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Fibroblasts co-expressing tyrosinase and the b-protein synthesise both eumelanin and phaeomelanin

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Abstract

Melanin synthesis in the mouse involves the interaction of many pigmentation loci. Tyrosinase, the product of the *albino* (c) locus, catalyses the first step of the pathway. The *brown* (b) locus protein has significant homology to tyrosinase and controls black/brown coat coloration, but its function is controversial. To investigate the function of the b-protein and its interaction with tyrosinase, we established cell lines expressing both tyrosinase and the b-protein by transfecting tyrosinase-expressing fibroblasts with a b-protein expression vector. The tyrosinase-expressing parent line does not have L-dopachrome tautomerase activity, but this enzyme is detectable in double transfectants as well as in fibroblasts expressing the b-protein alone. Cells expressing both proteins have a higher steady-state level of tyrosinase than fibroblasts expressing tyrosinase alone, and contain elevated levels of melanin intermediates. This is thought to result from interaction of tyrosinase with the b-protein. Only phaeomelanin is detectable in fibroblasts expressing tyrosinase alone, whereas double transfectants synthesise significantly more phaeomelanin and detectable eumelanin.

Keywords: Dopachrome tautomerase; Melanin synthesis; Pigmentation; Tyrosinase; Tyrosinase-related protein 1

1. Introduction

Pigment synthesis requires the interaction of a large number of proteins, many of which are expressed only by melanocytes. Over the past nine years the genes encoding several of these proteins have been cloned from the mouse, and this has greatly improved our understanding of their phenotypic effects [1–3]. Protein products of the *albino*, *brown* and *slaty* loci have been shown to function as melanogenic enzymes [4–12], whereas the *silver* locus protein may be a structural component of melanosomes, the specialised membrane-bound organelles in which pigment is synthesised [13,14]. The product of the *pink-eyed dilution* locus appears likely to act as an integral membrane transporter protein [15] and the *extension* locus protein is the melanocyte receptor for α-melanocyte stimulating hormone [16].

Expression and purification studies have been used to study individual cloned melanogenic enzymes. However, this is unlikely to give a true picture of their functions in vivo since several of the enzymes involved in melanin synthesis appear to be organised in a high molecular weight complex in the melanosomal membrane, and this is very likely to influence their activities [2,17,18]. We have previously used a mouse fibroblast expression system to study the functions of the proteins encoded at the *albino* (*c*) locus and *brown* (*b*) locus in isolation [12,19]. Both proteins are located in lysosomes in transfected fibroblasts, a finding consistent with recent evidence that melanosomes are closely related to lysosomes [17,20]. The aim of this

Abbreviations: AHP, 4-amino-3-hydroxyphenylalanine; Con A, concanavalin A; DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DMEM, Dulbecco's modified Eagle's medium; DO, dopa oxidase; 5H6MI2CA, 5-hydroxy-6-methoxyindole-2-carboxylic acid; 6H5MI2CA, 6-hydroxy-5-methoxyindole-2-carboxylic acid; HRP, horseradish peroxidase; MBTH, 3-methyl-2-benzothiazolinonehydrazone; NP-40, Nonidet P-40; PBSA, phosphate-buffered saline A; PTCA, pyrrole-2,3,5-tricarboxylic acid; 5-*S*-CD, 5-*S*-cysteinyldopa; 5-*S*-CGD, 5-*S*-cysteinylglycinedopa; 5-*S*-GD, 5-*S*-glutathionyldopa; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TDCA, Thiazole-4,5-dicarboxylic acid; TH, Tyrosine hydroxylase.

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study was to express both proteins simultaneously in fibroblasts to determine whether co-expression influences the function of either protein, and the phenotype of the cells.

The product of the *albino* locus is the enzyme tyrosinase (EC 1.14.18.1) which catalyses the first two steps of the melanin synthesis pathway, the hydroxylation of L-tyrosine to L-dopa and the oxidation of L-dopa to dopaquinone. It is the key enzyme in melanin synthesis and in null mutants no pigment is formed. In the presence of cysteine or other thiols, dopaquinone enters the pathway of phaeomelanogenesis [21,22], and fibroblasts transfected with a mouse tyrosinase cDNA have been shown to synthesise a phaeomelanic pigment [19].

If the supply of thiols is restricted, dopaquinone oxidises to dopachrome which in turn undergoes oxidative decarboxylation to form 5,6-dihydroxyindole (DHI). However, the enzyme dopachrome tautomerase (EC 5.3.2.3) or specific metal ions direct the tautomerisation of dopachrome to the carboxylated indole 5,6-dihydroxyindole-2-carboxylic acid (DHICA) [18,23–27]. In the mouse, both the *brown* locus protein (b-protein or TRP-1) and the *slaty* locus protein (TRP-2) have been shown to possess dopachrome tautomerase activity in vitro [11,12].

The wild-type b-protein has a clear phenotypic effect converting the brown mouse coat colour to black, but its precise function is still controversial. In addition to acting as a dopachrome tautomerase, evidence has been published that it functions as a second tyrosinase [6,7] or as a melanocyte-specific catalase [28]. We were, however, unable to detect either of these activities in fibroblasts expressing the b-protein in isolation, and these cells do not synthesise melanin [12]. Recently, the b-protein has been shown to be capable of oxidising DHICA in vitro [29] and hence may be involved in polymerisation of indoles to form eumelanin. Tyrosinase may also have a role in this process as it is able to oxidise DHI [30,31] and peroxidase has also been implicated [22,32]. By analysing enzyme activities and melanin intermediates in fibroblasts co-expressing tyrosinase and the b-protein, our aim was to gain further insight into the precise roles of these two enzymes in the complex process of melanin synthesis.

2. Materials and methods

2.1. Chemicals

All cell culture reagents were purchased from Gibco (Paisley, UK). PBSA tablets were obtained from Oxoid (Basingstoke, UK). Con A-Sepharose was from Pharmacia (Uppsala, Sweden) and amido black was from Sebia. Bovine serum albumin, 3,3′-diaminobenzidine, dithiothreitol, G418, hydrogen peroxide, hygromycin B, NP-40, SDS, Triton X-100 and Tween 20 were purchased from Sigma (Poole, UK), as were prestained molecular mass markers for polyacrylamide gel electrophoresis. Perchloric acid was obtained from BDH (Poole, UK). All other reagents were of analytical grade.

