B．研究方法

I. NPrCAP/M を用いた温熱療法の効果とそのメカニズムの解析

メラニン陽性のマウスメラノーマ細胞株である B16F1 細胞を用いて NPrCAP/M を用いた温熱療法の効果を検討する（分担研究者、高田らによる）。

1. NPrCAP/M, NPrCAP/M + hyperthermia の各々による治療後、残存腫瘍を外科的に切除したのち、再度 B16F1 細胞を接種し、腫瘍の増殖を観察した。
2. 上記の観察において、再チャレンジした腫瘍が拒絶されたマウスから、脾細胞を回収し、B16F1 を用いた MLTC を行い、５日後に細胞傷害性試験を行って、CTL 誘導について検討する。特に、B16F1 melanoma の腫瘍拒絶抗原として TRP (tyrosine related prtein)-2 由来の抗原ペプチド（配列を記載）が同定されているので、このペプチドに特異的な CTL が誘導されているかにつき検討することも重要な課題である。
3. B16F1 melanoma 細胞に NPrCAP/M を取り込ませ、その後磁場照射により hyperthermia を施行した細胞における各種熱ショック蛋白質 (heat shock proteins: HSPs)の発現変化を、Western blotting により検討する。
4. B16F 1melanoma 細胞に NPrCAP/M を取り込ませ、その後磁場照射により hyperthermia を施行した細胞からの Hsp70, Hsp90 の細胞外放出量を ELISA 法により測定する。
5. B16F1 melanoma 細胞に NPrCAP/M を取り込ませ、その後磁場照射により hyperthermia を施行した細胞を短期間培養する。その培養上清（細胞外に放出された HSP-抗原ペプチド複合体が含まれていると考えられる）を同系マウスに腹腔内免疫し、腫瘍特異的な免疫応答、特に CTL 誘導について検討する。
6. 上記の方法による CTL 誘導効果が確認されれば、この培養上清をあらかじめ誘導しておいた骨髄樹状細胞にパルスし、B16F1 細胞特異的 CTL との反応性を検討する。
7. さらに HSP-ペプチド複合体の関与を直接的に証明するために、この培養上清から免疫沈降法により HSP-peptide 複合体を除去し、骨髄樹状細胞にパルスし、B16F1 細胞特異的 CTL との反応性の変化を検討する。

II. NPrCAP/M を用いた温熱療法による全身性免疫応答の誘導と転移性腫瘍に対する治療効果

実際の臨床では、原発巣の治療のみならず、肺、肝臓、脳などの遠隔転移巣に対する治療が患者の生命予後を左右する。NPrCAP/M を用いた温熱療法による原発巣の治療により全身性の抗腫瘍免疫応答が誘導されているならば、T リンパ球をはじめとする免疫担当細胞が転移巣における

