The Immunoregulatory Mediator Macrophage Migration Inhibitory Factor (MIF) Catalyzes a Tautomerization Reaction

Evald Rosengren,* Richard Bucala,† Pierre Åman,‡ Lars Jacobsson,* Gerd Odh,* Christine N. Metz,+ and Hans Rorsman§

Departments of *Pharmacology, ‡Clinical Genetics, and §Dermatology, University Hospital, Lund, Sweden
+The Picower Institute for Medical Research, Manhasset, New York, U.S.A.

ABSTRACT

Background: Recent studies of melanin biosynthesis have uncovered an unusual enzymatic activity which converts the non-naturally occurring *D*-isomer of 2-carboxy-2,3-dihydroindole-5,6-quinone (dopachrome) into 5,6-dihydroxyindole-2-carboxylic acid (DHICA). The aim of the present investigation was to isolate and characterize the enzyme catalyzing this tautomerization reaction.

Materials and Methods: After we performed a tissue survey of *D*-dopachrome tautomerase activity, 10 bovine lenses were homogenized and used as a source of enzyme. A soluble fraction was obtained by high-speed centrifugation and subjected to successive FPLC chromatography on Phenyl-sepharose, Mono S cation-exchange, and Superdex gel-filtration. The isolated enzyme was electrophoresed, blotted onto PVDF membrane, and the N terminus analyzed by gas phase micro-sequencing.

Results: The protein catalyzing the conversion of *D*-dopachrome to DHICA was purified to homogeneity in 14% yield and showed a molecular weight of 12 kD when analyzed by SDS-PAGE. The first 27 amino acid residues of this protein were sequenced and found to be identical with those of bovine macrophage migration inhibitory factor (MIF). The catalytic activity of native MIF was confirmed by studies of purified recombinant human MIF, which showed the same tautomerase activity. While *L*-dopachrome was not a substrate for this reaction, the methyl esters of the *L*- and *D*-isomers were found to be better substrates for MIF than *D*-dopachrome.

Conclusions: MIF has been described recently to be an anterior pituitary hormone and to be released from immune cells stimulated by low concentrations of glucocorticoids. Once secreted, MIF acts to control, or counter-regulate, the immunosuppressive effects of glucocorticoids on the immune system. Although the tested substrate, *D*-dopachrome, does not occur naturally, the observation that MIF has tautomerase activity suggests that MIF may mediate its biological effects by an enzymatic reaction. These data also offer a potential approach for the design of small molecule pharmacological inhibitors of MIF that may modulate its potent immunoregulatory effects in vivo.

INTRODUCTION

The mediator originally described as macrophage migration inhibitory factor (MIF) has been redefined recently to be an anterior pituitary hormone, a macrophage cytokine, and a critical component of the host response to septic shock (1–4). MIF exists pre-formed in various cell types and in the last few years has been fortuitously identified in several other organ systems and physiological contexts (5–7). The present report describes an enzymatic activity for MIF that was uncovered during an investigation of the biochemical pathways of melanogenesis.

The late stages of melanin biosynthesis involve the enzymatic conversion of 2-carboxy-2,3-dihydroindole-5,6-quinone (dopachrome) into 5,6-di-

Address correspondence and reprint requests to: Hans Rorsman, Department of Dermatology, University Hospital, S-22185 Lund, Sweden.

