Agouti Signaling Protein Inhibits Melanogenesis and the Response of Human Melanocytes to α-Melanotropin

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In mouse follicular melanocytes, the switch between eumelanin and pheomelanin synthesis is regulated by the *extension* locus, which encodes the melanocortin-1 receptor (MC1R) and the *agouti* locus, which encodes a novel paracrine-signaling molecule that inhibits binding of melanocortins to the MC1R. Human melanocytes express the MC1R and respond to melanotropins with increased proliferation and eumelanogenesis, but a potential role for the human homolog of agouti-signaling protein, ASIP, in human pigmentation has not been investigated. Here we report that ASIP blocked the binding of α-melanocyte–stimulating hormone (α-MSH) to the MC1R and inhibited the effects of α-MSH on human melanocytes. Treatment of human melanocytes with 1 nM-10 nM recombinant mouse or human ASIP blocked the stimulatory effects of α-MSH on cAMP accumulation, tyrosinase activity, and cell proliferation. In the absence of exogenous α-MSH, ASIP inhibited basal levels of tyrosinase activity and cell proliferation and reduced the level of immunoreactive tyrosinase-related protein-1 (TRP-1) without significantly altering the level of immunoreactive tyrosinase. In addition, ASIP blocked the stimulatory effects of forskolin or dibutyryl cAMP, agents that act downstream from the MC1R, on tyrosinase activity and cell proliferation. These results demonstrate that the functional relationship between the agouti and MC1R gene products is similar in mice and humans and suggest a potential physiologic role for ASIP in regulation of human pigmentation. ***Key words: melanocortin-1 receptor/melanogenesis/agouti locus/extension locus. J Invest Dermatol 108:838–842, 1997***

The switch to produce eumelanin or pheomelanin in mouse follicular melanocytes is controlled by the *extension* (*e*) and *agouti* (*a*) loci (Silvers, 1958; Takeuchi *et al*, 1989). The former encodes the MC1 receptor, the melanocortin receptor, which is expressed on melanocytes (Tamate and Takeuchi, 1984; Mountjoy *et al*, 1992; Robbins *et al*, 1993; Suzuki *et al*, 1996). The *agouti* locus codes for the agouti-signaling protein (ASIP), an intrinsic factor expressed by dermal papilla cells within the mouse hair follicle, which functions as a paracrine regulator of mouse melanocytes (Miller *et al*, 1993). Although ASIP acts *in vitro* and *in vivo* as an antagonist of α-melanocyte-stimulating hormone (α-MSH) signaling mediated by the mouse melanocortin-1 receptor (MC1R), its exact mechanism of action is still controversial (Lu *et al*, 1994; Yen *et al*, 1994; Blanchard *et al*, 1995; Manne *et al*, 1995; Zemel *et al*, 1995). We and others have recently demonstrated a mitogenic and melanogenic response to α-MSH, β-MSH, and ACTH by human melanocytes in culture (De Luca *et al*, 1993; Hunt *et al*, 1994a, 1994b; Abdel-Malek *et al*, 1995; Suzuki *et al*, 1996). Recently, it was reported that α-MSH increases the synthesis of eumelanin in these cells (Hunt *et al*, 1995). Additional support for the notion that the MC1R has homologous functions in human and mouse pigmentation came from the recent observation that human MC1R variants are associated with fair skin and red hair (Valverde *et al*, 1995). A human homolog for the mouse *agouti* locus has been cloned, and its product was found to function similarly to the mouse protein *in vitro* and *in vivo* (Kwon *et al*, 1994; Wilson *et al*, 1995) but the response of human melanocytes to ASIP and its potential role in human pigmentation has not been investigated. Ectopic overexpression of ASIP in mice leads to obesity and increased tumor susceptibility [reviewed by Siracusa (Siracusa, 1994)] (Yen *et al*, 1994). It has been suggested that human ASIP may play a role in the regulation of these phenotypes as well (Manne *et al*, 1995).

