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Human placental protein/peptides stimulate melanin synthesis by enhancing tyrosinase gene expression

Chinmoy Sarkar,[1] Suman Kumar Singh,[1] Samir Kumar Mandal,[3] Bidisha Saha,[1] Rabindranath Bera,[1] Jagnyeswar Ratha,[1] Pijush Kanti Datta[2] and Ranjan Bhadra[1]

[1]*Department of Cellular Biochemistry, Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Jadavpur, Kolkata, India;* [2]*Department of Dermatology and STD, Medical College and Hospital, Calcutta, Kolkata, India;* [3]*Biomedical Research Division, The University of Texas Health Center at Tyler, Tyler, Texas, USA*

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Abstract

Placental protein/peptides as biological response modifier are well documented, but not much known about melanogenesis. We possibly for the first time, demonstrated melanogenesis in B16F10 mouse melanoma by a placental protein/peptide fraction (PPPF) prepared from a hydroalcoholic extract of fresh term human placenta. This study described the effect of PPPF on the induction of tyrosinase; the key enzyme of melanogenesis to investigate the basis of PPPF induced pigmentation in primary melanocyte and B16F10 melanoma. Tyrosinase induction by PPPF in B16F10 cells was found dose- and time dependent at the level of activity. Tyrosinase, at the level of transcription and protein expression when assessed by RT-PCR and Western blot analyses found to have considerable induction over untreated control. PPPF led to enhanced activation of tyrosinase promoter resulting higher transcription thus substantiating the role of PPPF as a stimulator of melanogenesis. Actinomycin D, the transcriptional inhibitor of protein synthesis, blocked the stimulatory action of PPPF since the induction of tyrosinase and melanin was markedly reduced in presence of this inhibitor. Thus the results suggested that PPPF mediated increase in tyrosinase expression occurred through transcriptional upregulation to stimulate melanogenesis in B16F10 cells and in primary melanocyte also. (Mol Cell Biochem **285:** 133–142, 2006)

Key words: B16F10 melanoma/melanocyte, melanogenesis, placental protein peptide fraction (PPPF), tyrosinase promoter

Introduction

Skin color depends on the synthesis and distribution of the pigment melanin by the specialized skin cell melanocytes, which along with adjacent keratinocytes constitute epidermal melanin unit [1–3]. Melanin produced within melanosome, a membrane bound organelle of melanocyte, is transferred to the neighboring keratinocytes and provides protection to the skin and the body against the deleterious effect of UV irradiation [2, 3]. Biosynthesis of melanin is a very complex phenomenon and controlled at various points. The synthesis and expression of different melanogenic enzymes and their inhibitors play a crucial role in accomplishing melanin synthesis inside melanosome. Tyrosinase, the copper containing key regulatory bifunctional enzyme in melanin biosynthesis, catalyses the conversion of essential amino acid L-tyrosine to

Address for offprints: R. Bhadra, Department of Cellular Biochemistry, Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Jadavpur, Kolkata-700 032, India (E-mail: rbhadra@iicb.res.in)

L-3, 4-dihydroxyphenylalanine (L-DOPA) and then its oxidation to L-dopaquinone to produce a highly polymerised melanin pigment [2–6]. In addition to tyrosinase, two other melanogenic enzymes identified as tyrosinase related protein-1 and -2 (TRP-1 and TRP-2) also play crucial role in this synthesis [2, 3, 7, 8]. Induction of melanogenesis in the skin is mainly influenced and controlled by the locally released peptide like stimulators or hormones such as ET-1, ACTH, α-MSH and β-endorphin. They impart such action by enhancing tyrosinase and other melanogenic enzyme activity and expression through receptor mediated mechanism [9–16].

Human placenta being served as a passage of nutrients from mother to foetus is enriched in numerous bioactive components among which some are melanocyte activity modulators. Presence of pro-opiomelanocortin peptides and corticotrophin releasing factor and other growth factors in human placenta has already been detected [17–21]. Among these FGF-2 is known to act as a strong mitogen for human melanocyte [22] whereas, pro-opiomelanocortin peptides such as adrenocorticotrophic hormone (ACTH), α-melanocyte stimulating hormone (α-MSH), β-endorphins are potent inducer of melanogenesis in melanocyte/melanoma system [10–16]. The term human placental extract prepared by heat alcohol treatment has been shown to induce melanin synthesis in both *in vitro* mouse melanoma and *in vivo* when studied with age onset hair graying C57BL/6J black mouse as animal model [23]. In an earlier study chemical analysis revealed the presence of lipids (including sphingolipids), small proteins or peptides of molecular weight less than 20 kDa, carbohydrates and nucleotides in the extract [24]. Before these studies in a patent [25] ET-1 like peptides and glycosphingolipids were predicted as the active factors in the extract as melanin inducer to claim the extract as a therapeutic material in treating hypomelanotic disease vitiligo. The resolution of the placental extract was made into protein/peptide and sphingolipid enriched lipid fraction stating as PPPF (placental protein/peptide fraction) and PTLF (placental total lipid fraction) respectively, to test their effect separately on the melanogenesis in B16F10 cell in culture. Traces of ACTH and ET-1, but not α-MSH were detected in the PPPF but not at a concentration effective for the induction of melanin synthesis. However PPPF induced melanin synthesis effectively [26] like PTLF [27]. PTLF induced melanogenesis by transcriptional activation of tyrosinase promoter through the stress specific p38 MAPK signaling pathway leading to higher expression of tyrosinase gene [28]. PPPF also enhanced melanin synthesis in the same cellular system posing to investigate the underlying mechanism involved. So this study was taken up to investigate the effect of PPPF on tyrosinase induction both at the level of activity and gene expression to clarify the basis of PPPF mediated increase in melanin production.