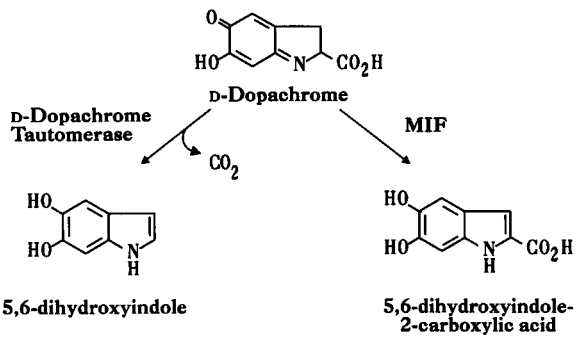

FIG. 1. Scheme for the conversion of *D*-dopachrome catalyzed by *D*-dopachrome tautomerase and by MIF

hydroxyindole-2-carboxylic acid (DHICA) (8,9). Two melanocytic enzymes catalyze the tautomerization of dopachrome to DHICA and are specific for the naturally occurring *L*-dopachrome stereoisomer (10–12). In a recent study of cultured melanoma cells, however, the conversion of *D*-dopachrome to 5,6-dihydroxyindole (DHI) was observed when the *D*-isomer of dopachrome was added as a control substrate (13). Subsequent investigations led to the isolation and the cloning of an enzyme, called *D*-dopachrome tautomerase, that catalyzes the conversion of *D*-dopachrome to DHI by a tautomerization and decarboxylation reaction (14) (Fig. 1).

We report herein the isolation from the bovine eye lens of a second, distinct enzyme which catalyzes the tautomerization of *D*-dopachrome to DHICA. Purification and N-terminal sequence analysis of the protein responsible for this activity identified it to be the bovine homolog of MIF. The enzymatic activity of purified, native MIF was confirmed by studies of recombinant human MIF, which also catalyzed the same tautomerization reaction.

MATERIALS AND METHODS

Reagents

DHI and DHICA were a gift from Professor G. Prota at the Department of Organic Chemistry, Naples, Italy. *D*-Dopa methylester was obtained from Dr. C. Hansson, Department of Dermatology, Lund, Sweden. Phenyl-Sepharose, PD-10 Sephadex, Mono S HR5/5, and Superdex 75 HR10/30 columns were purchased from Pharmacia-LKB (Uppsala, Sweden). Human recombinant MIF was purified from the pETIIb *Escherichia coli* plasmid expression system, denatured by dissolution in 8 M urea and 5 mM DTT, and carefully refolded by equilibrium dialysis (15). All other reagents were of highest grade commercially available.

Tissue Preparation and Extraction

Ten bovine eyes were obtained from an abattoir immediately after the sacrifice of the cows and transported to the laboratory on ice. The lenses were carefully excised, kept on ice, and Dounce homogenized (KEBO, Stockholm, Sweden) in 20 ml of KH_2PO_4 buffer, pH 7.2 (3 ml/lens). The homogenate was centrifuged at 15,000 × *g* for 45 min and the supernatant poured off and reserved. The sediment then was re-extracted twice with 20 ml of KH_2PO_4 buffer (pH 7.2) and the extracts pooled with the supernatant.

Purification of *D*-Dopachrome Tautomerase Activity

The bovine lens supernatant first was applied to an HIC phenyl-sepharose column (15 × 50 mm) equilibrated previously with 20 mM KH_2PO_4 buffer, pH 7.2. The column was washed with 50 ml of the same buffer and then eluted with 40% ethylene glycol in 20 mM KH_2PO_4 buffer (pH 7.2) at a flow rate of 0.3 ml/min. The fractions which catalyzed DHICA formation from *D*-dopachrome were pooled and concentrated with a Centricon-10 concentrator (Amicon, Beverly, MA, U.S.A.) following the manufacturer's instructions. The buffer then was changed to 50 mM NaAc (pH 5.5) and the protein-containing solution passed through a PD-10 Sephadex column (equilibrated previously with 50 mM sodium acetate buffer, pH 5.5).

The sample which eluted from the PD-10 column was applied to a Mono S HR5/5 FPLC column equilibrated with 50 mM NaAc buffer, pH 5.5. The column was washed with 5 ml of the equilibration buffer and then eluted with a linear gradient of 0.0–1.0 M NaCl in the same buffer. The flow rate was 1 ml/min and elution continued for 25 min. The fractions catalyzing DHICA formation were concentrated with a Centricon-10 concentrator and applied to a PD-10 Sephadex column where the buffer was changed to 20 mM Tris (pH 7.1) and 50 mM NaCl.