腫瘍細胞を感知し、これを傷害するものと考えられる。これを検証する。

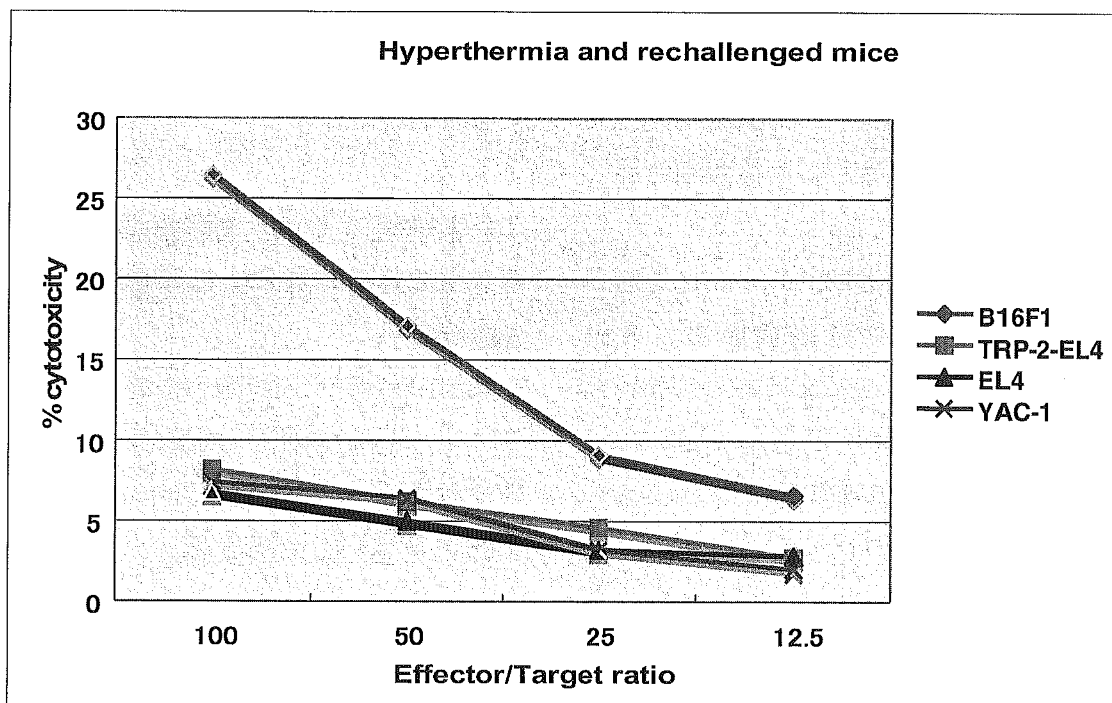
1. B16 melanoma を NPrCAP/M を用いて温熱療法を施行する。その1週後 B16 melanoma 由来の高転移株 B16F10 細胞を尾静脈内投与し、肺転移を形成させる。治療群、非治療群における肺転移巣の数および肺重量を計測する。
2. 上記実験において、肺転移に対する効果が確認されれば、免疫担当細胞のいずれが主要なエフェクターであるかを同定する。すなわち、あらかじめ CD4, CD8 および NK 細胞に対する単クローン抗体を腹腔内投与し、それぞれのポピュレーションを除去したのち、B16 melanoma を尾静脈内投与し、その後肺転移数を検討する。

C．研究結果

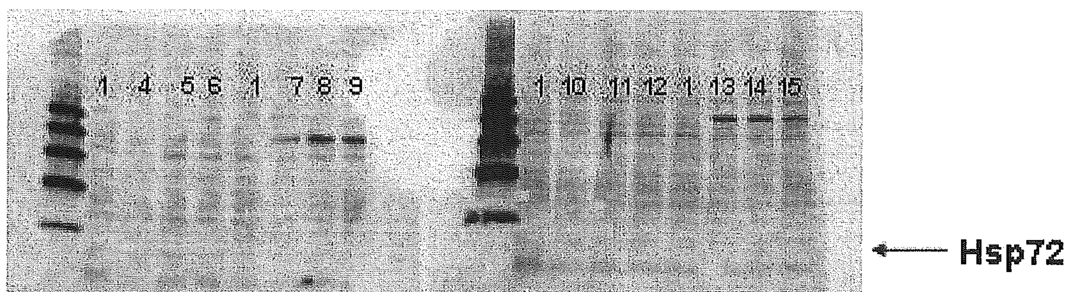
1．NPr/CAP/M 投与＋hyperthermia 治療マウスにおける B16F1 特異的細胞障害性T 細胞（CTL）の誘導

【方法】 NPrCAP/M 投与後磁場による hyperthermia を3回施行し、その後残存腫瘍を外科的に切除したマウスに対し、再度 B16F1 マウスを接種した。腫瘍拒絶を認めたマウスにおいて、B16F1 メラノーマ特異的な CTL が誘導されているかを ^{51}Cr release assay を用いて検討した。

【結果】下図に示すように、NPrCAP/M を投与し、hyperthermia を施行したマウスの脾細胞を用いて MLTC を行った結果、B16F1 メラノーマ特異的な CTL が誘導されていることが示された。YAC-1 に対する細胞傷害は認めず、メラノーマ特異的な腫瘍免疫応答が誘導されていることが示された。