Materials and methods

Reagents

Dulbecco's modified eagle medium (DMEM), foetal bovine serum (FBS), penicillin, streptomycin, neomycin (PSN) antibiotic, and trypsin/ethylenediaminetetraacetic acid (EDTA), OPTI-MEM reduced serum medium and other medium supplements were obtained from Gibco BRL (Grand Island, NY, USA). Plastic wares were purchased from NUNC, Roskilde, Denmark. MCDB 153, insulin, basic fibroblast growth factor (bFGF), bovine pituitary extract (BPE), isobutyl methyl xanthine (IBMX), 12-phorbol-13-myristate-acetate (PMA), mushroom tyrosinase, cycloheximide, actinomycin D, horseradish peroxidase-conjugated secondary antibodies, DAPI (4′,6′-diamidino-2-phenylindole) stain, DNase, diethyl pyrocarbonate, formaldehyde, 3-[N-Morphplino]propanesulfonic acid (MOPS) buffer, [Nle^4, D-Phe^7]-α-MSH and, antibiotic G418 (Geneticin disulfate) salt were procured from Sigma Chemical Co., St. Louis, MO, USA. Chemiluminescent Horseradish peroxidase Western blot detection system (LumiGLO Reagent and peroxide #7003) was purchased from Cell Signaling technology, Inc, MA, USA. All other chemicals used are of highest purity available.

Cell culture

B16F10 mouse melanoma cell line procured from National Center for Cell Science (Pune, India) was used throughout the study. Cells were grown in DMEM supplemented with 10% FBS and 1% penicillin, streptomycin and neomycin antibiotic at 37 °C in a humidified incubator with 5% CO_2. For induction study semi-confluent cells were harvested with 0.025% trypsin and 0.52 mM EDTA in phosphate buffered saline (PBS) and plated at a density of 1×10^6 cells/well in a 6-well plate. 24 h later medium was replaced with fresh DMEM containing 2% FBS and 1% penicillin, streptomycin and neomycin antibiotics and cells were incubated with or without the test substances. In some experiments, the transcriptional inhibitor actinomycin D (5 μg/ml) was added either alone or in combination with PPPF.

Normal human melanocyte culture was established from the surgical skin specimen according to the modified method of Eisinger and Marko [29]. The skin specimens after being cut into small pieces were kept overnight at 4 °C in a solution containing 0.25% trypsin, 0.05% EDTA, PSN in PBS. After incubation the epidermis was isolated from dermis by the means of forceps and the cell suspension was prepared as described by Eisinger and Marko. The cells were pelleted down by centrifugation at $500 \times g$ for 10 min and were resuspended and cultured in MCDB153 supplemented with 50 ng/ml

12-O-tetradecanoyl phorbol 13-acetate, 0.1 mM isobutylmethyl xanthine, 6 ng/ml bFGF, 5 μg/ml insulin, 1 μg/ml transferrin, 0.18 μg/ml hydrocortisone, 5% FBS and PSN at 37 °C in humidified atmosphere containing 5% CO_2. 24 h later medium was replaced and changed regularly every 2 days with fresh medium. When almost 70% confluency was reached cells were passaged and used for the experiment. To evaluate the effect of test substances on melanocytes the medium used in the experiment was depleted with TPA, BPE, IBMX and bFGF.

Preparation of placental protein/peptide fraction (PPPF) and placental total lipid fraction (PTLF)

The preparation of PPPF described before [26] was based on a published method [30]. Briefly the sticky mass left after the evaporation of the vehicle of the hydroalcoholic placental extract in a rotavapour (under vacuum, at 40 °C) was collected and then treated thrice with diethyl ether and then with ether-ethanol (3:1 *v/v*) mixture for three times at 4 °C. The residual material left was described as placental protein/peptide fraction or PPPF dissolved in water and used throughout this study. The amount of PPPF was determined by Lowry [31] using BSA as a standard. The supernatant thus obtained in all the steps was collected and evaporated to dryness in rotavopour. The dried mass was then dissolved in chloroform methanol mixture (2:1) to get the PTLF. Prior to addition to the cell culture media the PTLF solution was dried under reduced pressure in vacuum desiccator (Tarsons, India) and reconstituted in 2% FBS containing DMEM.

Tyrosinase activity

Tyrosinase activity was determined spectrophotomatrically according to the method of Nakazawa *et al*. 1998 [32], using L-DOPA as the substrate. Briefly, B16F10 cells cultured for 48 h with or without the test samples, were washed twice with ice-cold PBS and extracted by sonication in 100 μl of 0.1 M Tris-HCl buffer (pH 7.2) containing 1% Nonidet P-40, 0.01% SDS, 100 μM phenyl methyl sulfonyl fluoride, and 1 μg/ml aprotinin and then centrifuged at 10,000 g for 10 min at 4 °C. The supernatants collected were used for enzyme assay and its protein content was estimated by method of Lowry using BSA as standard [31]. Samples of cell extract supernatant were incubated in duplicate for 1 h at 37 °C in 1 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1% L-DOPA. The absorbance was then monitored at 450 nm in a Shimadzu Spectrophotometer (Model: UV-2401 PC, UV-VIS; Shimadzu Corporation, Kyoto, Japan) and compared with purified mushroom tyrosinase (Sigma). The standard curve was prepared limiting linearity within the range of experimental values.

