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Bioactive Motifs of Agouti Signal Protein

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The switch between the synthesis of eu- and pheomelanins is modulated by the interaction of two paracrine signaling molecules, α-melanocyte stimulating hormone (MSH) and agouti signal protein (ASP), which interact with melanocytes via the MSH receptor (MC1R). Comparison of the primary sequence of ASP with the known MSH pharmacophore provides no suggestion about the putative bioactive domain(s) of ASP. To identify such bioactive motif(s), we synthesized 15-mer peptides that spanned the primary sequence of ASP and determined their effects on the melanogenic activities of murine melanocytes. Northern and Western blotting were used, together with chemical analysis of melanins and enzymatic assays, to identify three distinct bioactive regions of ASP that down-regulate eumelanogenesis. The decrease in eumelanin production was mediated by down-regulation of mRNA levels for tyrosinase and other melanogenic enzymes, as occurs *in vivo,* and these effects were comparable to those elicited by intact recombinant ASP. Shorter peptides in those motifs were synthesized and their effects on melanogenesis were further investigated. The amino acid arginine, which is present in the MSH peptide pharmacophore (HFRW), is also in the most active domain of ASP (KVARP). Our data suggest that lysines and an arginine (in motifs such as KxxxxKxxR or KxxRxxxxK) are important for the bioactivity of ASP. Identification of the specific ASP epitope that interacts with the MC1R has potential pharmacological applications in treating dysfunctions of skin pigmentation.

Key Words: **agouti; melanocortin; receptor; G protein; antagonist.**

INTRODUCTION

G-protein coupled receptors are integral membrane proteins that contain seven transmembrane domains and that mediate cellular responses to a wide array of signaling molecules. In some G-protein coupled receptors, one ligand can activate different receptors within the same family, one example being the melanocortin family of receptors which are differentially activated by products of the *POMC* gene. The melanocortins are a group of melanotropic and adrenocorticotropic hormones produced mainly in the pituitary gland, but also in a number of extrapituitary sites, including the skin [1]. The effects of these peptides (known collectively as melanotropins) in different tissues are mediated by a family of specifically expressed melanocortin receptors (MCxR),[2] termed MC1R, MC2R, etc. [2, 3]. The intracellular action of melanocortin receptors, as well as other G-protein coupled receptors, is associated with the activation of adenylyl cyclase and the subsequent elevation of intracellular cAMP levels. In melanocytes, this pathway activates the expression of *tyrosinase* and other melanogenic genes [4, 5]. Mitogen activated protein kinases and various transcription factors are also part of the complex response pathway [6–8].

MC1R is primarily expressed by melanocytes and regulates their proliferation and melanogenesis, but it may also be expressed by monocytes, keratinocytes, fibroblasts, and endothelial cells [9–12]. In wild-type agouti mice, coat color is characterized by a yellow stripe on a black background in each hair as a consequence of a temporal (3-day) switch in the production of eu- to pheomelanin and then back again. Two interacting factors are known to be involved in regulating that melanogenic switch via the MC1R, namely agouti signal protein (ASP) and α-melanocyte stimulating hormone (MSH). MSH is a relatively short oligopeptide (SYSMEHFRWGKPV) and, in human skin, it may play important roles in regulating general epidermal cell function in addition to regulating pigmentation [13]. ASP is a much larger, 131 amino acid residue protein with several distinct sequence motifs (Fig. 1) that is expressed in skin. ASP interacts with at least four of the five known melanocortin receptors, and its best characterized interactions are with MC1R and MC4R

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[2] Abbreviations used: AGRP, agouti related protein; ASIP, human agouti signal protein; ASP, murine agouti signal protein; Dct, dopachrome tautomerase/TRP2; IGF, insulin-like growth factor; MCxR, melanocortin receptor; MSH, α-melanocyte stimulating hormone; Tyrp1, tyrosinase related protein 1/TRP1.

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[3, 14]. The interaction of ASP and MSH has been shown *in vivo* [15], as has the fact that ASP competes with MSH at the MC4R [16]. The human homolog of agouti signal protein (ASIP) contains 132 residues with an overall homology to murine ASP of 85%; its encoding gene is expressed in adipose tissue, heart, testis, and skin [17], and it is perhaps linked to regulation of lipid metabolism and/or hair color in humans [18–20]. Recombinant human ASIP and mouse ASP interact equally well with the human MC1R [21]. Agouti-related protein (AGRP) is structurally related to ASP and is 25% homologous to ASIP at the amino acid level [22, 23]. AGRP appears to antagonize binding of MSH to MC3R and MC4R. The *mahogany* locus plays an interactive role in ASP signaling via the MC1R and this locus has been recently cloned; its putative encoded product (termed attractin) contains a single transmembrane domain. Attractin has been proposed to function by stabilizing ASP in the microenvironment of the MC1R or perhaps by sequestering MSH allowing ASP to be more effective [24–26].

The functional domain(s) of ASP cannot be predicted by similarity with the known MC1R-MSH pharmacophore (HFRW) or by molecular modeling (our unpublished results). In order to characterize the bioactive domain(s) of ASP, we synthesized overlapping 15-mer peptides that encompass the entire ASP sequence. Murine melanocytes were treated with those peptides and Northern and Western blot analysis, enzyme assays, and measurements of melanin production were used to assess regulation of melanogenesis following treatment. Three potential bioactive regions were identified by this approach, and screening of shorter oligopeptides has resolved an active five-residue motif (KVARP). We confirmed that the peptides identified by down-regulation of melanogenic genes were indeed able to compete with MSH for binding to the MC1R and for stimulation of cAMP levels. Based on these results, a model is proposed for ASP interaction with the MC1R.

