Biochemical and Biophysical Research Communications **270,** 176–182 (2000)
doi:10.1006/bbrc.2000.2409, available online at http://www.idealibrary.com on IDEAL®

In Situ Localization of Agouti Signal Protein in Murine Skin Using Immunohistochemistry with an ASP-Specific Antibody

Naoko Matsunaga, Victoria Virador, Chie Santis, Wilfred D. Vieira, Minao Furumura, Jun Matsunaga, Nobuhiko Kobayashi, and Vincent J. Hearing

Pigment Cell Biology Section, Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Received February 25, 2000

Switching between production of eumelanin or pheomelanin in follicular melanocytes is responsible for hair color in mammals; in mice, this switch is controlled by the *agouti* locus, which encodes agouti signal protein (ASP) through the action of melanocortin receptor 1. To study expression and processing patterns of ASP in the skin and its regulation of pigment production in hair follicles, we have generated a rabbit antibody (termed αPEP16) against a synthetic peptide that corresponds to the carboxyl terminus of ASP. The specificity of that antibody was measured by ELISA and was confirmed by Western blot analysis. Using immunohistochemistry, we characterized the expression of ASP in the skin of newborn mice at 3, 6, and 9 days postnatally. Expression in nonagouti (a/a) black mouse skin was negative at all times examined, as expected, and high expression of ASP was observed in 6 day newborn agouti (A/+) and in 6 and 9 day newborn lethal yellow (A^y/a) mouse skin. In lethal yellow (pheomelanogenic) mice, ASP expression increased day by day as the hair color became more yellow. These expression patterns suggest that ASP is delivered quickly and efficiently to melanocytes and to hair matrix cells in the hair bulbs where it regulates melanin production.

Key Words: **agouti; pigmentation; melanogenesis; pheomelanin.**

Mammalian melanocytes can produce two distinct types of melanin, termed eumelanin and pheomelanin and the proportions of these determine hair and skin color in mammals (reviewed in (1)). These melanins are produced through a relatively well characterized biosynthetic pathway which involves the catalytic function of several melanogenic enzymes including tyrosinase, TRP1/Tyrp1 and TRP2/Dct (reviewed in (2)). The *agouti* locus encodes a paracrine signaling molecule that causes hair follicle melanocytes to synthesize pheomelanin instead of eumelanin. The product of that locus is termed agouti signal protein (ASP), which has 131 amino acids and an expected molecular mass of ~14.3 kDa. ASP is thought to be a secreted protein with a 23 residue amino-terminal signal sequence and a cysteine-rich carboxyl terminus (3). ASP has been shown by *in situ* hybridization to be produced by dermal papilla cells (4) and to function as a paracrine factor, controlling whether black/brown eumelanin or yellow/red pheomelanin is produced by melanocytes through its interactions with melanocyte stimulating hormone (MSH) and the melanocortin 1 receptor (MC1R). In agouti mice that carry the wild-type A allele, eumelanin is produced by all follicular melanocytes at the beginning of the hair growth cycle (from 0 to 4 days). Transient expression of ASP from days 4 to 7 of the hair growth cycle causes melanocytes to produce pheomelanin instead of eumelanin; after day 7, *agouti* gene expression is turned off and eumelanin is produced again. ASP expression is thus short-term and reversible in agouti mice. This pattern of pigment synthesis results in the yellow striped band near the tip of each black hair shaft.

There are more than 20 known *agouti* alleles in mice. Mutations at the *agouti* locus can elicit the production of all yellow or all black hair, depending on whether the mutation leads to overexpression/hyperfunction or nonexpression/nonfunction of ASP, respectively (5–7). For example, the dominant lethal yellow mutation (A^y) results in the production of completely yellow hairs (8–10), while the recessive *nonagouti* (*a*) mutation results in eumelanin production (3). This study was ini-

Abbreviations used: ASP, agouti signal protein; Dct, DOPAchrome tautomerase; ELISA, Enzyme-Linked ImmunoSorbent Assay; PBS, phosphate buffered saline.

0006-291X/00 $35.00

tiated to clarify the expression patterns and localization of ASP in hair follicles. Although ASP expression by dermal papilla cells has been shown by *in situ* hybridization, the secretion and localization of ASP in the follicular melanocyte microenvironment has not yet been directly shown. We now report the generation of a specific polyclonal antibody (termed αPEP16) in rabbits that recognizes mouse ASP and have used it in conjunction with immunohistochemistry to characterize the production and localization of ASP *in situ*.

