Kinesin and Kinectin Can Associate with the Melanosomal Surface and Form a Link with Microtubules in Normal Human Melanocytes[1]

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Microtubuli play an important role in the organization of organelles and membrane traffic. They are present in melanocytic dendrites through which melanosomes are transported towards keratinocytes. Besides the actin-based motility systems, microtubuli-associated motor proteins also play a critical role in melanosome movement, as has recently been confirmed in mouse melanocytes. We investigated the *in vitro* expression of two forms of human conventional kinesin and its receptor kinectin in normal human epidermal melanocytes, keratinocytes, and dermal fibroblasts by reverse transcription polymerase chain reaction and northern blot analysis. In an attempt to gain insight into the subcellular distribution of kinesin and kinectin in melanocytes, double immunofluorescent staining and immunogold electron microscopy were performed. In all studied skin cells ubiquitous and neuronal kinesin are expressed, as well as the kinectin receptor. Immunofluorescent staining shows distinct but partially overlapping distributions for kinesin heavy chain and melanosomes, suggesting that kinesin is associated with some but not all of the melanosomes. Similar observations for kinectin indicate that this receptor can colocalize with melanosomes, which was confirmed by immunoelectron microscopy. The latter technique allowed us to demonstrate a close association between kinesin heavy chain, microtubuli, and melanosomes. The combined data from reverse transcription polymerase chain reaction, northern blot analysis, double immunofluorescent staining, and immunogold electron microscopy suggest that kinesins and kinectin have an important role in microtubuli-based melanosome transport in human melanocytes. *Key words: cytoskeleton/melanosome transport/motor proteins. J Invest Dermatol 114:421–429, 2000*

Microtubuli (MT) play an important role in cellular membrane organization, membrane traffic, and vesicle transport, in part mediated by motor proteins that use the MT as a track for movement (for reviews see Vallee and Shpetner, 1990; Schroer and Sheetz, 1991; Bloom, 1992; Skoufias and Scholey, 1993; Cole and Lippincott-Schwartz, 1995; Lopez, 1996; Sheetz, 1996; Hamm-Alvarez, 1998; Lane and Allan, 1998). The kinesins form a superfamily of mechanochemical enzymes providing mechanical force as a result of adenosine-5′-triphosphate hydrolysis to move appropriate cargos. Conventional kinesins, also known as the ''KHC subfamily'', are anterograde motor proteins that transport vesicles from the slow growing minus towards the fast growing plus end of MT, in the direction of the cell periphery in most cells (Schroer *et al*, 1988, 1991; Hirokawa *et al*, 1991). Besides the conventional kinesins, a diverse array of vesicle/organelle motors as well as mitotic motors that participate in spindle separation and other mitotic events have been shown to belong to one of the subfamilies of the kinesin superfamily (Goldstein, 1993; Goodson *et al*, 1994; Hirokawa, 1996, 1998; Karsenti *et al*, 1996; Lafont and Simons, 1996; Moore and Endow, 1996; Hirokawa *et al*, 1998). Conventional kinesin (in the following called kinesin) is a tetramer, consisting of two heavy chains (130 kDa) and two light chains (65 kDa). In humans there are two genes encoding conventional kinesin heavy chain (KHC): ''ubiquitous'' KHC (uKHC), expressed in all cell types and tissues examined, and ''neuronal'' KHC (nKHC) whose expression has only been detected in neuronal cell types so far (Navone *et al*, 1992; Niclas *et al*, 1994). In chromosome mapping studies, the uKHC gene was localized on mouse chromosome 18, which is syntenic with human chromosome 18q, whereas the nKHC gene was localized on mouse chromosome 2, syntenic with human chromosome 2 (Niclas *et al*, 1994). A recent report, however, localized the nKHC gene to human chromosome band 12q13 by *in situ* hybridization (Hamlin *et al*, 1998). The globular N-terminal motor domain of KHC binds MT and contains a phosphate-binding loop to hydrolyze adenosine-5′-triphosphate (Kuznetsov *et al*, 1989; Yang *et al*, 1989; Vale and Fletterick, 1997). Kinesin light chain (KLC) binds to the C-terminal tail of the heavy chain (Hirokawa *et al*, 1989).

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Abbreviations: ER, endoplasmatic reticulum; KHC, kinesin heavy chain; KLC, kinesin light chain; MT, microtubuli; nKHC, neuronal kinesin heavy chain; uKHC, ubiquitous kinesin heavy chain.

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The tail is believed to couple the motor to its cargo via KLC (Stenoien and Brady, 1997; Khodjakov *et al*, 1998). Another function suggested for KLC is regulating kinesin's motor activity or interaction with MT (Matthies *et al*, 1993; Verhey *et al*, 1998).

New information is now emerging on the targeting of kinesin to cellular membranes. A putative transmembrane kinesin receptor, termed kinectin, has been identified, which possibly plays a role in regulation of motor activity as well (Kumar *et al*, 1995; Vallee and Sheetz, 1996; Sheetz, 1999). Human kinectin, a protein of 160 kDa, contains an N-terminal hydrophobic region, suggesting that it might be an integral membrane protein. It further contains domains for dimerization, which may produce a high-affinity binding site for kinesin. It has been shown to bind to the tail portions of kinesin (Toyoshima *et al*, 1992). Immunofluorescent staining for human or chicken kinectin reveals a perinuclear endoplasmatic reticulum (ER)-like pattern (Fütterer *et al*, 1995; Yu *et al*, 1995).

Kinesin itself has been shown to be associated with a variety of cellular structures and organelles (Tanaka *et al*, 1998; for reviews see above). It is involved in the maintenance of ER and Golgi at their appropriate location in the cell (Allan, 1996; Waterman-Storer and Salmon, 1998; Lane and Allan, 1999), and in transport of Golgi-derived vesicles towards the ER and the plasma membrane (Lippincott-Schwarz *et al*, 1995; Johnson *et al*, 1996; Minin, 1997; Fullerton *et al*, 1998). In neurons, fast anterograde axonal transport of synaptic vesicles is mediated by kinesin (Hirokawa *et al*, 1991; Senda and Yu, 1999). During pigmentation of the skin a similar directional transport process takes place. Melanocytes transfer their pigment granules, the melanosomes, to the surrounding keratinocytes, after they have been transported from the perinuclear area where they are synthesized from ER- and Golgi-derived vesicles, towards the peripheral dendrite tips (Jimbow *et al*, 1993; Orlow, 1998). The exact mechanism by which melanosomes are transported to the cell periphery is not completely understood. The dendrites of melanocytes consist of a central core of MT and a peripheral subcortical actin network (Lacour *et al*, 1992). There is accumulating evidence that the actin-associated motor protein myosin V is involved in melanosome transport (Provance *et al*, 1996; Wu *et al*, 1997; Lambert *et al*, 1998).