The human ASIP consists of 132 amino acids and is 80% identical in amino acid sequence to its mouse homolog (Kwon *et al*, 1994; Wilson *et al*, 1995). We have been investigating the effects of recombinant human and mouse ASIP and their interaction with α-MSH on cultured human melanocytes. Here we report that ASIP competes with α-MSH for binding to the MC1R and that both mouse and human ASIP inhibit basal and α-MSH–stimulated

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Abbreviations: MC1R, melanocortin-1 receptor; α-MSH, α-melanocyte stimulating hormone; TRP-1, tyrosinase-related protein 1; ASIP, agouti signaling protein; NDP-α-MSH, [Nle^4,D-Phe^7]-α-MSH; dbcAMP, dibutyryl cAMP.

tyrosinase activity and proliferation. Moreover, ASIP inhibits the activity of agents that mimic the effects of α-MSH and act downstream of the MC1R. The demonstration that human melanocytes respond to ASIP is an important step toward understanding the possible physiologic effects of this hormone and the mechanism(s) by which it interacts with melanotropins to affect human melanocytes as well as other target cells.

MATERIALS AND METHODS

Melanocyte Culture Conditions Primary cultures of normal human melanocytes were established from neonatal foreskins representing different skin types as described previously (Abdel-Malek *et al*, 1993). The complete growth medium used consisted of MCDB 153 supplemented with 4% fetal bovine serum, 1 μg human transferrin per ml, 5 μg insulin per ml, 1 μg α-tocopherol per ml, and the melanocyte mitogens human recombinant basic fibroblast growth factor (0.6 ng per ml), 12-*O*-tetradecanoylphorbol-13-acetate (8 nM) (all purchased from Sigma Chemical Co., St. Louis, MO), and bovine pituitary extract (13 μg per ml) (Clonetics, San Diego, CA), as previously described (Medrano and Nordlund, 1990). Since bovine pituitary extract contains high concentrations of α-MSH, we found it essential to remove it from the culture medium in order to assay the effects of exogenous melanocortins (Abdel-Malek *et al*, 1995).

Recombinant ASIP was purified from conditioned media of insect cells infected with a baculovirus construct that contained the mouse or human cDNA (Wilson *et al*, 1995). The purification protocol will be described elsewhere (Ollmann and Barsh, in preparation) but in brief is based on ion exchange chromatography similar to the protocols of Wilkison and colleagues (Lu *et al*, 1994; Blanchard *et al*, 1995). ASIP activity during purification was monitored by its ability to inhibit α-MSH–induced pigment granule dispersion in frog melanophores. The purity of recombinant ASIP, estimated at >90%, was based on analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Receptor Binding Assays: Competition of ^{125}I-[Nle^4,D-Phe^7]-α-MSH Binding to MC1R by α-MSH or ASIP [Nle^4,D-Phe^7]-α-MSH (NDP-α-MSH) (Sigma Chemical Co.), a potent and nonbiodegradable synthetic analog of α-MSH (Marwan *et al*, 1985; Suzuki *et al*, 1996), was labeled with ^{125}I as follows. Twenty microliters of 0.4 M sodium acetate (pH 5.6), 1.5 mCi ^{125}I (DuPont, NEN, Boston, MA), 4 μg NDP-α-MSH, 5 μl of 0.1 μg per ml lactoperoxidase, and 4 μl 30% H_2O_2 were added and vortexed lightly for 45 s. The reaction was stopped by the addition of 300 μl 0.1% acetic acid in phosphate-buffered saline. Subsequently, 300 μl Dowex solution in 0.2 N acetic acid were added and, following centrifugation, the supernatant was purified using G-25 column pre-swollen in 0.2 N acetic acid. Fractions were collected every 30 s, and the fractions with the highest radioactivity were utilized for receptor binding assays.