Measurement of melanin content

Melanin content was determined as per the previously reported method [33] with slight modifications. Briefly, after the treatment with the test substances cells were washed with PBS and then detached from the test plate using trypsin/ethylenediaminetetraacetic acid (EDTA). An aliquot was used for cell count and the remaining cells were pelleted down and lysed with 200 μl of 1 (N) NaOH. In an ELISA Reader (Model: Emax, Molecular device, USA) melanin was estimated by measuring the absorbance at 405 nm using synthetic melanin as standard.

Western blot analysis

To determine the amount of tyrosinase expressed as proteins, Western blot analysis was performed by the techniques as described earlier [10]. Briefly, 25 μg of total protein from each cell extract was electrophoresed in reducing 10% SDS-PAGE, as described by Laemmli [34]; and blotted on PVDF membranes (Immobilon-P, Millipore Corporation, Bedford, MA, USA) using a Transblot system (Transblot SD: Semidry transfer cell, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 2% BSA in PBS for overnight at 4 °C and were then probed with primary anti-tyrosinase (H-109) antibody (1:100 dilution, Santacruz Biotechnology) for 1 h at room temperature. After washing extensively the blots were incubated with horseradish peroxidase conjugated secondary antibody for 1 h and signals were visualized with enhanced chemiluminescences detection reagents. Loading control experiments were also carried out using antibody to β-actin. Membranes were processed for densitometric analysis using an imaging densitometer (Model: Image Scanner, Amarsham Pharmacia Biotech, Buckinghamshire, UK) and a Software (Image Master Total lab version 1.11).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cells using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription and polymerase chain reaction were carried out with 1 μg total isolated RNA sequentially in the same tube using Qiagen one step RT-PCR kit (Qiagen). All procedures were conducted following the manufacturer's instructions. The oligonucleotide primers used for PCR are as follows: tyrosinase upstream 5′-GGC CAG CTT TCA GGC AGA GGT-3′; downstream 5′-TGG TGC TTC ATG GGC AAA ATC-3′: actin upstream 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′; downstream 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′. The reaction was cycled 25 times through 60 s at 94 °C, 60 s at 56 °C, and 60 s at 72 °C. Fifty percent of reaction

mixture was analysed by electrophoresis on 1% agarose gels and stained with ethidium bromide. In order to check the reproducibility of the results, each experiment was carried out more than three times. Specific primers for actin were used as a control.

Transfection

The expression vector pTyrP. EGFP, plasmid encoding enhanced green fluorescent protein (EGFP) where cytomegalovirus (CMV) promoter in the pEGFP plasmid was replaced with the 2,500 bp Tyrosinase promoter, was a generous gift from Dr. Ruth Halaban, Yale University School of Medicine, New Haven, CT, USA. Vector included the neomycin gene conferring resistance to geneticin (G418) [33]. B16F10 cells were transfected using Lipofectamine 2000 (Invitrogen Life Technologies, Carisbad, California, USA). Briefly, cells were grown to 50–60% confluence in six well plates for 16 h before transfection, and 2 μg of expression vector was mixed with 5 μl of Lipofectamine in 1 ml of OPTI-MEM reduced serum medium. Cells were incubated with the transfection mixture for 6 h, washed three times with DMEM and incubated in fresh DMEM containing 10% FBS for 48 h. The transfected cells were selected for 20 days using 1.6 mg/ml G418. Then the stable transfected cells were cultured for 48 h with or without the 50 μg/ml PPPF. Effects of PPPF-induced transcriptional activation were analysed by GFP expression under inverted fluorescence microscope (Model: OLYMPUS 1X70, Olympus Optical Co. Ltd., Shibuya-ku, Tokyo, Japan). To visualize cellular population in the treated as well as untreated control, DAPI staining was carried out after fixing the cells with 2% paraformaldehyde and then stained with 1 μg/ml DAPI for 10 min at room temperature as per the standard technique.

HPLC analysis of PPPF

HPLC system was from Shimdzu (Japan) and was equipped with 2 pumps (LC-10AT), a PDA detector (SPD-M10Avp), and a controller (CBM-10A) to control the programming of the mobile phases. The analysis was performed using C18 column (300 $\times$ 4 mm I.D.;.) attached with a guard column (Vydac, USA) and a Rheodyne (7725i) injection valve with a 20-μl loop. A linear gradient elution was carried out from 100% water containing 0.1% (v/v) TFA to 100% acetonitrile containing 0.1% TFA (v/v) over a period of 60 min at a flow rate of 0.5 ml/min. The column was maintained at room temperature and the detection was performed at 214 nm.

Statistical analysis

All values were reported as mean $\pm$ SE and Student's *t* test was used for determining statistical significance.

