Identification of Aim-1 as the underwhite Mouse Mutant and Its Transcriptional Regulation by MITF*

Received for publication, September 21, 2001, and in revised form, November 1, 2001 Published, JBC Papers in Press, November 7, 2001 DOI 10.1074/jbc.M110229200

Jinyan Du and David E. Fisher‡
From the Division of Pediatric Hematology/Oncology, Children’s Hospital and Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

Animal pigmentation mutants have provided rich models for the identification of genes modulating pathways from melanocyte development to melanoma. One mouse model is the underwhite locus, alleles of which manifest altered pigmentation of both eye and fur, sometimes in an age-dependent fashion. Here we show that the mouse homolog of a recently identified gene whose mutation produces Japanese gold-colored fish, medaka b, maps to the mouse underwhite locus. We identify distinct mutations of this gene, known as Aim-1, in three underwhite mouse alleles and find that structure/function differences correlate with recessive versus dominant inheritance. The human ortholog of Aim-1 was originally identified as a melanocyte-restricted antigen that is recognized by autologous T cells from a patient with melanoma. We also provide evidence that Aim-1 is transcriptionally modulated by MITF, a melanocyte-specific transcription factor essential to pigmentation and a clinical diagnostic marker in human melanoma. Although Aim-1 appears to reside downstream of MITF, chromatin immunoprecipitations do not reveal binding of MITF to a 5′-flanking region containing histone 3 acetylation, indicating that MITF either acts indirectly on Aim-1 or it binds to a remote regulatory sequence. Nevertheless, MITF links Aim-1 expression and the underwhite phenotype to a transcriptional network central to pigmentation in mammals.

‡ Nirenberg Fellow in the Division of Pediatric Oncology at Dana Farber Cancer Institute. To whom correspondence should be addressed. Tel.: 617-632-4916; Fax: 617-632-2085; E-mail: david_fisher@dfci.harvard.edu.

This work was funded by National Institutes of Health Grant AR43369 to (D. E. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

A recent study reported a novel transporter protein Aim-1 that is responsible for the pigment phenotype of medaka b gold-fish mutants (1). In this fish, mutations in Aim-1 produce albino-like depigmentation of black melanophores, leaving gold-red xanthophores, white leukophores, and silver iridophores as the remaining pigmented cells. The gene product is predicted to be a 12-transmembrane-transporter protein that shows homology to yeast and plant sucrose transporters (1). Aim-1 is predicted to be a 12-transmembrane-transporter protein that shows homology to yeast and plant sucrose transporters (1). As the remaining pigmented cells. The gene product is predicted to be a 12-transmembrane-transporter protein that shows homology to yeast and plant sucrose transporters (1). As the remaining pigmented cells. The gene product is predicted to be a 12-transmembrane-transporter protein that shows homology to yeast and plant sucrose transporters (1). The major pigmentation hormone, melanocyte-stimulating hormone, up-regulates MITF expression through cAMP signaling followed by cAMP-response element-binding protein phospho-rylation and activation of the melanocyte-specific MITF promoter and may modulate multiple pigmentation genes through up-regulating MITF expression (4, 5). The three major pigmentation enzymes tyrosinase, TYRP1, and DCT, all contain consensys MITF DNA binding elements that are conserved across species and are thought to be transcriptional targets of MITF (6–8). In humans, germline heterozygous MITF mutation produces the pigmentation-deafness condition Waardenburg Syndrome IIA (9) and Tietz syndrome (10, 11), manifesting pigmentation disturbances and deafness due to inner ear melanocyte deficiency (12). Interestingly, MITF expression is usually (if not always) maintained in human melanoma specimens, and it is increasingly used as a histopathologic marker for melanoma diagnosis (13–17).

We BLASTed the human AIM-1 mRNA sequence (accession number AF172849) against the human genome and the gene to chromosome 5. Examination of the homologous region in the mouse genome revealed a previously described hypopigmentation locus, underwhite. Here experiments were carried out to explore a potential link between Aim-1 and the underwhite mutants. Distinct mutations in Aim-1 were found in three underwhite alleles, including a frameshift, as compared with wild-type controls. In addition, evidence is presented that suggests that the global transcriptional regulator of pigmentation, MITF, also resides upstream of Aim-1, linking this gene to the major pigmentation pathway in melanocytes.

