Melanin Monomers Within Coated Vesicles and Premelanosomes in Melanin Synthesizing Cells

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We have found substantial amounts (6.6–143 and 0.5–13 μg/mg. protein, respectively) of 5,6-dihydroxyindole (5,6-DHI) and 5,6-dihydroxyindole-2-carboxylic acid (5,6-DHI2C), which are key intermediate monomers for the formation of the eumelanin polymer, within coated vesicle fraction of pigment cells. In addition, the amounts of these eumelanin monomers have been found to decrease along with the process of eumelanin polymer formation from coated vesicles to premelanosomes and finally to melanosomes among melanogenic subcellular compartments. Our present findings seem to indicate that coated vesicles transfer not only highly glycosylated T_1-tyrosinase but also eumelanin monomers into premelanosomes. *J Invest Dermatol 91:181–184, 1988*

We have previously shown that among subcellular melanogenic compartments, coated vesicles contain the highest tyrosinase activity, which is often 5 to 10 times higher than that of premelanosomes [1]. However, electron microscopic observation reveals no observable electron density characteristic of melanin polymer in these vesicles, although they are strongly DOPA-positive [2]. Ito developed eumelanin and pheomelanin assay methods using pyrrole-2,3,5-tricarboxylic acid (PTCA) and aminohydroxyphenylalanine (AHP), respectively [3]. By these methods, we detected PTCA-demonstrable eumelanin and AHP-demonstrable pheomelanin in coated vesicles as well as in premelanosomes.

At first, we were puzzled by these results. However, it has also been shown that the "eumelanin" value obtained by PTCA method includes not only eumelanin polymer but also melanin precursor monomers such as 5,6-DHI and 5,6-DHI2C [3]. Furthermore, the "pheomelanin" value obtained by AHP method similarly includes pheomelanin polymer as well as its precursor monomers such as benzothiazine derivatives [3]. Thus, we have explored the possible function of coated vesicles as a carrier not only of tyrosinase but also as a carrier of melanin precursor monomers.

MATERIALS AND METHODS

Melanoma Tissues Greene's melanoma tissue was implanted in 20–30mg aliquots into the subcutaneous tissue (both shoulder and flank) of Syrian golden hamsters. Two weeks later the animals were killed and the grayish brown melanomas were removed for assay, four melanomas each from 10 hamsters being used for each experiment. A spontaneously developed 10 g deep black pig melanoma nodule from a Duroc pig was also studied.

Preparation of Subcellular Fractions Melanoma tissues were homogenized in an ice-cold MES buffer (pH 6.8) containing 0.1M MES, 1mM EGTA, 0.5mM $MgCl_2$, 0.02% sodium azide, and 3mM 2-mercaptoethanol. The coated vesicle fraction was isolated by the method of Usami, Takahashi, and Kadota [4]. Premelanosome and melanosome fractions were isolated by sucrose density gradient ultra-centrifugation, as described previously [5]. The purity of each fraction was examined by electron microscopy.

The activity of the marker enzyme tyrosinase was measured following the method of Hamada and Mishima [6].

Chemicals 5-S-Cysteinyldopa (5-S-CD), 5,6-DHI, 5,6-DHI2C, and PTCA were synthesized as described by Ito [7,8]. 3-amino-4-hydroxyphenylalanine was purchased from Sigma Chemical Co (St. Louis, MO). All other chemicals were purchased from Nakarai chemicals (Kyoto, Japan).

Detection of DOPA, 5-S-CD, Eumelanin, and Pheomelanin DOPA and 5-S-CD in each fraction were determined by the method of Ito et al [9]. Quantitative assays of eumelanin and pheomelanin were performed according to Ito's PTCA and AHP methods [3].

Detection of 5,6-DHI and 5,6-DHI2C 5,6-DHI and 5,6-DHI2C were detected by high-performance liquid chromatography. LC-5A model liquid chromatography (Shimadzu, Kyoto, Japan) was used with a SPD-6A model ultraviolet detector. The detector was set at 298 nm. Separation was achieved on a C_{18} reversed-phase column (Yanaco ODS-A, particle size 7 μm, 250 × 4.6 mm) at 40°C. The mobile phase was methanol-0.05M phosphate buffer (pH 6.5), containing ImM Na_2EDTA (5:95). The flow-rate was 0.7 ml/min. Under these chromatographic conditions, retention times of 5,6-DHI and 5,6-DHI2C were 5.19 and 10.52 min, respectively.