MATERIALS AND METHODS

Animals. Nonagouti black C57BL/6J mice (a/a), wild-type agouti C3H/HeJ mice (A/+) and lethal yellow C57BL/6J mice (A^y/a) were used in this study. Mice used for immunohistochemistry were 3, 6, or 9 days old and all mice were housed in the NCI/NIH animal facility. For Western blotting analysis, 11 or 15 day old nonagouti black (a/a) or lethal yellow (A^y/a) mice were used.

Peptide synthesis and antibody production. Peptide PEP16 (CFGSACTCRVLNPNC-COOH, which corresponds to the carboxyl terminus of ASP) was synthesized and conjugated to KLH as previously detailed (11–13). A polyclonal antibody (αPEP16) was raised in rabbits using the synthetic PEP16 peptide, as previously described (12) and titration of antibody production was tested by ELISA and by Western blot, as noted below.

Sample preparation. Mice were anesthetized and sacrificed by cervical dislocation, then sterilized by immersion in 70% ethanol and their dorsal skins were dissected. The dorsal skins were homogenized in NP40/SDS buffer (1% Nonidet P-40, 0.01% SDS, 0.1 M Tris-HCl, pH 7.2, and complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN)) on ice using a Microson ultrasonic cell disruptor Model XL2007 (Misonix Inc, New York), or were kept at −70°C until use. Following centrifugation at 20,800*g* for 15 min at 4°C, the supernatants were centrifuged again under the same conditions to remove insoluble protein. Soluble whole skin extracts were used for Western blotting analysis, as detailed below. For dermal cell extracts (which include hair follicles), we used 11-day-old mice, and dorsal skins were dissected as described above. Skins were then oriented with their dermal side up and fat tissue was removed. 100 μl of NP40/SDS buffer was put on each skin piece (~2 cm^2) and the dermis was removed from the epidermis by scraping with surgical blades, kept at 4°C overnight, and then centrifuged at 20,800*g* for 15 min. The supernatants were then used for Western blotting analysis, as detailed below.

Enzyme-linked immunosorbent assay (ELISA). ELISA was used to screen the polyclonal ASP αPEP16 antibody as previously reported (11, 14). Briefly, 20 μl coupling buffer (100 mM sodium bicarbonate, pH 9.6) containing 0.1 μg peptide was added to each well of a 96-well Immobilon II microtiter plate (DynaTech Laboratories, Alexandria, VA) and incubated at 4°C overnight. Following 6 washes with 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Tween-20 (PBS/Tween), 50 μl of antisera (at a 1:1000 dilution) was added to each well, as noted in the legend to Table 1. Following incubation at 23°C for 1 h, plates were then thoroughly washed in PBS/Tween, and incubated with 50 μl anti-rabbit IgG, peroxidase-linked whole antibody from donkey (Amersham, Arlington Heights, IL) at a 1:1000 dilution at 23°C for 30 min. Plates were then thoroughly washed again with PBS/Tween, and 100 μl *o*-phenylenediamine solution, containing 0.012% hydrogen peroxide, in 0.1 M citrate buffer (pH 4.5) was added to each well. Reactions were performed at 23°C and were stopped after 7 min by the addition of 100 μl 2 N sulfuric acid; absorbance of wells was measured at 492 nm using a SpectraMax 250 ELISA Reader (Molecular Devices, Sunnyvale, CA).

Western immunoblotting. Protein samples from skin extracts were separated on 15% polyacrylamide SDS gels, then transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA) and incubated with primary antibody (at a 1:1000 dilution). Subsequent visualization of antibody binding was performed using an appropriate secondary antibody with Enhanced ChemiLuminescence (Amersham Corp, Arlington Heights, IL), according to the manufacturer's instructions.

Immunohistochemical staining. Immunohistochemical staining for ASP was performed on 4% paraformaldehyde-fixed, paraffin embedded sections, using an avidin-biotin immunoperoxidase technique (Vector Laboratories, Burlingame, CA). In brief, histological sections were deparaffinized and rehydrated in two changes of xylene and an ethanol series. Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in anhydrous methanol for 20 min. For antigen retrieval, slides were microwaved for a total of 10 min in distilled water and blocked with 5% normal goat serum (Vector) in PBS for 10 min. Slides were then incubated with αPEP16 antibody at a 1:500 dilution in PBS at 4°C overnight. Biotinylated anti-rabbit IgG (Vector) was used at a dilution of 1:500 in PBS containing 2% normal goat serum at 23°C for 30 min. After 30 min incubation with avidin-biotinylated horseradish peroxidase complex (VectaStain ABC Reagent, Vector) in PBS, slides were incubated for 20 min at 23°C with a peroxidase substrate AEC (3-amino-9-ethylcarbazole) solution (Vector), which generates a red to brownish-red color. Sections were counterstained with hematoxylin and mounted in Immu-Mount (Shandon, Pittsburgh, PA).

