Role of Cytoplasmic Dynein in Perinuclear Aggregation of Phagocytosed Melanosomes and Supranuclear Melanin Cap Formation in Human Keratinocytes

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Cytoplasmic dynein is a microtubule-associated motor molecule involved in the retrograde transport of membrane-bound organelles. To determine whether the supranuclear melanin cap of transferred, phagocytosed melanosomes in keratinocytes is associated with cytoplasmic dynein, we performed immunofluorescent confocal microscopy on human keratinocytes *in situ*. We identified the intermediate chain of cytoplasmic dynein by immunoblotting and examined its distribution by confocal microscopy in relation to microtubules and melano-phagolysosomes *in vitro*. We also used antisense and sense oligonucleotides of the cytoplasmic dynein heavy chain 1 (Dyh1) and time-lapse and microscopy. The intermediate chain of cytoplasmic dynein was identified in extracts of human foreskin epidermis and in isolated human keratinocytes. The intermediate chain localized with the perinuclear melano-phagolysosomal aggregates *in vitro* and the supranuclear melanin cap *in situ*. Antisense oligonucleotides directed towards Dyh1 resulted in dispersal of the keratinocyte perinuclear melano-phagolysosomal aggregates after 24 to 48 h, whereas cells treated with diluent or sense oligonucleotides maintained tight perinuclear aggregates. Taken together, these findings indicate that in human keratinocytes, the retrograde microtubule motor cytoplasmic dynein mediates the perinuclear aggregation of phagocytosed melanosomes, participates in the formation of the supranuclear melanin cap or "microparasol" and serves as a mechanism to help protect the nucleus from ultraviolet-induced DNA damage.
Key words: epidermis/melanocytes/microtubules/phagosome/skin pigmentation. J Invest Dermatol 121:813–820, 2003

Melanin is responsible for skin color and provides protection against ultraviolet (UV) irradiation. Melanin is the major component of melanosomes, which are membrane-bound organelles synthesized by melanocytes. Melanosomes are transferred into adjacent keratinocytes singly or in aggregates as melano-phagosomes where they eventually become melano-phagolysosomes (Mottaz and Zelickson, 1967; Cohen and Szabo, 1968; Klaus, 1969; Wolff *et al*, 1974; Jimbow *et al*, 1976). The distribution of melano-phagolysosomes within human epidermis is concentrated in basal keratinocytes. In suprabasal keratinocytes, particularly in darkly pigmented individuals or after a tanning response in lightly pigmented individuals, melanin is aggregated into "supranuclear caps" (Gates and Zimmermann, 1953). The supranuclear melanin caps reside on the side of the nucleus closest to the skin surface, and similar to the tanning response, can be experimentally increased with aliphatic and alicyclic diols (Brown *et al*, 1998). Likewise, the supranuclear melanin caps of keratinocytes are identified in cultured skin equivalents (Archambault *et al*, 1995) and increase after UV irradiation or IBMX treatment (Gibbs *et al*, 2000). The perinuclear or supranuclear aggregation of melano-phagolysosomes therefore acts as an internal sunscreen. This nonrandom distribution of melanin increases its effective protection of the nucleus from UV-induced DNA damage. To date, we found one study that specifically investigated the "forces responsible for the establishment and maintenance of the supranuclear melanin cap" (Findlay and Liebenberg, 1976). By employing ultrastructural examination of the human epidermis after ultracentrifugation these investigators found a relatively shear prone tonofilament-free zone coinciding with the supranuclear cap. In this study, we postulate an important role for the retrograde microtubular motor cytoplasmic dynein in the formation and maintenance of the melano-phagolysosomal supranuclear cap in keratinocytes. This cap acts as a "microparasol" to protect the nucleus from UV-induced DNA damage.

Cytoplasmic dynein is an ATPase mechanochemical motor that ratchets along microtubules towards the centriolar region (minus-end directed motility). The ATPase mechanochemical transduction residues are found in two cytoplasmic dynein heavy chains of approximately 530 kDa each (Holzbaur and Vallee, 1994; Schroer, 1994). Associated light intermediate cytoplasmic dynein chains (55 kDa) and intermediate chains (74 kDa) participate in binding to the dynactin complex that attaches to membrane bound organelles (Gill *et al*, 1991; Schafer *et al*, 1994; Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). Subunit heterogeneity with multiple isoforms of cytoplasmic dynein heavy, intermediate, and light chains are believed to participate in coordinated membrane bound organelle transport or positioning in a variety

Manuscript received September 26, 2002; revised March 31, 2003; accepted for publication April 20, 2003

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Abbreviations: Dyh1 DHC1, cytoplasmic dynein heavy chain 1; Dyh2 DHC2, cytoplasmic dynein heavy chain 2; DHC3, cytoplasmic dynein heavy chain 3 IBMX, isobutyl-1-methyl-xanthine.