MATERIALS AND METHODS

Peptide synthesis. Fifteen-mer peptides, corresponding to the primary sequence of ASP, starting every third residue after the signal sequence (Fig. 1), were synthesized by continuous flow solid phase peptide synthesis using a MilliGen 9050 synthesizer based on the Fmoc/But strategy. Peptides were purified by HPLC and their purity was confirmed by electrospray mass spectroscopy. All peptides were soluble in aqueous solution, except ASP7, which was soluble in 2% DMSO. A 35-mer peptide (ASP33), corresponding to the cysteine-rich C-terminus, was partly oxidized after synthesis (ASP34), and the oxidized form was further purified by size exclusion chromatography (ASP35). Shorter peptides were similarly synthesized for further screening and the sequences of all peptides used in this study are shown in Fig. 3.

Cell lines. The melan-a melanocyte cell line [27], derived from C57BL/6 nonagouti mice, was a kind gift of Dr. Dorothy C. Bennett (St. George's Hospital Medical School, London, UK). Cells were grown in a humidified atmosphere with 10% CO_2 at 37°C and passaged in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 100 μM 2-mercaptoethanol, 2 mM L-glutamine (all from Gibco/BRL, Grand Island, NY), and 200 nM phorbol 12-myristate 13-acetate (Sigma Chemical Co., St. Louis, MO). Melan-a melanocytes were seeded at 4×10^6 cells/dish and were treated every day for 4 days with MSH (Sigma), recombinant ASP [28], or synthetic peptides; all were dissolved in water and were added to complete medium, as previously described [29]. ASP and MSH were routinely used at a concentration of 10 nM and the peptides at 100 nM, unless otherwise noted. ASP7 was added to complete medium at a final concentration of 0.0002% DMSO, and DMSO alone at 0.0002% was used as its control. Fresh medium and aliquots were added every day throughout the 4-day treatment period. Cells were harvested by brief treatment with trypsin/EDTA and were used for subculture or were processed for RNA, protein, or enzyme analysis, as detailed below.

RNA isolation and Northern blotting. Total RNA was isolated from cells using an RNeasy total RNA isolation kit (Qiagen, Santa Clara, CA) according to the manufacturer's instructions. Northern blotting was performed as previously described [29]. The probes used included a 2.0-kb *Eco*RI fragment of TYRS-J for tyrosinase (obtained from Drs. H. Yamamoto and T. Takeuchi, Sendai, Japan), a 1.7-kb *Hin*dIII fragment of pMT4 for Tyrp1 (also known as TRP1, obtained from Dr. S. Shibahara, Sendai, Japan), and a 1.75-kb *Eco*RI fragment of TRP2a for Dct (also known as TRP2, obtained from Dr. I. Jackson, Edinburgh, UK), and densities of bands were analyzed in a Storm 860 PhosphorImager with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). A GAPDH cDNA probe was used to standardize RNA loading.

Analysis of total melanin content. Absorbances of extracts of 10^7 treated or control cells were measured at 490 or 650 nm after solubilization with Soluene 350, as previously detailed [29, 30]. In some experiments, the absorbance of cell lysates prepared as detailed below was measured directly at 650 nm [31].

Western blotting. Cell lysates were prepared as described previously [29]. Equal amounts of protein were separated on 7.5% minigels under denaturing, reducing conditions. Proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA). Following blocking overnight at 4°C with 1% bovine serum albumin in PBS/Tween (0.1% Tween-20 in Dulbecco's phosphate-buffered saline), the blots were incubated with primary antibodies diluted 1:2000 in PBS/Tween. Peptide antibodies αPEP7, αPEP1, and αPEP13 were used which recognize the melanosomal proteins tyrosinase, Tyrp1, and Pmel17, respectively [32–34]. The secondary antibody used was donkey anti-rabbit immunoglobulin horseradish-peroxidase-linked antibody (Amersham Corp., Arlington Heights, IL) at 1:1500 dilution. Visualization of bound antibodies was carried out with enhanced chemiluminescence (Amersham) following the manufacturer's instructions. Protein concentrations were determined with the BCA assay kit (Pierce Chemical Co., Rockford, IL).

MTT assay. To measure cell viability, an MTT assay kit (Boehringer Mannheim, Indianapolis, IN) was used in the microtiter format, as described elsewhere [31].

Melanogenesis assays. Extracts of cells in NP-40 buffer were assayed for melanogenic activity by measuring the formation of radiolabeled melanin, as previously described [31, 32].