The sample obtained after PD-10 chromatography was applied to a Superdex 75 HR 10/30-FPLC column equilibrated previously with a

buffer consisting of 20 mM Tris (pH 7.1) and 50 mM NaCl. Elution was with the same buffer and at a flow rate of 0.5 ml/min. Molecular weight determination of the eluted proteins was with reference to BSA (67 kD), carbonic anhydrase (30 kD) and myoglobin (14.2 kD). All protein concentrations were determined by the Bradford method (16) using the Bio-Rad protein assay kit (Munich, Germany), or with Folin-Ciocalteu's Phenol reagent Merck (Darmstadt, Germany) as described by Lowry *et al.* (17). Bovine serum albumin (BSA) was used as a reference standard in both cases.

Protein Electrophoresis and N-Terminal Amino Acid Sequence Analysis

Protein purity was assessed by SDS-PAGE as described by Laemmli (18). Gels were stained with Coomassie brilliant blue and the relative content of chromatographed protein estimated by laser densitometry after comparison with known quantities of BSA (Personal Densitometer; SI Molecular Dynamics, Sunnyvale, CA, U.S.A.). For sequence analysis, proteins were electroblotted onto polyvinylidene difluoride membrane (Millipore, Bedford, MA, U.S.A.), using a semi-dry electroblotting apparatus (JKA-Biotech, Copenhagen, Denmark) and following the procedures of Matsudaira (19). The membrane was stained with Coomassie Brilliant blue and the protein bands excised and subjected to gas phase micro-sequencing analysis at the Biomolecular Resource Facility (Lund, Sweden). An ABI 477 protein/peptide sequencer was utilized and the Edman degradation products were identified by HPLC and on-line PTH detection.

D-Dopachrome Tautomerase Assay

D-dopachrome conversion to DHI or DHICA was measured in 1 ml sample cuvettes as previously described (12,20). *L*-dopachrome was used as a control substrate. The substrate specificity of MIF was determined spectrophotometrically by measuring the rate of the decrease of the iminochrome absorbance at 475 nm. The reactions were analyzed at room temperature in a Varian spectrophotometer with 0.42 mM substrate and 1.4 μg of human rMIF in 10 mM sodium phosphate buffer, pH 6.0, containing 0.1 mM EDTA. The substrates were prepared from the corresponding catecholic compounds by oxidation with sodium periodate (12,20).

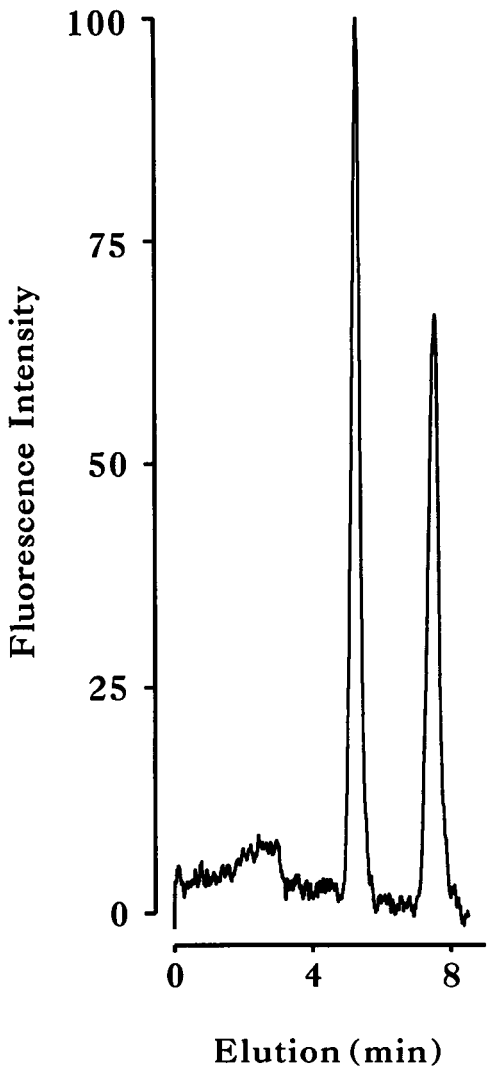

FIG. 2. Representative HPLC tracing of fluorophores formed by crude bovine lens homogenate after the addition of *D*-dopachrome as described in Materials and Methods