Melanocytes were inoculated into 24-well plates at a density of 4×10^5 cells/well and allowed to attach for 2 d. Cells were then washed twice with 1 ml of MCDB 153 medium containing 0.5% bovine serum albumin (fraction V, Sigma Chemical Co.) and incubated in 0.5 ml of MCDB medium containing 0.5% bovine serum albumin, 0.3 mM 1,10-phenanthroline (proteinase inhibitor; Sigma Chemical Co.) and 100,000–150,000 cpm of [^{125}I]NDP-α-MSH ([^{125}I[Nle^4,*D*-Phe^7-α-MSH]) and various concentrations of unlabeled α-MSH or human ASIP at room temperature for 2 h. After incubation, melanocytes were washed three times with 1 ml of ice-cold MCDB medium containing 0.5% bovine serum albumin and lysed with 1 ml of 0.5 N NaOH, 0.4% deoxycholate. Radioactivity was counted in a γ-counter and the data were analyzed by linear regression using software from Kaleidagraph.

Determination of Cellular Proliferation and Tyrosinase Activity Melanocytes maintained for 2–4 d in medium devoid of bovine pituitary extract were plated in 12.5 cm^2 flasks at a density of 1×10^5 cells per flask. Forty-eight hours later and every other day thereafter for a total of 6 d, melanocytes were treated with 10 nM mouse ASIP or 0.1 μM human ASIP in the presence or absence of 1 nM α-MSH, 0.1 μM forskolin, or 10 μM dibutyryl (db) cAMP. Previously we reported that the response of human melanocytes to α-MSH is maximal following 4–6 d of treatment (Abdel-Malek *et al*, 1995). On day 5 of treatment, each flask received 2.2 μCi [^{3}H]tyrosine (0.733 μCi per ml medium; Amersham, Arlington Heights, IL). Sixteen to eighteen hours later, cells from each flask were harvested and counted using a Coulter counter, and the tyrosine hydroxylase activity of tyrosinase was measured *in situ* using a modification of the charcoal absorption method of Pomerantz as described previously (Pomerantz, 1969; Abdel-Malek *et al*, 1992).

Cyclic AMP Assays Melanocytes were maintained in bovine pituitary extract-free growth medium for at least 2 d prior to and for the duration of the experiments. Cells were plated onto 24-well plates at a density of 3×10^5 cells per well. Forty-eight hours later fresh medium was added to each well, and on the next day the cAMP assays were performed as follows. The growth medium was removed from each well and replaced with 450 μl of growth medium containing 0.1 mM isobutyl methylxanthine, a phosphodiesterase inhibitor. The cultures were incubated for 10–20 min with this medium at 37°C and then treated with 10 nM or 0.1 μM α-MSH, 10 nM or 0.1 μM ASIP, or both for 45 min at 37°C in a total volume, in each well, brought up to 0.5 ml. The reaction was stopped by the addition of 50 μl of 1 N HCl. Duplicate 250-μl samples were removed, and 750 μl of 0.1 N HCl were added. Each sample was then acetylated by the addition of 40 μl of triethylamine and acetic anhydride (2.5:1 vol/vol), and the amount of cAMP was determined by radioimmunoassay as previously described by Liggett *et al* (1989).

Western Blot Analysis of Tyrosinase and Tyrosinase-Related Protein 1 (TRP-1) Cells were treated with 10 nM mouse ASIP in the presence or absence of 1 nM α-MSH for a total of 6 d, as described above. Cell lysates were prepared using RIPA buffer (Sambrook *et al*, 1989) containing the phosphatase inhibitor Na_3VO_4 (10 mM) and protease inhibitors methylsulfonyl fluoride (200 mM), aprotinin (10 μg per ml), and leupeptin (10 μg per ml). Equal amounts of protein (5–8 μg) were loaded on each lane and separated on a 7.5% polyacrylamide gel by electrophoresis. Following transblotting onto nitrocellulose membranes, the membranes were incubated with αhPEP-7 (1:1500), a polyclonal antibody raised against the carboxy terminus of human tyrosinase (a gift from Dr. Richard King, University of Minnesota), or with TA99 (1:1500), a mouse monoclonal antibody raised against human TRP-1 (kindly provided by Dr. Setaluri Vijayasaradhi, Rockefeller University, New York, NY). The membranes were then incubated with horseradish peroxidase–conjugated anti-rabbit IgG (Amersham; 1:3000 dilution) following incubation with αhPEP-7, or with anti-mouse IgG (Amersham; 1:15,000 dilution) following incubation with TA99. The immunoreactive bands were detected by chemiluminescence, using the Renaissance Kit (Dupont, NEN, Boston, MA). Under these conditions, αhPEP-7 recognizes immunoreactive material in lysates of normal or TRP-1–negative albino melanocytes, but not in lysates of tyrosinase-negative albino melanocytes that lack expression of tyrosinase (Boissy RE, Abdel-Malek ZA, Oetting WS, Hearing VJ, and King RA: manuscript in preparation).