EXPERIMENTAL PROCEDURES

BLAST—Human AIM-1 mRNA sequence (accession number AF172849) was BLASTed against the Human Genome. Mouse Aim-1 mRNA sequence (accession number AF360357) was BLASTed against the mouse Trace Archive.

Genomic DNA PCR—Genomic DNA from C57BL/6J and homozygous uwd, uwd, and Uwd mice was obtained from the Jackson Laboratory (Bar Harbor, ME). Genomic DNA from adult male BALB/c kidney tissue was purchased from CLONTECH. Primer pairs spanning each exon were designed as follows. Exon 1: 5′-CTG AGC ACC AGC CAA GAA GCC TAT T-3′ and 5′-CCA GCC TCG GGG TCA TCC AAA GGT G-3′. Exon 2: 5′-TAA AAC CCA ACC TAC AAA ACC AAA ACA-3′ and 5′-GCC ACT TCT TAT CAA CTG ACC CAT TC-3′. Exon 3: 5′-GAA GTG CTC TGC ATG GTG GGA AAT AAA GGA A-3′ and 5′-AGG CAA GAC AAC CAC TGA GCC ACA AAA T-3′. Exon 4: 5′-TCT GGC TGT GGC TCT GAC TAT GA-3′ and 5′-CAT GCC ATT CCT GTC TCC ACT TAG-3′. Exon 5: 5′-TGT CTG TCT GCT TGA ACT CTG G-3′ and 5′-ATA TAA AAT CTG CAT CCT GCT GCT A-3′. Exon 6: 5′-CTC AGT ATC AAA AGA AGT CGT CTA AAA-3′ and 5′-TGT GGC TCA TTA CA-3′.

This paper is available on line at http://www.jbc.org/
TCA TTG TCC TAA AA-3'. Exon 7: 5'–GCC CTG TGG GTT GCT GCC CTG TA-3' and 5'-AGT TGT GCT TGG TGC ATG AGA CTA CCT-3'.

**Chromatin Immunoprecipitation—Chromatin immunoprecipitation assay (ChIPs)** was performed in human primary melanocytes (provided by Dr. Ruth Halahan, Yale University), SKMEL5, or IMR90 cells (ATCC) grown in logarithmic phase. Cells were harvested by scraping, homogenized in a hypotonic buffer (10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 60 mM KCl, 1 m M EDTA, 0.1% Nonidet P40, 5% sucrose, 1 mM GTP), and concentrated adenovirus was added at multiplicity of infection of 100 for each virus. The cells were incubated at 37 °C for 20 min with 500 mM NaCl ChIPs buffer and once with TE, pH 8. The immunoprecipitates were released from the beads by incubating at 65 °C for 20 min in 1% SDS/TE, and proteins were digested by proteinase K without UNG (uracil-N-glycosylase), 0.625 l of each primer (10 μM stock), 0.25 l of the probe (5 μM stock) and 1 l of the template at 100 ng/μl. Reverse transcription proceeded at 48 °C for 30 min. Then 40 cycles of PCR reaction were carried out at 95 °C for 15 s and at 60 °C for 1 min. Real-time PCR was carried out using ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with analysis using the integrated Sequence Detection System Software Version 1.7.

### RESULTS

**Aim-1/B Is Mutated in *uw, uwld, and UwUwDbr* Mice—** We used human map viewer (ncbi.nlm.nih.gov/) to determine that the human ortholog of Aim-1 is located on chromosome 5p. We identified a region homologous to the human Aim-1 locus on mouse chromosome 15 and noted that this site maps to the murine *underwhite* locus (ncbi.nlm.nih.gov/Homology/ and Ref. 19). The complete mouse Aim-1 cDNA sequence (accession number AF360357) was BLASTed against the mouse Trace Archive to identify the exon/intron boundaries (Table I). Primer pairs located in the intron regions were designed to span individual exons. Each exon was amplified from the genomic DNA of *uw, uwld*, and *UwUwDbr* mice as well as the control C57BL/6J and BALB/c strains. All PCR products matched the predicted lengths (data not shown). DNA sequencing identified mutations in all three mutant alleles, but none of their wild-type controls (Fig. 1a). The *uw* mutant was found to harbor a 7-base pair deletion in Exon 3 of *Aim-1* (Fig. 1a), which results in a 43-amino acid frameshift followed by a premature stop at codon 308 (Fig. 1, b and c). The *uwld* allele thus encodes a protein that lacks the C-terminal 6 transmembrane domains of the predicted protein. The *uwld* mutant harbors a single nucleotide mutation from T to C in Exon 6 of *Aim-1* (Fig. 1a), which leads to a mutation from a conserved serine to proline in the tenth transmembrane domain (Fig. 1, b and c). The dominant allele *UwUwDbr* contains a nucleotide alteration from G to A in the second exon (Fig. 1a), which causes a missense point mutation of D153N (conserved residue from medaka to human) in the fourth transmembrane domain (Fig. 1, b and c). These studies thus identify *Aim-1* as the mutant gene in *underwhite* mice.