2. NPrCAP/ML＋hypertyhermiaによるHsp70の誘導効果

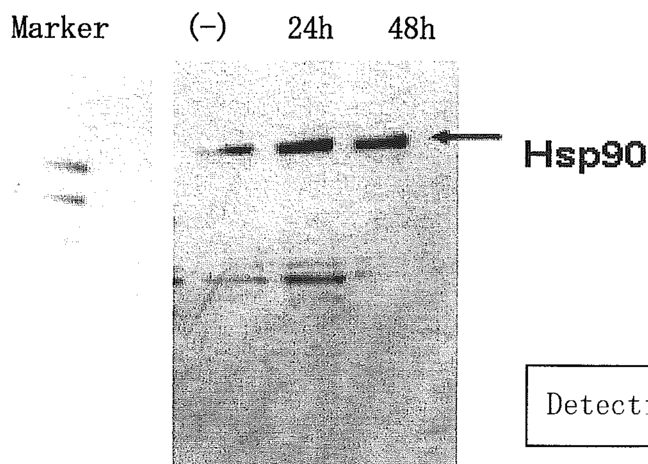


Detection: anti Hsp72 mAb

1：B16F1 cell	
4：磁場 (-), CAP/M(+), 12h	10：磁場 (-),M(+), 12h
5：磁場 (-), CAP/M(+), 24h	11：磁場 (-),M(+), 24h
6：磁場 (-), CAP/M(+), 36h	12：磁場 (-),M(+), 36h
7：磁場 (+), CAP/M(+), 12h	13：磁場 (+),M(+), 12h
8：磁場 (+), CAP/M(+), 24h	14：磁場 (+),M(+), 24h
9：磁場 (+), CAP/M(+), 36h	15：磁場 (+),M(+), 36h

【結果】B16F1 メラノーマにNPrCAP/MあるいはMagnetiteを取り込ませ、磁場照射した。いずれの場合でも12h, 24h, 36hと時間経過につれ、Hsp72の発現が増強した。

3. NPrCAP/ML＋hypertyhermiaによるHsp90の誘導効果

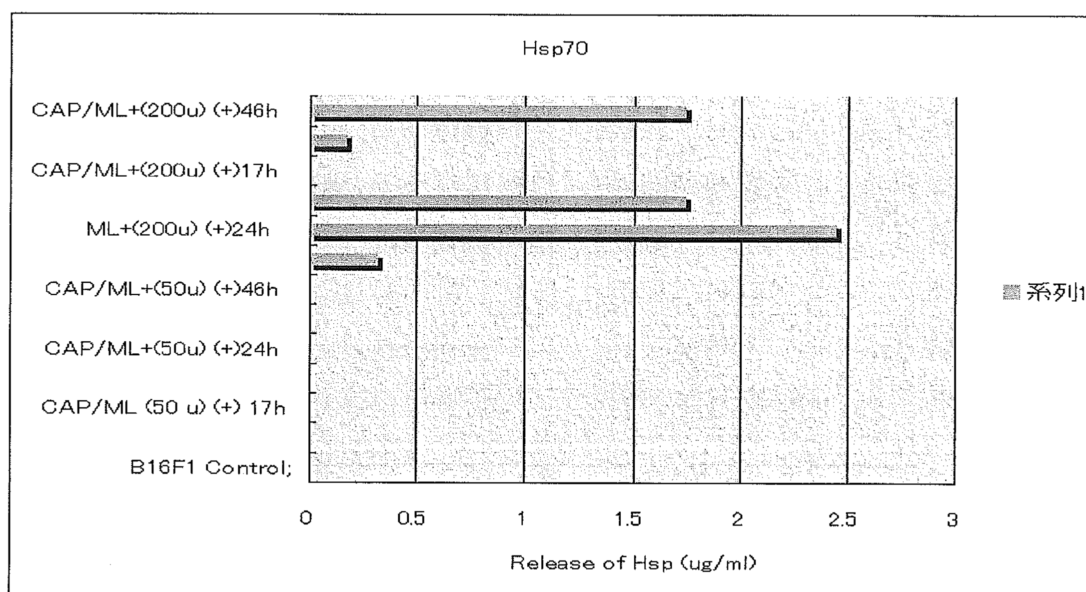


Detection: anti Hsp90

【結果】次にHsp90の発現変化について検討した。B16F1 メラノーマに NPrCAP/M を取り込ませ、磁場照射した。24h, 48h と時間経過につれ、Hsp90 の発現が増強した。このように NPrCAP/M を投与し、磁場照射すると細胞内加温により Hsp72 および Hsp90 の発現が明らかに増大することが示された。