2.2. Antibodies

Mouse monoclonal antibody TA99 [33] used in immunofluorescence was a gift from Dr K.O. Lloyd (Sloan-Kettering Cancer Center, New York, USA). The commercially available form of TA99 (MEL-5) was used in Western blotting (Signet Laboratories, Dedham, USA). Rabbit polyclonal antibodies αPEP5 and αPEP7 [34] were provided by Dr V.J. Hearing (NIH, Bethesda, USA). HRP-conjugated goat anti-mouse IgG was purchased from Bio-Rad (Hemel Hempstead, UK). ^{125}I-labelled goat anti-rabbit and rabbit anti-mouse second antibodies were a gift from Dr M.E. Bramwell (Sir William Dunn School of Pathology, University of Oxford, UK).

2.3. Cell culture

3T3 Swiss mouse fibroblasts were grown in DMEM supplemented with 10% (v/v) foetal calf serum and 2 mM L-glutamine. Clone c, a line of 3T3 Swiss fibroblasts expressing mouse *c* locus tyrosinase, has been described previously [19,35], as have line pKG4 (transfected with a G418-resistance gene) [19] and fibroblasts expressing the mouse b-protein (subclones $b_{1.2}$, $b_{2.1}$ and $b_{2.2}$) [12]. These transfected lines were cultured in the above medium supplemented with 0.8 mg ml^{-1} G418. All lines were passaged by incubation (37°C for 5 min) in PBSA/EDTA/trypsin (PBSA containing 0.02% (w/v) EDTA and 0.125% (w/v) trypsin) and incubated in a 5% CO_2 humidified atmosphere. Mycoplasma tests were done routinely and were always negative.

2.4. Transfection

Clone c cells (8×10^6) were seeded in medium without G418 into a 175 cm^2 Nunc tissue culture flask (Gibco) 24 h before transfection. A calcium phosphate DNA coprecipitate containing 18 μg pHDMT4 [8] and 2 μg pBabehygro [36] was added to the culture medium and the cells were incubated at 37°C for 5–7 h before treatment with glycerol [37]. In control flasks, clone c was transfected with 20 μg pBabehygro alone. Fresh medium lacking antibiotics was added 24 h after transfection and selection with hygromycin B (0.175 mg ml^{-1}) was started 24 h later. Clones were picked after 2–3 weeks.

2.5. Sample preparation

Method A: Cells were harvested using trypsin and washed twice in PBSA then resuspended in 4 volumes of buffer A (20 mM KH_2PO_4 [pH 7.5], 0.5% (v/v) Triton X-100). Lysates were sonicated for 10 s (MSE Ultrasonic

Disintegrator model 7100, setting 5 μm) then centrifuged (9000 × *g* for 5 min at 4°C). The centrifugation step was repeated using the supernatant, and the final supernatant was stored in aliquots at −70°C.

Method B: Cells were harvested and washed as in method A, then cell pellets were frozen in solid CO_2/ethanol. Approximately 10 volumes of Millipore-purified water were added to each pellet, the suspensions were centrifuged (2000 × *g* for 10 min), and the water poured off. Pellets were resuspended in approximately 10 volumes of buffer B (50 mM KH_2PO_4 [pH 7.2]) and homogenised by 10 strokes of an all-glass Potter-Elvehjem homogenizer.

Method C: Cells were harvested and washed as in method A, then cell pellets were frozen in solid CO_2/ethanol. Extracts were prepared by resuspending pellets in approximately ten volumes of Milli-Q-purified water for melanin determination, and 10 volumes of 0.4 M perchloric acid for catechol determination.

Method D: For analysis of cell culture medium, cells were seeded at a fixed density of 2×10^6 cells per 25 cm^2 tissue culture flask in 5 ml of medium. Representative flasks were taken at time points and the medium aspirated then centrifuged (350 × *g* for 3 min) to remove cell debris. Aliquots of the supernatant were immediately frozen in solid CO_2/ethanol and stored at −70°C.

2.6. Enzyme assays

Dopachrome tautomerase: Dopachrome tautomerase activity was determined by measuring the formation of DHICA and/or DHI from L-or D-dopachrome by HPLC. Preparation of Con A-binding fractions and dopachrome tautomerase assays were done as described previously [12].

Tyrosinase: The tyrosine hydroxylase activity of tyrosinase was determined using the $^{14}CO_2$ assay [38], and the dopa oxidase activity of tyrosinase was measured using both the continuous and stopped MBTH assays [38,39] in extracts prepared by method A. In addition, dopa oxidase activity was determined by measuring the formation of L-5-*S*-cysteinyldopa from L-dopa and L-cysteine in extracts prepared by method B, and in culture medium prepared by method D [40,41].

2.7. Protein determination

Protein concentrations were determined either by using a protein assay kit (Bio-Rad) based on the method of Bradford [42] or by the method of Lowry et al. [43]. Bovine serum albumin was used as a reference standard.

2.8. Growth curves

Growth curves were determined as described previously [19]. All cell lines were cultured in medium plus penicillin and streptomycin (both at 50 μg ml^{-1}) but without G418 or hygromycin B. At 24–48 h intervals cells were counted, pigmentation scored on an arbitrary scale as previously detailed [19], and extracts were prepared by method A then stored in aliquots at −20°C.

2.9. SDS-polyacrylamide gel electrophoresis and Western blotting

SDS-PAGE using 7.5% to 20% gradient gels and Western blotting were done as previously described [12] except that for gels run under reducing conditions 20 mM dithiothreitol was added to the sample buffer. Membranes were treated for 30 min with 1% (v/v) newborn calf serum in PBSA containing 0.2% (v/v) Tween 20 and 0.4 M NaCl (buffer C), rinsed in buffer C, and incubated at room temperature for 30 min with primary antibody diluted in buffer C (1:150, by vol. for TA99; 1:50, by vol. for αPEP5 and αPEP7).