The mobile phase consisted of 20 mM KH_2PO_4 buffer (pH 4.0) and 15% methanol. The flow rate was 1.2 ml min^{-1}. Fluorimetric detection was at 295/345 nm and is expressed in arbitrary units. Retention time: DHI 5.4 min, DHICA 7.8 min.

RESULTS

Crude bovine lens homogenates catalyzed the conversion of *D*-dopachrome but not *L*-dopachrome into two products which were identified by chromatographic comparison with authentic compounds to be DHI and DHICA (Fig. 2) (12). DHI and DHICA formed at a rate of 22 and 7.5 μmol/min respectively, from a homogenate prepared from 10 lenses. Treatment by boiling or

TABLE 1. Purification scheme for the protein catalyzing the conversion of *D*-dopachrome into DHICA

Step	Total protein (mg)	Enzyme Activity (μmol DHICA min^{-1})	Specific Activity (μmol DHICA min^{-1} mg^{-1})	Yield (%)
Homogenate	6980	7.5	0.001	100
High-speed supernatant	3770	6.6	0.002	88
Phenyl-sepharose	200	2.9	0.014	39
Mono S	0.90	1.6	1.8	22
Superdex	0.28	1.1	4.0	14
SDS-PAGE	0.10	—	—	—

The total starting material was a homogenate of 10 bovine lenses.

by adding subtilopeptidase A eliminated the catalytic activities. The formation of DHI was attributed to *D*-dopachrome tautomerase, the enzyme which catalyzes both tautomerization and decarboxylation (13), and the factor responsible for producing DHICA was investigated further by protein purification.

The protein catalyzing DHICA formation from *D*-dopachrome was found to be soluble and was recovered in the supernatant obtained by high-speed centrifugation of the crude lens homogenate (Table 1). Importantly, this catalytic activity was retained by Phenyl-Sepharose under conditions (20 mM KH_2PO_4 buffer, pH 7.2) which eluted *D*-dopachrome tautomerase. The protein catalyzing DHICA formation was subsequently eluted in 20 mM KH_2PO_4 buffer (pH 7.1) containing 40% ethylene glycol. Further purification was achieved by FPLC with Mono S, followed by Superdex 75. When analyzed by gel-filtration, the active enzyme had a molecular weight of approximately 25 kD.

The purity and homogeneity of the bovine lens enzyme were established by SDS-PAGE (Fig. 3). Under reducing conditions, a single polypeptide of molecular weight 12 kD was observed. A 27–amino acid N-terminal sequence was obtained and identified this protein to be the bovine homolog of MIF (Fig. 4) (6).

A procedure for the preparation of pure, bioactive recombinant MIF was recently described (15). The ability of native MIF to catalyze the tautomerization of *D*-dopachrome to DHICA thus was confirmed by demonstrating an identical enzymatic activity for human recombinant MIF (Fig. 5). As expected, this catalytic activity was specific for the *D*-stereoisomer of dopachrome. There was no detectable activity with dopaminochrome, which lacks a 2-carboxy group. Esterification of the 2-carboxy group was found to increase reaction velocity by almost 50-fold compared with *D*-dopachrome. Esterification also minimized the stereoselectivity of the reaction as the *L*-dopachrome carboxy ester was almost as effective a substrate as the *D*-dopa-