**MIF5 Regulates Endogenous Aim-1 mRNA Levels in Human Melanoma Cells**—Through recognition of E-1 box DNA elements (CA/C/TGTG) MIF5 is thought to modulate expression of many major pigmentation genes including tyrosinase, RACE, rapid amplification of cDNA ends.

### Table I

| Aim-1 exon no. | Exon size | Coded amino acid no. | 5'-end sequence of exon | 3'-end sequence of exon | Intron size |
|---------------|-----------|----------------------|-------------------------|-------------------------|-----------|
| human         | 410       | 128                  | GTGTGACAGGtataaggggcc   | GTCGTATCAAGtgattgc      | 1786      |
| mouse         | 389       | 128                  | tttcttttagCTTGTGTTGCG   | CTTTCAACAGtgaggaata     | 18215     |
| human         | 177       | 59                   | tttcttttagCTTGTGTTGCG   | CTTTCAACAGtgaggaata     | 9179      |
| human         | 326       | 109                  | tttcttttagCTTGTGTTGCG   | GTCGTATCAAGtgattgc      | 1979      |
| human         | 144       | 48                   | tttcttttagCTTGTGTTGCG   | CTTTCAACAGtgaggaata     | 2633      |
| mouse         | 124       | 41                   | tttcttttagCTTGTGTTGCG   | CTTTCAACAGtgaggaata     | 4178      |
| mouse         | 124       | 41                   | tttcttttagCTTGTGTTGCG   | CTTTCAACAGtgaggaata     | 2289      |
| human         | 212       | 71                   | tttcttttagCTTGTGTTGCG   | CTTTCAACAGtgaggaata     | 71        |
| human         | 212       | 71                   | tttcttttagCTTGTGTTGCG   | CTTTCAACAGtgaggaata     | 71        |
| human         | 248       | 74                   | tttcttttagCTTGTGTTGCG   | CTTTCAACAGtgaggaata     | 74        |
| mouse         | 597       | 74                   | tttcttttagCTTGTGTTGCG   | CTTTCAACAGtgaggaata     | 74        |

1 The abbreviations used are: ChIPs, chromatin immunoprecipitation assay; RACE, rapid amplification of cDNA ends.
TYRP1, and DCT (6–8). Therefore experiments were undertaken to investigate whether MITF regulates AIM-1 expression in the melanocyte lineage. Endogenous MITF activity was manipulated in human melanoma cells (SKMEL5) by infecting with a series of adenoviruses (see “Experimental Procedures” including null adenovirus, adenoviruses over-expressing a control green fluorescence protein fusion from the same promoter, wild-type MITF, or dominant-negative MITF (Arg-218 deletion, which preserves dimerization but ablates DNA binding by heterodimers) (6, 18). Western analyses revealed that expression of adenovirus-encoded MITF proteins is maximal at about 48 h after infection and lasts until at least 96 h (data not shown). Electrophoretic mobility shift assay demonstrated that the DNA binding activities of the wild-type protein and the dominant-negative effects of the mutant protein are strongest during this same period of time (data not shown). To examine the consequences of altering endogenous MITF on AIM-1 expression, RNA was harvested from virus-infected cells 48 and 72 h after infection. Real-time (quantitative) PCR was performed on the RNA samples, and AIM-1 expression values were normalized to GAPDH quantitative PCR signals. As shown in Fig. 2, wild-type MITF stimulated AIM-1 expression significantly over baseline control while dominant-negative MITF repressed its expression. Similar results were also observed in human primary melanocytes (data not shown). These effects were consistently seen at multiple time points examined. The data suggest that MITF can modulate the expression of AIM-1 in human melanoma cells.

