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Modulation of murine melanocyte function *in vitro* by agouti signal protein

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Molecular and biochemical mechanisms that switch melanocytes between the production of eumelanin or pheomelanin involve the opposing action of two intercellular signaling molecules, α-melanocyte-stimulating hormone (MSH) and agouti signal protein (ASP). In this study, we have characterized the physiological effects of ASP on eumelanogenic melanocytes in culture. Following exposure of black melan-a murine melanocytes to purified recombinant ASP *in vitro*, pigmentation was markedly inhibited and the production of eumelanosomes was decreased significantly. Melanosomes that were produced became pheomelanosome-like in structure, and chemical analysis showed that eumelanin production was significantly decreased. Melanocytes treated with ASP also exhibited time- and dose-dependent decreases in melanogenic gene expression, including those encoding tyrosinase and tyrosinase-related proteins 1 and 2. Conversely, melanocytes exposed to MSH exhibited an increase in tyrosinase gene expression and function. Simultaneous addition of ASP and MSH at approximately equimolar concentrations produced responses similar to those elicited by the hormone alone. These results demonstrate that eumelanogenic melanocytes can be induced in culture by ASP to exhibit features characteristic of pheomelanogenesis *in vivo*. Our data are consistent with the hypothesis that the effects of ASP on melanocytes are not mediated solely by inhibition of MSH binding to its receptor, and provide a cell culture model to identify novel factors whose presence is required for pheomelanogenesis.

Keywords: agouti/melanogenesis/pheomelanin/pigmentation/tyrosinase

Introduction

The *agouti* (a) locus is one of >60 distinct genes that regulate coat color in mice (Silvers, 1979). The product of that locus (termed agouti signal protein, ASP) is produced by dermal papillae cells (Millar *et al.*, 1995) and is a paracrine factor that modulates the production of pigment by follicular melanocytes (Bultman *et al.*, 1992; Miller *et al.*, 1993). More specifically, it controls whether black/brown eumelanin or yellow/red pheomelanin is produced, although the mechanism by which this switch is effected is as yet unknown. In mice that carry the A allele, eumelanin is produced by all follicular melanocytes at the beginning of the hair growth cycle (i.e. from 0 to 4 days). Transient expression of ASP from 4 to 6 days of the hair cycle causes melanocytes to produce pheomelanin instead of eumelanin; after 6 days, *agouti* gene expression is turned off and eumelanin is produced again. This pattern of pigment synthesis results in a yellow striped band near the tip of each hair shaft against a black background. Eumelanin and pheomelanin differ not only in their gross appearance, but also in their chemical composition and the ultrastructure of the melanosomes in which they are synthesized and deposited. Follicular melanocytes of 1- to 2-day-old agouti mice contain ellipsoid and fibrillar eumelanosomes, while follicular melanocytes of 4- to 6-day-old agouti mice contain ovoid and particulate pheomelanosomes. Similar changes in pheomelanosomes and eumelanosomes have been confirmed in a number of mouse mutants which produce one or the other type of melanin (Prota *et al.*, 1995).

Mutations at the *agouti* locus can cause the production of all yellow or all black hair, depending on whether the mutation leads to overexpression/hyperfunction or non-expression/non-function of ASP, respectively (Perry *et al.*, 1994; Siracusa, 1994; Vrieling *et al.*, 1994). As examples, the dominant lethal yellow mutation (A^y) results in the production of completely yellow hairs (Miller *et al.*, 1993; Duhl *et al.*, 1994; Michaud *et al.*, 1994), while the recessive non-agouti (a) (Bultman *et al.*, 1994), lethal non-agouti (a^x) (Miller *et al.*, 1994) and extreme non-agouti (a^e) (Hustad *et al.*, 1995) alleles cause the production of completely black hairs. Consequently, the *agouti* locus has been recognized to have an important role in regulating the switch between the production of eumelanin or pheomelanin by melanocytes.

The biochemical action of ASP is controversial and has been the source of continuing debate (Conklin and Bourne, 1993; Jackson, 1993; Yen *et al.*, 1994). Several studies (Lu *et al.*, 1994; Blanchard *et al.*, 1995; Willard *et al.*, 1995; Siegrist *et al.*, 1996) have shown that ASP antagonizes the action of α-melanocyte-stimulating hormone (MSH) in activating the melanocyte-specific MSH receptor (MC1-R), which suggests that the effect of the *agouti* locus on melanocytes is mediated by reduced signaling through the MC1-R, while its extrapigmentary effects may be mediated by reduced signaling through other melanocortin receptors. By contrast, the similarity of ASP

to the conotoxins and its ability to elevate intracellular calcium has also been noted (Manne *et al.*, 1995; Willard *et al.*, 1995; Zemel *et al.*, 1995; Perry *et al.*, 1996), suggesting that some effects of ASP might be mediated by an alteration in calcium channels, a mechanism supported by Hunt and Thody (1995) who found that ASP antagonizes the stimulation of melanogenesis by verapamil (a calcium modulator).

In vivo, we have reported (Kobayashi *et al.*, 1995) that the expression and enzyme activity of tyrosinase was reduced in follicular melanocytes of lethal yellow mice and of 5- to 7-day-old agouti mice but that there was little or no expression or enzyme activity of tyrosinase-related proteins 1 (TRP1) and 2 (TRP2) during pheomelanogenesis. This pattern of expression is consistent with the fact that tyrosinase is required for both types of pigment synthesis, but expression of the two tyrosinase-related proteins (TRP1 and TRP2) is required only for eumelanin synthesis (Tsukamoto *et al.*, 1992; Kobayashi *et al.*, 1994; Winder *et al.*, 1994).

We now describe studies examining the effect(s) of purified recombinant ASP on cultured melanocytes. Following treatment with ASP, eumelanogenic melanocytes in culture exhibit physiologic features characteristic of pheomelanogenesis *in vivo*, thus providing an *in vitro* model for characterization of the mechanisms and genes involved in this switch.