MSH binding assays. [^{125}I]MSH and [^{125}I]NDP-MSH were purchased from Peninsula Laboratories (San Carlos, CA) and Amersham Pharmacia Biotech (Piscataway, NJ), respectively, and were used in MSH binding studies, as previously detailed [21]. Preliminary studies were performed to optimize binding conditions according to previously published protocols [35–38]. Briefly, melan-a melanocytes were seeded in 24-well plates at 10^5 cells/well 1 day prior

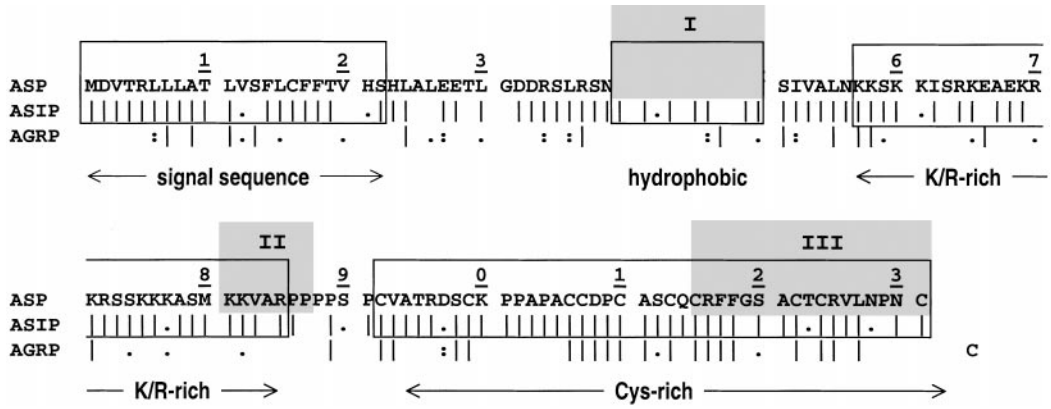

FIG. 1. Primary sequences of murine ASP, human ASIP, and murine AGRP. The signal sequence (residues 1–22, open box), a hydrophobic region (residues 40–50, open box), the K/R-rich basic domain (residues 57–85, open box) and the C-rich domain (residues 92–131, open box) are indicated. Residues 3–21, 64–71, and 79–82 are predicted to be in an α-helix while residues 123–126 are predicted to be in a β-strand. Shaded areas indicate bioactive regions I, II, and III, as discussed in the text.

to the binding studies. Cells were then rinsed and incubated in binding buffer (RIA kit buffer, Peninsula) containing unlabeled ASP, MSH, or peptides with 10^5 cpm [^{125}I]MSH at 4°C for 8 h. Following the incubation, cells were washed and solubilized and radioactivity was measured. Specific binding was determined by measuring the amount of radioactivity remaining after incubation with 10^{-5} M unlabeled MSH and subtracting that from the total bound radioactivity. Binding data were analyzed with GraphPad Prism software (San Diego, CA). Binding studies with [^{125}I]NDP-MSH were performed at 23°C for 2 h, following a previously published protocol [35].

cAMP assays. cAMP assays were performed as previously described [21]. Briefly, melan-a melanocytes were seeded in 24-well plates at 10^5 cells/well 1 day prior to the experiment. Cells were preincubated at 37°C for 20 min with growth medium containing 0.1 M isobutyl methylxanthine, a phosphodiesterase inhibitor. Cells were then treated with buffer, ASP (10 or 100 nM), or peptides (100 nM) for 10 min at 37°C in a total volume of 0.5 ml in each well in quadruplicate, as indicated in the figure legends. MSH (10 nM) was then added to the wells noted and the reactions were continued for 45 min. Reactions were terminated by the addition of 50 μl of 1 N HCl. Duplicate 250-μl samples were removed from each well, and cAMP levels were determined by radioimmunoassay following the manufacturer's instructions (NEN Life Science Products, Boston, MA); the acetylated procedure was used.

Modeling tools. To study ASP structure, the ProDom protein domain database was used (http://protein.toulouse.inra.fr/prodom.html). For sequence alignments, the GCG program (http:/molbio.info.nih.gov/molbio/gcglite) and the MultAlin program (http://www.toulouse.inra.fr/lgc/multalin/multalin.html) were used. For prediction of secondary structure, the PredictProtein server was used (http://www.embl-heidelberg.de/predictprotein/predictprotein.html). Helical wheel analysis was performed with the Java applet written by M. Turcotte (http://www.bmm.icnet.uk/people/turcotte/Java/HW/).

RESULTS AND DISCUSSION

Bioactive Domain(s) of ASP

The approach used to identify important bioactive motifs of ASP was based on the ability of ASP to down-regulate the eumelanogenic pathway *in vitro.* Thirty-five 15-mer peptides were synthesized which encompassed the complete amino acid sequence of ASP following the signal sequence (Fig. 1), as deduced from its mRNA. Northern blot analysis was used in the initial screening for bioactivity of these peptides since that is the most sensitive technique for detecting early cellular responses to ASP [29]. Western blot analysis, melanogenic activity, total melanin, and proliferation of cells treated with promising bioactive peptides were then performed in order to confirm the Northern blot results and to further elucidate the efficacy of various peptides.

Melan-a melanocytes were treated for 4 days with the synthetic 15-mer peptides (or MSH and ASP as positive and negative controls) and effects on melanogenic gene expression and melanin content were assessed; these are standard conditions used to assess effects of MSH and ASP on melanocytes [8, 15, 29, 39]. Representative Northern blots are shown in Fig. 2 and a summary of effects on melanin content and mRNA levels for tyrosinase, Tyrp1, and Dct is shown in Fig. 3. We have plotted those values as a continuum to highlight the fact that all peptides have 12 of their 15 residues overlapping with their neighbors on each side to show the consistency of effects in discrete regions. None of the peptides showed any significant effect on cell growth, as assessed by cell number, protein content, or MTT assay (data not shown). Peptides ASP4–ASP6 (region I) and, more dramatically, ASP13–ASP19 (region II), showed reproducible and significant down-regulation of mRNAs for all three melanogenic enzymes examined (tyrosinase, Tyrp1, and Dct). Intact ASP was able to elicit significant decreases (~40–50%) in levels of those same melanogenic enzyme mRNAs. Slight (~10%) decreases in total melanin production were elicited by many of those same peptides, compared to the ~25% decrease elicited by intact ASP; dramatic changes in melanin content are difficult to