It is possible, however, that melanosomes are transported for long distances along MT and afterwards through the actin network, each time by their respective motor protein. An interrelationship between the two motility systems in vesicle and organelle transport has been suggested and demonstrated previously in several cell types (Kuznetsov *et al*, 1992; Bearer *et al*, 1993; Fath *et al*, 1994; Langford, 1995; Morris and Hollenbeck, 1995; Bi *et al*, 1997; Evans *et al*, 1997; Gavin, 1997; Kelleher and Titus, 1998). For example in melanophores MT are necessary for pigment transport (McNiven *et al*, 1984; Haimo and Thaler, 1994; Nilsson *et al*, 1996), although *in vitro* their melanosomes can move both unidirectionally along actin filaments and bidirectionally along MT (Rogers *et al*, 1997; Rogers and Gelfand, 1998). Recently, in wild-type and *dilute* melanocytes, melanosomes were shown to undergo rapid MT-dependent movements to the cell periphery and back again. Probably, myosin V–dependent interaction of melanosomes with F-actin in the periphery prevents these organelles from returning on MT to the cell center, causing their distal accumulation (Wu *et al*, 1998). In fish melanophores, kinesin has been demonstrated to be responsible for pigment granule dispersion via microinjection of function blocking antibodies (Rodionov *et al*, 1991; Nilsson *et al*, 1996; Lane *et al*, 1998). Finally, subcellular fractionation of melanophores shows the presence of kinesin II (another member of the kinesin superfamily), cytoplasmic dynein, and myosin V on melanosomes (Rodionov *et al*, 1998; Rogers *et al*, 1998). These data support the hypothesis of a coordinated activity of the two motility systems during organelle transport.

In addition, MT-associated motor proteins have been shown to be essential in cell process formation. Not only are kinesin-related proteins likely to be involved (Sharp *et al*, 1996, 1997a, b; Morfini *et al*, 1997; Yu *et al*, 1997), but also members of the KHC subfamily. In neuroblastoma and phaeochromocytoma cell lines, nKHC is upregulated at the mRNA and protein level during differentiation and it is essential for neurite outgrowth (Vignali *et al*, 1996). Phosphorylation of KHC *in vivo* correlates with neurite outgrowth (Lee and Hollenbeck, 1995). Suppression of KHC expression by antisense oligonucleotides in cultured hippocampal neurons results in inhibition of neurite outgrowth (Ferreira *et al*, 1992). It is not clear yet, however, whether deficient vesicle transport is responsible for the effect on dendrite formation or whether kinesin is necessary for neurite elongation itself.

As a first step in examining a possible participation of MT-based motor proteins in human melanosome transport and dendrite formation, we have studied the expression and subcellular localization of kinesin and kinectin in human melanocytes. Additionally, the expression in other skin cells was examined. We find uKHC, nKHC, and the kinectin receptor to be expressed in all skin cells studied. The presence of KHC in the melanocytic dendrites and the association of KHC and kinectin with melanosomes further strengthen the hypothesis that this motor protein system plays a role at some stage of melanosome transport.

MATERIALS AND METHODS

Cell culture Primary epidermal melanocyte cultures were obtained from neonatal foreskins and cultured in Medium 199 supplemented with 2% fetal calf serum, 10^{-9} M cholera toxin, 10 ng basic fibroblast growth factor per ml, 10 μg insulin per ml, 1.4 μM hydrocortisone and 10 μg transferrin per ml, as described earlier (Naeyaert *et al*, 1991). Post-primary cultures were maintained in low calcium (0.03 mM) Medium 199 supplemented with the same additives and 10% fetal calf serum.

Cultures of human keratinocytes were initiated from adult skin biopsies and cultured in growth-factor- and serum-supplemented Dulbecco's modified Eagle's medium/Ham's F12 to confluency as previously described (Beele *et al*, 1991). Fibroblasts were established as explants from dermal portions of skin biopsies and cultured in Optimem I supplemented with 2% Ultroser G and 1% glutamine. A human neuroblastoma cell line (SK-N-SH, ATCC nr. HTB11) was cultured in RPMI, supplemented with 10% fetal calf serum. HeLa wild-type cell line (Hsu *et al*, 1976; Laboratory for Experimental Cancerology, Gent, Belgium) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. All culture media were obtained from Gibco BRL (Merelbeke, Belgium).

RNA preparation Poly(A)$^+$RNA was obtained from subconfluent cell cultures using Micro-Fast Track Kit (Invitrogen, Leek, The Netherlands). Purity of the obtained mRNA samples was checked by measuring the absorbance ratio at 260:280 nm, which was more than 1.7 in all samples.

Reverse transcription polymerase chain reaction (RT-PCR) About 400 ng of mRNA was reverse transcribed with random hexamers as primers and M-MLV Reverse Transcriptase (Gibco BRL). PCR amplification was performed with Platinum Taq DNA Polymerase (Gibco BRL). Two oligonucleotides (P1 and P2) for uKHC amplifying a 326 bp cDNA (nt 1389–1715, Genbank accession number X65873) were used as described earlier (Gudkov *et al*, 1994). The sequences of P1 and P2 were 5′-AGTGGCTTGAAAATGAGCTCA-3′ and 5′-CTTGATCCCTTC-TGGTAGATG-3′. Oligonucleotides in the human nKHC (nt 2031–2424) and kinectin (nt 848–1054) cDNA were chosen using Oligo 5.0 software (Genbank accession numbers U06698 and L25616). Considering the high probability of sequence overlap with other kinesin-related proteins the oligonucleotides were carefully checked for false priming sites using BLAST sequence similarity searching. False priming sites in human frequent sequences were excluded as well. The sequences of P3 and P4 (nKHC) were 5′-CCTGCCAGCTCCTCATCTC-3′ and 5′-TCTCGT-GTTCTTCGCTCTTCA-3′ defining a 393 bp cDNA. The sequences of P5 and P6 (kinectin) were 5′-AAACTGAAGACGGAAACTGAC-3′ and 5′-CACAGCAGCGAGTAACTTG-3′ defining a 225 bp band. PCR amplification conditions for uKHC and nKHC were: 95°C for 1 min, 59°C for 1 min, and 72°C for 1 min during 35 cycles after an initial denaturation at 95°C for 5 min and terminating the reaction with 7 min at 72°C. Conditions for kinectin were the same. In preliminary experiments, control RT-PCR reactions were performed for each primer set without adding reverse transcriptase to exclude amplification of genomic DNA. cDNA bands were visualized on a 1.5% TAE-agarose gel stained with ethidium bromide. The resulting cDNA bands were checked for specificity