Results

mRNA levels of melanogenic genes following treatment with ASP

We initially examined whether ASP had any effect on steady-state mRNA levels of melanogenic genes using Northern blotting of melan-a melanocytes treated with varying concentrations of ASP for 24 h (Figure 1). There were significant dose-dependent decreases in the expression of tyrosinase, TRP1 and TRP2 mRNAs following treatment with ASP at 10 and 1 nM. However, there was no significant effect on the level of mRNA for MC1-R at any ASP concentration tested. The numbers reported below the bands in each of the figures represent the quantitation of those bands by phosphoimager as a percentage of control (means ± SEM in seven independent experiments) following correction for loading against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The EC_{50} for ASP can be calculated at 5 nM based on these results; the EC_{50} for MSH is 10 nM (McLane *et al.*, 1987, and our unpublished results).

To examine the time course response of melanocytes to ASP, in subsequent experiments we treated the melan-a cells with 10 nM ASP for up to 11 days (data not shown). Dramatic decreases in the levels of tyrosinase, TRP1 and TRP2 mRNAs were again noted which approached 70–98% inhibition compared with controls following treatment for 2 or more days; maximum effects were usually noted within 2 days of treatment. Again, no effects were noted on MC1-R mRNA levels during the 11 day time course of these experiments.

Interaction between ASP and MSH

As noted in the Introduction, there is evidence using MC1-R-transfected cells that ASP can act as an antagonist of the MSH receptor. To examine directly the interaction between ASP and MSH in melanocytes, and to characterize further whether ASP might affect MSH signaling via a change in MC1-R receptor level, melan-a melanocytes were exposed to 10 nM ASP, 10 nM MSH or both for 5 days (Figure 2). This time was chosen since 4 days of treatment is the standard time to obtain the maximal response to MSH (Jiménez *et al.*, 1988; Abdel-Malek *et al.*, 1995) and since maximal responses to 10 nM ASP were also elicited by this time, as found in this study. We pre-treated the cells with ASP for 1 day prior to the start of MSH treatment in order to maximize the chances of seeing a competitive effect. Treatment with MSH alone produced a 2-fold increase in the level of tyrosinase mRNA, and lesser increases in TRP1 and TRP2 mRNA, but no significant change in the levels of MC1-R mRNA. On the other hand, treatment with ASP alone produced dramatic decreases in the levels of mRNAs for tyrosinase, TRP1 and TRP2, with no significant effect on the level of MC1-R mRNA. At equimolar 10 nM concentrations, addition of ASP and then MSH produced a response indistinguishable from that elicited by MSH alone, i.e. there was little or no antagonism of the MSH effect by ASP under these conditions. Measurements of the endogenous MSH concentration in the serum used for these experiments indicate that the residual MSH concen-

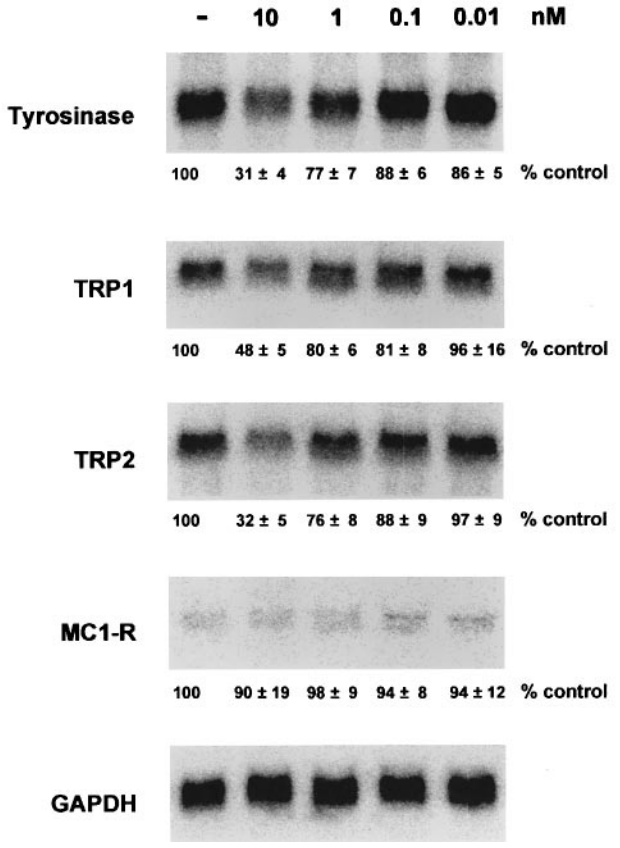

Fig. 1. Northern blot analysis of melanocytes exposed to different concentrations of ASP. Melan-a melanocytes were cultured in the absence (–) or presence of (10, 1, 0.1 or 0.01 nM) ASP for 24 h. RNA was isolated, electrophoresed, blotted to membranes and probed for expression of melanogenic genes as detailed in Materials and methods (% control corrected for loading by GAPDH is shown below each band as the mean ± SEM of seven independent experiments).