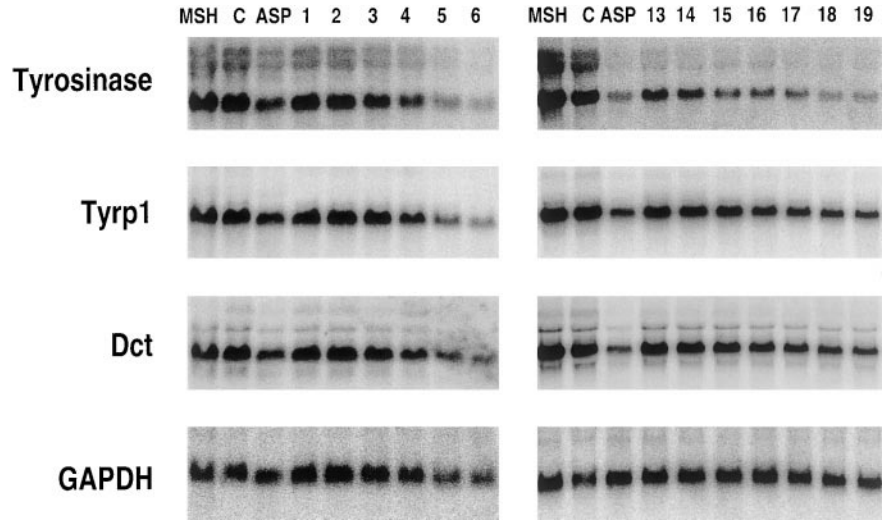

FIG. 2. Northern blot analysis of melanogenic enzyme mRNAs following peptide treatment. Levels of tyrosinase, Tyrp1, Dct, and GAPDH mRNAs are shown following treatment of melan-a melanocytes for 4 days with MSH, buffer control (C), ASP, peptides ASP1–ASP6, or peptides ASP13–ASP19. A summary of the quantitation of these bands for all treatments is shown in Fig. 3.

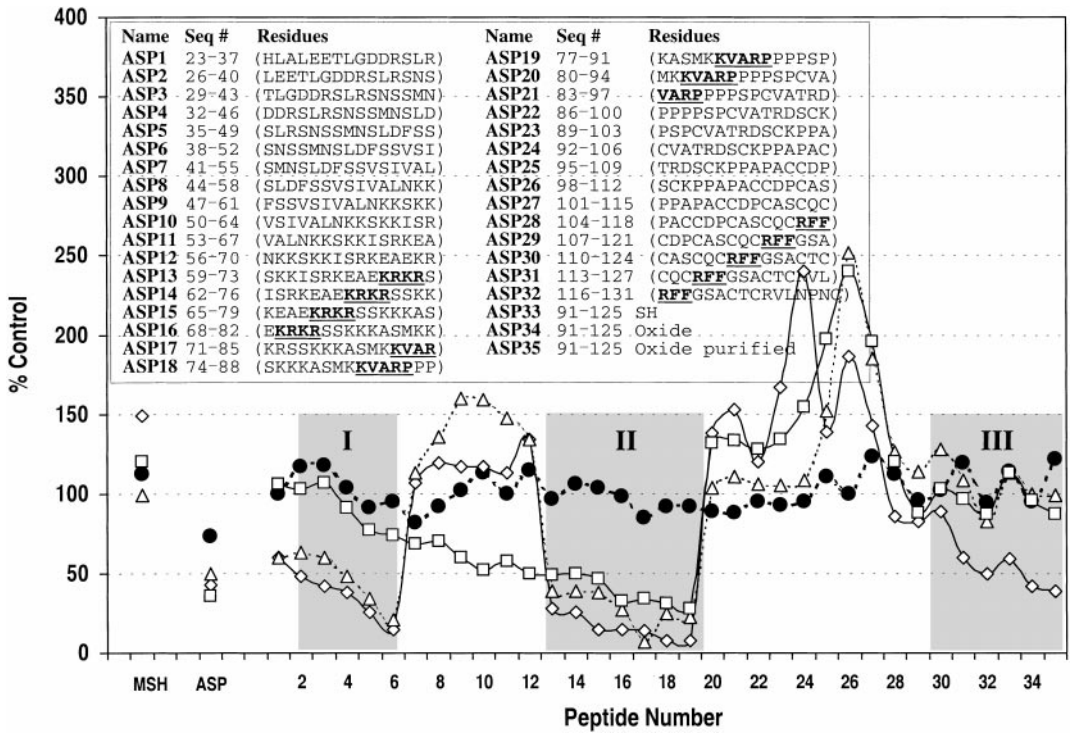

FIG. 3. Bioactivity measurements of synthetic ASP peptides on melanocytes. Melan-a melanocytes were treated with ASP or MSH at 10 nM or with peptides (ASP1–ASP35) at 100 nM for 4 days. Cells were then harvested and assayed for cell number, protein content, melanin content (●), or mRNA levels for tyrosinase (◇), Tyrp1 (△), and Dct (□). Northern blot values were normalized to GAPDH as a loading control and were then compared to untreated controls. Results for melanin are also presented as a percentage of the control compared to the untreated control. Numeric results plotted are the means of assays carried out at least in duplicate (experiments on the bioactive peptides (shaded) have been performed at least three times each with comparable results). Regions of peptides which showed reproducible significant decreases in mRNA levels for all three melanogenic enzymes are shown as shaded, region I being ASP2–ASP6, region II being ASP13–ASP19, and region III being ASP29–ASP34.