4. NPrCAP/ML＋hypertyhermia による Hsp70 の細胞外放出

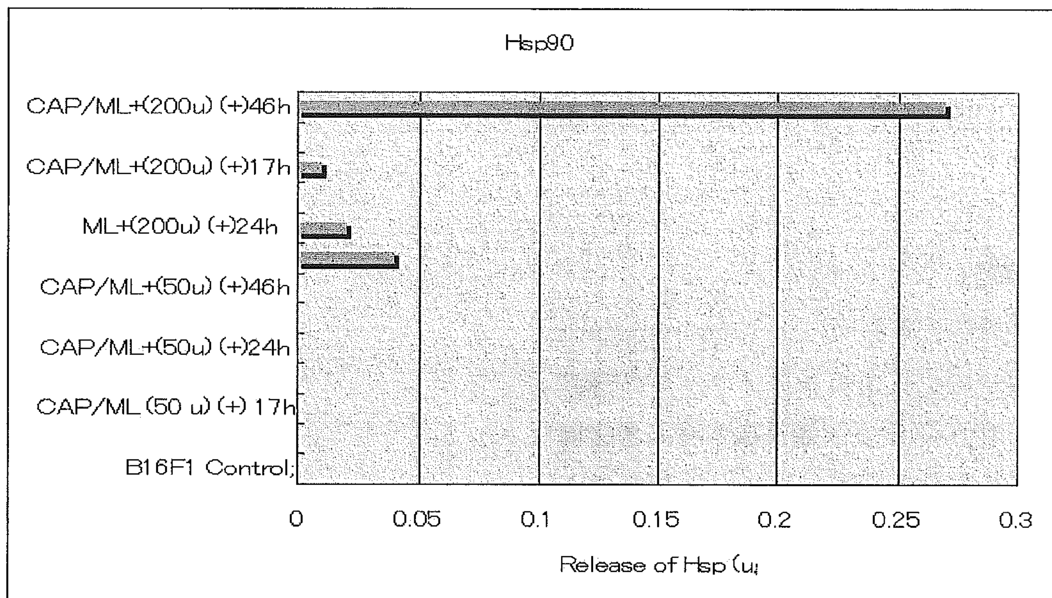
【方法】B16F1 メラノーマに NPrCAP/ML を取り込ませ、磁場照射した。その後、36 時間培養し、細胞外の培養液中に放出される Hsp72 を ELISA により測定した。



【結果】NPrCAP/ML (magnetoliposome)および Magnetoliposome を B16F1 に取り込ませて磁場照射すると熱ストレスが加わると同時に、細胞の壊死が誘導される。その際、細胞内の Hsp72 あるいは Hsp90 が細胞外に放出されると予想した。これを ELISA 法にて検討すると、いずれの場合でも B16 メラノーマの壊死がおこり、Hsp72 が細胞外に放出されることが確認された。

5. NPrCAP/ML＋hypertyhermia による Hsp90 の細胞外放出

【結果】同様に Hsp90 についても ELISA 法にて検討すると、NPrCAP/ML の用量依存性に B16 メラノーマの壊死がおこり、Hsp90 が細胞外に放出されることが確認された。



D．考察

(1) NPrCAP/M を投与し、hyperthermia を施行したマウスの脾細胞を用いて MLTC を行った結果、B16F1 メラノーマ特異的な CTL が誘導されていることが示された。YAC-1 に対する細胞傷害は認めず、メラノーマ特異的な腫瘍免疫応答が誘導されていることが示された。この結果は、上記の intracytoplasmic hyperthermia により、腫瘍特異的な全身性免疫応答が誘導されていることを示すものである。