After washing in buffer C for 30 min, membranes were incubated with second antibody diluted in PBSA. The HRP-conjugated second antibody was diluted 1:600 (by vol.) and after incubation for 30 min at room temperature membranes were washed briefly in buffer C, rinsed in PBSA then incubated with 0.5 mg ml^{-1} 3,3′-diaminobenzidine plus 0.15% (w/v) hydrogen peroxide in PBSA. The reaction was terminated by washing with water, and the membranes were dried and stored in the dark at room temperature until photographed.

^{125}I-Labelled second antibodies were diluted 1:300 (by vol.) and after incubation for 30 min at room temperature membranes were washed thoroughly in buffer C (five changes over 30 min) and rinsed twice in PBSA. Membranes were stained with amido black then air-dried, wrapped in Saran wrap, and exposed to X-ray film at −70°C for 2–7 days. The amount of protein in specific bands was quantitated using an LKB Ultroscan laser densitometer.

2.10. Analysis of catechols, indoles and melanin

Catechols: Cell extracts were prepared by method C and media samples by method D. The amounts of L-dopa, 5-*S*-cysteinyldopa, 5-*S*-glutathionyldopa and 5-*S*-cysteinylglycinedopa were determined by HPLC and electrochemical detection [44,45]. The detection limit for all four catechols is 1 pmol.

Indoles: Media samples prepared by method D were analysed for the presence of indoles by HPLC methods described previously [46,47]. Standards were kindly provided by Prof. G. Prota (Dept. of Organic Chemistry, University of Naples, Italy).

Melanin: Cell extracts were prepared by method C for phaeomelanin determination. The insoluble pellet was subjected to reductive degradation in hydriodic acid and the hydrolysis product AHP measured as described by Ito and Fujita [48]. The relative quantities of eumelanin and

phaeomelanin were determined by measuring the yields of PTCA and TDCA on oxidation with potassium permanganate. The TDCA/PTCA ratio correlates with the ratio of phaeomelanin to eumelanin [49].

2.11. Statistical analysis

Mean values were compared using Student's *t*-test, adopting 95% confidence intervals.

3. Results

3.1. Co-expression of tyrosinase and the b-protein

To investigate interactions between tyrosinase and the b-protein we transfected mouse fibroblasts expressing mouse *c* locus tyrosinase [19,35] with a b-protein expression vector. Clone c is a stable tyrosinase-expressing line established by co-transfection of 3T3 Swiss mouse fibroblasts with the tyrosinase expression vector pHDmcTyr1 [8] and a plasmid conferring resistance to G418, and was chosen for a second round of transfections because it contains a single integrated copy of the tyrosinase cDNA [19]. Clone c was co-transfected with the b-protein expression vector pHDMT4 [8] and a hygromycin-resistance plasmid; a second selectable marker being necessary because clone c is G418-resistant. As a control, clone c was transfected with the hygromycin-resistance plasmid alone.

Clones were screened for expression of the b-protein by indirect immunofluorescence staining with monoclonal antibody TA99, as described previously [12]. Antibody TA99 is specific for the b-protein [28,33] and does not stain untransfected fibroblasts or fibroblasts expressing tyrosinase alone, but does stain double transfectants expressing the b-protein (data not shown). Double transfectant lines are termed c + b clones since they have been transfected with both mouse *c* locus tyrosinase and the mouse *b* locus protein. In 6 clones ($c + b_1$ to $c + b_6$) more than 70% of cells reacted with antibody TA99, but these clones were somewhat heterogeneous and were subcloned to obtain homogeneous cell lines. Subclones derived from the original line $c + b_1$ are termed subclone $c + b_{1.1}$, $c + b_{1.2}$ etc., and notation is similar for other lines.

A correlation was observed between pigmentation and expression of the b-protein. Lines isolated by transfection of clone c with the hygromycin-resistance gene alone (denoted subclone c-$hyg_{1.1}$, c-$hyg_{1.2}$ etc.) were a similar light brown colour to the parent cell line, but transfectants expressing the b-protein were distinctly darker. Production of pigment by double transfectant clones indicates that they have retained a functional *c* locus tyrosinase since this is the rate-limiting enzyme for pigment synthesis. The darker pigmentation of c + b clones suggests that the b-protein they synthesise is functional since the wild-type b-protein converts the brown mouse coat colour phenotype to black.

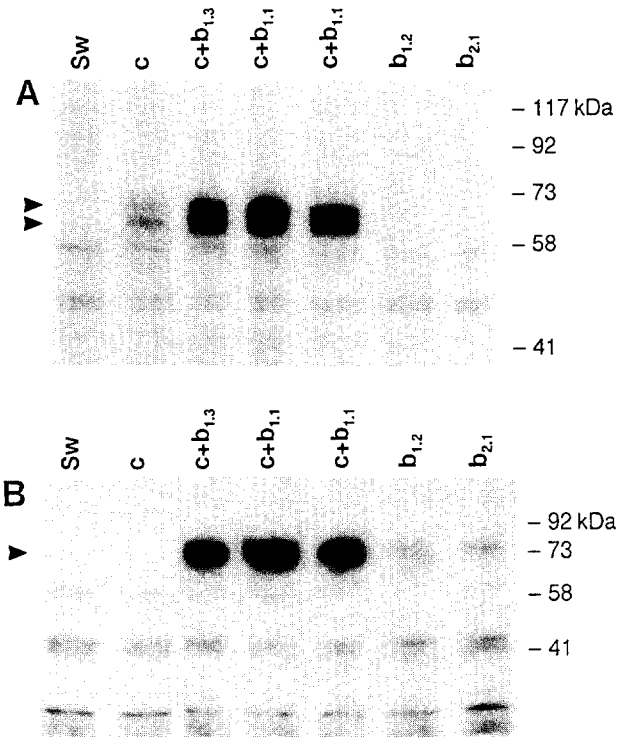

Fig. 1. Western blot analysis of steady-state levels of (A) tyrosinase and (B) the b-protein. Cell extracts (80 μg protein) from untransfected fibroblasts (Sw), fibroblasts expressing mouse *c* locus tyrosinase (clone c), double transfectants expressing both tyrosinase and the b-protein (subclones $c + b_{1.1}$ and $c + b_{1.3}$), and fibroblasts expressing the b-protein alone (subclones $b_{1.2}$ and $b_{2.1}$) were separated by SDS-PAGE under reducing conditions (A) or non-reducing conditions (B). Samples were blotted to nitrocellulose then probed with (A) antibody αPEP7 which detects membrane-bound tyrosinase or (B) antibody TA99 which detects the b-protein, followed by ^{125}I-labelled second antibodies. Arrowheads indicate (A) tyrosinase and (B) the b-protein.