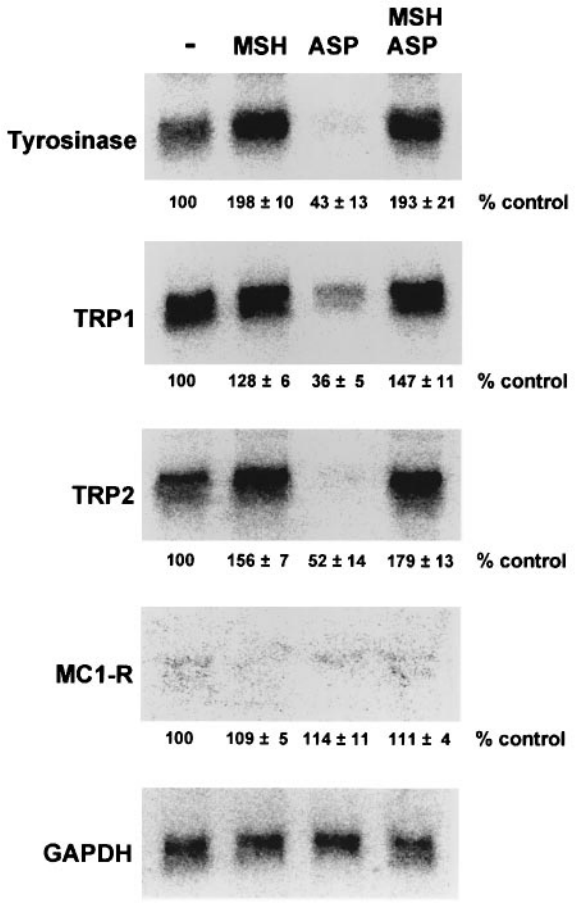

Fig. 2. Northern blot analysis of interactions between MSH and/or ASP. Melan-a melanocytes were cultured in the absence (–) or presence of 10 nM MSH, 10 nM ASP or both (MSH/ASP) for 5 days. Results are presented as detailed for Figure 1.

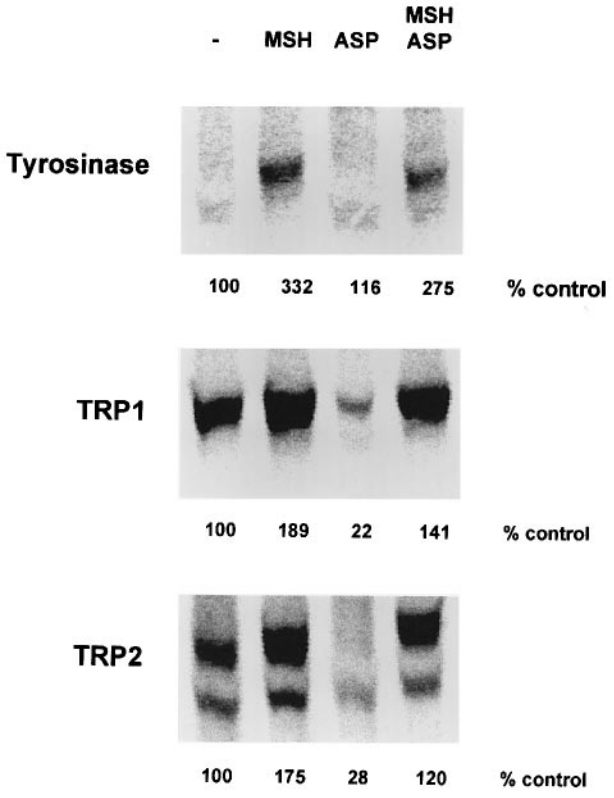

Fig. 3. Metabolic labeling and immunoprecipitation analysis of melanocytes exposed to MSH and/or ASP. Melan-a melanocytes were metabolically labeled with [^{35}S]methionine for 4 h after exposure to 10 nM MSH, 10 nM ASP or both (MSH/ASP) for 5 days. The cells were solubilized, immunoprecipitated by antibodies, separated by gel electrophoresis and visualized by autoradiography, as detailed in Materials and methods. This experiment has been repeated four times with comparable results.

tration in the medium is <2 pM, several orders of magnitude less than the EC_{50} for stimulation of tyrosinase activity or cAMP accumulation. Thus, these results confirm the ability of ASP to bring about physiologic changes in the absence of exogenously added MSH, and suggest further that the interaction of ASP and MSH is not mediated via an alteration in levels of expression of the MSH receptor itself.

Regulation of melanogenic protein expression by ASP

Steady-state levels of RNA as measured by Northern blot hybridization do not reveal alterations in the expression of gene products that might occur due to modulation of protein levels or protein function. To examine the effects of ASP at the translational level, melan-a cells were cultured in the presence or absence of MSH and/or ASP for 5 days, metabolically labeled for 6 h with [^{35}S]methionine and then subjected to immunoprecipitation analysis (Figure 3). Synthesis of tyrosinase in response to MSH treatment is significantly increased (>3-fold), while synthesis of TRP1 and TRP2 is also increased, but to a lesser extent. After exposure to ASP alone, synthesis of TRP1 and TRP2 was significantly suppressed, but we were unable to determine any inhibitory effect of ASP on tyrosinase synthesis, since, under these labeling conditions, the relatively slow synthesis of tyrosinase in the untreated control is virtually undetectable. Exposure to MSH and ASP together produced a pattern that did not differ significantly from MSH alone, results that are consistent with those obtained by Northern blot analysis.

Melanogenic enzyme function

To examine melanogenic enzyme levels following treatment of melanocytes with MSH and/or ASP, we performed Western blotting and enzyme assays under identical conditions. Western blotting (Figure 4) revealed that there were only moderate increases in the amount of tyrosinase, and little or no change in the levels of TRP1 or TRP2, in response to MSH treatment alone. After exposure to ASP alone, there were dramatic and significant decreases in the amounts of tyrosinase, TRP1 and TRP2 proteins; note that due to its relatively long half-life, tyrosinase protein in the untreated control is readily detectable by Western blotting. Enzymatic assays revealed that the catalytic functions of tyrosinase [i.e. tyrosine hydroxylase, 3,4-dihydroxyphenylalanine (DOPA) oxidase and melanin production] were increased dramatically after exposure to MSH, but were decreased to background levels after exposure to ASP (Table I). There was little impact of MSH on the protein levels of TRP1 or TRP2 (or the enzyme activity of the latter, i.e. DOPAchrome tautomerase), whereas ASP clearly diminishes the protein levels and catalytic function of both proteins to background