(2) この腫瘍免疫誘導のメカニズムとして、NPrCAP/M を用いた intracytoplasmic hyperthermia による熱ショック蛋白質 HSP の誘導と細胞の選択的な壊死により細胞外に放出された HSP-ペプチド複合体を介する免疫応答誘導の可能性を考えた。すなわち HSP-ペプチド複合体は樹状細胞などの抗原提示細胞に取り込まれ、HSP に結合している腫瘍抗原ペプチドが抗原提示細胞の MHC class I に提示され、これが T 細胞に認識されるものと考えられる。

(3) そこで実際、B16F1 腫瘍細胞を hyperthermia に供したところ、主要な HSP である Hsp72 および Hsp90 の明らかな発現誘導・増強を確認しえた。

(4) さらに、腫瘍壊死に伴って Hsp72 および Hsp90 が細胞外環境に放出されるのかを ELISA 法にて確認したところ、いずれの HSP も細胞外に放出されることが確認された。

(5) 以上の事実は、NPrCAP/M を用いた

intracytoplasmic hyperthermia によるメラノーマの治療により、HSP-ペプチド複合体を介する腫瘍特異的免疫応答が誘導されている可能性を示唆するものである。

E．結論

NPrCAP/M を用いた intracytoplasmic hyperthermia によるメラノーマの治療により、HSP-ペプチド複合体を介する腫瘍特異的免疫応答が誘導されている可能性がある。今後、細胞外に放出された HSP-ペプチド複合体が実際に免疫応答を誘導しえるかにつき、重点的に検討する予定である。

F．健康危険情報

特になし。

G．研究発表

現在のところ、なし。

H．知的財産権の出願・登録状況

現在のところ、なし。

III．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表【書籍・雑誌】

【書籍】

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
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Inoue H, Yamashita T, Jin HY, Sakauchi F, Kageshita T, Takata M, Saida T, Jimbow K	Unique sequence of MC1R gene in Japanese melanoma; Only Bal92Met is associated with higher susceptibiligy.	**Sapporo Med J**		in press	2006
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Ito A, Kobayashi T, Honda H	A mechanism of antitumor immunity induced by hyperthermia.	**Japanese Journal of Hyperthermic Oncology,**	21 (1)	1-11	2005
Tanaka K, Ito A, Kobayashi T, Kawamura T, Shimada S, Matsumoto K, Saida T, Honda H	Intratumoral injection of immature cells enhances antitumor effect of hyperthermia using magnetic nanoparticles.	**Int J Cancer**	116	624-633	2005
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Tanaka K, Ito A, Kobayashi T, Kawamura T, Shimada S, Matsumoto K, Saida T, Honda H	Heat immunotherapy using magnetic nanoparticles and dendritic cells for T-lymphoma.	**J Biosci Bioeng.**	100 (1),	112-115	2005
Ito A, Fujioka M, Tanaka K, Kobayashi T, Honda H	Screening of cytokines to enhance vaccine effects of heat shock protein 70-rich tumor cell lysate.	**J Biosci Bioeng.**	100 (1)	36-42	2005

Ito A, Shinkai M, Honda H, Kobayashi T	Medical application of functionalized magnetic nanoparticles.	**J Biosci Bioeng.**	100 (1),	1-11	2005
Kawai N, Ito A, Nakahara Y, Futakuchi M, Shirai T, Honda H, Kobayashi T, Kohri K.	Anticancer effect of hyperthermia on prostate cancer mediated by magnetite cationic liposomes and immune-response induction in transplanted syngeneic rats.	**Prostate.**	64 (4),	373-381	(2005)
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Tanaka K, Ito A, Kobayashi T, Kawamura T, Shimada S, Matsumoto K, Shimada T, Honda H	Immunotherapy for melanoma using hyperthermia with magnetic nanoparticles and dendritic cells	12th European Congress on Biotechonology	コペンハーゲン	デンマーク	2005 8月