Expression of both tyrosinase and the b-protein by c + b subclones was confirmed by Western blot analysis (Fig. 1). Antibody αPEP7 was raised against a C-terminal peptide of tyrosinase and recognises only the membrane-bound form of the enzyme [34,41,50]. Using this antibody, the steady-state level of tyrosinase was found to be 2-to 3-fold higher in several c + b subclones than in clone c from which they were derived (Fig. 1A and Table 1). A similar result was obtained using antibody αPEP5 which recognises the N-terminus of the protein [34] and detects both membrane-bound and soluble forms of tyrosinase (data not shown). The level of b-protein detected with the highly-specific antibody TA99 was 4-to 6-fold greater in several c + b subclones than in lines expressing the b-protein alone (Fig. 1B). Increased steady-state levels of both proteins are thought to result from interaction of tyrosinase and the b-protein in a complex which enhances their stability [29].

Table 1
Correlation of tyrosine hydroxylase and dopa oxidase activities of transfected fibroblasts with the amount of membrane-bound tyrosinase protein

Cell line	Enzyme activity (nmol min^{-1} mg^{-1} ± S.D.)		Tyrosinase protein (A.U. [a] ± S.D.)
	TH	DO	
Clone c	0.78 ± 0.14	3.10 ± 0.10	8.3 ± 0.4
Subclone c + $b_{1.1}$	4.64 ± 0.59	26.28 ± 2.66	26.5 ± 1.6
Subclone c + $b_{1.3}$	n.d. [b]	17.07 ± 0.52	21.4 ± 1.1
Subclone c + $b_{1.4}$	4.07 ± 0.16	18.10 ± 1.13	n.d.

Tyrosine hydroxylase (TH) activity was determined using the $^{14}CO_2$ assay and dopa oxidase (DO) activity was measured using the continuous MBTH assay. Specific activities are expressed per mg of protein. The amount of membrane-bound tyrosinase protein was determined by laser densitometer scanning of Western blots probed with antibody αPEP7. Data represent mean ± S.D. for three determinations.
[a] A.U., absorbance units
[b] n.d., not determined.

3.2. Physiology of pigment synthesis

Pigmentation of c + b subclones increases significantly when the culture medium is not changed for several days. To investigate whether this increase in pigmentation correlates with alterations in tyrosinase activity and/or expression of the b-protein, cells were seeded at a fixed density and pigmentation, cell number, and tyrosinase activity were measured over a time course during which the medium was not renewed (Fig. 2). The growth of double transfectant subclone c + $b_{1.2}$ was compared to that of clone c which expresses only tyrosinase, and to subclone $b_{2.2}$ which expresses the b-protein alone [12].

The growth curve for subclone $b_{2.2}$ (Fig. 2A) is virtually identical to that of untransfected 3T3 Swiss fibroblasts (data not shown), and clone c grows slightly more slowly. In contrast, subclone c + $b_{1.2}$ cells grow poorly when the