RESULTS

Antagonism of the Effects of α-MSH on Human Melanocytes by ASIP Recombinant mouse ASIP inhibits the binding of radiolabeled NDP-α-MSH to mouse melanoma cells (Blanchard *et al*, 1995) or heterologous cells that express the mouse MC1R (Lu *et al*, 1994). To determine whether human ASIP would act in a similar manner, we measured the binding of [^{125}I]NDP–MSH to intact human melanocytes in the presence of increasing concentrations of human ASIP or α-MSH **(Fig 1)**. The IC_{50} values of ASIP and α-MSH were 3.128 ± 0.13 nM (mean ± SEM) and 1.71 ± 0.56 nM, respectively, which suggests that the short-term action of human ASIP on human melanocytes is similar to the action of mouse ASIP on their mouse counterparts.

We next examined the long-term functional consequences of ASIP on human melanocytes. Melanocyte lines derived from individuals of different pigmentary phenotypes express different basal tyrosinase activities and may vary in the extent of their response to α-MSH (Abdel-Malek *et al*, 1993; Hunt *et al*, 1994a; Abdel-Malek *et al*, 1995). We have shown previously, however, that 1 nM α-MSH elicits a near-maximal stimulation of proliferation and tyrosinase activity in human melanocyte lines derived from donors with different pigmentary phenotypes after a 6-d treatment period (Abdel-Malek *et al*, 1995; Suzuki *et al*, 1996). In three separate experiments carried out with different melanocyte lines, complete abrogation of these effects was observed upon addition of 10 nM mouse or 0.1 μM human ASIP. Representative data from two experiments are shown in **Fig 2** where, in Experiment I, 1 nM α-MSH caused a 70% increase in cell proliferation and a 140% increase in tyrosinase activity, and 10 nM mouse ASIP reduced both effects to controls levels. In **Fig 2**, Experiment II, using another melanocyte line, 1 nM α-MSH caused an approximately 75% increase in proliferation and a 62% increase in tyrosinase activity. Again, both effects were abolished in the presence of 0.1 μM human ASIP. Treatment with 10 nM mouse or 0.1 μM human

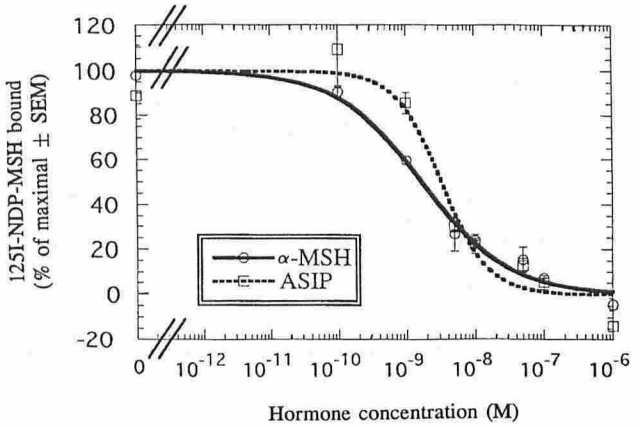

Figure 1. Displacement of [^{125}I]NDP–α-MSH binding to the MC1R by increasing concentrations of human ASIP or α-MSH. Melanocytes were incubated in the presence of 100,000–150,000 cpm of [^{125}I]-NDP–MSH alone (for total binding) or in the presence of increasing concentrations (10^{-11}–10^{-6} M) of α-MSH or human ASIP for 2 h at room temperature, as described in *Materials and Methods*. The amount of radioactivity was counted in a γ-counter, and the data were analyzed by Kaleidagraph software. Each value represents the mean counts per minute of three determinations ± SEM. Maximal binding was obtained in the absence of any cold ligand. This experiment was repeated twice with similar findings using two different melanocyte lines.