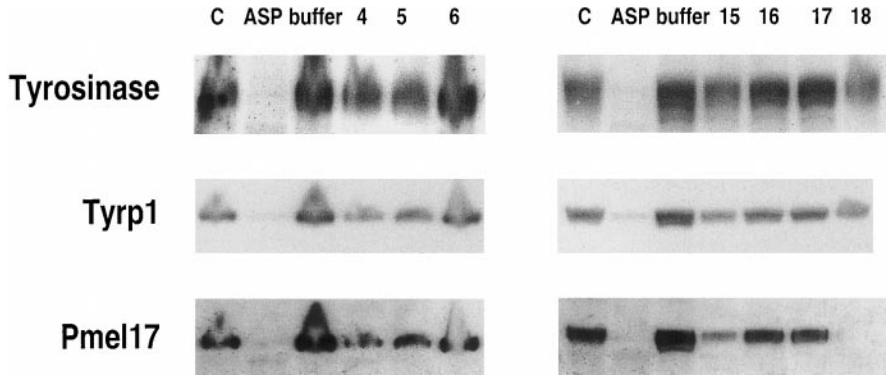

FIG. 4. Western blot analysis of melanogenic proteins following peptide treatment. Protein levels for tyrosinase, Tyrp1, or Pmel17 are shown for melanocytes treated for 4 days with medium only (C), ASP, buffer, peptides ASP4–ASP6, or peptides ASP15–ASP18.

elicit within 4 days because of its highly stable nature and the relatively long half-life of tyrosinase required for its synthesis. In contrast, changes in melanogenic gene expression occur relatively quickly.

None of the three synthetic 35-mer peptides at the carboxyl terminus of ASP (ASP33, ASP34, and ASP35) resulted in any significant inhibition of melanin production or levels of Tyrp1 or Dct mRNAs, although all three elicited decreases in tyrosinase mRNA levels. The high cysteine content in this region suggests that correct disulfide bonding may be important to secondary structure (cf. below). ASP28–ASP32 (region III) also elicited decreases in tyrosinase mRNAs, although melanin content and mRNA levels for Tyrp1 and Dct were again unaffected, suggesting that those two regions (I and III) may have different binding sites or affinities to the MC1R. Binding of ASP and ASIP mutant proteins to recombinant MC receptors expressed in COS cells demonstrated that several substitutions (R116A, F117A, and F118A) interfered with inhibition of MSH binding [40]. Similarly, the homologous residues RFF in the C-terminal domain of human AGRP have been identified as critical for binding to MC3R and MC4R [41]. That RFF sequence is contained in each of the 15-mer peptides of ASP28–ASP32 of region III and in the 35-mer (ASP33–ASP35), which decreased tyrosinase expression in this study.

Figure 4 shows a representative Western blot analysis after treatment of melanocytes with several of the putative bioactive peptides. Intact ASP and peptides in region I (e.g., ASP4 and ASP5) and in region II (e.g., ASP15–ASP18) elicited significant decreases in one or more of the melanogenic enzymes–tyrosinase, Tyrp1, and/or Pmel17. Following treatment with intact ASP, eumelanogenic proteins disappeared almost completely by Western blot analysis, although such a dramatic decrease was not evident at the mRNA level (cf. Fig. 2), suggesting that pheomelanogenesis might correlate with melanogenic protein instability. Enzyme stability has been shown to be an important regulatory point in pigmentation, although it is not known whether this is mediated by proteolytic degradation within the melanosome or by another mechanism. It is known that basal levels of tyrosinase expression lead to pheomelanin synthesis, while elevated levels of tyrosinase switch the pathway to production of eumelanin [42, 43]. Tyrp1 is also extremely important to tyrosinase stability [34, 44], and the decrease in tyrosinase activity noted could result, at least in part, from the lower number of eumelanogenic melanosomes observed in pheomelanogenesis [29]. Progression of melanosomes to stages 3 and 4 is mediated by an increase in melanogenesis and melanin deposition.

Following the identification of those bioactive regions in ASP, shorter oligopeptides (ASP1′ through ASP11′) were synthesized to further characterize the specific and minimal epitope(s) that elicit down-regulation of melanogenesis. Regions I and II were examined in this fashion since the most significant down-regulation of tyrosinase, Tyrp1, and Dct mRNAs was noted in those regions, as detailed above (cf. Fig. 2). A summary of the results obtained with ASP1′–ASP11′ is shown in Table 1. Treatment with intact ASP elicited a significant decrease in Tyr and Tyrp1 mRNA levels (~40% of controls) and in tyrosinase catalytic function (~20% of controls). Oligopeptides from region I (i.e., ASP1′–ASP5′) showed no significant inhibitory effects on mRNA levels for melanogenic enzymes and had only slight inhibitory effects on melanin content or on tyrosinase activity. In contrast, oligopeptides from region II (i.e., ASP7′–ASP11′) showed reproducible decreases in melanin content (10–15%) and in mRNA levels for tyrosinase and Tyrp1. Although a tripeptide (ASP6′, VAR) had no significant bioactivity, the addition of a single residue to its carboxylic end (i.e., ASP7′, VARP) elicited bioactivity, and adding a residue to both ends (i.e., ASP9′, KVARP) was optimal. Other oligopeptides in that region (ASP8′, KVAR, and ASP10′, KKVARPP) elicited similar decreases in tyrosinase and Tyrp1 mRNA levels and enzyme activity, which strongly sug-