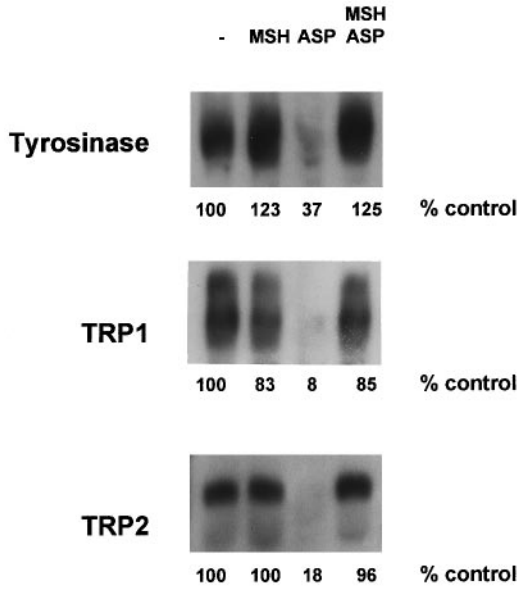

Fig. 4. Western blot analysis of expression of melanogenic proteins by melan-a cells exposed to MSH and/or ASP. Melan-a melanocytes were exposed to 10 nM MSH, 10 nM ASP or both (MSH/ASP) for 5 days; cells were then solubilized, and levels of melanogenic proteins were analyzed by Western immunoblotting, as detailed in Materials and methods.

levels. Again, simultaneous addition of both ASP and MSH produced a response indistinguishable from that elicited by MSH alone.

Structural characteristics

After incubation of melan-a cells with 10 nM ASP, the color of the cell pellets was changed from black to light brown, reflecting the decreased pigmentation evident in ASP-treated cells at the light and electron microscopic levels (Figure 5). After treatment with MSH, pigmentation of the cells was markedly increased and eumelanosomes were more numerous. However, after exposure to ASP, the number of eumelanosomes in the melan-a melanocytes was decreased significantly, and pheomelanosome-like structures were more predominant. As might be expected from the results presented above, visible pigmentation and melanosome structure of melanocytes treated with the combination of ASP and MSH were indistinguishable from those treated with MSH alone (not shown).

Chemical analysis

Chemical analysis of the types of melanins being produced in response to ASP and/or MSH reveals that while MSH treatment increases the amount of eumelanin production at least 3-fold, it elicited only a slight (~10%) but statistically insignificant decrease in pheomelanin content (Table II). Interestingly, however, treatment with ASP led to a dramatic 10-fold reduction in eumelanin content while the amount of pheomelanin produced increased slightly (~5%, but again not statistically significant).

cAMP responses

The ability of ASP to inhibit eumelanin synthesis in the absence of exogenous MSH could be mediated by an MC1-R-independent mechanism, by antagonism of residual MSH present in the culture media or by inverse agonism, i.e. a direct effect on the MC1-R itself independent of and opposite to that of MSH. In any of those mechanisms, the effects of ASP are likely to inhibit downstream effectors of cAMP such as protein kinase A, since the MC1-R is a G protein-coupled receptor which activates adenylate cyclase *in vitro* and *in vivo* (Mountjoy *et al.*, 1992; Jackson, 1993; Suzuki *et al.*, 1996). To determine whether ASP could bring about a decrease in cAMP levels in the absence of exogenous MSH, we measured cAMP accumulation in melanocytes exposed for 40 min to ASP alone or in combination with 10 nM MSH or 20 μg/ml cholera toxin (which increases cAMP levels by ribosylation of G_s protein). As shown in Figure 6, basal levels of cAMP accumulation in melan-a melanocytes (4.9 ± 0.1 pmol/10^6 cells) were decreased slightly by the addition of ASP at 10 nM (3.9 ± 0.3 pmol/10^6 cells) or 100 nM (3.4 ± 0.1 pmol/10^6 cells). There was a dramatic (35-fold) stimulation of cAMP following treatment of melanocytes with MSH, and this stimulation was partially inhibited by a 10-fold excess of ASP (i.e. at 100 nM ASP), and was reduced to a 13-fold increase above control. Cholera toxin elicited a 30-fold increase in cAMP accumulation, and this effect was reduced to 20- or 16-fold above control by simultaneous treatment with 10 or 100 nM ASP, respectively.

Table I. Enzyme activity of melanocytes following treatment with MSH and/or ASP

Activity	MSH only (× control)	ASP only (× control)	MSH + ASP (× control)
Tyrosine hydroxylase	20.4 ± 7.8	0.0 ± 0.1	12.6 ± 4.5
DOPA oxidase	11.4 ± 1.0	0.0 ± 0.2	11.7 ± 1.4
DOPAchrome tautomerase	1.5 ± 0.4	0.0 ± 0.2	1.4 ± 0.2
Melanin production	10.4 ± 3.0	0.1 ± 0.0	9.0 ± 2.3

Melan-a melanocytes were treated with 10 nM MSH and/or 10 nM ASP for 5 days and then were harvested and solubilized; melanogenic enzyme activities of the extracts were then measured as detailed in Materials and methods. Results for the MSH, ASP and MSH + ASP treatments are reported as *n*-fold of control values ± SEM ($n \geqslant 4$ independent experiments). Control values were: tyrosine hydroxylase, 7.4 ± 0.6; DOPA oxidase, 17.3 ± 5.1; DOPAchrome tautomerase, 148 ± 54; and melanin production, 1.2 ± 0.4 (all in pmol/μg protein/h).