Double immunodetection of ASP and tyrosinase with immunofluorescence. To further evaluate the localization of ASP, we perform the same protocol described above, but using αPEP7 (a tyrosinase antibody (11)) as the primary antibody followed by Texas Red-conjugated secondary antibody. Following that, the slides were incubated with Alexa 488-conjugated αPEP16 (Alexa 488 Protein Labeling Kit, Molecular Probes, Eugene, OR), and immunofluorescence was detected as detailed above.

RESULTS

Reactivity of the αPEP16 antibody with ASP. In order to clarify the expression and localization of ASP, we synthesized a peptide (termed PEP16) that corresponds to the carboxyl terminus of ASP and raised an antibody (termed αPEP16) against it in rabbits. The titer and specificity of the αPEP16 antibody was measured by ELISA (Table 1). Prebleed normal rabbit serum and PBS showed only background binding to PEP16 but the αPEP16 antibody had a high reactivity with the immunizing PEP16 peptide, at a 1:1000 dilution. We also examined the cross-reactivity of αPEP16 against other synthetic melanogenic peptides, including PEP1 (the C-terminus of Tyrp1/TRP1), PEP7 (the C-terminus of tyrosinase), PEP8 (the C-terminus of Dct/TRP2) and PEP13 (the C-terminus of Pmel17/silver). No binding activity above background was detectable against any of those peptides. The sum of these data show that the αPEP16 antibody reacts specifically only with the PEP16 peptide used to generate it.

We then examined the reactivity of αPEP16 with intact purified recombinant ASP using Western blot-

TABLE 1

Peptide Sequence and Specificity of Antibody

	Peptide bound to plate				
Antibody	PEP16	PEP1	PEP7	PEP8	PEP13
Phosphate buffered saline	0.05	0.04	0.04	0.04	0.04
Normal rabbit serum	0.09	0.07	0.08	0.08	0.07
αPEP16	1.87	0.07	0.07	0.05	0.07

Note. Specificity of the αPEP16 antibody was tested by ELISA; data shown in the table report the absorbance at 492 nm as the means of assays performed in quadruplicate (±SEM was less than ±5% in all cases). The αPEP16 antibody was used at a 1:1000 dilution. Peptides bound to the plate (100 ng/well) are shown above each column, and reactivity against normal rabbit serum taken as a prebleed from the immunized rabbit, phosphate buffered saline or αPEP16 antibody was measured. PEP1, the C-terminus of Tyrp1/TRP1; PEP7, the C-terminus of tyrosinase; PEP8, the C-terminus of Dct/TRP2; and PEP13, the C-terminus of Pmel17/silver protein.

ting analysis (Fig. 1A). Recombinant ASP was generated in a baculovirus expression system and was then purified by FPLC as previously described (15). Recombinant ASP includes minor protein contaminants (as shown by silver staining), but the αPEP16 antibody recognized only ASP (normal rabbit serum showed no activity). It should be noted that in separate experiments (data not shown), we found that reactivity of αPEP16 with ASP requires prior denaturation of ASP, e.g. by treatment with SDS, 2-mercaptoethanol and heat.

We next examined ASP expression in murine skin using Western blot analysis and αPEP16 (Fig. 1B). We made protein extracts of dermal cells (hair follicles) and of whole skins from newborn lethal yellow (A^y/a) and black (a/a) mice and analyzed them by Western immunoblotting. The αPEP16 antibody reacted with a 16 kD band, similar in size to that observed with recombinant ASP, in extracts of whole skin from 15-day-old lethal yellow mice. An even stronger reactivity existed in extracts from dermal cells of 11-day-old lethal yellow mice. In both types of samples, staining of comparable extracts from black mice were negative. In addition to the full length (~16 kD) band, 1 or 2 smaller immunoreactive bands were noted in the lethal yellow tissue extracts. These results suggest that ASP exists at a higher concentration in dermal cells, which includes hair follicles, compared with whole skins of lethal yellow mice (as expected), and that ASP remains essentially intact in the skin, and can be recognized by the αPEP16 antibody following denaturation.