TABLE 1

Analysis of ASP Oligopeptide Bioactivity on Melanocytes

Rx	Residue No.	(Sequence)	% Cell No.	% Melanin	% Tyr mRNA	% Tryp1 mRNA	% Tyr Enzyme
Control			100	100	100	100	100
ASP			41	93 ± 10	37 ± 13**	46 ± 9**	19 ± 17**
ASP1′	40–50	(SSMNSLDFSSV)	131	104 ± 7	94 ± 17	104 ± 40	109 ± 18
ASP2′	41–49	(SMNSLDFSS)	122	99 ± 10	100 ± 13	106 ± 44	92 ± 12
ASP3′	42–48	(MNSLDFS)	130	95 ± 13	105 ± 30	114 ± 56	104 ± 14
ASP4′	43–47	(NSLDFS)	108	97 ± 5	114 ± 38	118 ± 54	80 ± 9*
ASP5′	43–47	(NSLDFS)	111	87 ± 11	122 ± 54	132 ± 68	95 ± 13
ASP6′	83–85	(VAR)	140	88 ± 17	123 ± 75	136 ± 79	99 ± 18
ASP7′	83–86	(VARP)	96	83 ± 13*	92 ± 1*	81 ± 2**	96 ± 25
ASP8′	82–85	(KVAR)	98	94 ± 10	75 ± 12*	104 ± 25	98 ± 10
ASP9′	82–86	(KVARP)	82	85 ± 9*	59 ± 16**	75 ± 20*	78 ± 7**
ASP10′	81–87	(KKVARPP)	146	86 ± 10*	57 ± 21*	80 ± 39	97 ± 12
ASP11′	Ac83–85	(AcVARam)	153	93 ± 1*	65 ± 38	89 ± 38	95 ± 21

Note. Results reported are the means ± SEM of two (in the case of Northerns) or three independent experiments; assays for cell number and melanin content and Northern blotting were performed in quadruplicate as detailed under Materials and Methods. Statistically significant difference from control at *<0.05; **<0.01.

gests that region II (residues 81–87) is indeed an important bioactive domain of ASP. However, the reduced efficacy of these peptides as they become shorter, especially when compared to intact ASP, suggests that peptide length may be an important determinant in binding efficiency. These data suggest that the minimal functional domain comprises five amino acids (KVARP, residues 82–86) that can elicit significant down-regulation of melanogenic enzyme expression and function. Residues V83, R85, P86, and P89 were important to the binding of ASP to MC3R, MC4R, and MC5R while only the substitution V83A significantly inhibited binding of ASP to the MC1R [45].

cAMP Levels and MC1R Binding Analysis

In order to study the physiological effects of the most active ASP peptides on melanocyte function, we examined whether ASP18 and ASP9′ (15- and 5-mer representatives of bioactive region II) could displace MSH from binding to the MC1R and whether they were able to inhibit the accumulation of intracellular cAMP. Figure 5 shows the effects of MSH, ASP, ASP18, and/or ASP9′ on intracellular cAMP levels in melanocytes. MSH elicits a rapid and significant stimulation of cAMP and it can be seen that ASP (at 10 or 100 nM) is able to abrogate that effect in a dose-dependent manner, as has recently been shown by several groups for murine and human melanocytes [21, 29, 35, 46]. Peptides ASP18 and ASP9′ had similar inhibitory effects on cAMP accumulation as did the full-length ASP, albeit requiring a 10-fold higher concentration to achieve comparable effects. Binding displacement studies of MSH or the superpotent NDP-MSH analog using these same factors showed that intact recombinant ASP displaced MSH binding with an estimated EC_{50} of 20 nM, while ASP18 had an EC_{50} of 300 nM and ASP9′ was similar (data not shown), results that are consistent with the effects on intracellular cAMP. An EC_{50} of 2 nM for MSH in B16 F10 murine melanoma cells and normal human melanocytes has been previously reported [35, 36], and both of those papers reported similar K_d's of about 3 nM for murine ASP and human ASIP. To our knowledge, there are no published data on binding of MSH or ASP to melan-a melanocytes, although titration of ASP effects on *tyrosinase* gene transcription in those cells would suggest an EC_{50} in the range of 10–20 nM [29].

Structure/Function Relationships in ASP

ASP and AGRP share a signal sequence (Fig. 1), suggesting that they are secreted, a relatively conserved internal sequence (ASP residues D46–S59) and a highly conserved cysteine-rich region from C105 to the C terminus. A BLAST search for ProDom domains [47, 48] showed 50% similarity between ASP and the keratin type I HA1 intermediate filament coiled coil heptad repeat, which is consistent with the α-helical structure of the putative secretory signal sequence. The cysteine-rich domain of ASP is homologous to toxin venom plectoxins, as has been noted previously [49], which has lead to speculation about the possible involvement of ASP in the regulation of intracellular calcium [50]. A specific disulfide bridging arrangement is clearly necessary to stabilize the tertiary structure of secreted proteins. Interestingly, this region shows some homology with the binding motif of growth factor binding protein precursor signal insulin-like growth factor (IGF) binding glycoproteins (IGFBP) (Pro-