An E-Box Repeat Is Present in the Putative Promoter Region of the Human Aim-1 Gene—Examination of the upstream region of the human Aim-1 gene revealed a 1.2-kb repetitive sequence containing 55 E boxes (CACGTG or CATGTG). Fig. 3a depicts representative smaller repeat units found in this region. It is unclear whether this promoter structure (or specific sequence) is conserved in the mouse. A CENSOR (20) search on this repeat against RepBase (21–23) showed no homology to known human repetitive sequences. Also, a BLAST search against the human genome revealed only a few shorter copies of this repeat distributed in non-coding regions. Repeats can arise very quickly during genome propagation, especially if they by chance contain short transcription, recombination, or
Aim-1 Mutations in underwhite Mouse

**FIG. 3.** E-box repeats in the putative promoter of human Aim-1 and chromatin immunoprecipitation. 

**a.** Diagram of the putative promoter of human Aim-1. Representative repeat units in the human E box-containing element are presented. 

**b.** Chromatin immunoprecipitation of the putative human Aim-1 promoter region. Neither of two regions in the AIM-1 promoter (termed 5′- and 3′-fragments on promoter map) was bound by MITF or the other E box-binding proteins USF1, USF2, and c-Myc. A positive control chromatin immunoprecipitation from a separate genomic location is shown for MITF and USF1. The experiment was repeated three times. 

**c.** Endogenous AIM-1 mRNA levels were plotted after normalization to GAPDH.

**DISCUSSION**

The studies described here identify the Aim-1 gene as the locus responsible for the mouse *underwhite* mutations. Truncation of more than 40% of the protein in the recessive *uw* mutant leads to complete loss of pigmentation and exhibits the greatest pigment deficiency of the three alleles in *uw*/*uw* mice. In contrast, the S435P mutation in *uw*/*uw* allele produces a mild coat color dilution in homozygotes. Thus, the *uw* mutation is a strong loss-of-function mutant, which might behave as a functional null while the *uw* mutant leads to complete loss of pigmentation and exhibits the greatest pigment deficiency of the three alleles in *uw*/*uw* mice.

The dominant inheritance pattern of *UwDbr* strongly suggests that Aim-1 functions in a molecular complex with other species, possibly as a homo- or hetero-oligomer. *Aim-1* bears remote sequence homology to the sucrose transporter family (1) whose oligomerization properties are not well studied although dimer or tetramer formation has been reported for other transporter families and genetic data even suggest interactions between different transporters in yeast (24). Since the *UwDbr* mutation occurs within a putative transmembrane motif, it is likely that the altered charge of the asparagine side chain (versus aspartate) disrupts a salt bridge or other necessary secondary structure that contributes to function in a manner that does not ablate intermolecular interactions. However, despite its dominant genetic behavior, this mutation produces a milder phenotype in homozygotes as compared with the frame-shift allele (*uw*), indicating that even in homozygous form the mutant protein retains some wild-type function.

The putative transcriptional start described in this study is...
based on published data using 5'-RACE (1). However, more definitive methods (such as primer extension) will be needed to ascertain the transcriptional start site in mammals. This is of particular importance because the medaka AIM-1 mRNA contains an N-terminal region that is not present in the currently defined human and mouse sequences. Furthermore, no in-frame stop codon is present prior to the putative initiation ATG for human or mouse, suggesting that the current sequence may not contain the true 5'-untranslated region.

Identification of a large E-box containing insertion within the human 5'-flanking region did not correlate with binding by the bHLH zipper protein MITF, despite the fact that MITF could regulate endogenous AIM-1 expression levels. Thus, MITF likely regulates AIM-1 expression via an indirect mechanism (such as an intermediate transcription factor) or via an enhancer element located at a distance from this region. It is also possible that the reported transcriptional start site is incorrect (as discussed above), in which case a different promoter region may be subject to direct MITF binding and regulation. Nonetheless, AIM-1's position downstream of MITF places it into the 'uw' locus to human oculocutaneous albinism type 4 (OCA4) (28). Thus, underwhite mice appear to provide a model for this or other types of oculocutaneous albinism in humans.

Acknowledgments—We thank Dr. George Church for helpful discussion on repetitive sequences. We also thank Dr. Ruth Halaban for providing human primary melanocyte cultures. RepBase Update is thanked for access to their data base of human and rodent repetitive sequences.