In situ localization of ASP. In order to investigate the expression of ASP in murine skin, we analyzed *in vivo* expression patterns of ASP using αPEP16 and immunohistochemistry (Fig. 2). We used AEC as a substrate to make immunoreactivity visible as a red color readily distinguishable from melanins. Eumelanins are black and can be easily seen in the sections from black mice, while pheomelanins are relatively soluble and are poorly fixed in these sections (note that yellow pheomelanin is just barely visible as a light yellow color in the lethal yellow sections stained with normal rabbit serum in the far right column of Fig. 2). In nonagouti black mouse hair follicles (left column), only black melanin granules were evident at all times of the hair growth cycle examined (at days 3, 6, and 9), and ASP expression was undetectable by αPEP16 staining, as expected. Immunostaining with normal rabbit serum showed negative staining of tissue sections with a suitably low background.

We next examined ASP expression in agouti mouse skin at ages when they are producing eumelanin (at day 3), pheomelanin (at day 6) and eumelanin again (at day 9). In hair follicles of 3-day-old agouti mice only black eumelanin is evident, and only a few cells, which seem to be hair matrix cells (i.e. keratinocytes) that are devoid of melanin, stained slightly positive for ASP. Skin from 6 day old agouti mice has hair follicles in the pheomelanogenic phase, and these have now become quite positive for ASP. Nine-day-old agouti mouse hair follicles contain only eumelanin again, and no ASP positive cells detected by αPEP16 staining could be seen. To confirm the expression of ASP in pheomelanogenic hair follicles, we confirmed these staining pat-

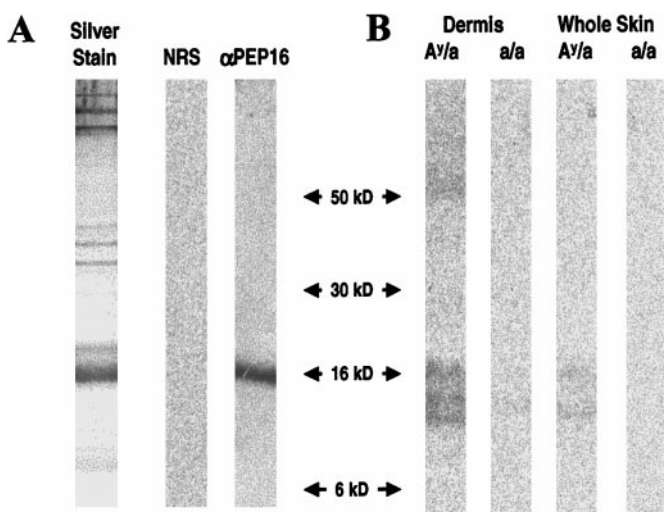

FIG. 1. Western blot analysis of αPEP16 reactivity and specificity. Western blot analysis was performed to test the reactivity and specificity of αPEP16. (A) 1 μg/lane purified recombinant ASP was electrophoresed in 15% SDS-PAGE gels, and transferred to PVDF membranes as detailed under Materials and Methods. From left to right, silver staining, immunostaining with normal rabbit serum (NRS, at 1:1000), and immunostaining with αPEP16 (at 1:1000 dilution). (B) Extracts of skin were separated by electrophoresis as described above and were immunostained with αPEP16 antibody (at 1:1000 dilution). Extracts (30 μg/lane) from the dermis of lethal yellow (A^y/a) or black (a) 11-day-old mice or from whole skins of lethal yellow (A^y/a) or black (a/a) 15-day-old mice were used.

terns with other chromophores, e.g. blue-gray, which also contrasted well with the color of eumelanin and pheomelanin (data not shown).

We also examined the expression of ASP in hair bulbs from 3-, 6-, and 9-day-old lethal yellow mice which become progressively pheomelanogenic. Three-day-old lethal yellow mice produce a small amount of eumelanin; dark brown pigment could be observed in hair follicles, although the amount of eumelanin was significantly less than that produced by 3-day-old black or agouti mice (compare the top row of Fig. 2). At 6 days, the production of pheomelanin in lethal yellow newborn mice had become more evident and hair bulbs that contained pheomelanin were strongly positive for ASP. With continued growth, production of pheomelanin became even more predominant, as seen in hair bulbs of 9-day-old lethal yellow mice, where all hair bulbs had only yellow melanin and were positive for αPEP16 staining. Thus, in lethal yellow mice, expression of ASP continues to increase with age, at least during the first hair cycle.

