a neutral pH. There are several well-characterized substances available that neutralize intracellular acidic organelles that could be used to test this hypothesis. These include the selective vacuolar type proton pump inhibitors, bafilomycinA1 (BafA1) and concanomycinA (ConA), the H+/Na + exchanger monensin, and ammonium chloride (NH_4Cl). Although Puri *et al* used monensin in their experiments to demonstrate the specificity of their method to visualize acidic organelles, they made no mention of its effects on melanogenesis. This was surprising since there are reports that treatment of mouse melanoma cells with monensin or NH_4Cl will cause immediate and large increases in melanogenesis (Saeki and Oikawa, 1985; Fuller *et al*, 1993). These findings are consistent with the view that mammalian tyrosinase has optimal conditions at neutral pH (Hearing and Ekel, 1976; Townsend *et al*, 1984) but contrasts with the hypothesis that acidic conditions favor melanogenesis (Devi *et al*, 1987). In fact we also have observations that support the view that melanin production is optimal at near neutral pH. We have reported that the proton pump inhibitors, BafA1 and ConA, induced melanogenesis in tyrosinase positive but amelanotic human and mouse melanoma cells (Ancans and Thody, 2000) and our observations have recently been confirmed (Fuller *et al*, 2001). Interestingly, one of the melanoma cell lines (FM3) used in our study failed to express mRNA for the P-protein. Melanogenesis was also restored in response to monensin and NH_4Cl and in all cultures this correlated with neutralization of acidic organelle pH. We carried out similar experiments using the mouse melanocyte line Melan-p1 (p^{cp}/p^{25H}) as well as wild-type P locus cell lines Melan-a and Melan-b (kindly provided by Dr. D. Bennett). Neutralization of acidic organelles resulted in a 4–5-fold increase in the melanin content of Melan-p1 cells while there was no significant change with the Melan-a cells (wild type). Melan-b cells that have wild-type P locus but reduced melanogenesis, as a result of mutated b locus, responded with a slight increase in melanin content (**Fig 1**). These results were not affected by the protein synthesis inhibitor cycloheximide suggesting an activation of pre-existing tyrosinase.

Our findings are consistent with the idea that P protein has a role in regulation of melanosomal pH but argue against the hypothesis of acidification. We would suggest the alternative hypothesis that the P-locus protein has a role in the neutralization of melanosomal pH and that this change facilitates tyrosinase activity. P-locus protein is homologous to the *E. coli* Na+/H + antiporter and in the light of our findings it is reasonable to propose that the P-protein functions as a channel that acts to reduce the proton concentration inside the melanosome (as these organelles are related to lysosomes). The observation made by Puri *et al* that both p null melanocyte lines had a 25% increase in the total number of acidic vesicles would be in keeping with this. Finally, our hypothesis does not contradict the fact that melanosomes can be acidic; however,

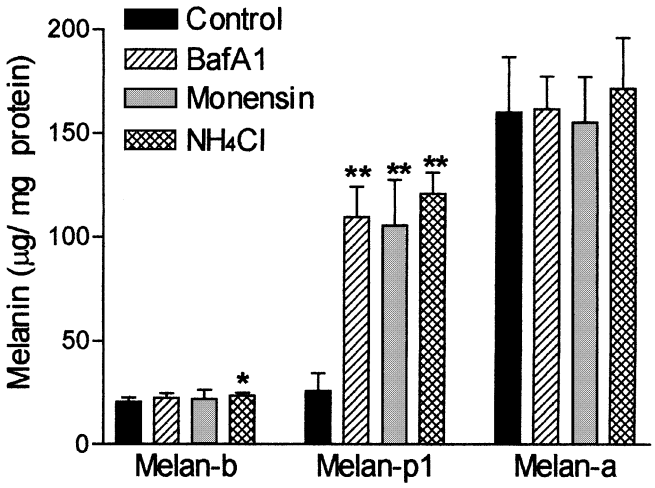

Figure 1. Effect of acidic intracellular organelle neutralization on melanogenesis in mouse melanocytes with wild type p-locus (Melan-a and b) and the p^{cp}/p^{25H} line Melan-p1. The cells were treated with BafA1 (20 nM), monensin (10 μM), or NH_4Cl (10 mM) for 24 h and mean values of three experiments ± SD are represented. *p < 0.05; **p < 0.001.

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we would propose that stages of melanin formation that require tyrosinase enzymatic activity will proceed optimally at near neutral pH and that it is the p-locus product that functions to provide these conditions. It is possible that the P-protein creates a neutral local microenvironment and this is important in maintaining the high molecular weight melanogenic complex of tyrosinase, TRP-1 and -2. This would explain the absence of this complex in p-locus mutated mouse cells (Lamoreux *et al*, 1995).