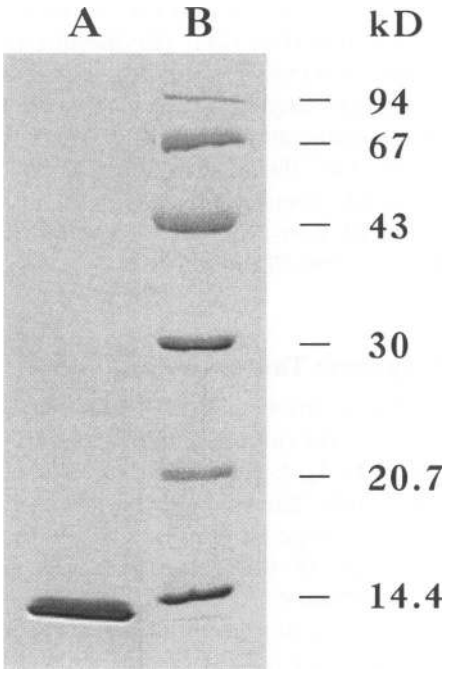

FIG. 3. SDS-PAGE analysis of the protein isolated from bovine lens catalyzing the conversion of *D*-dopachrome into DHICA (Lane A) and protein molecular weight markers which ranged in size from 14.4 to 94 kD (Lane B)

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          1                  10                  20          27
I.   PMFVVNTNVPRASVPDGLLSELTQQLA
     | | | | | | | | | | | | | | | | | | | | | | | | | | | |
II.  PMFVVNTNVPRASVPDGLLSELTQQLA
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FIG. 4. N-terminal amino acid sequence of the purified bovine lens protein catalyzing the tautomerization of *D*-dopachrome into DHICA (I.), and of bovine MIF (II.)

chrome methyl ester. The highest reaction velocity was 0.3 mmoles min^{-1} per mg protein and was achieved with *D*-dopachrome methyl ester as substrate (0.4 mM, pH 6.0, 25°C). Substrate inhibition was observed at higher substrate concentrations.

DISCUSSION

The present study demonstrates MIF to be a tautomerase that can convert *D*-dopachrome to DHICA. The enzymatic tautomerization of *D*-dopachrome first was observed in studies of cultured melanoma cells in which the *D*-isomer was utilized as a control for the conversion of the naturally occurring substrate, *L*-dopachrome. The liver manifests a high level of *D*-dopachrome tautomerization activity (13), a result that is consistent with recent immunohistochemical data showing hepatocytes to be an abundant source of pre-formed MIF protein (M. Bacher et al., unpublished observations). Although *D*-dopachrome does not occur naturally, the strict substrate requirements for the MIF-catalyzed conversion of *D*-dopachrome to DHICA nevertheless suggest that MIF may mediate its biological effects by an enzymatic reaction. Identification of the true biological substrate for MIF is likely to provide important information about the mechanism of action of this immunoregulatory mediator.

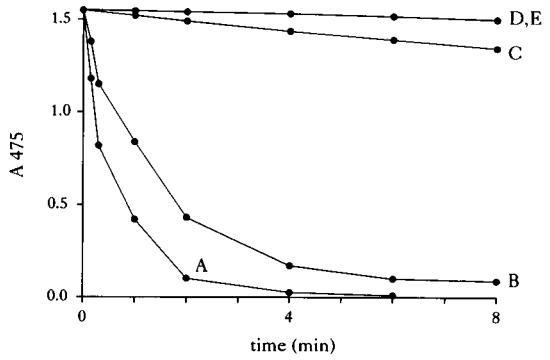

FIG. 5. *D*-dopachrome tautomerase activity of human recombinant MIF

Changes in absorption at 475 nm after incubation of the indicated iminochromes (0.42 mM) with MIF (1.4 μg). A, *D*-dopachrome carboxy methyl ester; B, *L*-dopachrome carboxy methyl ester; C, *D*-dopachrome; D, *L*-dopachrome; E, dopaminochrome.