Colocalization of ASP and tyrosinase. The specific localization patterns of ASP expression in hair follicles were interesting since reactivity was not detected in dermal papilla cells as expected but appeared to be localized only in hair matrix cells and melanocytes of hair follicles. The identity of those ASP-positive cells in the hair bulbs was then further examined using double immunofluorescence (Fig. 3). Green fluorescence was used to identify cells that expressed ASP, while red fluorescence of the same field identified melanocytes which expressed tyrosinase. In a superimposed image, all melanocytes were yellow in color showing that all were positive for tyrosinase and ASP. However, some cells in the hair matrix were ASP-positive by tyrosinase-negative.

DISCUSSION

Mammalian coat color is determined by the relative proportion of eumelanin and pheomelanin in the hair (16, 17). This eumelanin/pheomelanin ratio is regulated by the *agouti, POMC,* and *extension* loci, which encode ASP, melanocyte stimulating hormone (MSH) and the MSH receptor (MC1R), respectively. Many mutations at the murine *agouti* locus have been described, and mice that carry *agouti* mutations can range in coat color from black to yellow, depending on whether the mutation leads to hypo- or hyper-function of ASP, respectively. In mammalian hair bulb melanocytes, αMSH increases the production of eumelanin, while ASP has an opposite action, eliciting the production of pheomelanin. Purified recombinant ASP added to cultured melan-a cells decreases the expression of several eumelanogenic genes, reduces the production of eumelanin and eumelanosomes, and increases the production of pheomelanin and pheomelanosome-like structures (18). Other studies have shown that ASP inhibits αMSH stimulated expression of tyrosinase, Tyrp1/TRP1 and Dct/TRP2 by inhibiting the transcriptional activity of their respective promoters (15). It has been shown that this regulation results from ASP interfering with the binding of αMSH to the MC1R (19), and that ASP acts as an antagonist of that receptor (20). The *agouti* locus encodes a protein of 131 amino acids; that sequence includes a 22 residue putative secretion signal, an internal basic region, and a C-terminal domain (containing 10 cysteines) (3, 8). The N-linked glycosylation site and the C-terminal Cys-rich motif are important for full biological activity of ASP (18). The lysine-rich basic domain has been suggested to be dispensable for normal function (5), although a more recent report (21) suggests that this domain may have a distinct function in regulating melanocyte differentiation. Regardless of the specific mechanism(s) involved, ASP is a paracrine-signaling molecule whose mRNA expression is observed in the dermal papillae of hair follicles (4).

The production and deposition of pheomelanin in the growing hair shaft of agouti mice occurs during days 4–7 of the hair growth cycle, which is coincident with the appearance of the subapical yellow band. Expression of the *agouti* gene is maximal during days 4–7 and correlates closely with pheomelanin production. Whereas the *agouti* gene is normally expressed only in skin and perhaps in testis, the A^y allele is associated with overproduction of agouti RNA transcripts in neonatal skin and in all adult tissues such as brain, liver, lung, spleen, and kidney (3). Lethal yellow mice ($A^y/+$) have dark hair initially after birth, but their hair color changes to yellow soon after that, and this progress suggests that pheomelanogenesis gradually becomes more dominant after birth. Interestingly, our antibody recognizes high levels of expression of ASP in dorsal body fat tissues of lethal yellow mice as they age, and there are decreases in the expression of ASP in their skin with age (data not shown), and the pattern of ASP in various tissues with age will be the subject of a future report.

In this study, the αPEP16 antibody was developed specifically against mouse ASP to clarify its expression and localization in the skin. We tested the specificity of αPEP16 by ELISA and confirmed that this antibody reacts only with PEP16 and not with other known melanogenic peptides. This antibody also specifically reacted with mouse ASP in Western blotting analysis using extracts of skin and in immunohistochemical studies using paraffin embedded skin sections. However, it should be emphasized that αPEP16 requires prior denaturation of ASP for recognition by αPEP16 using Western blotting analysis or immunohistochemical staining. This suggests that the C-terminus of ASP is inaccessible under native conditions, perhaps by be-