by restriction enzyme analysis, eluted from the gel (QiaEx gel extraction kit, Westburg, The Netherlands), and after purification used as a hybridization probe in northern blot. To check the specificity of the mRNA signals in northern blot experiments, another set of primers was designed for uKHC (P7-P8; P7: ATTCTGATGACACCGGAGGC, P8: TCACAGCGGAGATCTGCATTA), nKHC (P9-P10; P9: GGAACT-CCAGACCCTCCACA, P10: GGAAGCTCACAACGCAGATCT), and kinectin (P11-P12; P11: GCATCTTCTTTTCCCCCTCAT, P12: AAATCCATGCAACCATTCACC), chosen in other regions of the cDNA, defining a 124 bp (nt 2766–2889), 210 bp (nt 2515–2724), and 246 bp (nt 3002–3247) probe, respectively. PCR conditions were the same as described above, with an annealing temperature of 59°C for P7-P8 and of 58.5°C for P9-P10 and P11-P12.

Northern blot Northern blot was performed as previously described (Wintzen *et al*, 1996). Briefly, 2 μg of poly(A)$^+$RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. RNA was transferred overnight onto a nylon membrane (Hybond N$^+$, Amersham, Gent, Belgium) and immobilized by ultraviolet irradiation in a UV-Stratalinker (Stratagene, La Jolla, CA). Prehybridization of the blot was in a solution containing 50% deionized formamide, 5× Denhardt's solution, 0.02% sodium dodecyl sulfate (SDS), and 0.2 mg salmon sperm DNA per ml at 45°C during 2 h. The cDNA probes were labeled with ^{32}P-dCTP using Oligolabeling kit (Pharmacia, Roosendaal, The Netherlands) and hybridized to the blot overnight at 45°C in a solution containing 50% deionized formamide, 2.5× Denhardt's solution, 0.02% SDS, 5 × saline sodium phosphate EDTA, and 0.02 mg salmon sperm DNA per ml together with a radiolabeled glyceraldehyde-3-phosphate dehydrogenase control probe (for northerns with P1-P2, P3-P4, and P5-P6). Washes were performed at room temperature once for 5 min and three times for 20 min in 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS, and subsequently twice for 20 min at 45°C and twice at 55°C in 15 mM NaCl, 1.5 mM sodium citrate, 0.1% SDS. Blots were exposed to Hyperfilm MP (Amersham) in intensifying screens at −70°C for 72 h.

Indirect immunofluorescence and confocal microscopy Cells were grown on coverslips and fixed for 20 min at room temperature with 3% paraformaldehyde in phosphate-buffered saline. Cells were permeabilized by treatment with 0.2% Triton X-100 in phosphate-buffered saline for 5 min. Staining for KHC or kinectin was performed by incubating for 1 h at room temperature with a 1:15 dilution of a mouse monoclonal antibody against the N-terminal head portion of bovine brain KHC (specificity for uKHC or nKHC not known; Chemicon, CA; Pfister *et al*, 1989) or a 1:10 dilution of a mouse monoclonal antibody directed against the carboxyterminal end (residues 373–923) of human kinectin (CT-1, obtained from Professor M. Krönke, Department of Immunology, University of Kiel, Germany; Fütterer *et al*, 1995). After three washes in Tris-buffered saline, the coverslips were incubated in the dark in a mixture of fluorescein isothiocyanate (FITC) labeled sheep antimouse (1:20) (Amersham) and DAPI 1:100 dilution for 1 h at room temperature. For negative controls incubation with an irrelevant isotype-matched antibody was performed. For double labeling, cells were first incubated with a mouse monoclonal IgG2b NKIbeteb antibody (1:40) against the (pre)melanosomal *silver* protein (Monosan, Uden, The Netherlands), followed by biotinylated rat antimouse IgG2b antibody (1:50) (Pharmingen, San Diego, CA) and streptavidin-texas-red (1:50) (Amersham). After washing, cells were finally incubated with KHC antibody (1:15) or CT-1 antibody (1:10) followed by FITC-labeled rat antimouse IgG1 antibody (1:20) (Pharmingen). In preliminary experiments, the specificity of the secondary antibodies was tested by incubating slides with NKIbeteb followed by FITC-labeled anti-IgG1 and KHC followed by biotin-labeled anti-IgG2b, which were negative. Slides were coverslipped in Prosan fluorescence mounting fluid and confocal images were photographed with a Bio-Rad MRC 1024 confocal laser microscope.

Immunoelectron microscopy Confluent cell cultures were fixed for 1 h at room temperature in 0.1% glutaraldehyde in 0.14 M cacodylate buffer, pH 7.3, and processed for immunogold labeling as described elsewhere (Mommaas *et al*, 1992). Briefly, cells were pelleted and embedded in 10% gelatin, cut into 1 mm^3 cubes, cryoprotected in 2.3 M sucrose for 25 min, and snapfrozen in liquid nitrogen. Ultrathin cryosections were prepared and incubated with the mouse monoclonal anti-KHC antibody (1:80) or with the CT-1 or NT-1 antibodies (directed against the amino-terminal end, residues 46–260, of human kinectin, Professor M. Krönke, Kiel) diluted 1:10. After washing, sections were incubated in rabbit antimouse antibody (1:200) (Dako) followed by 10 nm protein A-gold. For double labeling with MT, sections were finally incubated with a mouse monoclonal antibody against α-tubulin (Sigma, Belgium) conjugated with 5 nm gold. For negative controls the primary antibody step was omitted and incubation with an irrelevant isotype-matched antibody was performed. After immunolabeling sections were embedded and contrasted in methylcellulose/uranyl acetate and viewed with a Philips EM 410 electron microscope.