MIF exhibits an extremely high sequence conservation among the various species which have been examined so far (1,6,7,15). The mouse and human MIF sequences show 90% identity at the amino acid level, which among cytokines is the highest mouse to human homology described to date. Interestingly, MIF and *D*-dopachrome tautomerase, the enzyme catalyzing the tautomerization and decarboxylation of *D*-dopachrome to DHI, share a low level of structural homology. The two proteins are 117 and 115 amino acids in length, respectively, show conservation in the position of a cysteine residue (Cys^{56}), and exhibit an overall 27% sequence identity at the amino acid level (14). If conservative amino acid substitutions are considered, the overall sequence homology between the two proteins rises to 54%. Neither MIF nor *D*-dopachrome tautomerase share any apparent structural similarities with the membrane-bound, melanocytic enzymes that catalyze the conversion of *L*-dopachrome into DHICA (11,12,21–23).

D-dopachrome remains the only substrate known for *D*-dopachrome tautomerase (13) which shows a maximum velocity of 0.5 mmoles min^{-1} per mg protein. MIF is less active than *D*-dopachrome tautomerase in catalyzing the conversion of *D*-dopachrome, but is more active than this enzyme in converting *D*-dopachrome methyl ester. In the case of MIF, a reaction velocity of 0.3 mmoles min^{-1} per mg protein was observed. Dopachrome conversion to indoles may occur by two routes (24). The first proceeds via the initial conversion of dopachrome to indolenine and subsequent rearrangement to an indole. The second route, by contrast, involves the formation of a quinone methide intermediate which then is transformed further into an

indole. Whether the natural substrate for MIF proceeds through similar intermediates is worthy of consideration and may assist in the search of the true in vivo substrate of the MIF-catalyzed reaction.

The observation that MIF can act as an enzyme potentially opens a new dimension in our understanding of this mediator, which appears to function physiologically as a counter-regulator of glucocorticoid action within the immune system. In addition to being secreted by the anterior pituitary, MIF is released from immune cells by both pro-inflammatory stimuli and by glucocorticoids (1–4). Once released, MIF overrides the immunosuppressive effects of glucocorticoids on inflammatory cytokine production and acts to modulate the potent anti-inflammatory properties of glucocorticoids which are necessarily produced as part of the host response to infection and tissue invasion. The finding that MIF, a soluble immunoregulatory mediator, also exhibits an isomerization activity is reminiscent of the protein cyclophilin, which is secreted in response to pro-inflammatory stimuli (25), but which also functions intracellularly as a peptidyl-prolyl *cis-trans* isomerase and is a target for the powerful immunosuppressant drug cyclosporin (26).

In conclusion, this study identifies MIF to have tautomerase activity and suggests that elucidation of its true substrate may offer important insight into the mechanism of action of this potent, immunoregulatory protein. These data also provide an approach for the rational design of small molecule, pharmacological inhibitors of MIF that can act in an anti-inflammatory capacity by increasing the therapeutic efficacy of glucocorticoids.