Electron Microscopy Melanoma specimens were fixed with 2.5% glutaraldehyde 0.1M phosphate buffer (pH 7.4) and 1% OsO_4. After dehydration in graded ethanols, specimens were em-

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Abbreviations:
- 5,6-DHI: 5,6-dihydroxyindole
- 5,6-DHI2C: 5,6-dihydroxyindole-2-carboxylic acid
- 5-S-CD: 5-S-cysteinyldopa
- 5H6MI2C: 5-hydroxy-6-methoxyindole-2-carboxylic acid
- AHP: aminohydroxyphenylalanine
- DCOR: dopachrome oxidoreductase
- DOPA: 3,4-dihydroxyphenylalanine
- GERL: golgi-associated endoplasmic reticulum of lysosome

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bedded in Epon. Ultrathin sections stained with uranyl acetate and lead citrate were examined using JEOL 100S electron microscopy. Isolated coated vesicle fractions were observed using the technique of negative staining [10].

Protein Quantitative Analysis Protein content of each sample was measured by the Lowry method [11]. Bovine serum albumin was used as a standard protein.

RESULTS

Figure 1*a,b* shows the coated vesicle fraction prepared from hamster Greene's melanoma by negative staining electron microscopy. The fraction consists of small spheroid bodies and is almost free from other cytoplasmic organelles. The diameter and shape of these coated vesicles are the same as those shown by thin section electron microscopy [Fig 1*c*]. This coated vesicle fraction has not only higher tyrosinase activity compared to whole tissue homogenate and premelanosome fraction but also consistently substantial amounts of PTCA and AHP (Table I). It was also noted [12] that a moderate amount of 5-S-CD was present in premelanosome and melanosome fractions.

Further, we have found that the coated vesicle fractions that were isolated from hamster Greene's melanoma and spontaneous pig melanoma contained a considerable amount of 5,6-DHI and 5,6-DHI2C (Table II). These indolic compounds were also detected in premelanosome and melanosome fractions. Furthermore, the amount of 5,6-DHI2C has decreased in parallel with the degree of maturation and melanization (CV → PMS → MS) (Fig 2).

DISCUSSION

The maturation and activation of tyrosinase synthesized by ribosomes have been shown to occur primarily as a glycosylation process in the GERL-coated vesicle system [1,2,13], which is a separate melanogenic subcompartment from the premelanosome forming Golgi apparatus or smooth endoplasmic reticulum. Glycosylated mature T_1- or T_3-tyrosinase is transported from the GERL to premelanosomes by budding-off of coated vesicles. For this transporting process, carbohydrate moieties [14] of T_3-tyrosinase should play an integral role as signal for the intracellular recognition process. Coated vesicles, after fusing into premelanosome, transfer their highly concentrated T_1-tyrosinase leading to the formation of membrane-bound T_3-tyrosinase [13,14] and melanin polymer within premelanosomes. Our present findings (Fig 2, Table II) seem to indicate that eumelanin monomers such as 5,6-DHI2C, which has been shown to be the core for the eumelanin polymer [15], are already present within coated vesicles in the pigment cells. In accordance with this, electron microscopic observation of pigment cells reveals the presence of electron dense melanin polymer only in some premelanosomes, and in all melanosomes, but not in coated vesicles. It has been shown that 1 mg of 5,6-DHI2C and 5,6-DHI give 0.6 and 0.015 mg of eumelanin (PTCA × 50), respectively, by Ito's eumelanin assay method [16]. It is thus further suggested that coated vesicles may contain not only melanin monomer but also melanin oligomer that shows no observable electron density in electron microscopy. The absence of melanin polymer formation in coated vesicles in spite of the presence of high tyrosinase activity and melanin monomer is of considerable interest.

The new findings presented here could be explained by the action of several regulatory factors found recently. We have previously shown that T_3-tyrosinase is essential for melanin polymer formation within premelanosomes of living pigment cells in vivo and in a culture system [1].

The absence of T_3-tyrosinase activity within coated vesicles has been shown by glycosylation inhibitor experiments using tunicamycin and glucosamine [2,13]. Korner and Pawelek demonstrated a critical role of tyrosinase in the conversion of 5,6-DHI to melanin [17,18]. Further, the T_3-tyrosinase isozyme has been shown to be involved in the conversion process of melanin monomer to polymer [19]. These findings and our results indicate that melanin polymer formation should not take place in the coated vesicles due to the lack of T_3-tyrosinase.