Table I. KHC is present on a significant number of melanosomes in normal human melanocytes

Melanocyte	Total number of melanosomes	Number of positive melanosomes	% of positive melanosomes
KHC-labeled			
Melanocyte I	235	72	31
Melanocyte II	36	11	30.5
Melanocyte III	74	20	27
Melanocyte IV	67	14	21
Melanocyte V	82	37	45
Melanocyte VI	71	9	13
Median	72.5	17	28.75
$p < 0.01$			
Kinectin-labeled			
Melanocyte I	237	28	12
Melanocyte II	66	11	16
Melanocyte III	203	32	15
Melanocyte IV	265	35	13
Melanocyte V	120	13	11
Melanocyte VI	46	12	26
Median	161.5	20.5	14
$p < 0.01$			
Controls			
Melanocyte I	237	7	3
Melanocyte II	47	2	4
Melanocyte III	62	3	4
Melanocyte IV	87	5	5
Melanocyte V	73	1	1
Melanocyte VI	140	7	5
Median	80	4	4

Immunoelectron microscopy quantitation For quantitation of KHC or kinectin labeling of melanosomes immunoelectron microscopy photographs of whole melanocytes ($n = 6$) were made at a final magnification of 55,000×. The total number of melanosomes and the number of positive melanosomes were scored per cell (**Table I**). Melanosomes were assigned positive when the distance of the colloidal gold particles to the melanosome surface was within the range of 120 nm (kinesin = 80 nm + 2 × IgG = 20 nm) on the outside or within 40 nm (2 × IgG) on the inside of the membrane for KHC labeling (Hirokawa *et al*, 1990). As the dimension of the kinectin tail on the outside of the membrane is not known, the distance of colloidal gold particles for kinectin was taken as 40 nm (2 × IgG) on the outside and 40 nm on the inside of the membrane. Results obtained for various experimental groups were compared using the nonparametric Wilcoxon rank sum test (Mann–Whitney U test) for comparison of median values of two populations. p values less than 0.05 were considered to be statistically significant.

RESULTS

uKHC, nKHC, and kinectin are expressed in human skin cells *in vitro* In human melanocytes, keratinocytes, fibroblasts, and neuroblastoma cells, RT-PCR with primers P1-P2, P3-P4, and P5-P6 revealed the appropriately sized cDNA bands for uKHC, nKHC, and kinectin (**Fig 1*a–c***, respectively), suggesting that the mRNAs for the two types of human KHC and for the kinectin receptor are expressed in all the cell types studied. Neuroblastoma mRNA was included as a positive control (Niclas *et al*, 1994). Northern blot analysis with the corresponding probes confirmed the RT-PCR results and showed a complex transcription pattern for both uKHC and nKHC (data not shown). Hybridization with the second set of probes (P7-P8, P9-P10) gave similar results (**Fig 2*a***). Hybridization with the uKHC probe revealed several strong signals of 1.2, 1.5, 5.2, and 6.7 kb in

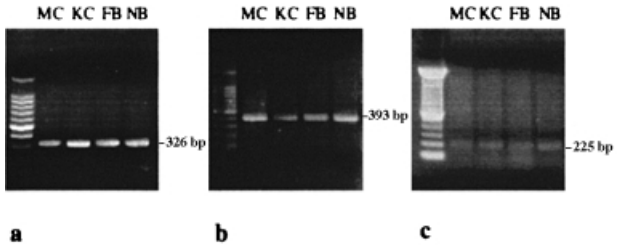

Figure 1. Kinesin and kinectin mRNA is expressed in human skin cells and tumor cell lines, RT-PCR. mRNA was extracted from cultured cells of a normal individual, or from human tumor cell lines. 400 ng of mRNA was reverse transcribed with random hexamers and M-MLV Reverse Transcriptase. PCR amplification was performed with uKHC (*a*), nKHC (*b*), or kinectin (*c*) specific primers (P1-P2, P3-P4, and P5-P6, respectively), resulting in appropriately sized bands. The first lane represents a 100 bp ladder with a more intense band of 500 bp DNA. NB, human neuroblastoma SK-N-SH; MC, melanocytes; KC, keratinocytes; FB, fibroblasts.

all the cell types studied. Another band of 14 kb was visible in all the lanes, although less intense. Hybridization with the nKHC probe gives similar signals of 1.2, 1.5, 5.2, and 6.7 kb, with additional bands at 14 kb in all cell types. These results were reproduced in three separate experiments. Northern blot hybridization with two different specific kinectin probes (P5-P6; P11-P12) (**Fig 2*b***, only hybridization with P11-P12 shown) gave the same strong signals of 1.2, 2.3, and 6.5 kb in all cell types, with an additional weak signal of 8.5 kb, confirming mRNA expression in all skin cell types examined.

KHC is present throughout the cytoplasm and in the melanocytic dendrites, whereas kinectin has a perinuclear distribution Because mRNA expression was positive in all cell types, we investigated the distribution of KHC by immunofluorescent labeling. In melanocytes KHC showed a punctate staining pattern distributed throughout the cell body and the dendrites, with accentuation in the perinuclear area (**Fig 3*a***). In some cells label could be observed reaching into the tip. Given this distribution for KHC, we investigated the cellular localization of kinectin in melanocytes (**Fig 3*b***). We observed a perinuclear reticular pattern, similar to what has been previously described in NIH3T3 cells, COS cells, and CV1 cells (Fütterer *et al*, 1995; Yu *et al*, 1995). The intensity of the staining was less bright in the cell periphery, although in some cells the reticular pattern extended somewhat into the dendrites.

KHC and kinectin colocalize with melanosomes in human melanocytes In order to detect a possible association of kinesin with melanosomes, we performed double labeling experiments with the anti-KHC antibody and NKIbeteb antibody, which marks melanosomes (**Fig 4*a***, ***b***, respectively). Partially overlapping but distinct distributions of KHC and silver protein were discerned (**Fig 4*c***). Yellow dots indicating colocalization could mainly be seen in the perinuclear area, along the dendrites, and in the tips of the dendrites. Some red label could still be seen in the perinuclear area. Higher magnification images of a peripheral dendrite of another cell are shown in **Fig 5**, where yellow dots of colocalization of melanosomes with KHC can be seen along the dendrite and in the tip (**Fig 6**). Double immmunofluorescent labeling for kinectin and NKIbeteb is shown in **Fig 5**. In the overlay images we saw overlapping distributions for kinectin and melanosomes with yellow dots indicating colocalization in the perinuclear area (**Fig 5*c***).