Results

PPPF induces melanogenesis in melanoma and melanocyte cells

To gain insight about the pigment inducing ability of PPPF as demonstrated earlier in B16F10 cells [26] we assessed its effect on the induction of key melanogenic enzyme – tyrosinase in the same cellular system. Dose and time dependent change in the tyrosinase activity in PPPF treated B16F10 cells was shown in Fig. 1. Maximum enhancement in tyrosinase

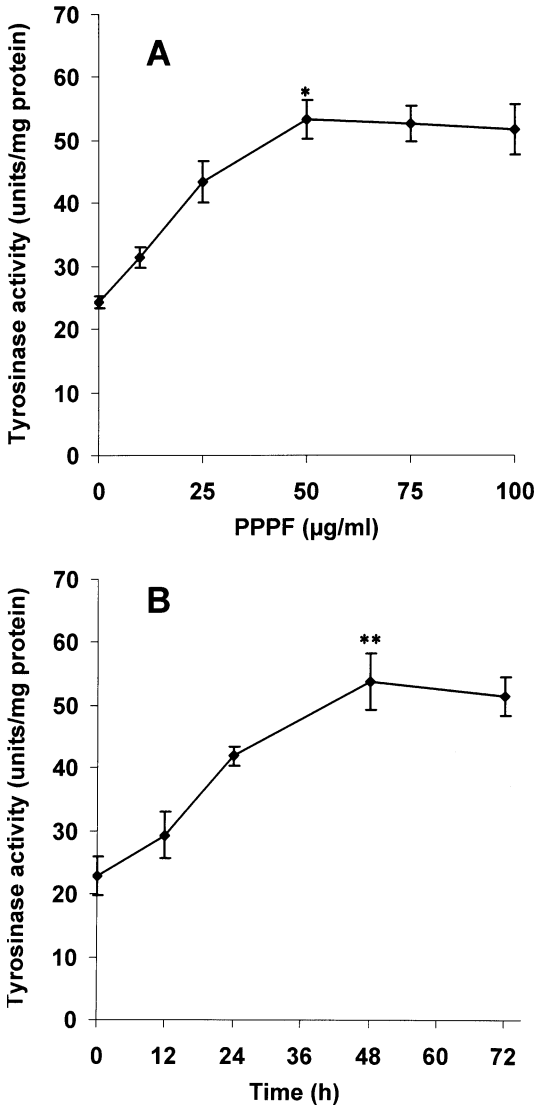

Fig. 1. Dose (A) and time kinetics (B) for the effect of PPPF on the induction of tyrosinase in B16F10 cells. [Results expressed as mean $\pm$ SE from triplicate determination in each experiment (of total five experiments). ($^{*}P < 0.01$; $^{**}P < 0.02$)].

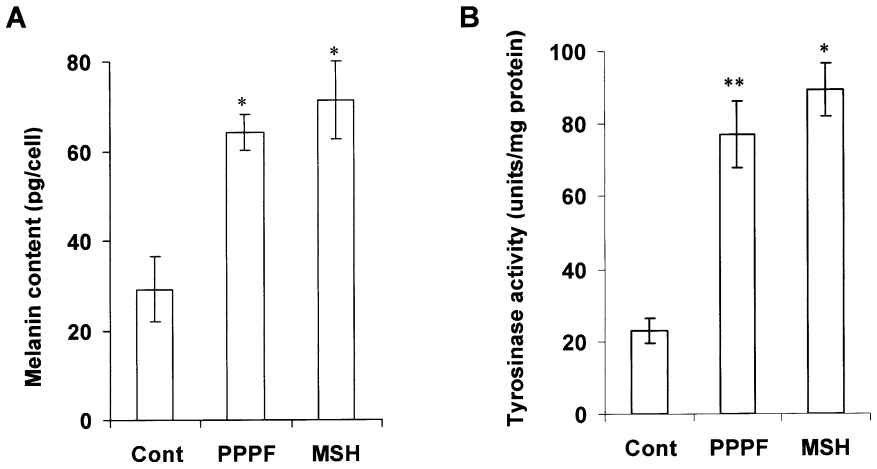

Fig. 2. Effect of PPPF on melanin synthesis (A) and tyrosinase activity (B) in normal human melanocyte cells. [Results expressed as mean ± SE determined from triplicate experiments. ($^{*}P < 0.001$; $^{**}P < 0.05$)].

activity (53.3 units/mg protein as compared to 24.3 units/mg protein of untreated control, $P < 0.01$) was observed after 48 h of treatment with PPPF at a concentration 50 μg/ml as the optimum dose and this was used throughout the study in all the experiments.

Induction of tyrosinase in PPPF treated B16F10 cells prompted us to evaluate its effect on normal human melanocytes. Increase in tyrosinase activity from 23.1-units/mg proteins to 77-units/mg protein in melanocyte followed by the treatment of PPPF (Fig. 2) indicated clearly the induction of tyrosinase by PPPF. Likewise in B16F10 [26] melanin synthesis was also stimulated in melanocyte from 29.3 pg/cell of control to 64.1 pg/cell by PPPF treatment. Thus we saw that the response of melanocyte and B16F10 to PPPF was extremely comparable for both the tyrosinase induction and melanin formation. Nonetheless all data presented hence onward in this study was generated from B16F10 mouse melanoma to study the effect of PPPF due to convenience and time saving as the culture of melanocyte from human skin is very time consuming.

PPPF increases tyrosinase protein expression

Enhanced enzyme formation at the protein level is obvious at the first instance to account the increase in tyrosinase activity. Thus the effect of PPPF on tyrosinase protein expression in B16F10 cells was assessed by Western blot analysis. The extent of tyrosinase enhancement at the protein level in B16F10 cells after treating with PPPF (50 μg/ml for 48 h) was highly significant compared to untreated control (Fig. 3.) PPPF treated cells showed 2.13-fold increase in tyrosinase at the protein level while α-MSH treatment as a positive control induced 2.81-fold taking untreated control as 1-fold.