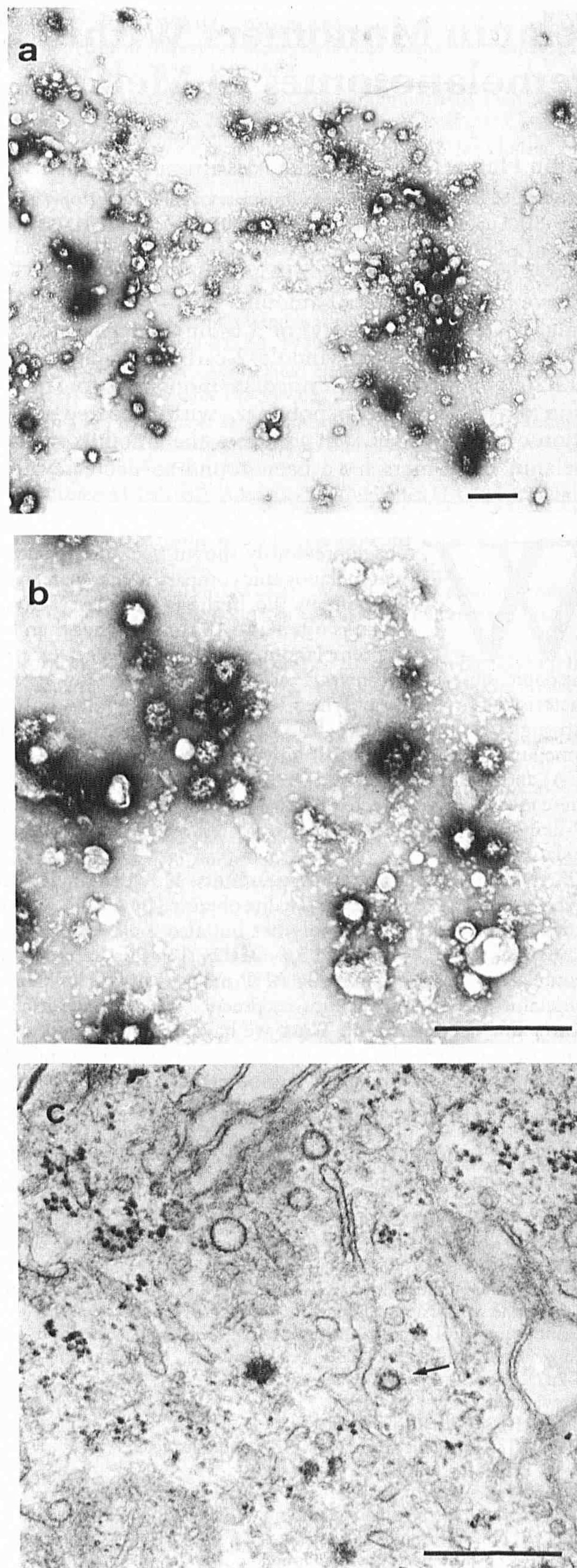

Figure 1. Electron microscopic examination. *a,b:* Coated vesicle fraction isolated from hamster Greene's melanoma revealing its high degree of purity and characteristic coated structure using negative staining at low *(a)* and high *(b)* magnification. *bar* = 0.5 μm. *c:* In order to confirm the integrity of the coated vesicles in the fraction, their ultrastructure in the melanoma cell is shown in an ultrathin section at the same magnification *(arrow)*. GTA-OsO_4, *bar* = 0.5 μm.

Table I. Analysis of Eumelanin, Pheomelanin, and its Precursors in Coated Vesicles Isolated from Hamster Greene's Melanomas[a]

	μg/mg · protein			
Tyrosinase Ratio (CV/homo)[b]	Eumelanin (PTCA ×50)[c]	Pheomelanin (AHP ×5)	5-S-CD	DOPA
154.3	1.35 ± 0.41	0.50 ± 0.11	0.05 ± 0.01	0.02 ± 0.01
129.0	0.85 ± 0.27	4.82 ± 1.20	—	0.48 ± 0.07
109.0	0.60 ± 0.18	1.93 ± 0.44	—	0.08 ± 0.01
127.5	0.80 ± 0.22	9.31 ± 2.14	0.04 ± 0.01	0.05 ± 0.01

[a] This table shows data of four separately performed independent experiments with duplicate assays of each sample (mean ± SE). Each isolated coated vesicle fraction has exhibited 100 ~ 150 times higher tyrosinase activity than tissue homogenate.
[b] Ratio of tyrosinase activity (ΔE 475 nm/10 min/mg · protein) between coated vesicle fraction and tissue homogenate.
[c] See Ref 3.