Ⅳ．研究成果の刊行物・別冊

Dominant Negative Rab7 Inhibits Intracellular Transport of Tyrosinase-Related Protein-1, but not Tyrosinase or gp100, from Golgi to Melanosomes

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Key words: endosome, microtubule, protein transport

Short Title: Rab7 and transport of melanosomal proteins

Abbreviations: AP, adaptor protein; EEA1, early endosome antigen-1; mAb, monoclonal antibody; MPR, mannose phosphate receptor; MTOC, microtubule-organizing center; RILP, Rab7-interacting lysosomal protein; TYR, tyrosinase; TYRP, tyrosinase-related protein; TGN, *trans*-Golgi network; wt, wild-type

Abstract

We have previously shown that Rab7, a small GTP-binding protein, is involved in melanogenesis via participation in the transport of melanosomal proteins. This study further examined the biological role of Rab7 and compared the vesicular transport of tyrosinase (TYR), tyrosinase-related protein-1 (TYRP1) and gp100 in COS7 cells or melan-a (murine melanocyte cell line) cells, which were transfected with plasmid carrying flag-tagged wild-type Rab7 (Rab7wt) or dominant negative mutant Rab7 (Rab7N125I). In immunocytochemistry of COS7 using a confocal laser scanning microscope, Rab7wt was distributed in the perinuclear area, especially microtubule-organizing center, and was co-localized with a late endosomal maker. In contrast, Rab7N125I was distributed in both the perinuclear area and the peripheral cytoplasm, and formed conglomerated structures and small granules, respectively, which were co-localized with an early endosomal maker. The two Rab7 proteins were found to be localized along microtubules in the cytoplasm. In melan-a cells, the distribution of melanosomal proteins was analyzed in the presence or absence of Rab7 function. In the Rab7wt-expressing melan-a cells, TYR, TYRP1 and gp100 were detected in both the perinuclear and peripheral areas. In the Rab7N125I-expressing melan-a cells, however, TYR and gp100 were distributed at the cell periphery while TYRP1 was not detected in the entire cytoplasm. These results indicate that Rab7N125I perturbs endosomal vesicular transport of melanosomal proteins and TYRP1 was most significantly affected by the expression of Rab7N125I. Thus, the transport of TYRP1 from Golgi complex to melanosomes is different from that of TYR and gp100.

Introduction

More than 95 genetic loci have been reported to regulate mammalian melanogenesis (Jackson, 1997). These loci include the genes encoding proteins involved in the vesicular transport of melanosomal proteins. Previously we have reported that Rab7 is localized on the melanosomal membrane of B16 murine melanoma cells (Gomez *et al*, 2001). The cells treated with Rab7 antisense-oligonucleotide revealed tyrosinase-related protein-1 (TYRP1) confined to the perinuclear area, indicating that Rab7 is involved in the transport of TYRP1 from the *trans*-Golgi network (TGN) to melanosomes, possibly passing through late endosome-delineated compartments. We have also reported that, through use of adenovirus vectors, amelanotic melanoma cells with exogenous expression of TYRP1 and Rab7N125I revealed TYRP1 to be localized to early endosomes (Hirosaki *et al*, 2002). Thus, it is suggested that the transport of TYRP1 may require functional Rab7 and that TYRP1 may pass through early to late endosomal compartments.

Rab7 is a member of Rab small GTP-binding protein family, and is essential for the regulation of endosomal vesicular transport, assembling the general tethering, docking, or fusion of transport vesicles (Chavrier *et al*, 1990; Feng *et al*, 1995; Vitelli *et al*, 1997; Press *et al*, 1998). Moreover, Rab7 is partly associated with lysosomal membranes (Meresse *et al*, 1995). It is essential for the maintenance of a functional lysosomal compartment (Bucci *et al*, 2000). Also Rab7 has been reported to play an important role in other lysosome-oriented pathways such as phagocytosis (Vieira *et al*, 2003) and autophagocytosis (Gutierrez *et al*, 2004). Recently, four Rab7-binding

molecules have been found and, through binding with these molecules, Rab7 regulates organelle motility, phospholipid signaling pathways and cytosolic degradative machineries (Cantalupo *et al*, 2001; Jordens *et al*, 2001; Mizuno *et al*, 2003; Stein *et al*, 2003; Dong *et al*, 2004).