PPPF stimulates tyrosinase mRNA

In order to account the induced tyrosinase at the protein level tyrosinase mRNA expression in PPPF treated B16F10 cells was assessed by RT-PCR analysis. Similar to tyrosinase protein induction the level of tyrosinase mRNA was also raised significantly in PPPF treated cells with respect to the untreated control (Fig. 4). Almost 2.46-fold increase in tyrosinase mRNA content was observed in PPPF treated cells as compared to the control cells. However such stimulation in tyrosinase mRNA expression by PPPF was slightly lower than that of α-MSH mediated response (3.09-fold versus 2.46-fold).

PPPF upregulates tyrosinase promoter activity

The activation of tyrosinase promoter is a prerequisite to justify increased synthesis of tyrosinase both at the mRNA as well as protein level so we attempted to visualise this using with a transfectant B16F10 carrying the plasmid pTyrP.EGFP (a generous gift from Dr. Ruth Halaban). Expression of enhanced green fluorescent protein (EGFP) coding sequence as a reporter gene under the control of tyrosinase promoter (of 2.5 Kbp fragment) was investigated to see the effect of PPPF on tyrosinase promoter activation. Stable transfectants after incubation with PPPF (50 μg/ml for 48 h) showed remarkably higher GFP expression as compared to the basal level of its expression in untreated control (Fig. 5) while DAPI

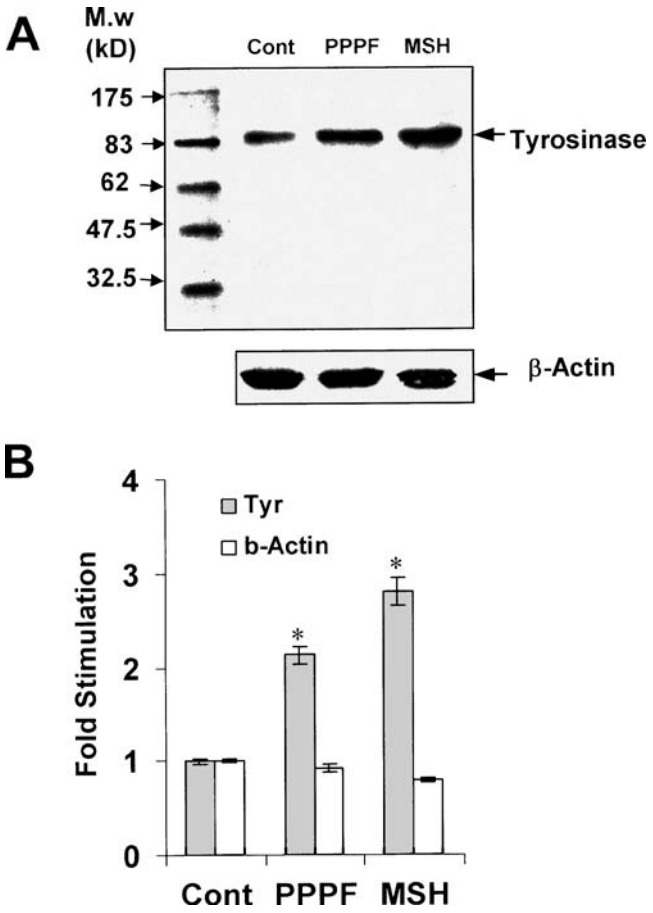

Fig. 3. (A) Western blot analysis of tyrosinase for B16F10 cells treated with PPPF (50 μg/ml) and α-MSH (100 nM), and untreated control (representative one of five experiments). (B) Densitometric scanning; data analysed from figures of five experiments and presented as mean $\pm$ SE. ($^*P < 0.01$).

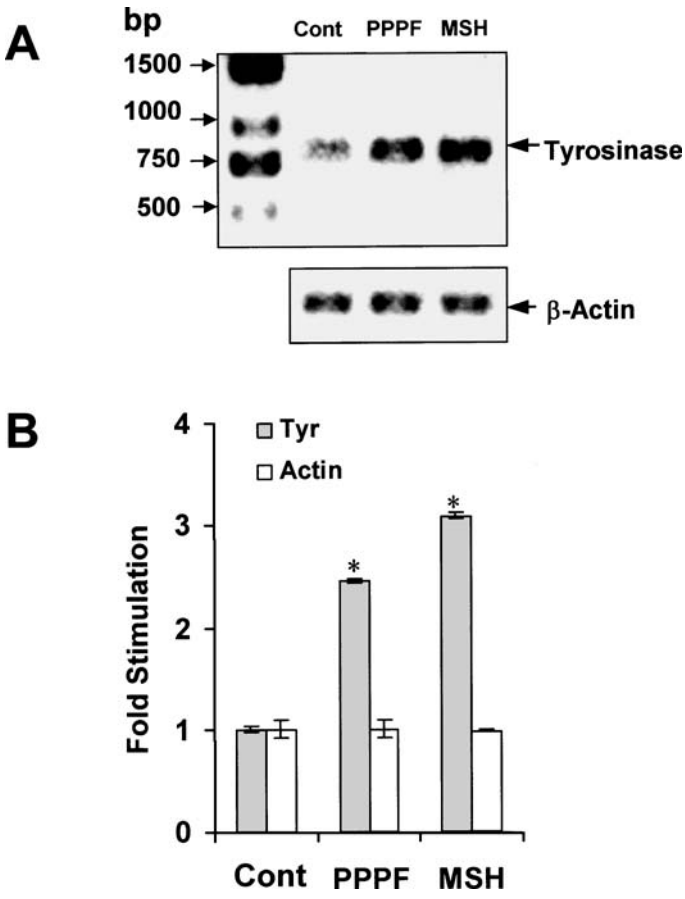

Fig. 4. (A) RT-PCR analysis for tyrosinase mRNA expression in B16F10 cells treated with PPPF (50 μg/ml) and α-MSH (100 nM) and untreated control (representative one out of five). (B) Densitometric scanning, data analysed from figures of five experiments and presented as mean $\pm$ SE ($^*P < 0.001$).

staining visualised clearly the presence of identical cell population in all cases. Thus PPPF mediated upregulation of tyrosinase promoter activity was clearly evidenced and thereby establishing PPPF as a strong mediator of tyrosinase gene expression leading to the melanin production.