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REFERENCES

1. Bernhagen J, Calandra T, Mitchell RA, et al. (1993) MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. *Nature* **365:** 756–759.
2. Calandra T, Bernhagen J, Mitchell RA, Bucala R. (1994) The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. *J. Exp. Med.* **179:** 1895–1902.
3. Nishino T, Bernhagen J, Shiiki H, Calandra T, Dohi K, Bucala R. (in press) Localization of macrophage migration inhibitory factor (MIF) to secretory granules within the corticotrophic and thyrotrophic cells of the pituitary gland. *Mol. Med.*
4. Calandra T, Bernhagen J, Metz CN, et al. (1995) Identification of MIF as a glucocorticoid-induced mediator and counter-regulator of glucocorticoid Inhibition of cytokine production. *Nature* **377:** 68–71.
5. Wistow GJ, Shaughnessy MP, Lee DC, Hodin J, Zelenka PS. (1993) A macrophage migration inhibitory factor is expressed in the differentiating cells of the eye lens. *Proc. Natl. Acad. Sci. U.S.A.* **90:** 1272–1275.
6. Galat A, Riviere S, Bouet F. (1993) Purification of macrophage migration inhibitory factor (MIF) from bovine brain cytosol. *FEBS Lett.* **319:** 233–236.
7. Sakai M, Nishihira J, Hibiya Y, Koyama Y, Nishi S. (1994) Glutathione binding rat liver 13k protein is the homologue of the macrophage migration inhibitory factor. *Biochem. Mol. Biol. Int.* **33:** 439–446.
8. Prota G. (1992) *Melanins and Melanogenesis.* Academic Press, San Diego.
9. Pawelek JM. (1991) After dopachrome? *Pigment Cell Res.* **4:** 53–62.
10. Pawelek JM. (1990) Dopachrome conversion factor functions as an isomerase. *Biochem. Biophys. Res. Commun.* **166:** 1328–1333.
11. Tsukamoto K, Jackson IJ, Urabe K, Montague PM, Hearing VJ. (1992) A second tyrosinase-related protein, TRP-2, is a melanogenic enzyme termed DOPAchrome tautomerase. *EMBO J.* **11:** 519–526.
12. Winder AJ, Wittbjer A, Rosengren E, Rorsman H. (1993) The mouse *brown* (b) locus protein has dopachrome tautomerase activity and is located in lysosomes in transfected fibroblasts. *J. Cell Sci.* **106:** 153–166.
13. Odh G, Hindmith A, Rosengren A-M, Rosengren E, Rorsman H. (1993) Isolation of a

new tautomerase monitored by the conversion of *D*-dopachrome to 5,6-dihydroxyindole. *Biochem. Biophys. Res. Commun.* **197:** 619–624.

14. Zhang M, Åman P, Grubb A, et al. (1995) Cloning and sequencing of a cDNA encoding rat *D*-dopachrome tautomerase. *FEBS Lett.* **373:** 203–206.
15. Bernhagen J, Mitchell RA, Calandra T, Voelter W, Cerami A, Bucala R. (1994) Purification, bioactivity and secondary structure analysis of mouse and human macrophage migration inhibitory factor (MIF). *Biochemistry* **33:** 14144–14155.
16. Bradford MM. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
18. Laemmli UK. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277:** 680–685.
19. Matsudaira P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262:** 10035–10038.
20. Aroca P, Solano R, Garcia-Borron JC, Lozano JA. (1991) Specificity of dopachrome tautomerase and inhibition by carboxylated indoles. *Biochem. J.* **277:** 393–397.
21. Shibahara S, Tomita Y, Sakakura T, Nager C, Chadhuri B, Müller R. (1986) Cloning and expression of cDNA encoding mouse tyrosinase. *Nucleic Acids Res.* **14:** 2413–2427.
22. Jackson JJ. (1988) A cDNA encoding tyrosinase-related protein maps to the *brown* locus in mouse. *Proc. Nat. Acad. Sci. U.S.A.* **85:** 4392–4396.
23. Jackson JJ, Chambers DM, Tsukamoto K, et al. (1992) A second tyrosinase-related protein, TRP-2, maps to and is mutated at the mouse *slaty* locus. *EMBO J.* **11:** 527–535.
24. Sugumaran M, Semensi V. (1991) Quinone methide as a new intermediate in eumelanin biosynthesis. *J. Biol. Chem.* **266:** 6073–6078.
25. Sherry B, Yarlett N, Strupp A, Cerami A. (1992) Identification of cyclophilin as a proinflammatory secretory product of lipopolysaccharide-activated macrophages. *Proc. Natl. Acad. Sci U.S.A.* **89:** 3511–3515.
26. Schreiber SL, Crabtree GR. (1992) The mechanism of action of cyclosporin A and FK506. *Immunol. Today* **13:** 136–142.

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