In the present study, we investigated the role of Rab7 in the control of vesicular transport of three representative melanosomal proteins, i.e., tyrosinase (TYR), TYRP1 and gp100. TYR is a critical enzyme for melanin synthesis and is transported from TGN to early-stage melanosomes via endosomal compartments (Oetting and King, 1999; Jimbow *et al*, 2000). TYRP1, functioning as dihydroxyindole carboxylic acid oxidase in mice, also passes through endosomes to melanosomes (Jimenez-Cervantes *et al*, 1994; Jimbow *et al*, 2000). It has been reported that gp100, a melanosomal structural protein, is transported from *cis*-Golgi to early stage melanosomes (Yasumoto *et al*, 2004). Here, we constructed recombinant plasmids carrying cDNA of wild-type Rab7 (Rab7wt) or mutant Rab7N125I and analyzed their effects on the intracellular distribution of melanosomal proteins. We found that the transport pathway of TYRP1 from Golgi complex to melanosomes may be different from that of TYR and gp100 and that Rab7 is a crucial regulator of TYRP1 transport.

Materials and Methods

Vector Constructions cDNAs of Rab7wt and Rab7N125I (Feng *et al*, 1995) were amplified from the Rab7 plasmids by using the N-terminal flag sequence-containing primer with *Hin*dIII site and C-terminal primer with the same restriction site.

Oligonucleotides used for the amplification were 5'-CCC AAG CTT ACC ATG *GAC TAC AAG GAT GAC GAT GAC AAG* ACC TCT AGG AAG AAA GTG TTG-3' and 5'-CCC AAG CTT TCA GCA ACT GCA GCT TTC CGC-3' (underlines, *Hin*dIII site; italic, flag sequence). The fragments of flag-tagged-Rab7 were separately inserted into the *Hin*dIII site of pcDNA3.1/Hygro (+) (Invitrogen, Carlsbad, CA). Parts of the inserted cDNAs in pcDNA3.1/Hygro (+) were verified by nucleotide sequencing by the dideoxy termination method.

Cell culture and transient transfection COS7 cells were maintained in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO) supplemented with 5% fetal calf serum (FCS; Sigma) and Penicillin-streptomycin solution (Sigma), and grown in a 5% CO_2 incubator at 37°C. Melan-a cells, kindly provided by Dr D. C. Bennett, UK, were cultured in RPMI 1640 medium (Sigma) supplemented with 10% FCS, 200 nM phorbol myristate acetate, 0.1 mM 2-mercaptoethanol and Penicillin-streptomycin solution in 10% CO_2 atmosphere. Transfection was performed using FuGENE6 (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol.

Antibodies Rabbit polyclonal antiserum against tyrosinase, anti-PEP7, was kindly provided by Dr V. J. Hearing, USA. Mouse monoclonal antibody (mAb) against TYRP1, HMSA5, was developed previously (Der *et al*, 1993). Mouse mAb against gp100 (clone HMB45) was from DAKO (Carpinteria, CA). Mouse mAb against flag (clone M2) and rabbit anti-flag polyclonal antibody were purchased from Sigma.

Mouse mAb against α-tubulin (clone TU-01) and rabbit antiserum against Rab7 were purchased from Zymed Laboratories (San Francisco, CA) and Cytosignal Research Products (Irvine, CA), respectively. Mouse mAb against p230 trans Golgi (clone 15) and EEA1 (clone 14) were purchased from BD-Biosciences (San Diego, CA). Mouse mAb against mannose phosphate receptor (clone 2G11) was purchased from Research Diagnostics (Flanders, NJ). Goat anti-mouse and anti-rabbit IgG antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 were products of Molecular Probes (Eugene, OR). For immunoblotting, anti-rabbit horseradish peroxidase-conjugated antibody (Amersham Biosciences, Piscataway, NJ) was used.