Actinomycin D inhibits the PPPF induced melanogenesis

It is clear from the above experiments that the induction of melanogenesis in B16F10 cells by PPPF is regulated at the level of tyrosinase gene expression. To prove it further we examined the effect of transcriptional inhibitor actinomycin D (5 μg/ml) on PPPF stimulated melanin production in B16F10 cells (Fig. 6). In presence of actinomycin D, PPPF-induced melanin synthesis in B16F10 cells was reduced markedly from 7.8 to 3.6 pg/cell. Under similar condition the level of tyrosinase activity was also lowered from 51.5 to 25.3-units/mg protein. Thus these results clearly confirmed that PPPF stimulated melanogenesis in B16F10 cells by enhancing tyrosinase gene expression at the transcriptional level.

HPLC analysis of PPPF

To gain some idea about the peptides present in PPPF reversed phase HPLC analysis was carried out with it. The chromatogram revealed that all the peptides present in the preparation were eluted within 26.62 min. Under the similar condition α-MSH was eluted at 29.03 min at which no peaks were detected in the chromatogram of PPPF. This result clearly indicated that α-MSH was absent in this preparation.

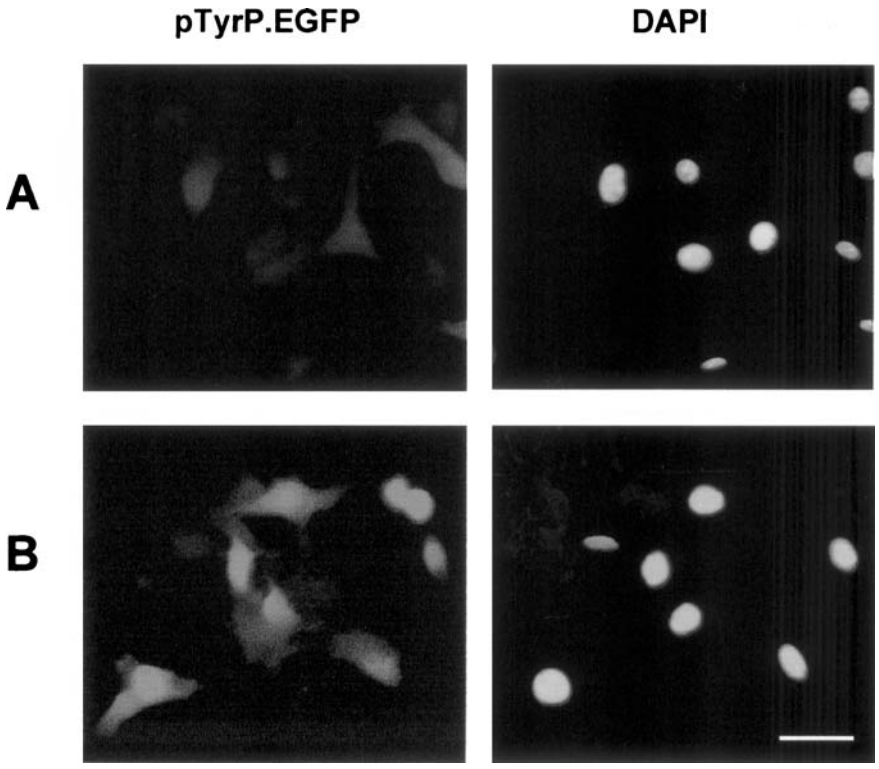

Fig. 5. Activation of tyrosinase promoter using green fluorescent protein (GFP) as reporter in pTyrP.EGFP transfected B16F10 cells after treating with (B) and without PPPF (A). The pTyrP.EGFP coded GFP expression (left panel) and DAPI staining (right panel) photographed under fluorescence microscope (equal magnification -bar represents 25 μm).

Discussion

Placental protein/peptide as biological response modifier studied before as cellular activity modulator [35–37] other than melanogenesis while we reported earlier with a protein/peptide preparation made from a hydroalcoholic extract of term fresh human placenta showing it as inducer of melanin synthesis [26]. This study extended our early observation and provided basis of the induced melanin formation. Here a correlation between the induction of melanin and the increase in tyrosinase activity, the key to the production of melanin was clearly established by the action of PPPF. The response of B16F10 and human primary melanocyte to PPPF was of the similar extent with respect to the induction of melanin synthesis and tyrosinase activity. As the culture of primary melanocyte from the skin specimen is very time consuming we used B16F10 mouse melanoma to make detailed study to find how the induction of tyrosinase was mediated by PPPF.