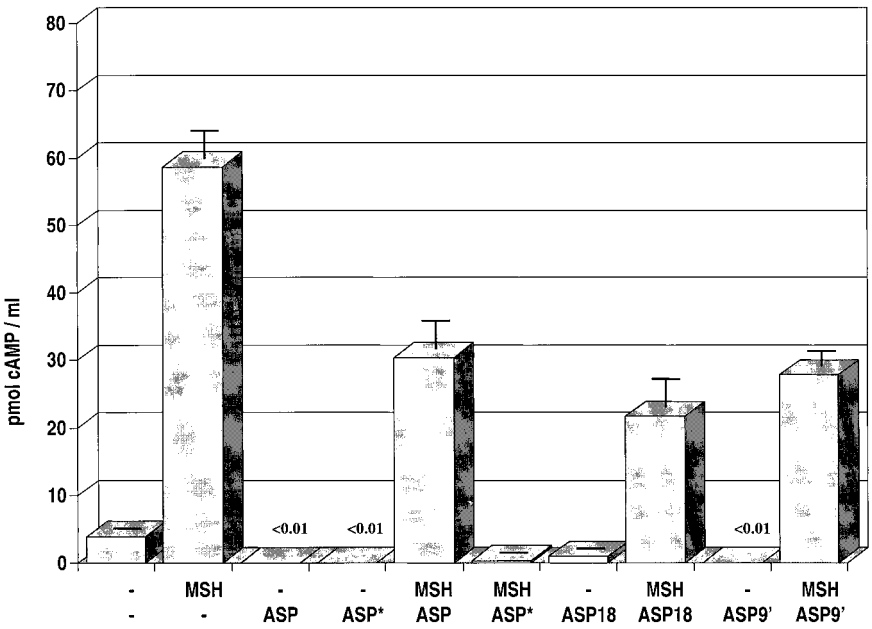

FIG. 5. Intracellular cAMP levels in melanocytes treated with MSH, ASP, and/or ASP-derived peptides. Melanocytes were cultured in multiwell plates and treated with ASP or the ASP peptides for 10 min and then MSH was added and the incubations were continued for 40 min, after which cAMP levels were measured, as detailed under Materials and Methods. Results are reported as means ± SEM (n = 8) of one experiment; similar results were obtained in an independent experiment. Concentrations used were MSH—10 nM; ASP—10 nM; ASP*—100 nM; ASP9′, ASP18, and ASP34′—100 nM.

Dom36, domain ID 579). IGFBPs alter the interaction of IGF with their cell surface receptors. Homology of ASP to IGFBP-3 occurs mainly in a polyproline stretch from residues 85 to 91 (RPPPPSP), which may be responsible for protein–protein interactions [51]. IGF1 plays an important role in the hair follicle cycle by inhibiting apoptosis and entry in catagen [52] and a putative interaction between ASP and IGF1 may signal the entry into catagen. It has also been suggested that the basic region of ASP may protect it from degradation or might allow it to interact with cell surface acidic glycosaminoglycans as a low-affinity receptor [39] or with the product of the *mahogany* locus [24]. This would allow for protein stabilization *in situ* prior to binding to MC1R. Helical wheel analysis of ASP residues 80–88 suggests that residues K81, K82, and R85 would align on one side of the helical wheel and might thus be responsible for binding to an extracellular loop in the MC1R through electrostatic interactions. This has also been pointed out by Kiefer *et al.* [40] in their homology model. Disulfide analysis of recombinant AGRP further supports this hypothesis since residue R86 (equivalent to R85 in ASP) is located adjacent to the first disulfide bond [53]. Residue R85 is conserved between ASP, ASIP, and AGRP, and therefore we suggest that this residue is most likely involved directly in binding.

The region between residues 40 and 50, and a wider region including FSSVSIVAL, is again a relatively conserved motif between ASP and AGRP homologs. This sequence, which includes a predicted β-strand, is in the region of peptides ASP7–ASP10, which have no significant effect on tyrosinase or Tyrp1 expression. However, this sequence conservation may be relevant to ASP and AGRP functions. Hydrophobic sequences with β-sheet structure may be important for binding to lipids [54] or to membrane phospholipids.

An inspection of sequences contained in ASP13–ASP19 reveals that the pattern KxxR is present in all of them in motifs such as KxxxxKxxR or KxxRxxxxK, which strongly suggests that those two residues are responsible for the binding. The KxxR sequence is conserved in human ASIP, but is not present in AGRP, which might explain the differences in melanocortin receptor specificity reported by other groups. Our model (Fig. 6) depicts MSH as binding to extracellular loop(s), which is consistent with published data and models for MC1R and MC4R [38, 55, 56]. However, Miltenberger *et al.* [57], Ollmann and Barsh [58], and our study show that interaction of ASP with MC1R and that of AGRP with MC4R may not be identical and our characterized region II of ASP is involved in the agouti-mediated down-regulation of tyrosinase activity through direct interaction with the MC1R. Therefore, we also suggest that it may be possible for ASP to bind to a separate site than does MSH, e.g., the N-terminal loop, which would be consistent with what is known