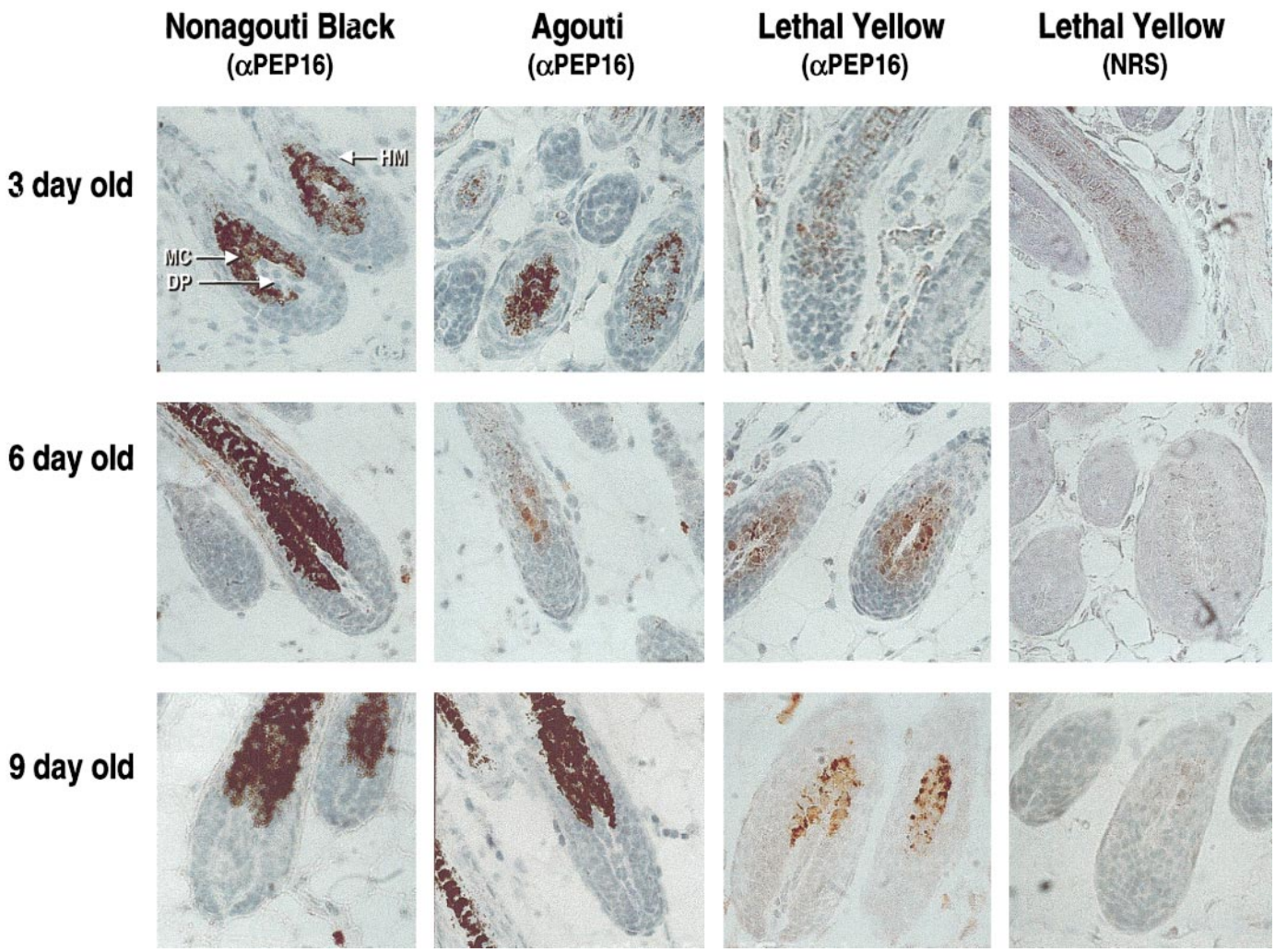

FIG. 2. Immunohistochemical staining with αPEP16. ASP expression in 3 day (top), 6 day (middle), and 9 day (bottom) old dorsal skins from nonagouti black (a/a), wild-type agouti (A/+), or lethal yellow (A^y/a) mice were examined using immunohistochemical staining of paraformaldehyde fixed, paraffin-embedded sections. αPEP16 was used at a 1:1000 dilution, as was normal rabbit serum (NRS). Positive stains are shown as a brownish red color. HM, hair matrix; MC, melanocytes; DP, dermal papilla. Original magnification, 400×.

ing folded within the tightly coiled cys-rich domain. In Western blotting analysis, αPEP16 recognizes ASP as 2 or 3 different bands in extracts of 11 day and 15 day old lethal yellow mouse skin, although Western blotting analysis of purified recombinant ASP shows only a single band. This suggests that ASP undergoes some posttranslational processing *in vivo* in the skin and that processing must occur at the amino terminus since the epitope recognized by αPEP16 is at the carboxyl terminus, although a study by Ollmann and Barsh (21) suggests that doesn't happen. The molecular masses of the identified forms were estimated to be around 12–16 kDa by SDS-PAGE by Western blotting analysis, which is slightly larger than that calculated for ASP (~12 kD after the signal sequence is cleaved). This result further suggests that ASP exists at higher concentrations in dermal cells, probably in hair follicles, of lethal yellow mice compared with the skin in general. The sum of those results suggest that 1 or 2 small fragments are cleaved from the amino terminus of ASP but remain associated with ASP and are only released under denaturing conditions.