It must be mentioned here that PPPF is consisted of small peptides [24] in which ET-1 and ACTH, two important melanocyte/melanoma activating peptides [26] are present at detectable level, not effective in bringing any change in cellular activity. α-MSH, a powerful peptide hormone of melanocyte activity modulator [10, 11, 13, 15] is not present at all in PPPF (Fig. 7). In addition to that upon fractionation the melanogenic activity of PPPF retained in the fraction having proteins/peptides of molecular weight greater than 3 kDa (Data not shown). Since α-MSH is a very small peptide (1.66 kDa), presence of α-MSH or any of its active fragments in PPPF could be ruled out. Hence the effect of PPPF was always compared with α-MSH as parallel positive control throughout the studies.

Induction of tyrosinase activity in response to PPPF was established by Western blot analysis in terms of enhanced protein expression. It was further supported by increased transcriptional activity of the gene encoding the tyrosinase enzyme protein. Here significant enhancement in the level of tyrosinase mRNA provided clear evidences in this respect. Thus PPPF treated cells supported strongly its role as potent inducer of tyrosinase. We have exploited a transfected melanoma system having GFP encoding gene down stream to the tyrosinase promoter to show the involvement of promoter activation in relation to the increased transcriptional activity. The significant enhancement of GFP expression in PPPF-treated cells (Fig. 5) compared to untreated one again provided the evidence of higher upregulation of tyrosinase gene expression by PPPF. So the induced formation of tyrosinase appeared to be transcriptionally regulated in exposure

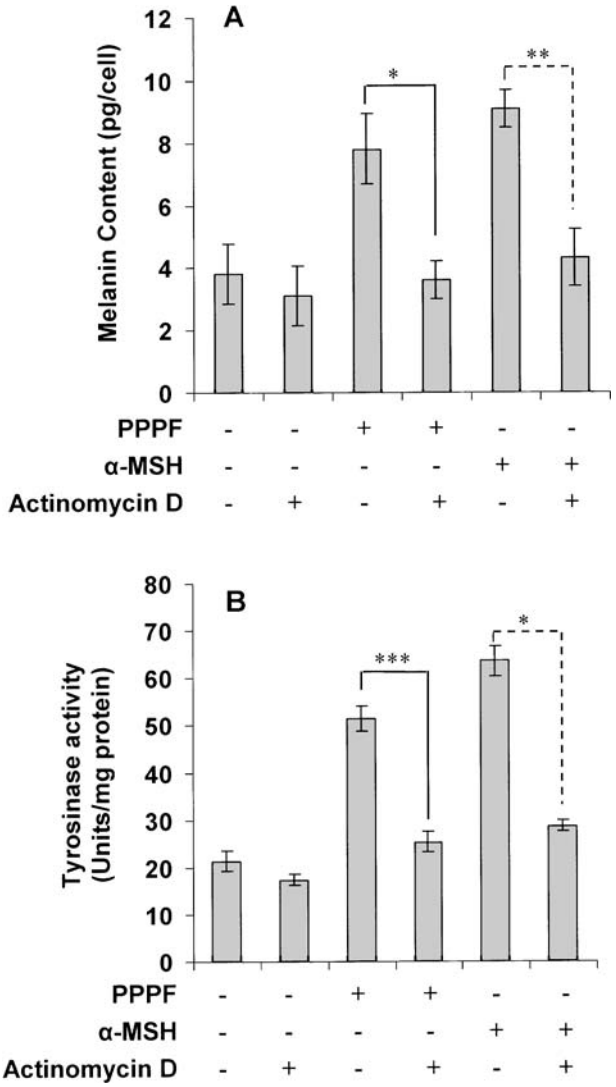

Fig. 6. Effect of actinomycin D on PPPF induced melanin formation (A) and tyrosinase activity (B) in B16F10 cells. Data analysed from triplicate for each dose taking total of five experiments and expressed as mean ± SE ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.02$).

to PPPF treatment. In presence of well-known transcriptional blocker, actinomycin D, PPPF mediated tyrosinase induction was close to untreated control (Fig. 6). So the effect of PPPF is undoubtedly mediated through upregulation of transcriptional activity of tyrosinase gene.

We have already reported that placental total lipid fraction (PTLF) prepared from the same hydroalcoholic extract was effective for melanogenesis in B16F10 cells [27]. This was very similar to the action of PPPF with respect to tyrosinase induction. So we were curious to see the effect of both these two combined and cells were so treated accordingly. Here the extent of increase in the activity of tyrosinase and melanin formation was slightly higher but not additive. The results

Table 1. Comparative evaluation of PPPF and PTLF mediated increase in melanin content and tyrosinase activity in B16F10 cells (the culture condition was as stated under 'Cell culture' in Method and Materials)

Stimulants	Melanin content (pg/cell)	Tyrosinase activity (units/mg protein)
Control	3.1 ± 0.32	21.2 ± 2.26
PPPF (50 μg/ml)	8.3 ± 0.19^{a}	58 ± 1.99^{b}
PTLF (100 μg/ml)	9.7 ± 0.91^{a}	67.3 ± 4.91^{a}
PPPF (50 μg/ml) + PTLF (100 μg/ml)	12.3 ± 0.79^{b}	78.5 ± 2.86^{a}

$^{a}P < 0.01$.
$^{b}P < 0.001$.

(Table 1) suggested that PPPF and PTLF acted possibly on the same target in a kind of overlapping fashion, thus asserting the involvement of tyrosinase promoter activation and it was shown for PTLF [28].