Discussion

Mammalian hair color is determined primarily by the relative proportions of eumelanin and pheomelanin produced by follicular melanocytes (Ozeki *et al.*, 1995; Prota *et al.*, 1995). Ratios of eumelanin:pheomelanin of >1 generally result in brown or black hair, the intensity of the color depending upon the total amount of melanins present. Eumelanin:pheomelanin ratios of <1 result in the production of yellow or red hair. By those criteria, the melanins being produced by melan-a melanocytes under basal conditions, or following treatment with MSH, would be black or brown (i.e. eumelanic) while those produced following treatment with ASP would be yellow or red (i.e. pheomelanic). This result is quite reasonable since melan-a melanocytes were generated from non-agouti black mice and, in the absence of ASP, would be expected to produce eumelanin. Conversely, treatment of melan-a cells with ASP would be expected to induce pheomelano-

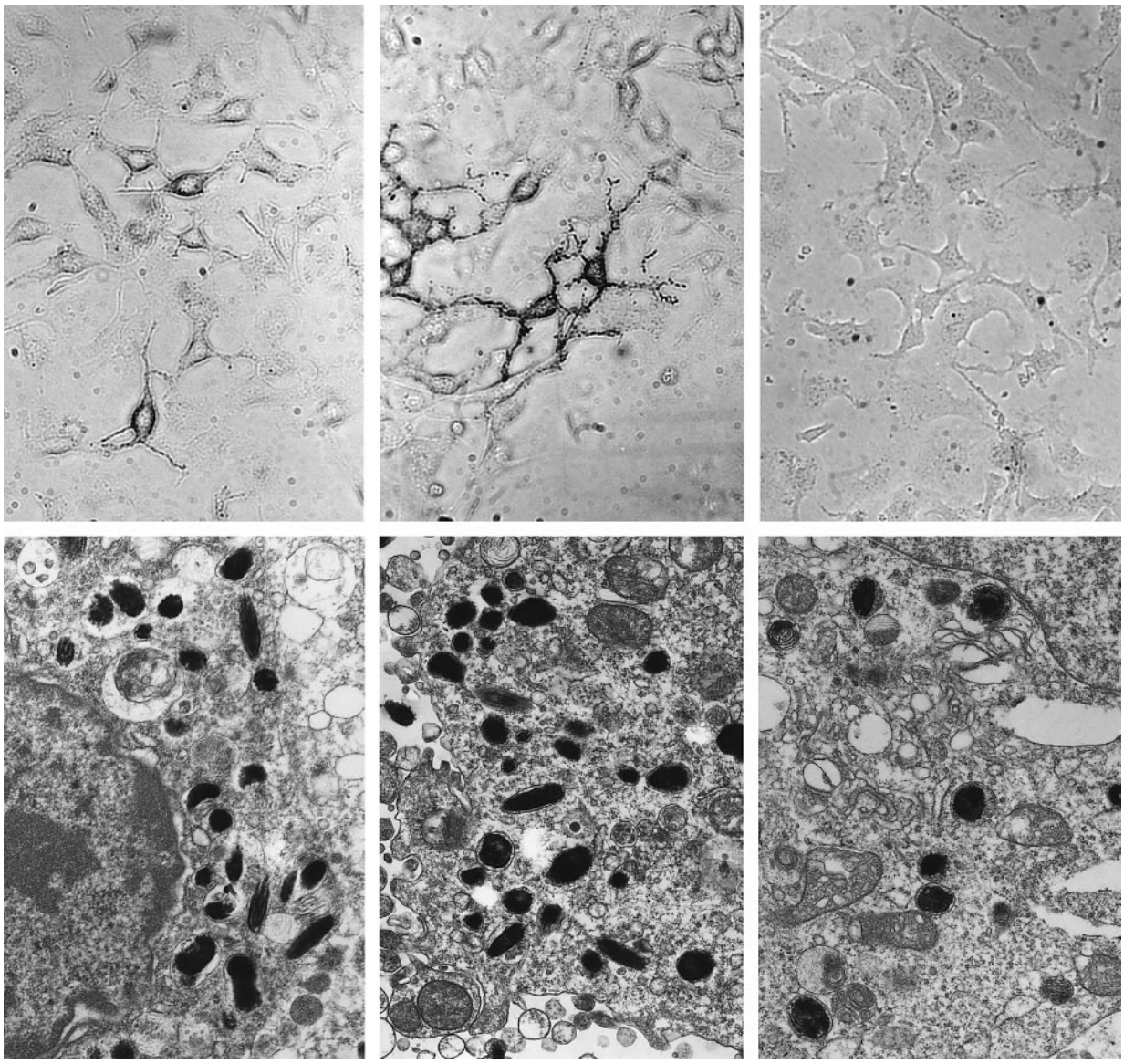

Fig. 5. Light and electron microscopy of melanocytes treated with MSH and/or ASP. Light (top row, all at initial magnification of 200×) and electron (bottom row, all at initial magnification of 80 000×) microscopy of melan-a melanocytes that were untreated (left), treated with 10 nM MSH (center) or with 10 nM ASP (right) for 5 days. Many melanosomes are found in the untreated control and the MSH-treated cells; at higher magnification, it can be seen that most have the typical elliptical shape with striated filaments that are characteristic of eumelanosomes. Following treatment with ASP for 5 days, the numbers of melanosomes are significantly decreased, and many have pheomelanosome-like ovoid shapes with a more particulate internal matrix.

genesis *in vivo*, and this occurs in tissue culture as well, suggesting that this *in vitro* system is an appropriate one mimicking physiological conditions.

Much is known about the biochemistry and cell biology of eumelanogenesis, but a similar level of understanding has not been achieved for pheomelanogenesis due, in part, to the lack of an appropriate cell culture system. *In vivo*, MSH promotes the production of eumelanin, while expression of the *agouti* gene promotes the production of pheomelanin. In this study, we now show that purified recombinant ASP added to melan-a melanocytes in culture decreases the expression of several eumelanogenic genes, reduces the production of eumelanin and eumelanosomes and increases the production of pheomelanin and pheomelanosome-like structures. These results establish an important tool with which to study pheomelanogenesis and, in addition, suggest that the biochemical action of MSH is not completely reciprocal to that of ASP. MSH stimulates the expression and function of tyrosinase but has little or no effect on TRP1 or TRP2; ASP on the other hand down-regulates all three of these melanogenic enzymes.