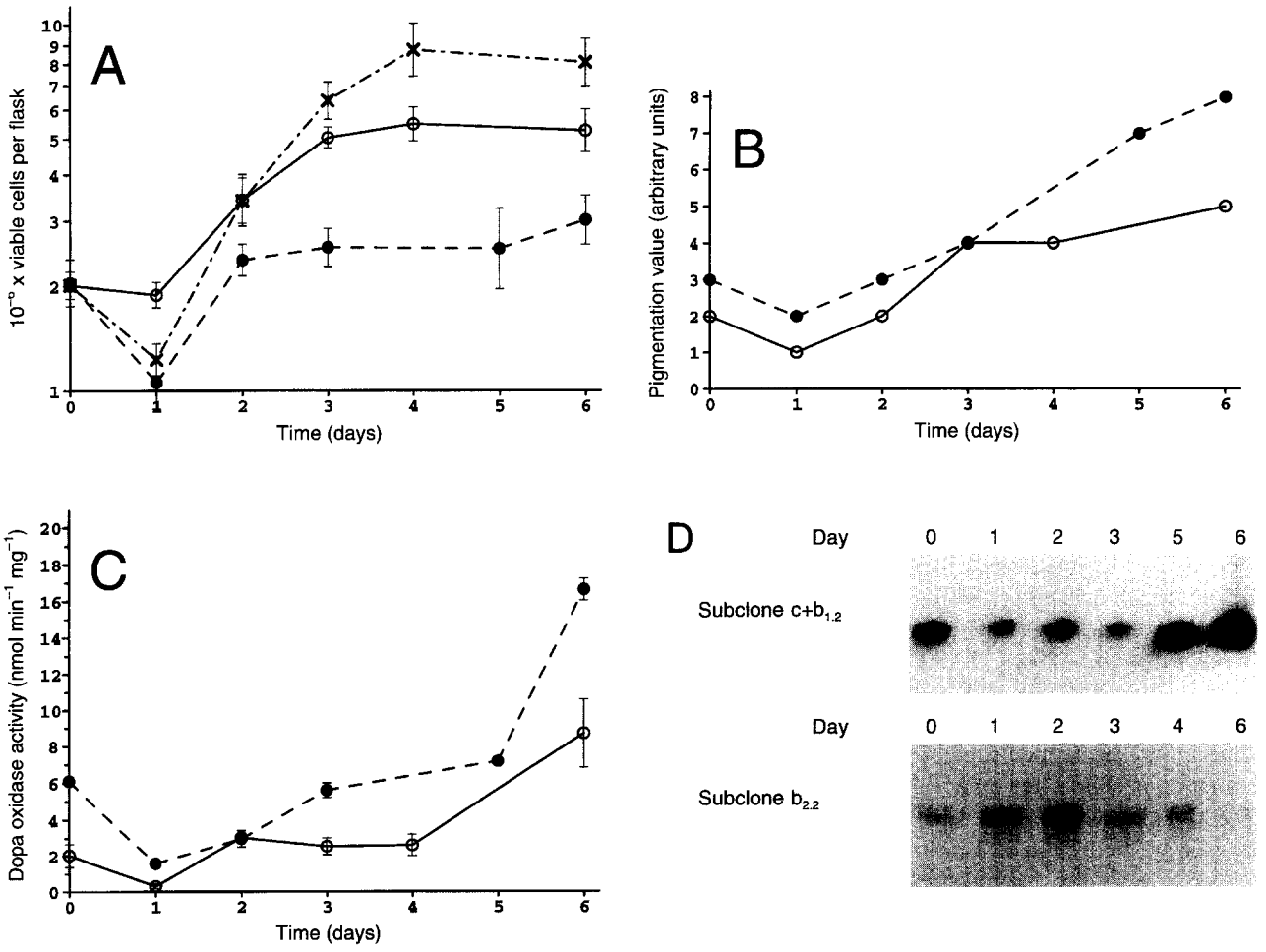

Fig. 2. Effect of cell density on tyrosinase activity, pigmentation and b-protein levels of transfected fibroblasts. Cells were seeded at a fixed density and measurements of (A) viable cell number, (B) pigmentation, (C) dopa oxidase activity, and (D) b-protein level, were made over a time course. Cell lines are (○) clone c expressing tyrosinase alone, (×) subclone $b_{2.2}$ expressing the b-protein alone, and (●) subclone c + $b_{1.2}$ expressing both tyrosinase and the b-protein. Medium was not changed during the experiment. Values for cell numbers and tyrosinase activity are the means for three samples ± S.D. Error bars not shown are smaller than the symbols. Pigmentation values are estimated from observation of three pellets. For analysis of b-protein levels, cell extracts (80 μg protein) were separated by SDS-PAGE under non-reducing conditions, blotted to nitrocellulose and probed with antibody TA99 followed by second antibodies labelled with ^{125}I for subclone c + $b_{1.2}$ or conjugated to HRP for subclone $b_{2.2}$.

culture medium is not changed regularly (Fig. 2A). For all three cell lines there is little difference between total and viable cell numbers throughout the time course (data not shown), and hence it appears that the low viable cell numbers for subclone c + $b_{1.2}$ reflect a failure to multiply. When the medium is changed at 48-h intervals, growth of subclone c + $b_{1.2}$ is virtually identical to that of subclone $b_{2.2}$ and clone c, all lines reaching a saturation density of 7–9 × 10^6 viable cells per 25 cm^2 flask (data not shown).

Pigmentation of both tyrosinase-expressing lines clone c and subclone c + $b_{1.2}$ decreases 24 h after seeding (Fig. 2B). This is not due to cell multiplication (Fig. 2A), but may be due to changes induced in the cells by subculturing procedures. Pigmentation of both lines increases over the remainder of the time course, and subclone c + $b_{1.2}$ is considerably darker than clone c by day 6 (Fig. 2B). Subclone $b_{2.2}$ remains unpigmented throughout the time course. The tyrosinase activity of both clone c and subclone c + $b_{1.2}$ increases in parallel to pigmentation (Fig. 2C) which is expected since tyrosinase is the rate-limiting enzyme for melanin synthesis. At no stage of the time course could tyrosinase activity be detected in subclone $b_{2.2}$.

When the culture medium is changed at 48-h intervals, pigmentation of both clone c and subclone c + $b_{1.2}$ on day 6 is the same as on day 0, and an increase in tyrosinase activity over the time course is not observed (data not shown). This suggests that when the medium is not renewed, accumulation of metabolites may induce the increase in tyrosinase activity and pigmentation of these lines.

The amount of b-protein was measured over the same time course by quantifying the protein detected on Western blots with antibody TA99 (Fig. 2D). In subclone c + $b_{1.2}$ the amount of b-protein increases in parallel to tyrosinase activity, both reaching a maximum on day 6: tyrosinase activity increases 9-fold and the amount of b-protein 6-fold between days 1 and 6 (Fig. 2C,D). In contrast, in subclone $b_{2.2}$ the amount of b-protein reaches a maximum on day 2 then decreases, returning to its original level by day 6 (Fig. 2D). This difference in b-protein kinetics suggests that the presence of tyrosinase may influence the steady-state level of the b-protein.

3.3. Enzyme activities of double transfectants

Assays for tyrosinase in extracts of confluent cultures showed that double transfectants expressing the b-protein have between 3-and 7-fold higher activity than clone c (Table 1 and data not shown). In contrast, c-hyg subclones derived by transfection of clone c with the hygromycin-resistance plasmid alone, have approximately equal or slightly lower tyrosinase activity than clone c (data not shown). The enhanced tyrosinase activity of c + b subclones applies to both tyrosine hydroxylase (TH) and dopa oxidase (DO) activities. The DO/TH ratios for several c + b subclones are not significantly different ($P > 0.1$) from the ratio for clone c (data not shown), confirming that the enhancement of tyrosinase activity affects both functions of the enzyme equally. For a given cell line, the ratio of enzyme activity to tyrosinase protein is constant, but the relative amount of tyrosinase protein in clone c and c + b subclones does not correlate completely with their relative enzyme activity (Table 1 and data not shown). For example, the tyrosinase activity of subclone c + $b_{1.1}$ is approximately 7-fold that of clone c, but the amount of tyrosinase protein is only approximately 3-fold greater. Hence, the enhanced tyrosinase activity of c + b subclones cannot be completely accounted for by a higher steady-state level of tyrosinase protein.