REFERENCES

1. Fukamachi, S., Shimada, A., and Shima, A. (2001) Nat. Genet. 29, 381–385
2. Harada, M., Li, Y. F., El-Gamil, M., Rosenberg, S. A., and Robbins, P. F. (2001) Cancer Res. 61, 1089–1094
3. Sweet, H. O., Brilliant, M. H., Cook, S. A., Johnson, K. R., and Davison, M. T. (1980) J. Hered. 89, 546–551
4. Price, E. R., Horstmann, M. A., Wells, A. G., Weilbaecher, K. N., Takemoto, C. M., Landis, M. W., and Fisher, D. E. (1998) J. Biol. Chem. 273, 33042–33047
5. Bertolotto, C., Abbe, P., Hemesath, T. J., Bille, K., Fisher, D. E., Ortonne, J. P., and Baldi, R. (1998) J. Cell Biol. 142, 827–835
6. Hemesath, T. J., Steinriessmann, E., McGill, G., Hansen, M. J., Vaught, J., Bogachkin, C. A., Arneuitteler, H., Copeland, N. G., Jenkins, N. A., and Fisher, D. E. (1994) Genes Dev. 8, 2770–2780
7. Bentley, N. J., Eisen, T., and Goding, C. R. (1994) Mol. Cell. Biol. 14, 7996–8006
8. Yasumoto, K., Yokoyama, K., Shibata, K., Temita, Y., and Shihahara, S. (1994) Mol. Cell. Biol. 14, 8058–8070
9. Tassabehji, M., Newton, V. E., and Read, A. P. (1994) Nat. Genet. 8, 251–255
10. Amiel, J., Watkin, P. M., Tassabehji, M., Read, A. P., and Winter, R. M. (1995) Clin. Dysmorphol. 12, 17–20
11. Smith, S. D., Kelley, P. M., Kenyon, J. B., and Hoover, D. (2000) Neuron 30, 15–18
12. Busam, K. J., Iversen, K., Coplan, K. C., and Jungbluth, A. A. (2001) Am. J. Surg. Pathol. 25, 197–204
13. King, R., Weilbaecher, K. N., McGill, G., Cooley, E., Mihm, M., and Fisher, D. E. (1999) Am. J. Pathol. 155, 731–738
14. Koch, M. B., Shih, I. M., Weiss, S. W., and Felpe, A. L. (2001) Am. J. Surg. Pathol. 25, 58–64
15. Miettinen, M., Fernandez, M., Franssila, K., Gatalica, Z., Lasota, J., and Sarlomo-Rikala, M. (2001) Am. J. Surg. Pathol. 25, 205–211
16. Salti, G. I., Manougian, T., Farolan, M., Shilkaitis, A., Majumdar, D., and Das Gupta, T. K. (2000) Cancer Res. 60, 5012–5016
17. Wu, M., Hemesath, T. J., Takemoto, C. M., Horstmann, M. A., Wells, A. G., Price, E. R., Fisher, D. Z., and Fisher, D. E. (2000) Genes Dev. 14, 301–312
18. Blake, J. A., Eppig, J. T., Richardson, J. E., Bult, C. J., and Kadin, J. A. (2001) Nucleic Acids Res. 29, 91–94
19. Jurka, J., Klonowski, P., Dagman, V., and Pelton, P. (1996) Comput. Chem. 20, 119–121
20. Jurka, J. (1998)Curr. Opin. Struct. Biol. 8, 333–337
21. Jurka, J. (2000) Trends Genet. 16, 418–420
22. Smit, A. F. (1999) Curr. Opin. Genet. Dev. 9, 657–663
23. Van Belle, D., and Andre, B. (2001) Curr. Opin. Cell Biol. 13, 389–398
24. Dickie, M. (1964) Mouse Newsletter 20, 30
25. Lehman, A. L., Silvers, W. K., Puri, N., Wakamatsu, K., Ito, S., and Brilliant, M. H. (2000) J. Invest. Dermatol. 115, 601–606
26. King, R. A., Wirtshafter, J. D., Ols, D. B., and Brumbaugh, J. (1986) Clin. Genet. 23, 42–50
27. Newton, J. M., Cohen-Barak, O., Hagihara, N., Gardner, J. M., Davison, M. T., King, R. A., and Brilliant, M. H. (2001) Am. J. Hum. Genet. 68, 981–984
Identification of Aim-1 as the underwhite Mouse Mutant and Its Transcriptional Regulation by MITF
Jinyan Du and David E. Fisher

J. Biol. Chem. 2002, 277:402-406. doi: 10.1074/jbc.M110229200 originally published online November 7, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M110229200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 28 references, 9 of which can be accessed free at http://www.jbc.org/content/277/1/402.full.html#ref-list-1