KHC and kinectin can be detected on the melanosomal surface To obtain more information on the exact subcellular localization of kinesin and kinectin, immunoelectron microscopy was performed on cultured human melanocytes (**Fig 7**). Labeling with the KHC antibody showed gold particles present in the cytoplasm near membranous structures such as ER, Golgi, and on

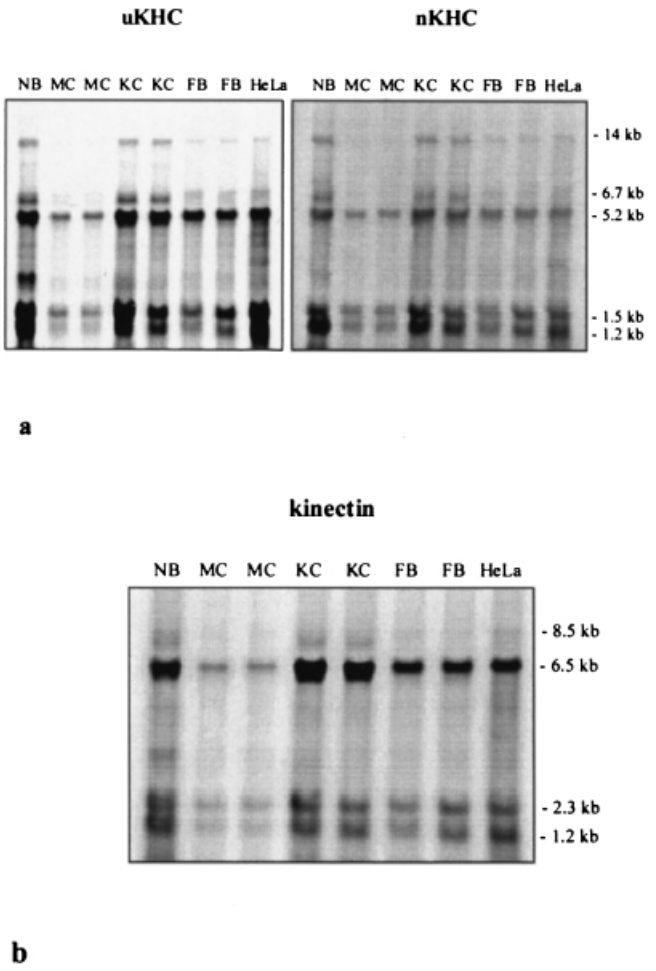

Figure 2. Northern blot analysis reveals similar different length transcripts for uKHC and nKHC in human skin cells and tumor cell lines and confirms kinectin mRNA expression in all cell lines. Two micrograms of mRNA was loaded into each lane. Hybridization was performed with an α^{32}P-labeled fragment of human uKHC (P7-P8) or nKHC (P9-P10) (*a*) or kinectin (P11-P12) (*b*) cDNA PCR product. (*a*) Exposure of 72 h reveals messages of 1.2 kb, 1.5 kb, 5.2 kb, 6.7 kb, and 14 kb. A similar transcription pattern is detected for nKHC. (*b*) Strong signals are detected at 1.2, 2.3, and 6.5 kb in all cell lines. An additional weak signal is detected at 8.5 kb. NB, human neuroblastoma SK-N-SH; MC, melanocytes; KC, keratinocytes; FB, fibroblasts; HeLa, wild-type HeLa cell line.

mitochondria as described previously (Jellali *et al*, 1994; Lippincott-Schwarz *et al*, 1995; Johnson *et al*, 1996; Fullerton *et al*, 1998; Tanaka *et al*, 1998; Waterman-Storer *et al*, 1998) (data not shown). KHC was also regularly detected on melanosomes (**Fig 7*a***). Not all the melanosomes were positively labeled. After labeling for kinectin with NT-1 antibody 10 nm gold particles could clearly be detected on several melanosomes (**Fig 7*b***), although again not all melanosomes were positively labeled. Similar results were obtained with the NT-1 antibody (**Fig 7*c***). Throughout the cytoplasm and on melanosomes kinectin labeling was less abundant than KHC labeling. Negative controls labeled with irrelevant IgG1 showed almost no gold particles in the cell (**Fig 7*d***).

A quantitation of number of melanosomes stained positively for KHC or kinectin gives a more accurate idea about the significance of the association (**Table I**). Comparison of KHC-labeled or kinectin-labeled melanocytes versus control cells revealed a significantly higher ($p < 0.01$) proportion of positively labeled melanosomes in the first group versus control cells.

KHC can form a link between melanosomes and MT Immunogold double labeling was performed for KHC (10 nm gold

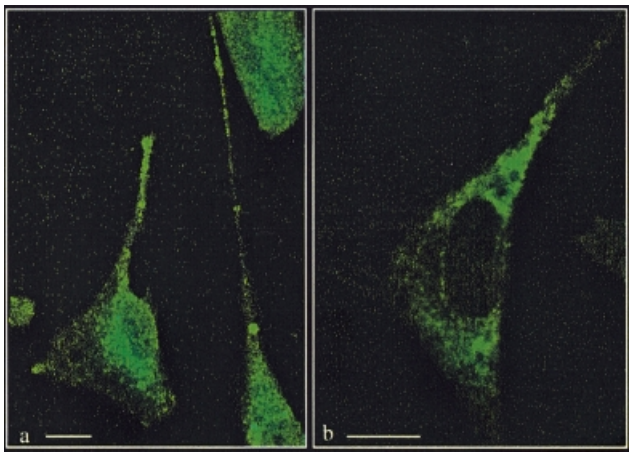

Figure 3. Distribution of antigen recognized by the KHC and kinectin antibodies in human melanocytes. Melanocytes were cultured in coverslips for 24 h, fixed, and stained with a mouse monoclonal anti-KHC antibody (*a*) or a mouse monoclonal anti-kinectin CT-1 antibody (*b*). (*a*) KHC is present throughout the cell body, with accentuation in the perinuclear area. Label can also be detected along the dendrites and in the tips. (*b*) Kinectin is concentrated in the perinuclear area, although the reticular network could be seen extending somewhat into the dendrites. *Scale bar:* 10 μm.