In melanocytes, tyrosinase exists in both membrane-bound and soluble forms, the soluble form being produced by proteolytic cleavage of the membrane-bound enzyme close to the C-terminus [41,50]. In clone c, 60–70% of tyrosinase activity is found to be soluble after cellular fractionation [19], whereas only 40–50% of the enzyme from subclone c + $b_{1.2}$ is soluble. It is known that tyrosinase is released from living melanoma cells [51], and to further investigate the processing of the enzyme in transfected fibroblasts we analysed culture medium for the presence of tyrosinase activity. Cells were seeded at a fixed density and activity was measured over a time course of four days during which the medium was not changed. At all time points, the level of tyrosinase activity in the medium from clone c was higher than in the medium from subclone c + $b_{1.2}$ (Fig. 3). Due to the slower multiplication of subclone c + $b_{1.2}$, the number of clone c cells per flask is 1.5-to 2-fold that of subclone c + $b_{1.2}$ cells over much of the time course, but this is counterbalanced by the fact that the specific tyrosinase activity of subclone c + $b_{1.2}$ is 2-to 3-fold greater (Fig. 2). Hence, the 2-to 5-fold higher enzyme activity in medium from clone c suggests that this cell line releases proportionately more tyrosinase. Interac-

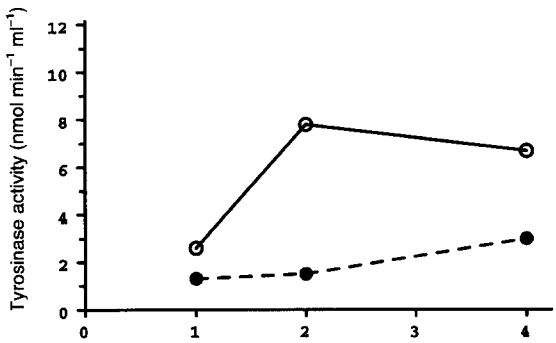

Fig. 3. Comparison of tyrosinase activity in the culture medium of clone c and subclone c + $b_{1.2}$. Cell lines are (○) clone c expressing tyrosinase alone, and (●) subclone c + $b_{1.2}$ expressing both tyrosinase and the b-protein. Cells were seeded at a fixed density and media samples prepared by method D over a time course. Medium was not changed during the experiment. Tyrosinase activity was assayed by the method of Wittbjer et al. [41] and values are expressed per ml culture medium.

tion of the b-protein with tyrosinase in some form of high molecular weight complex [17,18] may explain the increased amount of tyrosinase in the membrane-bound fraction of subclone c + $b_{1.2}$, and the decreased release of enzyme to the medium.

The distribution of the b-protein between membrane-bound and soluble fractions in subclone c + $b_{1.2}$ was analysed by Western blotting using antibody TA99 (data not shown). More than 95% of the b-protein is membrane-bound, as is the case in human melanoma cells [52], and hence we assume that the b-protein interacts primarily with membrane-bound tyrosinase rather than the soluble form of the enzyme.

We recently reported that the b-protein functions as a stereospecific L-dopachrome tautomerase [12], and hence tested for this activity in c + b subclones. In common with other cell types, c + b subclones possess a high level of D-dopachrome tautomerase activity which does not bind to Con A-Sepharose and produces DHI [53]. However, c + b subclones also contain a dopachrome tautomerase activity which binds to Con A-Sepharose and catalyses the conversion of L- but not D-dopachrome to DHICA. For subclone c + $b_{1.2}$ the specific activity of the L-dopachrome tautomerase is approximately 0.9 nmol min^{-1} per mg of Con A-purified protein. This L-dopachrome tautomerase is present in fibroblasts expressing the b-protein alone at a similar level, but not in untransfected fibroblasts or clone c [12]. The finding of activity in c + b lines is consistent with our previous proposal that the b-protein functions as an L-dopachrome tautomerase [12].

3.4. Characterisation of the pigment synthesised by double transfectants

Clone c expresses tyrosinase and contains the melanin synthesis intermediates L-dopa, 5-*S*-cysteinyldopa and 5-*S*-glutathionyldopa. It produces a phaeomelanin-like pigment, but does not contain detectable eumelanin [19]. Fibroblasts expressing both tyrosinase and the b-protein are more darkly pigmented than clone c, and we tested whether this change in colour correlates with alterations in the levels of melanin intermediates and in the nature of the melanin polymer.

Table 2
Catechol levels in extracts of fibroblasts expressing tyrosinase alone (clone c) or tyrosinase and the b-protein (subclone c + $b_{1.4}$)

Cell line	Compound concn. (nmol/mg protein)			
	L-dopa	5-*S*-CD	5-*S*-GD	5-*S*-CGD
Clone c	0.03	1.40	0.23	0.00
Subclone c + $b_{1.4}$	0.14	8.60	5.85	0.10

Catechol content of cell extracts prepared from confluent cultures was measured as described in Section 2. The results of a typical experiment are shown. Similar results were obtained each time.

Table 3
Melanin synthesis intermediates in culture medium of fibroblasts expressing tyrosinase alone (clone c) or tyrosinase and the b-protein (subclone c + $b_{1.4}$)

Cell line	5-*S*-CD concn. (μmol ml^{-1})		6H5MI2CA concn. (μmol ml^{-1})	
	day 1	day 4	day 1	day 4
Clone c	0.02	0.46	0.00	0.01
Subclone c + $b_{1.4}$	0.01	0.57	0.00	0.55

Cells were seeded at a density of 2×10^6 per 25 cm^2 flask on day 0 and media samples were prepared over a time course. Levels of melanin intermediates were determined as described in Section 2 and are expressed per ml of culture medium. The results for one of three experiments are shown. Similar results were obtained each time.

The amounts of the melanin synthesis intermediates L-dopa, 5-*S*-cysteinyldopa and 5-*S*-glutathionyldopa are all considerably higher in extracts of subclone c + $b_{1.4}$ than in clone c (Table 2): the levels of L-dopa and 5-*S*-cysteinyldopa are approximately 5-fold greater and the level of 5-*S*-glutathionyldopa is 25-fold higher. We were also able to detect 5-*S*-cysteinylglycinedopa in subclone c + $b_{1.4}$, but this compound was not present at a detectable level in clone c. It is formed on the hydrolysis of 5-*S*-glutathionyldopa by the enzyme γ-glutamyl transpeptidase [54]. The higher levels of melanin intermediates in subclone c + $b_{1.4}$ suggest that the tyrosinase activity of this line is greater than that of clone c in vivo as well as in vitro (Tables 1 and 2).