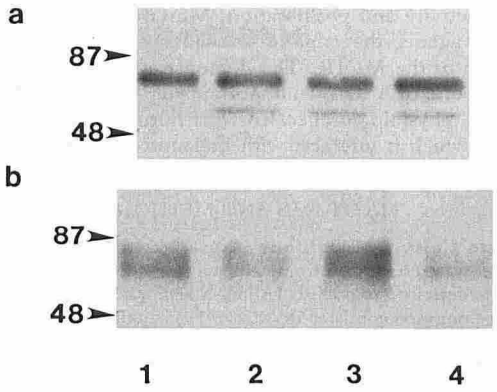

Figure 3. Inhibition of TRP-1 but not tyrosinase levels following treatment with mouse ASIP, in the absence or presence of α-MSH. Melanocytes were treated with 10 nM mouse ASIP with or without 1 nM α-MSH as described for **Fig 2**, and cell lysates were prepared 48 h after the final treatment. Proteins were separated by electrophoresis using 7.5% polyacrylamide gels, and western blotting analysis for tyrosinase *(A)* and TRP-1 *(B)* was performed using αhPEP-7 polyclonal antibody and TA99 mouse monoclonal antibody, respectively. The immunoreactive bands were detected using chemiluminescence. Lanes 1–4 represent control, ASIP, α-MSH, and ASIP + α-MSH, respectively. This experiment was repeated three times with similar results.

ASIP alone reduced basal tyrosinase activity by 50% and 13%, respectively, and also had a small but significant inhibitory effect on cell proliferation.

Modulation of Expression of Tyrosinase and TRP-1 by ASIP

In mouse melanocytes, the effects of α-MSH and ASIP on tyrosinase activity are mediated primarily at the level of mRNA (Sakai *et al*, 1997). To investigate the level at which ASIP inhibited tyrosinase activity in human melanocytes, we carried out western blot analysis of cells treated with ASIP and/or α-MSH using an antibody (αhPEP-7) that detects all tyrosinase isozymes. The level of tyrosinase protein remained constant after exposure to either 1 nM α-MSH or 10 nM ASIP, suggesting that the effects of ASIP on tyrosinase activity are posttranslational or are mediated by the activation of a tyrosinase inhibitor **(Fig 3)**. Interestingly, the level of TRP-1 (detected by TA99 antibody), which was increased by 1 nM α-MSH, was significantly reduced in response to addition of 10 nM ASIP **(Fig 3)**. Northern blot analysis of tyrosinase, TRP-1, and TRP-2 revealed no change in the steady state mRNA levels of these enzymes following treatment for 4 d with ASIP or ASIP and α-MSH (data not shown). These results suggest that the inhibitory effect of ASIP on melanogenesis in human melanocytes is not elicited at the transcriptional level.

Inhibition of α-MSH–Induced cAMP Formation by ASIP

Blanchard *et al* (1995) have shown previously that treatment of mouse B16F10 melanoma cells with recombinant agouti protein does not significantly alter basal levels of cAMP production. In contrast, normal mouse melanocytes treated with ASIP in the absence of exogenous α-MSH exhibited a small but significant decrease in cAMP accumulation (Sakai *et al*, 1997). We found that treatment of human melanocytes with 10 nM or 0.1 μM mouse ASIP did not significantly affect the basal level of cAMP accumulation **(Fig. 4)**. When mouse ASIP and α-MSH were added