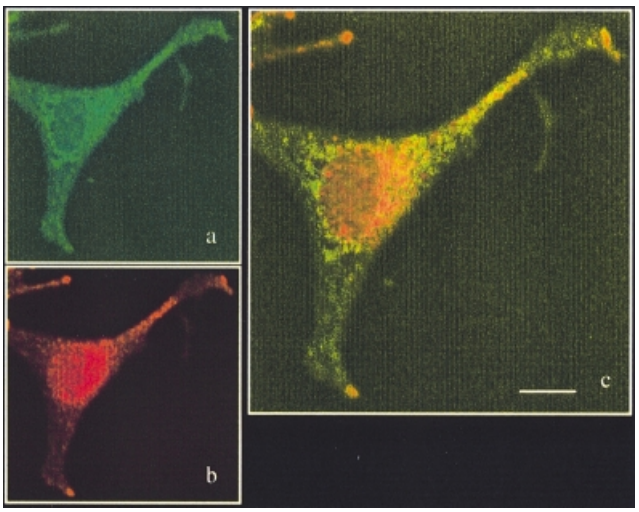

Figure 4. KHC colocalizes partially with melanosomes. Human melanocytes were cultured on coverslips for 24 h, fixed, and double stained with a mouse monoclonal anti-KHC antibody (FITC signal) (*a*) and a mouse monoclonal NKIbeteb antibody (texas-red signal) (*b*). In the overlay image (*c*) yellow staining indicates colocalization, which can be detected in the perinuclear area, along the dendrites, and in the dendrite tips. *Scale bar:* 10 μm.

particles) and α-tubulin (5 nm gold particles) showing the association of KHC with α-tubulin. In the dendrites, we regularly detected KHC associated with α-tubulin on the melanosomal surface (**Fig 8*a***), where KHC serves as a linking molecule between the melanosomal surface and the MT. KHC and α-tubulin could also be observed on nonmelanized organelles (**Fig 8*b***).

DISCUSSION

This study demonstrates the presence and subcellular localization of an MT-associated motor protein, kinesin, and its receptor, kinectin, in normal human epidermal melanocytes. The expression of mRNA for uKHC, nKHC, and kinectin in all cell types as demonstrated by RT-PCR was confirmed by northern blot

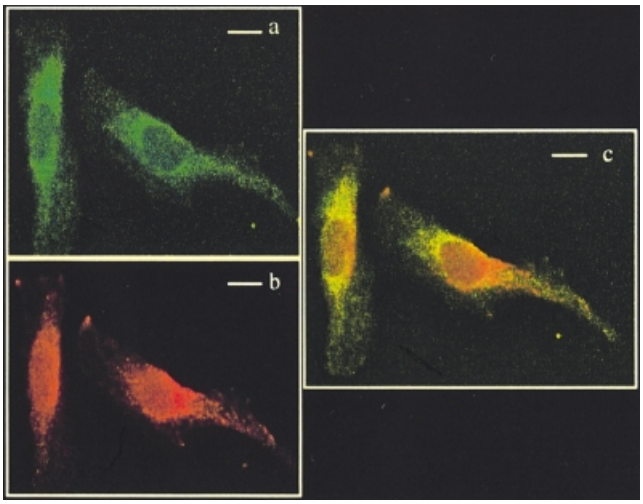

Figure 5. Kinectin colocalizes with a minority of melanosomes. Human melanocytes were cultured on coverslips for 24 h, fixed, and double stained with a mouse monoclonal anti-kinectin antibody (FITC signal) (*a*) and a mouse monoclonal NKIbeteb antibody (texas-red signal) (*b*). In the overlay yellow dots indicating colocalization are detected in the perinuclear area (*c*). *Scale bar:* 10 μm.

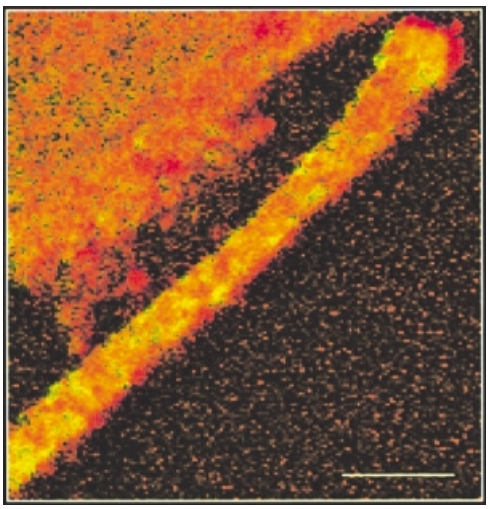

Figure 6. Colocalization of KHC with melanosomes in a peripheral dendrite tip. Human melanocytes were cultured on coverslips for 24 h, fixed, and double stained with a mouse monoclonal anti-KHC antibody (FITC signal) and a mouse monoclonal NKIbeteb antibody (texas-red signal). In the overlay image yellow dots indicating colocalization can be detected along the dendrite and in the dendrite tip. *Scale bar:* 10 μm.

analysis. Primers were checked in the NCBI Genbank by BLAST sequence similarity search to exclude amplification of any other known human sequence. Amplification of unknown homologous sequences cannot be excluded, however. Northern blot analysis showed a complex transcription pattern for the two kinesin heavy chains, which are very similar to one another. By performing hybridization for each sequence with two specific probes against different parts of the coding sequence (uKHC P1-P2 and P7-P8; nKHC P3-P4 and P9-P10), detection of nonspecific signals was excluded. There are several different length transcripts for each heavy chain. Transcription patterns of the nKHC and uKHC genes are highly similar, leading to the hypothesis that both genes most probably arose from duplication of a common ancestral gene, maintaining similar transcription initiation/processing signals (Niclas *et al*, 1994). The same group showed that nKHC was exclusively expressed in newborn and adult rat brain but not in other rat tissues or human HeLa cells, whereas uKHC was

ubiquitously expressed. Their results differ from our observations, as we detected nKHC expression not only in neural-crest-derived cells such as epidermal melanocytes, but also in non-neuronal cell types such as epidermal keratinocytes, dermal fibroblasts, and HeLa cells.