In early neonatal (~3 day) hair follicles of agouti mice, almost all hair bulbs had black melanin granules, and only a few hair bulbs were slightly ASP positive. Three days later, approximately half of the hair follicles were producing eumelanin and the other half were producing pheomelanin. ASP positive cells existed only in hair bulbs in the pheomelanogenic phase, suggesting that many of these other (ASP-negative) hair bulbs had already returned to eumelanogenesis. Nine-day-old agouti mice were uniformly producing eumelanin again, and no expression of ASP is seen. In contrast, in 3-day-old lethal yellow mice,

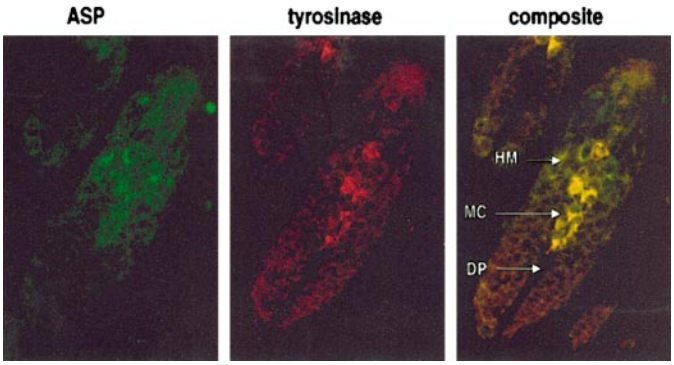

FIG. 3. Double immunostaining with αPEP16 and αPEP7 antibodies. ASP and tyrosinase localization in hair bulbs was examined using double label immunohistochemistry in skin sections, as detailed under Materials and Methods. ASP expression was detected by αPEP16 and is shown as green fluorescence (left) while tyrosinase, expressed only by melanocytes, is identified by αPEP7, and is shown as red fluorescence (center). In a superimposed image (right), all melanocytes appear yellow, indicating that ASP is colocalized there. However, some cells in the surrounding hair matrix cells can also be seen to express only ASP. HM, hair matrix; MC, melanocytes; DP, dermal papilla. Original magnification, 400×.

almost all hair bulbs produced dark eumelanin granules as described before (22), although the amount of melanin in the hair bulbs of 3-day-old mice was much less than in black or agouti mice at the same age. Similar to agouti, 3 days later (i.e. at 6 days) some hair bulbs (less than half) were still producing eumelanin but more than half were now ASP positive. After that, pheomelanin was produced progressively, and by day 9, all hair bulbs contained only pheomelanin and were strongly ASP positive.

ASP positive cells appear to be melanocytes and hair matrix cells. Melanocytes can be easily recognized since they contain melanin and exist above the dermal papillae. Other ASP-positive but melanin-negative cells are recognized as hair matrix cells and these might be 'resting' melanocytes in the outer root sheath, which don't have melanin. In black mice, eumelanin is produced constantly and there is no detectable expression of ASP (as expected). It has been reported that the *agouti* gene of nonagouti black mice has an 11 kb insertion which interferes with normal transcription and and/or splicing of the agouti message (6). Our observations are consistent with that report, and it seems quite reasonable that black mouse skin is ASP negative.

Immunohistochemical staining reveals that ASP localizes in hair matrix cells and in melanocytes within hair bulbs. The αPEP16 antibody developed in this study specifically stained only ASP localized in melanocytes and in hair matrix cells of hair follicles. Double staining showed clearly that ASP localizes not only to melanocytes but also to hair matrix cells. Surprisingly, we were unable to detect ASP positive dermal papilla cells where it is transcribed. One possible interpretation of this is that after being produced in the dermal papillae, ASP is transferred quickly and efficiently, and is localized in the hair matrix cells and melanocytes soon afterward. It may also be that unbound ASP (e.g. as secreted by dermal papilla cells) might be lost during fixation and processing, and that only ASP bound to the MC1R is detectable. Since follicular melanocytes are the target cells for ASP, but ASP also colocalizes with hair matrix cells, ASP may play some role in the latter type of cells as well. Hair matrix cells are keratinocytes, and some reports (23, 24) have suggested that keratinocytes also express MC1R, and might therefore also be a target of ASP action. Alternatively, hair matrix cells may have picked ASP up by indiscriminate phagocytosis.