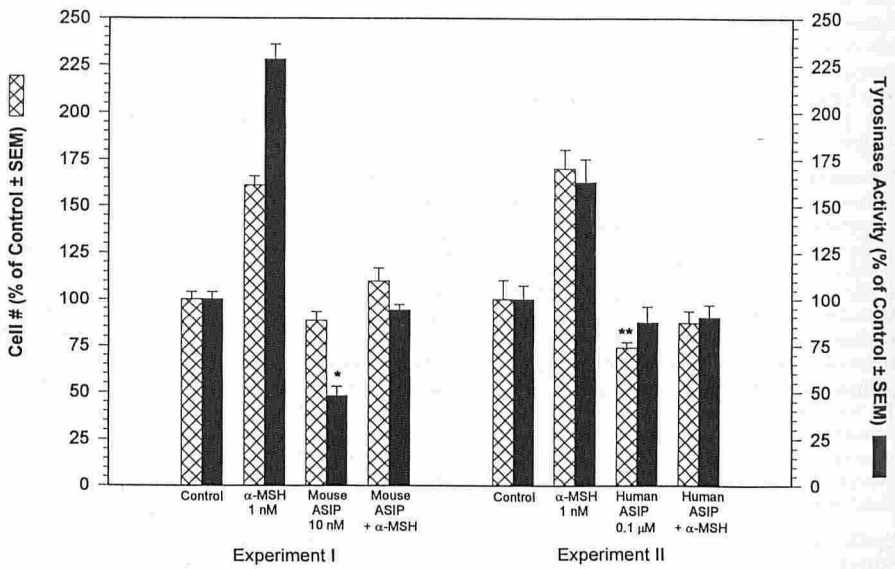

Figure 2. Inhibition of human melanocyte proliferation and tyrosinase activity by mouse (Experiment I) and human (Experiment II) ASIP, in the presence or absence of α-MSH. Melanocytes were treated 48 h after plating and every other day thereafter for a total of three treatments for 6 d with 10 nM mouse ASIP, or 0.1 μM human ASIP, in the presence or absence of 1 nM α-MSH. Forty-eight hours after the final treatment, cell numbers and tyrosinase activities were determined. The experiments presented were repeated three times using different melanocyte lines each time, with similar findings. * Indicates that the difference between the experimental and control groups is statistically significant at $p \leq 0.001$. ** Denotes that the experimental and control groups are significantly different at $p = 0.07$, as determined by unpaired Student's t test.

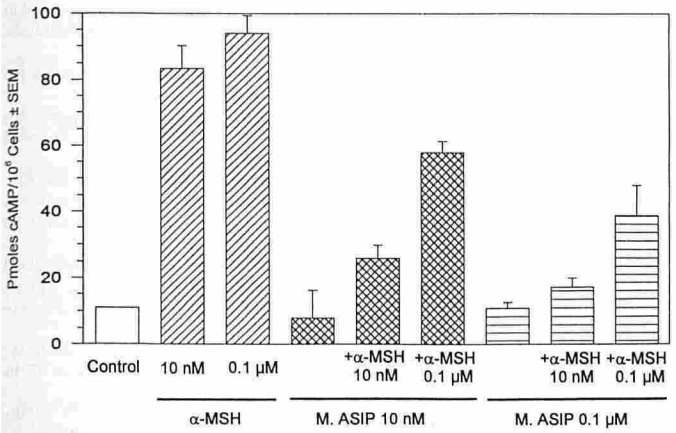

Figure 4. Downregulation of basal and α-MSH–induced cAMP levels by mouse ASIP. Melanocytes were incubated for 45 min with 10 nM or 0.1 μM mouse ASIP in the presence or absence of 10 nM or 0.1 μM α-MSH, in a medium containing 0.1 mM isobutyl methylxanthine. Cyclic AMP levels were determined by radioimmunoassay. Similar results were obtained in four independent experiments.

together, however, ASIP caused a dose-dependent inhibition of the α-MSH induced cAMP accumulation **(Fig 4)**. Treatment of melanocytes with 10 nM or 0.1 μM α-MSH resulted in a 7- or 8.5-fold increase in cAMP, respectively. Concomitant treatment with 1 nM mouse ASIP reduced the effects of 10 or 0.1 μM α-MSH by 80% or 43%, respectively. Similarly, treatment with 0.1 μM mouse ASIP diminished the above effects of α-MSH by 83% or 58%, respectively.