The punctate distribution of KHC in melanocytes and other skin cells as seen with immunofluorescent labeling indicates an association with vesicular structures. Earlier observations describe numerous staining patterns for kinesin, depending on the type of antibody used, the cell type examined, and the type of fixation, ranging from punctate to evenly distributed diffuse staining (Hollenbeck, 1989; Pfister *et al*, 1989; Navone *et al*, 1992; Niclas *et al*, 1994; Lin *et al*, 1996; Tanaka *et al*, 1998). Our results indicate that KHC is present throughout the melanocyte cell body and dendrites and appears to be more concentrated in the perinuclear area, which is the region of the cell where anterograde transport initiates. Similar observations for kinesin were made in neuronal cells (Carson *et al*, 1997). The overlapping but distinct distributions of KHC and melanosomes that we observed can be explained as follows: (i) kinesin is a molecular motor associating with and transporting other cargos besides melanosomes (see Introduction); (ii) the association with only a part of the melanosomes suggests that other motors may be present on melanosomes, e.g., myosin V (Lambert *et al*, 1998), or that there is a pool of "stationary" melanosomes, not taking part in the transport process (Sheetz, 1999). Previous observations in fish melanophores also showed that there is no complete colocalization of kinesin with melanosomes and confirmed its presence on other organelles (Nilsson *et al*, 1996). Quantitation of our immunoelectron microscopy data, however, gives very strong evidence for a meaningful association of KHC with melanosomes. This can perhaps be explained by the higher resolution of the technique. Lack of apparent immunofluorescent colocalization does not rule out a role for a motor in transport, as a single kinesin or dynein motor molecule can generate processive movement (Goodson *et al*, 1997). On the other hand, morphological association of a motor protein with an organelle has to be interpreted carefully. Presence of a motor protein is no evidence of active participation in transport, as they can be transported passively while residing in an inactive form on organelles (Lin *et al*, 1996; Evans *et al*, 1997; Goodson *et al*, 1997). In turn, their activity can be switched on at a certain point where they take over the transport of their cargo. Furthermore, our immunoelectron microscopy studies confirm earlier reports of kinesin being present on membranous structures such as Golgi membranes or ER. Previous studies have proved the functional importance of kinesin in the organization of these cell compartments (Lippincott-Schwarz *et al*, 1995; Allan, 1996). The presence of KHC on mitochondria also supports earlier results suggesting a kinesin-mediated transport mechanism (Jellali *et al*, 1994; Tanaka *et al*, 1998). The H1 mouse monoclonal antibody we used is directed against the N-terminal head portion of conventional KHC, originally described by Pfister *et al* (1989). Due to the high sequence similarity in this region between the different members of the kinesin superfamily, cross-reaction with other kinesin-related proteins cannot totally be excluded. Besides conventional kinesin, other plus end directed members of the superfamily could be present on melanosomes and participate in transport. A recent paper by Tuma *et al* (1998) showed that the heterotrimeric kinesin II is responsible for pigment dispersion in Xenopus melanophores. Studies on the Atlantic cod, however, suggest that conventional kinesin is present on pigment granules (Nilsson *et al*, 1996). It remains to be established whether different

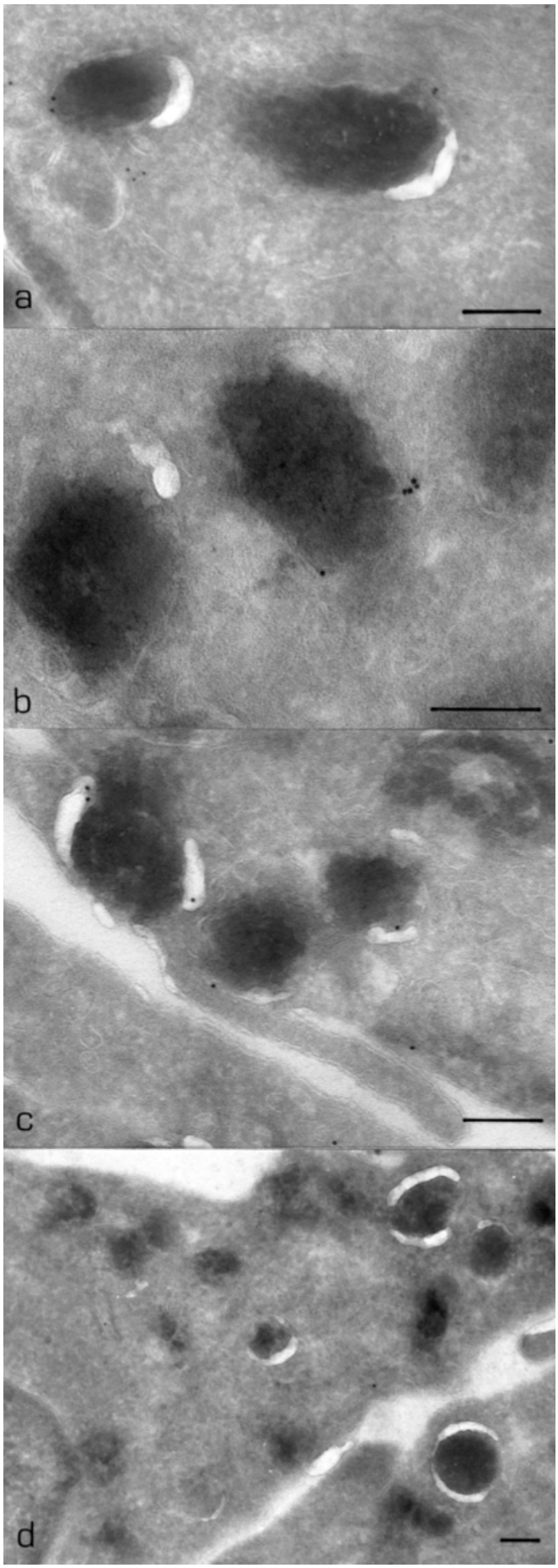

Figure 7. KHC and kinectin can associate with melanosomes. Glutaraldehyde-fixed ultrathin cryosections of cultured normal melanocytes were labeled with anti-KHC (*a*), or anti-kinectin NT-1 (*b*), or anti-kinectin CT-1 antibodies followed by incubation in protein A-10 nm gold complex. Gold particles are seen in close association with the melanosome membrane in (*a*), (*b*), and (*c*). Hardly any label was seen in the cytoplasm or on melanosomes in the negative control shown in (*d*). *Scale bar:* 0.2 μm.