5-*S*-Cysteinyldopa in the culture medium from clone c and subclone c + $b_{1.4}$ was analysed over a time course during which the medium was not changed. The level increases over the time course and is similar in the two lines at all time-points (Table 3). The ratio of concentrations of 5-*S*-cysteinyldopa in the medium compared to cell extracts is higher for clone c than for subclone c + $b_{1.4}$, even when the slower growth of subclone c + $b_{1.4}$ is taken into account (Tables 2 and 3). No melanin intermediates were detectable in cell extracts or medium from untransfected fibroblasts or fibroblasts expressing the b-protein alone.

Analysis of medium from subclone c + $b_{1.4}$ also revealed the presence of several carboxylated indoles: DHICA, 5-hydroxy-6-methoxyindole-2-carboxylic acid (5H6MI2CA) and 6-hydroxy-5-methoxyindole-2-carboxylic acid (6H5MI2CA) (Fig. 4A). None of these compounds were detectable in medium from clone c. We quantitated the amount of 6H5MI2CA in the medium by comparison with a synthetic standard (Fig. 4B) over a time course of four days during which the medium was not changed. 6H5MI2CA was not found at levels significantly above background in medium from clone c at any time-point, but increased markedly by day four in medium from subclone c + $b_{1.4}$ (Table 3). Carboxylated indoles are only expected to be predominant in cells containing an active

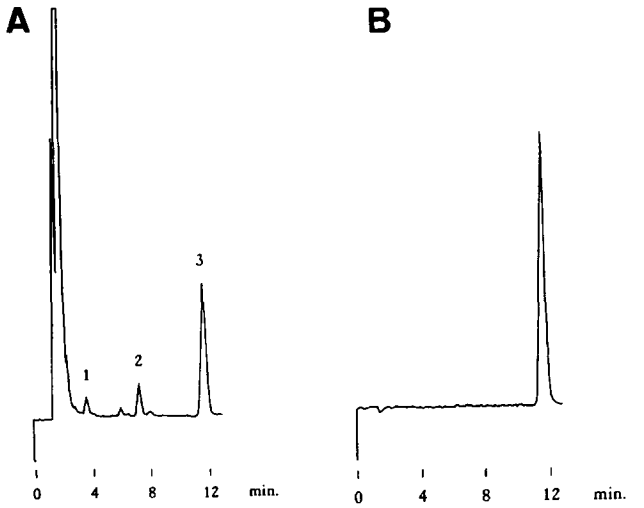

Fig. 4. HPLC analysis of carboxylated indoles in the culture medium of fibroblasts co-expressing tyrosinase and the b-protein. (A) A representative sample of culture medium from subclone $c+b_{1.2}$, grown for 4 days without the medium being changed, was analysed by HPLC as described in Section 2. Retention times for carboxylated indoles are: (1) DHICA, 3.63 min; (2) 5H6MI2CA, 7.21 min; (3) 6H5MI2CA, 11.67 min. (B) Trace for a synthetic 6H5MI2CA standard used for quantifying this carboxylated indole.

L-dopachrome tautomerase since spontaneous oxidation of the L-dopachrome produced by tyrosinase generates decarboxylated products. Hence, these results suggest that the b-protein acts as a dopachrome tautomerase in vivo as well as in vitro [12].

Clone c synthesises a phaeomelanic pigment and its hydrolysis product AHP is present at a concentration of 0.15–0.19 μg per mg of protein [19]. The yield of AHP is 0.72 μg per mg of protein for subclone $c + b_{1.4}$, indicating that these cells contain 3–4 times more phaeomelanic pigment than clone c.

The eumelanin degradation product PTCA is produced in very low yield on oxidation with potassium permanganate, limiting the sensitivity of the assay for this form of melanin. No PTCA could be detected on analysis of clone c, but trace amounts were detected in hydrolysates of subclone $c + b_{1.4}$.

On oxidation with potassium permanganate, TDCA is produced from phaeomelanin, and hence the ratio of TDCA to PTCA can be used to judge the ratio of phaeomelanin to eumelanin [49]. This ratio was estimated to be approximately 4 to 1 for subclone $c + b_{1.4}$.

4. Discussion

4.1. Interaction of tyrosinase and the b-protein

On transfection of clone c with a b-protein expression vector, we observed that fibroblasts expressing both tyrosinase and the b-protein have higher tyrosinase activity than the parent line that expresses tyrosinase alone. Although all double transfectant lines described in this report were derived from the same parent line, clone c, we have observed a similar increase in tyrosinase activity when several other tyrosinase-expressing lines were transfected with the b-protein (Winder, A.J., unpublished data), and believe the results reported here to be generally applicable. The higher in vitro tyrosinase activity of c + b subclones correlates with increased levels of the melanin intermediates dopa, 5-*S*-cysteinyldopa and 5-*S*-glutathionyldopa in cell extracts, demonstrating that enzyme activity is also greater in vivo. We have previously shown that the higher tyrosinase activity of c + b suclones is not due to rearrangement of the transfected tyrosinase cDNA or to alterations in transcription of this sequence [55]. The increased enzyme activity results primarily from a higher steady-state level of tyrosinase protein which in turn is due to stabilisation by interaction with the b-protein [55]. However, data presented in this report suggest that an increased level of tyrosinase protein cannot completely account for the measured increase in enzyme activity, implying that presence of the b-protein may also activate the enzyme. In addition, a comparison of kinetics of accumulation of the b-protein in fibroblasts expressing this protein alone, or in combination with tyrosinase, suggests that the presence of tyrosinase influences the steady-state level of the b-protein.

Tyrosinase and the b-protein co-localise in lysosomes in c + b subclones [55] and a large proportion of both proteins are membrane-bound in double transfectants, favouring the concept that the two proteins interact in a membrane-bound complex, as has been previously reported to be the case in melanoma cells [17,18]. In melanocytes, tyrosinase and the b-protein are located in melanosomes, and recent evidence suggests that these organelles are closely related to lysosomes [12,17,20].