As PPPF does not contain α-MSH and ACTH or ET-1 not at a level effective for stimulated production of melanin and tyrosinase, and it is prepared by heat alcohol treatment so the presence of other protein/peptide stimulator of melanogenic activity namely bFGF, SCF, HGF are ruled out in this fraction. HPLC profile of the peptides present in PPPF clearly indicated the presence of 3 major regions (I, II and III) consisting of multiple peaks (Fig. 7). Examination of each region as a fraction revealed the biological activity in all the three (data not shown). But molecular cut based fractionation showed that only components of PPPF present in greater than 3 kDa molecular size were biologically active and these are distributed apparently in the regions of HPLC profile. So it led us to think that more than one peptide (>3 kDa mol. size) may be involved in biological response modification ruling out the occurrence of α-MSH or its fragments.

The pigment induction is considered as the result of stress specific event. For example UV-induced DNA damage led the melanocyte to switch on the melanization [38]. The involvement of stress responsive p38 MAPK has already been demonstrated for melanogenic activation by PTLF in B16F10 [28]. As we have shown in this study that PPPF and PTLF appears to act apparently in an overlapping fashion for melanin production in B16F10. So it is very likely that PPPF might act through the similar signaling pathway. However our studies are currently pursued to this direction.

Hence potent pigment inducing activity of placental protein/peptide fraction or PPPF as shown in this study together with the sphingolipid rich fraction having melanogenic property could be considered as the basis to the melanogenic potency of hydroalcoholic human placental extract. This provides strong support to the usefulness of this placental extract for pigment recovery in treating hypopigmentary disorder like vitiligo.

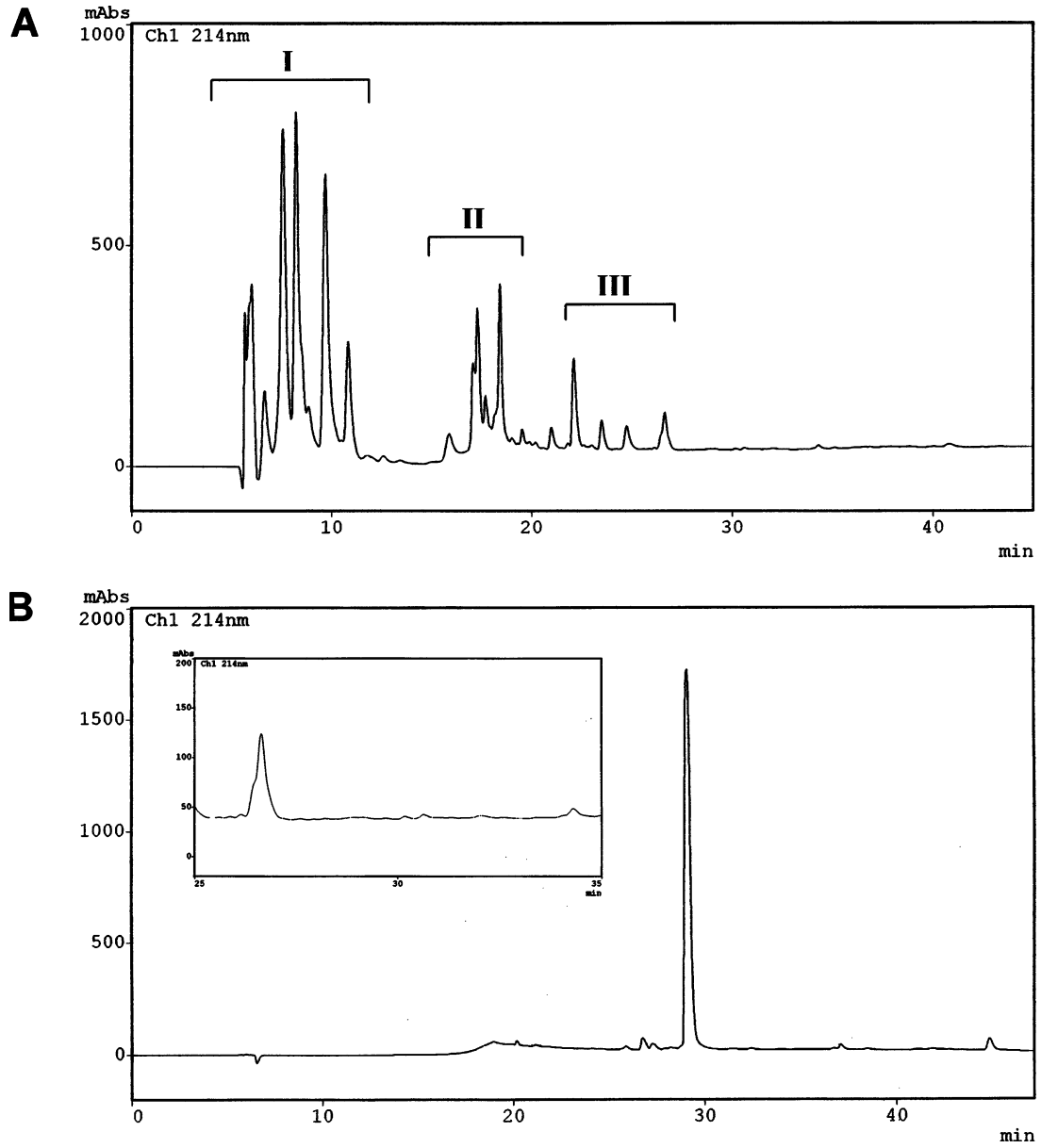

Fig. 7. Reversed phase HPLC analysis of PPPF (A) indicating three major regions as I, II and III consisting of multiple peaks and that of α-MSH (B) (Inset: chromatogram of PPPF between 25–35 min).
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