The sum of these findings demonstrates clearly that ASP expression coincides with pheomelanogenesis in murine skin and is localized in hair follicles there. The αPEP16 antibody should prove to be important in the future to further characterize ASP production, processing and function.

ACKNOWLEDGMENT

The authors thank Dr. Gregory S. Barsh for his gift of the ASP expression vector.

REFERENCES

1. Prota, G. (1992) Melanins and Melanogenesis, Academic Press, New York.
2. Hearing, V. J. (1998) *in* The Pigmentary System: Physiology and Pathophysiology (Nordlund, J. J., Boissy, R. E., Hearing, V. J., King, R. A., and Ortonne, J. P., Eds.), pp. 423–438, Oxford Univ. Press, New York.
3. Bultman, S. J., Michaud, E. J., and Woychik, R. P. (1992) *Cell* **71,** 1195–1204.
4. Millar, S. E., Miller, M. W., Stevens, M. E., and Barsh, G. S. (1995) *Development* **121,** 3223–3232.
5. Perry, W. L., Nakamura, T., Swing, D. A., Secrest, L., Eagleson, B., Hustad, C. M., Copeland, N. G., and Jenkins, N. A. (1996) *Genetics* **144,** 255–264.
6. Siracusa, L. D. (1994) *Trends Genet.* **10,** 423–428.
7. Vrieling, H., Duhl, D. M. J., Millar, S. E., Miller, K. A., and Barsh, G. S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5667–5671.
8. Miller, M. W., Duhl, D. M. J., Vrieling, H., Cordes, S. P., Ollmann, M. M., Winkes, B. M., and Barsh, G. S. (1993) *Genes Devel.* **7,** 454–467.
9. Duhl, D. M. J., Vrieling, H., Stevens, M. E., Saxon, P. J., Miller, M. W., Epstein, C. J., and Barsh, G. S. (1994) *Development* **120,** 1695–1708.
10. Michaud, E. J., Bultman, S. J., Klebig, M. L., van Vugt, M. J., Stubbs, L. J., Russell, L. B., and Woychik, R. P. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2562–2566.
11. Jiménez, M., Maloy, W. L., and Hearing, V. J. (1989) *J. Biol. Chem.* **264,** 3397–3403.
12. Jiménez, M., Tsukamoto, K., and Hearing, V. J. (1991) *J. Biol. Chem.* **266,** 1147–1156.

13. Tsukamoto, K., Jackson, I. J., Urabe, K., Montague, P. M., and Hearing, V. J. (1992) *EMBO J.* **11,** 519–526.
14. Tomita, Y., Montague, P. M., and Hearing, V. J. (1985) *J. Invest. Dermatol.* **85,** 426–430.
15. Ollmann, M. M., Lamoreux, M. L., Wilson, B. D., and Barsh, G. S. (1998) *Genes Devel.* **12,** 316–330.
16. Ozeki, H., Ito, S., Wakamatsu, K., and Hirobe, T. (1995) *J. Invest. Dermatol.* **105,** 361–366.
17. Prota, G., Lamoreux, M. L., Muller, J., Kobayashi, T., Napolitano, A., Vincenzi, R., Sakai, C., and Hearing, V. J. (1995) *Pigment Cell Res.* **8,** 153–163.
18. Sakai, C., Ollmann, M., Kobayashi, T., Abdel-Malek, Z. A., Muller, J., Vieira, W. D., Imokawa, G., Barsh, G. S., and Hearing, V. J. (1997) *EMBO J.* **16,** 3544–3552.
19. Jackson, I. J. (1993) *Nature* **362,** 587–588.
20. Lu, D., Willard, D., Patel, I. R., Kadwell, S., Overton, L., Kost, T., Luther, M., Chen, W., Woychik, R. P., Wilkison, W. O., and Cone, R. D. (1994) *Nature* **371,** 799–802.
21. Ollmann, M. M., and Barsh, G. S. (1999) *J. Biol. Chem.* **274,** 15837–15846.
22. Kobayashi, T., Vieira, W. D., Potterf, S. B., Sakai, C., Imokawa, G., and Hearing, V. J. (1995) *J. Cell. Sci.* **108,** 2301–2309.
23. Jiang, J., Sharma, S. D., Hruby, V. J., Bentley, D. L., Fink, J. L., and Hadley, M. E. (1996) *Pigment Cell Res.* **9,** 240–247.
24. Chakraborty, A. K., Funasaka, Y., Pawelek, J. M., Nagahama, M., Ito, A., and Ichihashi, M. (1999) *J. Invest. Dermatol.* **112,** 853–860.