4.2. The b-protein acts as a dopachrome tautomerase in vivo

Conversion of the light brown phenotype of fibroblasts expressing tyrosinase alone to dark brown on co-expression of the b-protein suggests that the expressed b-protein is functional. There has been considerable controversy regarding the function of the b-protein, proposals having been made that it acts as a second tyrosinase, a catalase, a dopachrome tautomerase or a DHICA oxidase [6,7,12,28,29]. Our previous studies of fibroblasts expressing the b-protein alone showed that this protein can act as an L-specific dopachrome tautomerase [12], but due to the presence of high D-dopachrome tautomerase activity in cell extracts [53] we were only able to demonstrate this function in fractions enriched in the b-protein by Con A-Sepharose chromatography. This Con A-binding L-dopachrome tautomerase activity is present in fibroblasts co-expressing tyrosinase and the b-protein, but not in fibroblasts

expressing tyrosinase alone, providing additional evidence that the b-protein has this function.

In the absence of dopachrome tautomerase, spontaneous oxidation of L-dopachrome generated by tyrosinase produces predominantly decarboxylated indoles which are very unstable and cannot be detected in culture medium. The presence of carboxylated indoles in the medium of c + b subclones therefore strongly suggests that these cells contain an L-dopachrome tautomerase enzyme. No carboxylated indoles are found in the medium from clone c, even though the tyrosinase product 5-*S*-cysteinyldopa is readily detectable, and hence it appears that the b-protein acts as an L-dopachrome tautomerase in vivo as well as in vitro. This does not, however, rule out the possibility that the b-protein may also have other enzyme activities, as has been demonstrated for tyrosinase [30,31]. Furthermore, it should be borne in mind that the mechanism of conversion of dopachrome to DHICA is a matter of debate [26,56–58], and further work is needed to define the precise catalytic activity of the b-protein with respect to dopachrome tautomerisation.

4.3. Fibroblasts co-expressing tyrosinase and the b-protein synthesise both eumelanin and phaeomelanin

Detection of higher levels of phaeomelanin and traces of eumelanin in c + b subclones is consistent with the darker phenotype of these cells compared to clone c in which only low levels of phaeomelanin are detected [19]. It has been proposed that the switch between the synthesis of eumelanin and phaeomelanin is controlled by the level of tyrosinase activity and that eumelanin is only produced when the level of tyrosinase activity is high enough to saturate the supply of thiols [21,59]. The higher in vitro tyrosinase activity of c + b subclones compared to clone c, and the higher levels of intermediates within the cells, are consistent with this hypothesis.

Del Marmol et al. [60] recently reported a correlation between expression of b-protein mRNA and synthesis of eumelanin in melanoma cells and concluded that the b-protein is involved only in eumelanin and not in phaeomelanin synthesis. However, no data on protein levels were presented, and our results suggest that the situation is not quite so straightforward, presence of the b-protein having at least two effects. Firstly, its presence results in higher tyrosinase activity which in turn leads to the synthesis of increased amounts of phaeomelanin, and formation of eumelanin when the supply of thiols is saturated. Secondly, presence of L-dopachrome tautomerase activity will affect eumelanin synthesis since the ratio of DHI to DHICA is important in determining both the rate of melanin formation and the nature of the pigment [18,61]. A high level of dopachrome tautomerase activity relative to tyrosinase accelerates melanin synthesis and results in the formation of a more ordered polymer [61], and DHICA has itself been shown to be incorporated into mammalian melanins [62].

4.4. The b-protein may have a protective role

Growth of c + b subclones is strongly inhibited when the culture medium is not renewed regularly, although the majority of cells remain viable. Fibroblasts expressing the b-protein alone have similar growth kinetics to untransfected cells, whereas clone c, expressing tyrosinase at a low level, grows slightly more slowly. The strong inhibition of growth of c + b subclones is therefore very likely to be related to the high tyrosinase activity of these lines. Increased tyrosinase activity results in elevated intracellular levels of cytotoxic melanin intermediates and the depletion of thiols, particularly glutathione. In c + b subclones the level of 5-*S*-glutathionyldopa, formed by non-specific oxidation of L-dopa in the cytoplasm, is much higher than that found in melanoma cells such as IGR 1 [59]. Its formation probably reflects the lack of true melanosomes which normally retain the highly-reactive quinones produced during melanin synthesis. Considerable oxidative stress is induced by the generation of cytotoxic melanin intermediates, and by the production of hydrogen peroxide in the medium on their extracellular oxidation [54,63].

Indirect evidence suggests that presence of the b-protein is necessary for the survival of fibroblasts with high tyrosinase activity, since attempts to increase the tyrosinase activity of clone c by re-transfection with a second tyrosinase expression vector have failed to produce any clones with significantly higher enzyme activity (Winder, A.J., unpublished data). This suggests that in fibroblasts expressing tyrosinase alone there is a maximum level of enzyme activity compatible with survival, but that when the b-protein is co-expressed with tyrosinase, transfected fibroblasts can survive with up to 7-fold higher tyrosinase activity. This protective function of the b-protein may be related to its dopachrome tautomerase activity, since DHICA, the product of L-dopachrome tautomerase, is much less cytotoxic than the spontaneous oxidation product DHI [11,18].

4.5. Interactions of other melanogenic proteins

Co-expression of tyrosinase and the b-protein in fibroblasts has provided insight into the functions and interactions of these two proteins and their roles in melanin synthesis. Following the recent cloning of cDNAs encoding other proteins involved in regulating melanin synthesis, it will be useful to extend this co-expression approach to build up a picture of how multiple melanogenic proteins interact. Fibroblast expression studies may also prove useful in relating specific mutations in melanogenic proteins to phenotypic effects. The limitations of using fibroblasts to study proteins normally synthesised by cells of a different lineage must always be borne in mind. In particular, lack of true melanosomes may limit the usefulness of this system, but given recent evidence of the close relationship

between melanosomes and lysosomes, in which melanin is synthesised in transfected fibroblasts [12,17,20], this may not be the case.

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