Table II. Analysis of Eumelanin, Pheomelanin, and Precursor Monomers in Melanogenic Compartments of Pigment Cells[a]

	μg/mg · protein					
	Eumelanin (PTCA ×50)[e]	Pheomelanin (AHP ×5)[e]	5,6-DHI	5,6-DHI2C	5-S-CD	DOPA
Hamster melanoma						
CV[b]	1.6 ± 0.1	8.2 ± 0.1	6.6 ± 1.0	0.50 ± 0.08	—	0.24 ± 0.06
PMS[c]	4.4 ± 0.8	26.9 ± 0.4	0.7 ± 0.1	0.42 ± 0.07	0.04 ± 0.01	0.20 ± 0.06
MS[d]	4.8 ± 1.1	24.2 ± 0.4	13.1 ± 1.8	0.23 ± 0.03	0.08 ± 0.03	0.10 ± 0.04
Pig melanoma						
CV	85 ± 7	—	143 ± 20	13.1 ± 2.5	—	—
PMS	8.1 ± 1.1	1492 ± 55	4.7 ± 0.7	2.9 ± 0.5	0.06 ± 0.02	0.18 ± 0.04
MS	663 ± 49	16.1 ± 0.6	2.3 ± 0.3	0.09 ± 0.02	0.02 ± 0.01	0.11 ± 0.02

[a] Data of Greene's hamster melanoma obtained from two independent performed experiments with duplicate assays of each sample (mean ± SE). Data of a naturally occuring pig melanoma were obtained from one experiment with duplicate assays (mean ± SE).
[b] Coated vesicle.
[c] Premelanosome.
[d] Melanosome.
[e] See Ref 3.

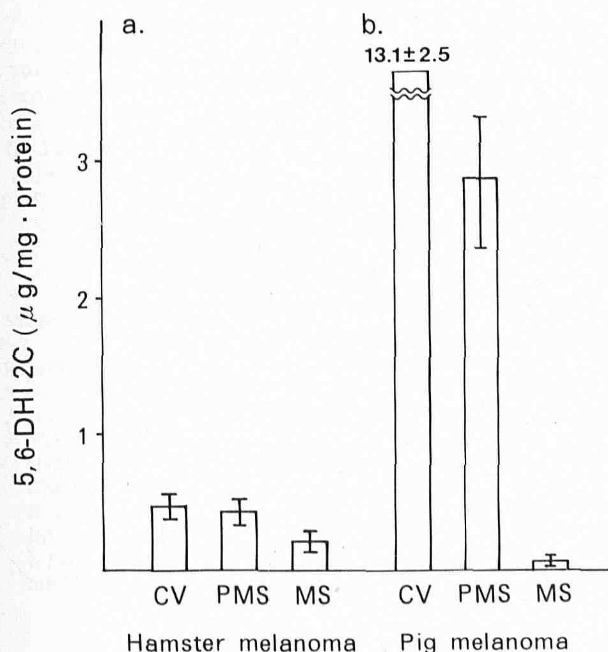

Figure 2. Distribution of 5,6-DHI2C within melanogenic subcellular compartments of less eumelanic hamster Greene's melanoma and highly eumelanic pig melanoma. The value of 5,6-DHI2C has decreased in the parallel to the process of eumelanin polymer formation from coated vesicles to premelanosomes and finally to melanosomes.

Barber et al have shown that dopachrome oxidoreductase (DCOR) blocks the conversion of indoles to melanin polymer in in vitro fractionation experiments [20]. Therefore, it may be possible that coated vesicles contain high DCOR activity which blocks the polymerization process.

Recently, Palumbo and Prota showed that metal ions (Cu^{2+}, Ni^{2+}, Co^{2+}), which were present at high levels in melanoma tissues, catalyzed the conversion of dopachrome to 5,6-DHI2C rather than to 5,6-DHI [15]. Moreover, Westerhof and Pavel reported that an Asian subject exhibited higher urinary excretion of 5-hydroxy-6 methoxyindole-2-carboxylic acid (5H6MI2C), which was formed from DHI2C by its methylation, as compared to Caucasian and albino subjects. They concluded that 5H6MI2C was an important melanin related metabolite in the skin pigmentary system [21]. In accordance with this, our present data (Table II) indicate a much higher content of 5,6-DHI2C in eumelanic pig melanoma as compared to less eumelanic hamster Greene's melanoma. Furthermore, it should be noted that 5,6-DHI2C content is the highest in coated vesicles and decreases linearly as melanin polymer is formed in premelanosomes and melanosomes (Fig 2).

It seems reasonable to assume that the linear decrease of 5,6-DHI2C from coated vesicle to melanosome may be due to its consumption during the melanin polymer formation process in melanogenic compartments. The real inhibitory mechanisms of factors that regulate 5,6-DHI2C to melanin polymer in coated vesicles require further investigation, for elucidation.

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