Abrogation of the Mitogenic and Melanogenic Effects of Forskolin and dbcAMP by ASIP The effects of ASIP on normal human melanocytes were evident in the presence as well as in the absence of exogenous α-MSH. To further investigate the effects of ASIP in the absence of α-MSH, we determined whether ASIP could inhibit the forskolin- or dbcAMP-induced melanocyte proliferation and tyrosinase stimulation **(Fig 5)**. We found that 0.1 μM forskolin or 10 μM dbcAMP stimulated melanocyte proliferation by 82% or 52%, respectively, and increased tyrosinase activity by 30%. These effects were completely inhibited by 10 nM mouse ASIP, and tyrosinase activity was reduced even below control values. These results confirm the ability of ASIP to affect human melanocytes by antagonizing the cAMP-dependent signaling pathway.

DISCUSSION

The genetics of mouse coat color have provided great insights into the biochemical control of melanogenesis and the interaction between different gene products. In many cases, the products of human pigmentation genes have been shown to carry out functions similar to those of their mouse homologs *in vitro* and *in vivo*. Whether the products of the human and mouse *extension* (*MC1R*) and *agouti* (*A*) loci have functions similar to their mouse counterparts is not yet resolved.

In mice, activation of the MC1R promotes eumelanin synthesis, whereas mutations that inactivate the MC1R gene result in pheomelanin formation. Expression of the agouti protein induces a switch from eumelanin to pheomelanin formation (Silvers, 1958). This effect appears to be mediated, in part, by the ability of ASIP to act as an inhibitor of α-MSH binding to the MC1R (Lu *et al*, 1994; Blanchard *et al*, 1995; Siegrist *et al*, 1996). Other studies (Yen *et al*, 1994; Hunt and Thody, 1995; Zemel *et al*, 1995; Sakai *et al*, 1997) have suggested, however, that agouti protein may act via a receptor distinct from the melanocortin receptors. In addition, the human homolog of *agouti*, ASIP, is normally expressed in nonpigmented tissues including the heart, gonads, and adipose tissue (Kwon *et al*, 1994; Wilson *et al*, 1995), and it has been suggested that ASIP may play a role in normal regulation of cellular processes other than pigmentation (Manne *et al*, 1995). It was demonstrated recently that ASIP is an antagonist for the MC4R, which accounts for the obesity associated with ectopic expression of *agouti* protein in A^y and A^{vy} mice (Lu *et al*, 1994; Yen *et al*, 1994; Fan *et al*, 1997).

Here we report that normal epidermal human melanocytes treated with recombinant mouse or human ASIP exhibit physiologic effects predicted by the genetics of mouse coat color. These effects can be attributed to a large extent to the ability of ASIP to inhibit binding of α-MSH to the MC1R **(Fig 1)**. Both the human and mouse proteins block the capacity of α-MSH to stimulate mitogenesis and melanogenesis, although in long-term assays the mouse protein is approximately 10-fold more potent than the human protein **(Fig 2)**. This difference is not due to experimental variation in the donor melanocytes or the preparation of agouti protein, inasmuch as it was observed in several independent experiments with various concentrations of ASIP and multiple donor melanocytes. In addition, these identical preparations of human or mouse ASIP have been tested for their capacity to inhibit α-MSH binding to heterologous cells that express the mouse and human MC1R and exhibit similar K_D values (Yang and Gantz,