The mechanism by which ASP acts remains controversial (Conklin and Bourne, 1993; Jackson, 1993; Zemel *et al.*, 1995). While ASP clearly acts as a competitive antagonist of the MC1-R in heterologous cells, this mechanism alone cannot easily explain why ubiquitous overexpression of the *agouti* gene *in vivo* in *lethal yellow* mice affects pigmentation and regulation of body weight differently from *recessive yellow* mice which have a loss-of-function mutation of the MC1-R. Much of the controversy stems from the lack of a suitable *in vitro* assay system that accurately reflects the biological activity of the protein *in vivo*. Our results not only provide such

Table II. Chemical analysis of melanins following treatment with MSH and/or ASP

	MSH only (× control)	ASP only (× control)	MSH + ASP (× control)
Pheomelanin content	0.91 ± 0.13	1.05 ± 0.02	0.84 ± 0.10
Eumelanin content	3.11	0.10	2.55
Eumelanin/pheomelanin	3.42	0.09	3.04

Melanins produced in the samples as described for Table I were subjected to chemical analysis as detailed in Materials and methods; pheomelanin content is estimated by analysis of aminohydroxyphenylalanine (AHP) derivative in the degraded sample, whereas eumelanin content is estimated by quantitation of the pyrrole-2,3,5-tricarboxylic acid (PTCA) derivative. In each experiment, AHP analyses were performed routinely in duplicate, whereas PTCA analyses could only be performed once due to the sample size required. Results for the MSH, ASP and MSH + ASP treatments are reported as *n*-fold of control values (averages of two independent measurements). Control values were: pheomelanin content, 169 ng/mg protein; eumelanin content, 179 ng/mg protein.

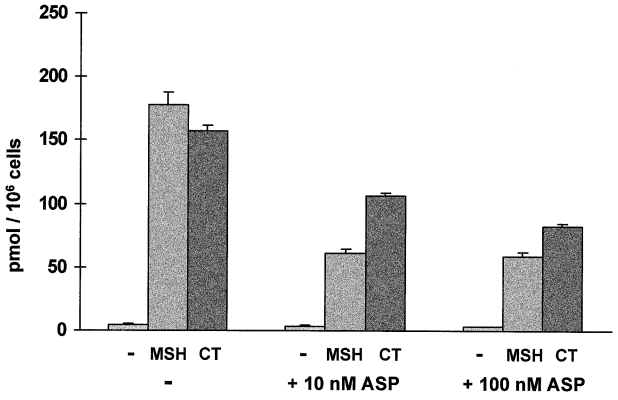

Fig. 6. Effects of ASP, MSH and cholera toxin on cAMP accumulation. Melan-a melanocytes were cultured to subconfluence, then incubated for 40 min in the presence of MSH (at 10 nM), cholera toxin (CT, at 20 μg/ml) and/or ASP (at 10 or 100 nM), then assayed for cAMP concentration as detailed in Materials and methods; results are reported as pmol/10^6 cells ± SEM (n = 6).

an assay system, but also shed insight into the underlying mechanism, since the ability of purified ASP to induce pheomelanogenesis in eumelanic melanocytes demonstrates that ASP alone is sufficient to elicit such changes and that cytokines, growth factors, endothelins and other constituents of the epidermis or hair bulbs are not required. This study now confirms the antagonism of ASP on MSH-induced stimulation of cAMP in melanocytes, but also shows that ASP has physiologic effect(s) in the absence of exogenous MSH.

Two of the genes we have examined, TRP1 and TRP2, encode enzymes that catalyze specific distal steps in the eumelanogenic pathway, and we have shown previously that expression of these genes ceases during pheomelanogenesis *in vivo*. The enzymatic activity of the third member of the TRP family, tyrosinase, is required for both types of pigment synthesis; tyrosinase is also down-regulated during pheomelanogenesis *in vivo*, though not to the same extent as TRP1 and TRP2. The changes in gene expression we have described here for melan-a cells treated with ASP are similar to those that occur during pheomelanogenesis *in vivo*, although *in vitro* ASP has a comparable down-regulatory effect on tyrosinase, TRP1 and TRP2. This discrepancy may be caused by intrinsic differences between melan-a cells, which were derived from neonatal epidermis, and hair follicle melanocytes. Epidermal and hair follicle melanocytes arise from the same pool of precursor melanoblasts, but could later acquire changes in cell surface receptors or intracellular signaling that affect their pheomelanogenic potential. In addition, extrinsic differences could help to explain why pheomelanin is produced within and not between hair follicles, and treatment of melan-a cells with paracrine factors unique to the follicular microenvironment may identify molecules that function in addition to and/or downstream of ASP that promote pheomelanogenesis.

The results of chemical analyses of melanins produced in the presence or absence of MSH and/or ASP *in vitro* clearly demonstrate the effect of MSH in stimulating eumelanin synthesis with a negligible effect on pheomelanin production. MSH thus elicits a marked increase in the percentage of eumelanin produced and thus dramatically decreases the percentage contribution of pheomelanin to the total melanin. ASP, on the other hand, caused a significant decrease in eumelanin production while at the same time slightly stimulating pheomelanin synthesis, resulting in a dramatic increase in the percentage contribution of pheomelanin (91%) to the total melanin content. The lack of correlation of pheomelanin production with tyrosinase mRNA synthesis or enzyme function argues strongly that an as yet undiscovered enzyme or regulatory point is at least partly responsible for pheomelanin content. Why eumelanogenic melanocytes, when introduced into culture, produce significant levels of pheomelanin in the absence of exogenously added ASP, is not known at this time, although it has been noted previously (Sato *et al.*, 1985a; Hunt *et al.*, 1995). The latter report showed that human melanocytes introduced into culture produced significant levels of pheomelanin that did not correlate with the racial origin of those melanocytes. It is possible that nutrient concentrations in the media that are not present in the epidermal microenvironment play a role in this determination, but further study will be necessary to resolve this point.