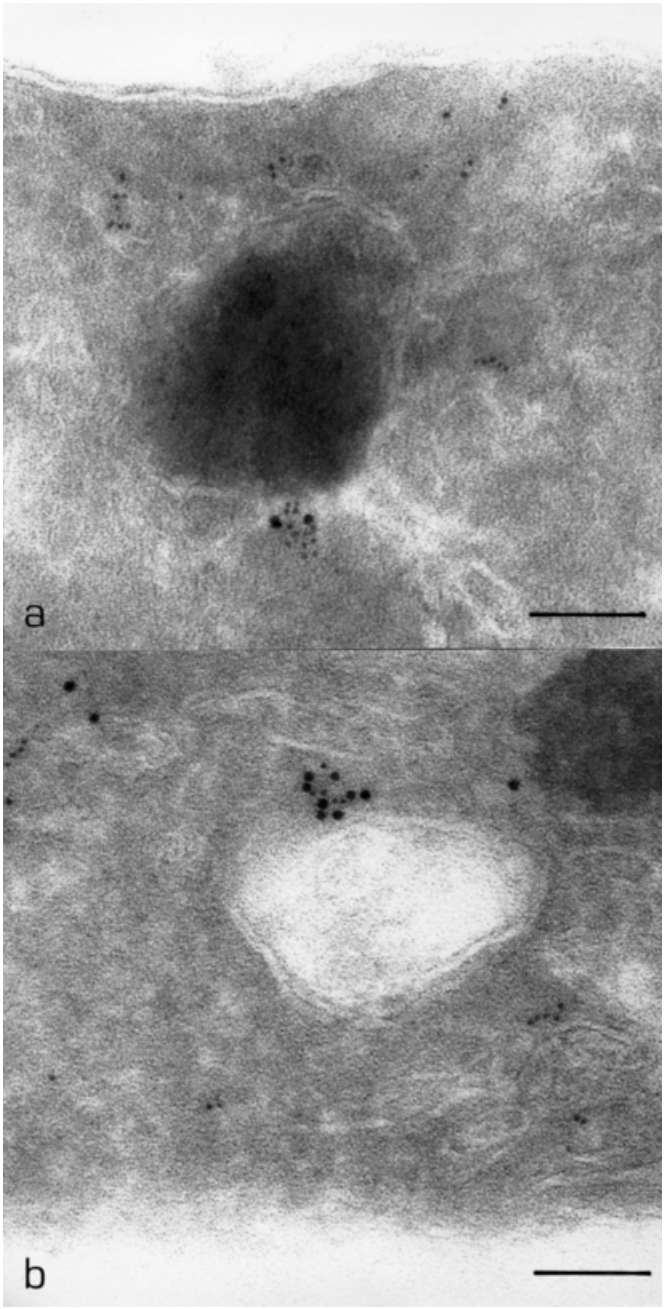

Figure 8. KHC forms a link between melanosomes and MT, but can be seen on nonmelanized organelles as well. Glutaraldehyde-fixed ultrathin cryosections of normal human melanocytes were labeled with anti-KHC (10 nm gold particles) and anti-α-tubulin (5 nm gold particles). Kinesin is often seen forming a link between melanosomes and MT (*a*) but can be detected on other nonmelanized organelles and vesicles as well (*b*). *Scale bar:* 0.1 μm.

organisms use different motors for pigment granule movement (Lin *et al*, 1996).

The distribution of kinectin as seen by immunofluorescent labeling seems quite different from the results for KHC. Earlier observations revealed indeed an ER-like perinuclear reticular staining pattern for kinectin (Fütterer *et al*, 1995; Yu *et al*, 1995). Immunofluorescent double labeling shows only colocalization with melanosomes in the perinuclear area. Our immunoelectron microscopy results show association of kinectin with a subpopulation of melanosomes, with the labeling being much less abundant than for KHC. Quantitation of the immunoelectron microscopy data reveals, however, a meaningful association of kinectin with melanosomes. This could mean that kinectin is probably not the only receptor for kinesin on membranous organelles, as suggested previously (Fütterer *et al*, 1995; Burkhardt, 1996).

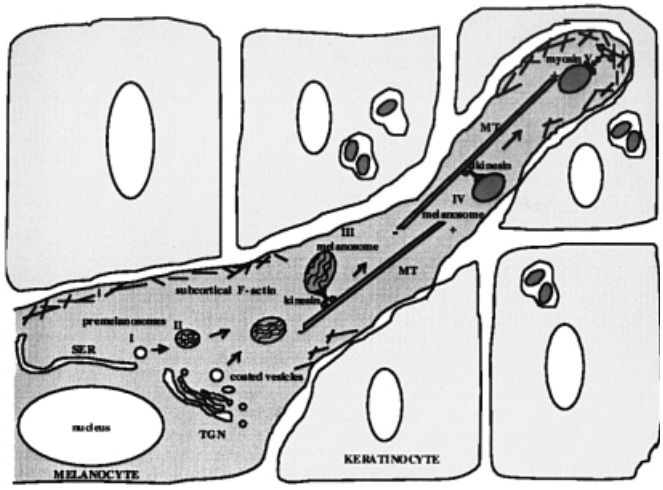

Figure 9. Schematic presentation of the possible cooperation of actin-based and MT-based melanosome transport. Melanosomes could be transported for long distances along MT through the dendrite and afterwards be captured and transferred through the subcortical actin network. SER, smooth endoplasmatic reticulum.

The importance of motor proteins in melanosome transport in human melanocytes is illustrated by our previous work demonstrating that myosin V is expressed in human melanocytes and is localized on the melanosome membrane (Lambert *et al*, 1998). The present data support the hypothesis that both actin-based and MT-based motility systems cooperate in melanosome transport. There are several arguments to support this hypothesis (Lambert *et al*, 1998). For example, in Griscelli patients where myosin V is mutated, there is still a limited transfer of melanosomes towards the surrounding keratinocytes. Moreover, in cultured melanocytes from Griscelli patients, melanosomes accumulate in the perinuclear area but a limited number can still be detected along the dendrites (personal observations, Wu *et al*, 1998). This suggests that there is some defect in the transport machinery, although transport is not completely abrogated. We have also demonstrated that myosin V colocalizes with actin bundles mainly in the subcortical cell area (Lambert *et al*, 1998). The most reasonable mechanism of cooperation, given the cytoskeletal arrangements in the dendrites, is that melanosomes are transported for long distances along MT and are afterwards captured in the periphery by myosin V and transported through the subcortical actin network (Wu *et al*, 1998) (**Fig 9**). The idea has been suggested before that, in animal cells, MT-based motility provides long-range organelle transport whereas microfilaments provide local movement (Lane *et al*, 1998).

In conclusion, we have demonstrated the presence and subcellular localization of kinesin and kinectin in normal human melanocytes. The close spatial association of KHC, kinectin, melanosomes, and α-tubulin strongly suggests that this type of MT-based motor proteins is involved in some stage of melanosome movement. To provide evidence for an active role of kinesin in melanosome transport in melanocytes and perhaps in dendrite formation, more dynamic studies are needed, e.g., observation of melanosome transport in living melanocytes. We are currently studying the motor proteins in a more dynamic way by using reporter genes as markers to follow their expression and distribution in living melanocytes.

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