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A Novel a(– 4)-to-g Acceptor Splice Site Mutation Leads to Three Bases Insertion in Ferrochelatase mRNA in a Patient with Erythropoietic Protoporphyria

To the Editor:

Erythropoietic protoporphyria (EPP; MIM 177000) is an inherited disorder caused by a partial deficiency of ferrochelatase (FECH), the last enzyme in the heme biosynthetic pathway (McKusick, 1998). In EPP patients, protoporphyrin accumulated in red blood cells, skin, liver, and other organs causes photosensitivity and occasionally life-threatening liver failure. Flushing, blister formation, pigmentation, and scarring of areas of skin exposed to sunlight are the dermatologic symptoms of EPP (Todd, 1994). The level of protoporphyrin in erythrocytes and feces is increased, but usually it is not secreted in the urine. Human FECH cDNA contains 2443 bp. The FECH protein consists of 423 amino acids and has a molecular weight of 40–42 kDa (Nakahashi *et al*, 1990). The FECH gene spans at least 45 kb on the chromosome 18q21.3 and contains 11 exons (Taketani *et al*, 1992). Over 38 mutations in the FECH gene in EPP patients have been reported (Rufenacht *et al*, 1998). The characteristic FECH gene mutation in patients with liver failure had been thought to be abnormal splicing leading to exon skipping; however, it has been found that other mutations resulting in a null allele are involved in EPP pedigrees with liver complications (Rufenacht *et al*, 1998). Here we report a novel mutation in the exon–intron boundary in a case of EPP in which FECH activity in lymphocyte is 39.4% of normal.

A 23-y-old Japanese male who had photosensitivity visited our University Clinic for a thorough examination. He had the first symptoms of painful erythematous swelling on his face, hands, and feet at the age of 15 when he came home from a school trip. His symptoms were getting worse. His parents were asymptomatic and he had no siblings. There was no history of photosensitivity in other family members. Routine blood test values and liver function data were within normal limits. The minimal erythema doses of ultraviolet (UV) A and UVB were normal. Examination of the urine with a Wood light revealed no fluorescence. Blood protoporphyrin was 1424 μg per dl (30–86), blood coproporphyrin 2 μg per dl (< 1), and urine coproporphyrin 30 μg per $G_{creatinine}$ (47–151). Lymphocyte FECH activity in the patient measured by the method (Li *et al*, 1987) was 24.4 nmol zinc-mesoporphyrin formed per mg protein per h in contrast to 62.0 in a normal control.

After informed consent, blood samples were collected from the patient. Total RNA was extracted from the white blood cells by the TRIZOL method (Life Technologies, Gibco BRL, Gaithersburg, MD) and was subjected to oligo(dT) primed cDNA synthesis using M-MuLV reverse transcriptase (RT) (Amersham Pharmacia Biotech, Cleveland, OH). One microgram of the first strand cDNA was used as a template for PCR amplification with the primer pair, FCH-F; 5′-CGGGCCGAGGCTGCCCAGGC-3′ and FCH-E5-B; 5′-CTG-TGGTGGAGCAGCTGTACTG-3′ [cDNA nucleotide positions –21~–2 and 567–588 (GenBank accession no. D00726), respectively]. Amplification conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min, and then final extension at 72°C for 5 min. The PCR product (619 bp) was cloned into pCR2.1-TOPO TA-cloning vector (Invitrogen, Carlsbad, CA). Sequencing was carried out using the Dye Terminators Cycle Sequencing Ready Reaction (Perkin Elmer, Warrington, U.K.) on an ABI 310 genetic analyzer. To confirm the heterozygosity of the mutation, direct sequencing was done with a smaller size of PCR product with primers, E4DF2; 5′-AAGAGCAGTACCGCAGGATT-3′ [cDNA nucleotide position 359–378 (GenBank accession no. D00726)] and FCH-E5-B.

Genomic DNA was extracted from white blood cells with a QIAamp Blood Kit (Qiagen, Hilden, Germany). Genomic DNA from normal healthy Japanese people was used as controls. Specific primers, FCint4up; 5′-CTCTGAGGAATCTATATAAGG-3′ (unpublished data) and FCex5dn; 5′-GTGAAAGCAATAGCC-CTTTC-3′ [cDNA nucleotide position 547–566 (GenBank accession no. D00726)] were designed for amplification of the intron 4–exon 5 boundary. Amplification conditions were 95°C for

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