Several hypotheses have been put forward to explain the mechanism of ASP action, including competitive antagonism for MSH binding, binding to an as yet unidentified 'agouti receptor' or modulation of calcium flux. Distinguishing between these alternatives is difficult since virtually all of the effects of ASP, including those reported here, can be reversed by the addition of exogenous MSH. Our results demonstrate that ASP antagonizes MC1-R signaling, but effects of ASP treatment can be observed in the absence of exogenous MSH. Our findings are unlikely to be explained by the presence of residual MSH in the culture medium since the concentration of endogenous MSH in the medium is 1000-fold below the EC_{50} for MSH, but could be accounted for by inverse agonism, particularly if the MC1-R has a significant degree of constitutive activity. The hypothesis that the action of ASP is independent of MSH but not of the MC1-R could be addressed by studies of normal melanocytes cultured from non-mutant animals and those that carry the *recessive yellow* (*Mc1r*^{e}) mutation. The stage is now set for critical studies to characterize the regulation of gene expression

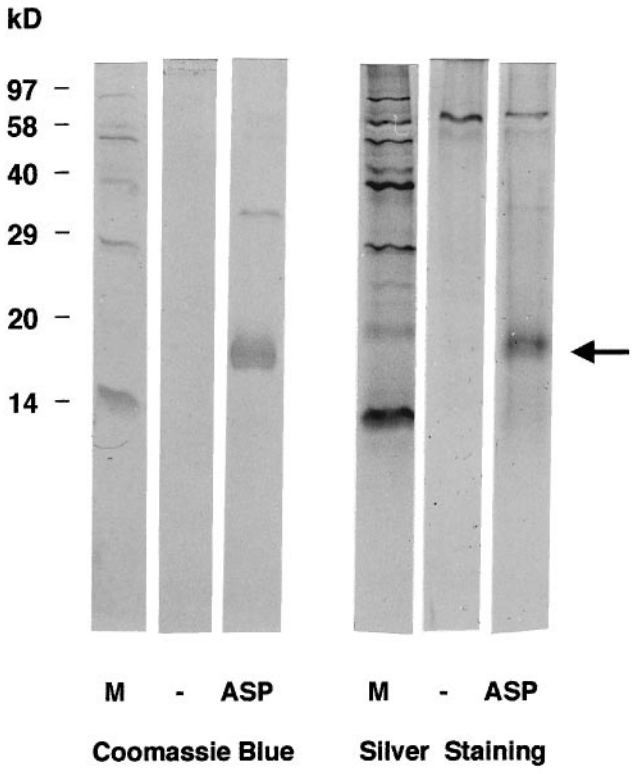

Fig. 7. Purity of recombinant ASP used in these studies. ASP was expressed in a baculovirus system, purified and separated by SDS electrophoresis on 15% acrylamide gels as detailed in Materials and methods. The position of molecular weight markers (M) is shown, as is a buffer control (–); 1 μg of ASP was used on the Coomassie Blue-stained gel (left) while 0.1 μg of ASP was used in the silver-stained gel (right).

and cellular signaling mechanisms triggered by MSH and ASP that modulate the pheomelanogenic switch in melanocytes.

Materials and methods

Cells and cell culture conditions

The melan-a melanocyte line, derived from C57Bl non-agouti black mice, was a kind gift from Dr Dorothy Bennett, London, and was cultured in Dulbecco's minimal essential medium with additives as described by Bennett *et al.* (1987). Cells were usually seeded at 1.5×10^6 cells per 15 cm diameter dish. For 24 h experiments, we added ASP and/or MSH immediately, while for the 5 day experiments, we added ASP immediately, and MSH was added starting on the next day. The concentrations of ASP and MSH used ranged from 0.01 to 10 nM, as detailed in the figure and table legends. The cells were cultured routinely at 37°C in a humidified incubator with 5% CO_2, and all media were changed daily. Cells were harvested by brief treatment with trypsin/EDTA, and used for subculture, or were processed for RNA, protein or enzyme analysis, as detailed below.

Agouti signal protein

Recombinant mouse ASP was generated and purified using a baculovirus expression system as described in Ollmann *et al.* (in preparation). The ASP used for the experiments is ⩾90% pure, as estimated by analysis of gels stained with Coomassie Blue or silver stain (Figure 7), and inhibits activation of the MC1-R with a K_i of 2.2×10^{-10} M. At 37°C, ASP retains activity for >48 h in water or tissue culture media. The experiment described in Figure 2 has also been repeated with an ASP preparation ⩾99% pure with virtually identical results.

Electron microscopy

Cells were harvested, centrifuged for 5 min at 14 000 *g* at 4°C, and fixed for 2 h at 23°C in 2% glutaraldehyde–2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3. The samples were stored in phosphate-buffered saline (PBS) containing 2% sucrose at 4°C, then processed with graded alcohols and embedded in epoxy resin in the usual manner. Thin sections were stained with uranyl acetate and lead citrate, viewed and photographed with a Zeiss EM10 electron microscope, as previously detailed (Prota *et al.*, 1995).