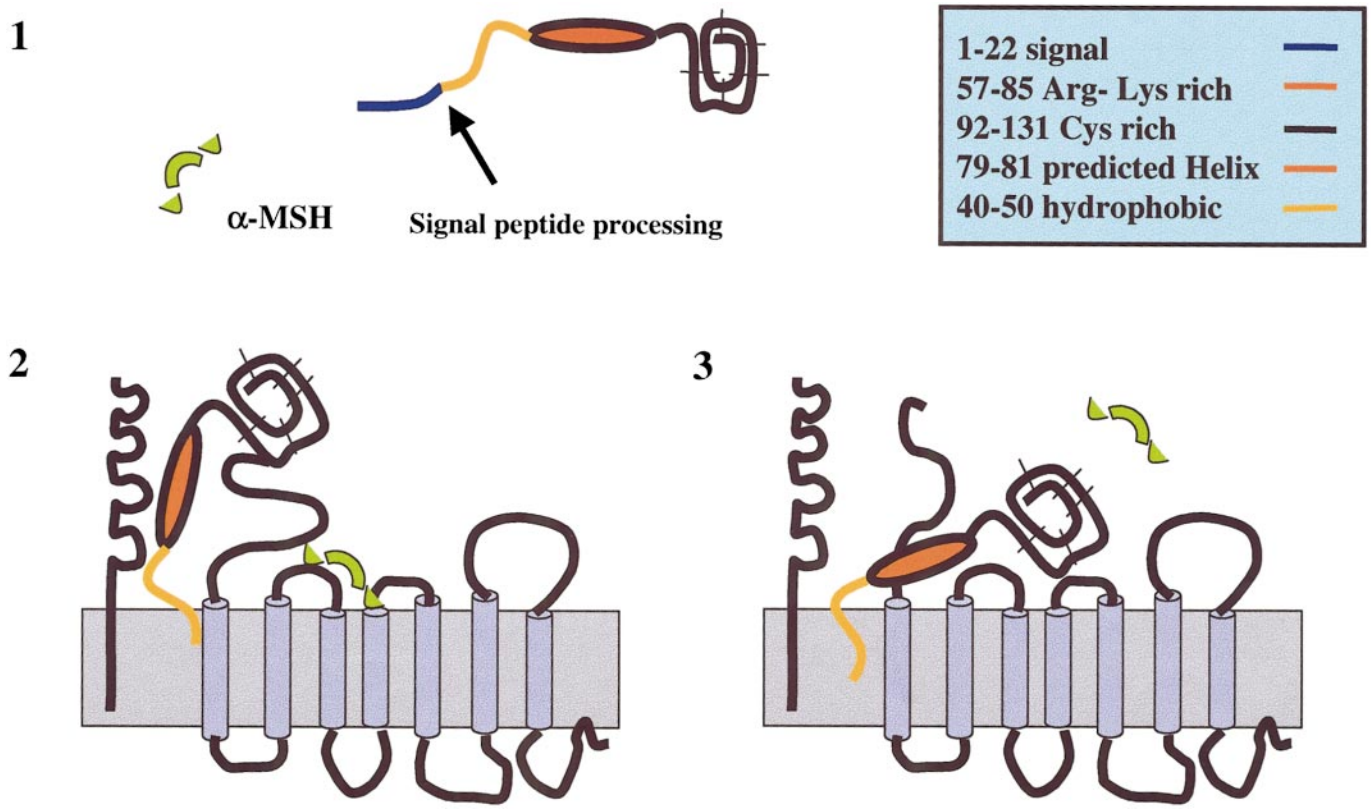

FIG. 6. Schematic showing the putative interactions of ASP with the MC1R. (1) ASP is generated *de novo* and then secreted, leaving behind its signal sequence and moving to the vicinity of melanocytes. (2) ASP might interact at the melanocyte membrane with phospholipids and extracellular matrix components and/or with the mahogany/attractin protein through hydrophobic and basic domains which would provide local concentration and stability of ASP. The C-terminus of ASP may be proteolytically cleaved (not shown). The ASP epitope located in a loop preceding the disulfide bonds may interact with the N-terminal loop of MC1R. (3) The C-terminus of ASP may alternatively interact with extracellular loops 2 and 3 of the MC1R as suggested for AGRP [41].

about the binding of larger peptides to G-protein-coupled receptors [59].

Mechanism of ASP and MC1R Interaction

Some groups have reported that ASP interacts with MC receptors as a competitive antagonist [16], while others have suggested that ASP acts as an inverse agonist, or at least have failed to see direct antagonist effects [36, 60, 61]. The C terminus of ASP showed competitive antagonism of MSH in melanophores while intact recombinant ASP exhibited a more complicated pattern [58]. Figure 6 shows a model consistent with our results in which ASP and its smaller bioactive peptides (ASP18 and ASP9′) bind at a different site than does MSH. Our previously published data suggest that ASP may act as an inverse agonist rather than as an antagonist of the MC1R since ASP by itself is able to elicit changes in cell shape and in the number and type of melanosomes produced [29]. Inverse agonists bind preferentially to the inactive form of their receptors, thereby reducing the population of receptors in the activated form. Inverse agonists promote structural changes in G-protein-coupled receptors [62, 63]. The fact that the shorter ASP peptides do not elicit strong down-regulation of tyrosinase and Tyrp1 mRNAs suggests either that those peptides are too short for optimal interaction with the MC1R or that other regions of ASP (such as the identified region I) are necessary to improve binding and elicit conformational changes in the receptor. Longer peptides should fold more closely to the physiologically relevant structure since the effects caused by terminal residues become more negligible. Our current data, however, do not identify the exact binding site of ASP to the MC1R. Experiments with labeled ASP may help clarify this point. Work is in progress in our laboratory to further elucidate the mechanisms of ASP signaling.

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