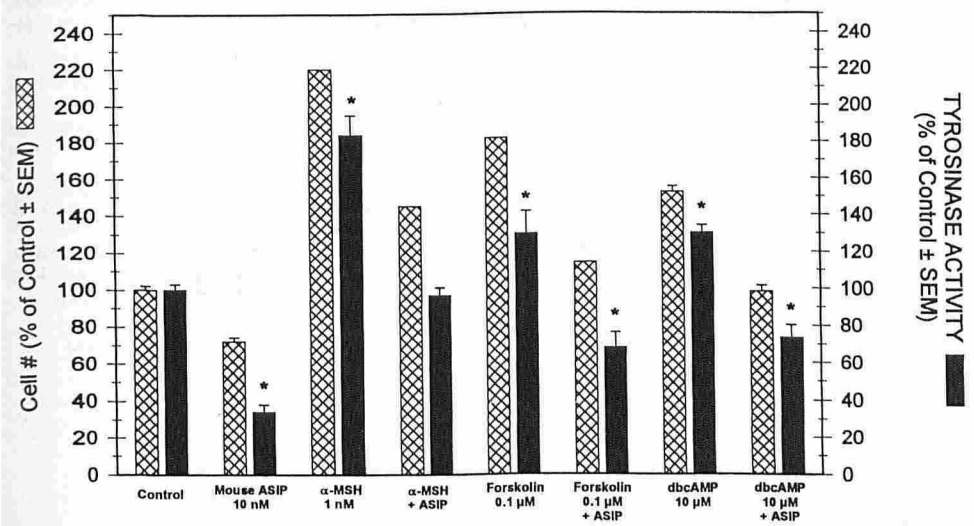

Figure 5. Abrogation of the forskolin and dbcAMP induced melanocyte proliferation and tyrosinase activity by ASIP. Melanocytes were treated every other day for a total of 6 d with 0.1 μM forskolin, 10 μM dbcAMP, or 1 nM α-MSH in the presence or absence of 10 nM mouse ASIP, as described in the legend for **Fig 2**. Cell numbers and tyrosinase activities were determined 48 h after the final treatment. This experiment was repeated twice with similar results. * Indicates that the difference between the experimental and control groups is statistically significant ($p \leq 0.001$) as determined by unpaired Student's t test.

unpublished data). ASIP was a more potent inhibitor of TRP-1 than tyrosinase in human melanocytes, an effect that appears to be important for the switch from eumelanin to pheomelanin synthesis (Sakai *et al*, 1997). Whether ASIP functions as a positive effector of pheomelanin synthesis or only modulates the effects of α-MSH by blocking its signaling pathway is currently being investigated. The ability of ASIP to increase pheomelanin synthesis may have important implications on the response of melanocytes to ultraviolet light and on the photoprotective role of epidermal melanin, particularly because pheomelanin is more susceptible to photodegradation than eumelanin (Chedekel *et al*, 1978; Menon *et al*, 1985).

Several possibilities have been proposed to explain ASIP action, including competitive inhibition of melanocortin binding and stimulation of a putative "agouti receptor" (Yen *et al*, 1994; Blanchard *et al*, 1995; Hunt and Thody, 1995; Manne *et al*, 1995). We found that, in general, ASIP antagonizes the effects of α-MSH and inhibits [^{125}I]NDP–α-MSH binding. We also found that ASIP could inhibit the stimulation of tyrosinase activity and cell proliferation in the absence of exogenous α-MSH and in response to agents such as forskolin and dbcAMP that act downstream of the MC1R. These results could be explained by inverse agonism or an effect of ASIP on MC1R desensitization. Alternatively, ASIP may also bind a receptor distinct from MC1R that antagonizes the signaling pathway mediated by cAMP. This putative receptor is yet to be identified.

Regardless of the precise biochemical mechanism(s) by which ASIP acts, our observations have implications for understanding the factors that control normal human pigmentation. Recent studies byf Valverde *et al* (1995) demonstrated an increased frequency of certain MC1R sequence variants among Caucasian individuals with red hair and fair skin. Our results provide functional evidence to support the idea that MC1R signaling affects the behavior of human melanocytes and suggest that genetic variation in ASIP might also influence human pigmentation.

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