RNA isolation and Northern blotting

Total RNA was extracted from cells using an RNeasy total RNA isolation kit (QIAGEN, Catsworth, CA), following the manufacturer's instructions. Twenty μg of total RNA were denatured, electrophoresed through 1.0% agarose gels and transferred overnight at 23°C to SureBlot nylon hybridization membranes (Oncor, Gaithersburg, MD) in the standard manner. Filters were pre-hybridized for 3 h at 45°C with Hybrisol I solution (Oncor), and then hybridized with ^{32}P-labeled probes. A 2.0 kb *Eco*RI fragment of TYRS-J, a 1.7 kb *Hin*dIII fragment of pMT4, a 1.75 kb *Eco*RI fragment of TRP2a and a 2.1 kb *Bam*HI–*Sal*I fragment of A26 were used to detect tyrosinase, TRP1, TRP2 and MC1-R mRNAs, respectively. TYRS-J was obtained from Drs Hiroaki Yamamoto and Takuji Takeuchi, Sendai, Japan; pMT4 was obtained from Dr Shigeki Shibahara, Sendai, Japan; TRP2a was obtained from Dr Ian Jackson, Edinburgh, UK; A26 was obtained from Dr Roger Cone, Oregon. A commercially available cDNA probe specific for GAPDH was used to standardize RNA loading on the blots. The cDNA probes were labeled using random primer extension and heated to 100°C for 10 min, then cooled on ice for 10 min prior to adding to the hybridization solution. Hybridization was performed with the radiolabeled probes in Hybrisol I (3×10^7 c.p.m./10 ml) overnight at 45°C with gentle shaking. Following incubation, the blots were washed for 10 min at 23°C with 2× SSC/10% SDS, for 10 min with 0.2× SSC/0.5% SDS and finally for 10 min with 0.1× SSC/0.1% SDS. Blots were exposed in phosphoroimager cassettes at 23°C for 1 h and the densities of the bands were scanned using ImageQuant software. The percentage control for each probe was corrected for initial loading using comparison with the GAPDH standard. Residual probes were then removed by repeated washings for 15 min at 100°C in 0.1× SSC/0.1% SDS in 10 mM Tris, pH 7.0 until no remaining probe could be detected.

Metabolic labeling and immunoprecipitation

These techniques were performed as previously reported (Tsukamoto *et al.*, 1992; Aroca *et al.*, 1993). Briefly, subconfluent cells growing in 10 cm diameter dishes were pre-incubated for 1 h at 37°C in methionine-free medium, and then radiolabeled for 6 h with 0.4 mCi/flask of [^{35}S]methionine. The cells were then harvested and solubilized for 1 h at 4°C with NP-40/SDS buffer (1% NP-40, 0.01% SDS, 0.1 M Tris–HCl, pH 7.2, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin). The cell lysates were then centrifuged for 15 min at 14 000 *g* at 4°C, and the supernatants were pre-treated overnight at 4°C with normal rabbit serum and GammaBind G Sepharose (Pharmacia/LKB, Piscataway, NJ) to reduce background. Then 5×10^6 c.p.m. of each pre-absorbed supernatant were incubated with 10 μl of rabbit antibodies generated against peptides corresponding to the unique carboxyl sequences of the three melanogenic proteins studied; they are termed αPEP1, αPEP7 and αPEP8, which recognize TRP1, tyrosinase and TRP2, respectively (Tsukamoto *et al.*, 1992). Following incubation at 37°C for 1 h, 50 μl of GammaBind G Sepharose was mixed in each tube for 20 min at 23°C, and the immune complexes were washed four times with NP-40/SDS buffer at 23°C and then denatured in SDS sample buffer by heating to 100°C for 3 min. Specifically bound proteins were then analyzed by SDS–PAGE and visualized by autoradiography.

Western immunoblotting analysis

Cells in tissue culture were harvested and solubilized for 1 h at 4°C with NP-40/SDS buffer, then centrifuged at 14 000 *g* for 15 min at 4°C, and the supernatants were recovered. Proteins from the NP-40/SDS-solubilized cells were separated on 7.5% SDS gels, and then transferred electrophoretically to polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA). Following blocking overnight at 23°C in 3% bovine serum albumin in TBS/Tween (0.1% Tween-20 in Tris-buffered saline), the blots were incubated with primary antibodies (at 1:1000 dilution in TBS/Tween). Following four washes in TBS/Tween to reduce non-specific binding, subsequent visualization of specific antibodies bound was carried out with Enhanced ChemiLuminescence (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

Melanogenic enzyme assays

Melanogenic assays were carried out routinely on NP-40/SDS-soluble extracts (obtained as above) at pH 6.8, 37°C for 60 min. To determine tyrosinase activity, the tyrosine hydroxylase assay was used to measure tritiated water produced during the hydroxylation of L-[3,5-^{3}H]tyrosine to DOPA (Hearing, 1987). For DOPA oxidase activity, the production of acid-insoluble melanin product from [3-^{14}C]DOPA was measured (Aroca *et al.*, 1993). To determine DOPAchrome tautomerase activity, the disappearance of DOPAchrome substrate and the production of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) rather than 5,6-dihydroxyindole (DHI) was measured by HPLC (Tsukamoto *et al.*, 1992). To determine melanin production, the [U-^{14}C]tyrosine assay (Hearing, 1987) was used. All radioactive precursors were obtained from DuPont-New England Nuclear. DOPAchrome was prepared using the silver oxide method, and DHI and DHICA used as standards were obtained from Pierce Chemical Co. (Rockford, IL) and from Professor Shosuke Ito, Nagoya, Japan. The pmoles of product of the assays were calculated from radioactivity measured or by comparison with standard curves.

cAMP assays

These assays were performed as previously detailed (Suzuki *et al.*, 1996). Briefly, cells were plated into 24-well plates at 3×10^{5} cells per well and allowed to grow for 48 h with a single change of medium. The media were then removed from each well, and the cells were incubated for 40 min in the presence of MSH, ASP and/or cholera toxin, following which the reactions were stopped with 1 M HCl. Each sample was then acetylated by the addition of triethylamine and acetic anhydride, and the amount of cAMP was determined by radioimmunoassay as previously detailed (Liggett *et al.*, 1989).

Chemical analysis

Chemical degradation and analysis of eumelanin and pheomelanin contents were performed and quantitated as previously reported (Ito and Fujita, 1985).

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