Immunoprecipitation and immunoblot analysis Immunoprecipitation was performed by using anti-Flag M2 affinity gel (Sigma) according to the manufacturer's protocol. Briefly, COS7 cells grown in 10 cm dishes were transfected with 5 μg of plasmid DNA and cultured for 48 h. Then, cells were harvested by a brief exposure to trypsin, washed and incubated in lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing Protease inhibitor cocktail (Sigma). The lysate was centrifuged at 20,000 x *g* for 30 min. The supernatants were incubated with the anti-Flag M2 affinity gel for 2 h and flag-fused proteins were eluted by 0.1 M glycine HCl, pH 3.5. An equal amount of extracted flag-fused proteins was separated by 14%-16% gradient SDS polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a PVDF membrane (Millipore, Bedford, MA), and reacted with rabbit polyclonal anti-flag antibody. Immunoblot analysis was carried out using anti-rabbit horseradish peroxidase-conjugated antibody and

chemiluminescent substrate (Pierce, Rockford, IL) according to the manufacturer's instructions.

Immunofluorescent staining and confocal laser scanning microscopy

Immunofluorescent staining was performed as described previously with a slight modification (Hirosaki *et al*, 2002). Cells grown on round coverslips coated with 1% porcine skin gelatin (Sigma) were transfected with 0.5 μg of plasmid DNA and cultured for 48 h. Microtubules were, when necessary, disrupted by treating cells with 10 μg/mL of nocodazole (Sigma) for 30 min prior to the fixation. Then, the cells were fixed in a mixture of acetone and methanol (1:1) at –20°C for 5 min. The following antibody dilutions were used for primary reactions; HMSA5, 1:1000; anti-flag M2, 1:1000; polyclonal anti-flag, 1:2000; anti-α-tubulin, 1:100; Rab7 antiserum, 1:2000; anti-p230 trans Golgi, 1:500; anti-EEA1 1:100; anti-syntaxin8 1:100. The secondary fluorescent-labeled antibodies were used at a dilution of 1:600. The coverslips were mounted with Permafluor (Immunotech, Marseille, France). Fluorescent images were obtained with a confocal laser scanning microscope, Axiovert 100M (Carl Zeiss, Thornwood, NY) equipped with LSM510 V2.5 (Carl Zeiss).

For estimation of transfection efficiency, cells transfected with the plasmids encoding Rab7wt or Rab7N125I were incubated 48 h and stained with anti-flag polyclonal antibody and with secondary fluorescent antibody, then observed by using a confocal laser scanning microscope. The percentage of flag-positive cells in approximately 100 cells depicted in a digital image was calculated.

Statistics The distribution of melanosomal proteins between Rab7wt- and Rab7N125I-expressing melan-a cells were compared by using Pearson's chi-square test (significant level, 1%).

Results

Expression of flag-tagged Rab7 proteins in COS7 cells Two plasmids encoding Rab7wt or Rab7N125I were constructed with a flag-tag at the N-terminus and introduced into COS7 cells by DNA transfection. The addition of such tag sequences to Rab proteins does not appear to alter their function or subcellular localization (Bucci *et al*, 2000). The cells transfected with the plasmids were lysed, immunoprecipitated and analyzed by immunoblot as described in the Materials and Methods (Fig 1*A*). The Rab7wt was detected as an approximately 25 kDa polypeptide, whereas the band of Rab7N125I moved faster than that of Rab7wt on SDS-PAGE. The reason for this migration change was unclear, but point mutation could have caused anomalous migration (Press *et al*, 1998). The pilot immunoblot analysis revealed that the amount of extracted Rab7N125I was much smaller than that of Rab7wt (data not shown). To elucidate the reason why this result was obtained, the transfection efficiency of each plasmid was calculated (Fig 1*B*). The transfection efficiency of the plasmids encoding Rab7wt and Rab7N125I was 23.2% ± 4.5% and 3.2% ± 0.7%, respectively. To exclude the possibility that exogenous Rab7N125I was modified in the COS7 cells, the cells transfected with the plasmid encoding Rab7N125I were stained with anti-flag mAb and Rab7 antiserum (Fig 1*C*-*D*).