of cell types (Koonce *et al*, 1992; Paschal *et al*, 1992; Mikami *et al*, 1993; Zhang *et al*, 1993; Tanaka *et al*, 1995; Pfister *et al*, 1996a, b; Vaisberg *et al*, 1996; Criswell and Asai, 1998; King *et al*, 1998; Maeda *et al*, 1998; Nurminsky *et al*, 1998; Brill and Pfister, 2000; Tynan *et al*, 2000; Tai *et al*, 2001). In fish pigment cells, microinjection of anti-dynein antibody inhibits minus-end microtubule-directed melanosome transport and results in pigment dispersion (Nilsson and Wallin, 1997). Similarly, in human melanocytes, cytoplasmic dynein is concentrated in a perinuclear pattern (Byers *et al*, 2000) and colocalizes with melanosomes (Vancoillie *et al*, 2000b). In addition, antisense directed towards cytoplasmic dynein heavy chain release melanosomes from the perinuclear region (Byers *et al*, 2000). These findings indicate cytoplasmic dynein transports melanosomes towards perinuclear microtubule organizing centers. Finally, the dynactin subunits $p150^{Glued}$ and dynamitin (p50) also colocalize with melanosomes (Vancoillie *et al*, 2000a), findings that further support a role for cytoplasmic dynein-mediated melanosome transport. In opposition to the cytoplasmic dynein mediated centripetal forces, the centrifugal forces acting on melanosomes include the actin-based motor myosin V (Provance *et al*, 1996; Wu *et al*, 1997, 1998; Lambert *et al*, 1998) and the plus-end microtubule motor kinesin (Hara *et al*, 2000; Vancoillie *et al*, 2000c). In nerve axons, there is bidirectional organelle transport; however, there is net outward transport of organelles as plus-end directed motors are observed to override minus-end motors (Muresan *et al*, 1996; Byers *et al*, 2000). Likewise, there is bidirectional melanosomal movement also in melanocyte dendrites and the net outward transport is subtle (Byers *et al*, 2000; Hara *et al*, 2000). In contrast, in keratinocytes the perinuclear and supranuclear aggregation of melano-phagolysosomes suggests a dominant minus-end directed force acting on these organelles. Therefore, to test the hypothesis that cytoplasmic dynein is the motor behind this force, we used immunoblotting techniques to identify cytoplasmic dynein intermediate chain in keratinocytes and confocal microscopy of cells *in vitro* and *in situ* to characterize its distribution with respect to melano-phagolysosomes and microtubules. Finally, we tested the effect of sense and antisense oligonucleotides in time-lapse functional assays to examine their effect on melano-phagolysosomal distribution.

MATERIALS AND METHODS

Melanosome or melano-phagolysosome visualization *In situ* melanosomes or melano-phagolysosomes were identified by phase contrast or bright field microscopy and staining with the Fontana–Masson silver method. Briefly, 5 μm paraffin sections from darkly pigmented or tanned individuals were deparaffinized, hydrated and treated with 10% silver nitrate at 56°C for 1 to 2 h and 0.2% gold chloride for 1 min with distilled water rinses. The sections were then dehydrated with ethanol, cleared in xylene, and mounted. Densitometry was performed comparing supranuclear cytoplasm with infranuclear cytoplasm of 50 cells by image analysis and statistically analyzed using the paired t test (see *Video Time-Lapse and Image Analysis* and *Statistical Analysis* sections below).

Cell culture Keratinocyte and melanocyte cocultures were obtained from human foreskin as described previously (Gilchrest, 1979) with modifications. Briefly, after cutting the foreskin into 1 mm squares and incubating for 45 min at 37°C and then overnight in 0.25% trypsin at 4°C, the epidermis was separated from the dermis and primary cocultures were obtained by placing keratinocytes and melanocytes in primary keratinocyte media 60% DME (Gibco BRL, Grand Island, New York), 20% F12 nutrient mix (Sigma, St Louis, Missouri; 10.7 g mix and 2.2 g $NaHCO_3$ per liter), 0.18 mM adenine (Sigma), 10% fetal bovine serum, 1.4×10^{-6} M hydrocortisone (Calbiochem-Behring Corp., La Jolla, California), and 10 μg per mL epidermal growth factor (Calbiochem-Behring Corp.).

Isolated human keratinocytes without melanocytes were subsequently obtained at second passage in secondary keratinocyte medium KBM-2 with supplied growth factors (Clonetics, San Diego, California).

Isolated human melanocytes were isolated from neonatal foreskin as described previously (Gilchrest *et al*, 1984; Park *et al*, 1993) with modifications. Briefly, foreskins were processed as above and melanocytes were initially established in Medium 199 (Gibco) supplemented with 10 μg epidermal growth factor per mL (Bethesda Research Laboratories, Gaithersburg, Maryland), 10^{-9} M triiodothyronine (Sigma), 10 μg transferrin per mL (Sigma), 10 μg insulin per mL (Sigma), 10 ng basic fibroblast growth factor per mL (Amgen, Thousand Oaks, California), 1.4×10^{-6} M hydrocortisone (Calbiochem-Behring Corp.), and 5% fetal bovine serum. Post-primary maintenance cultures were in a similar medium but calcium free to support selectively melanocyte growth (Naeyaert *et al*, 1991). Melanocytes at passages 3 to 6 were used for all experiments. For video time-lapse image analysis, keratinocyte and melanocyte cocultures were seeded on to 35 mm Petri dishes. For confocal immunofluorescent studies cells were seeded on to eight-chamber slides (Permanox Tissue Tek, Nunc, Inc., Naperville, Illinois).

Video time-lapse and image analysis Intracellular motility of melanosomes or melano-phagolysosomes was analyzed by time-lapse computerized microscopy as described previously with modifications (Byers *et al*, 2000). Briefly, keratinocytes and melanocyte cocultures were viewed with the Nikon MicroPhot inverted microscope equipped with a Plexiglas housing, Nikon incubator and CO_2/airflow mixer to maintain constant pH and temperature (37°C). Intracellular transport of phagocytosed melanosomes was recorded using the 40 × objective under bright field illumination with a Cohu High performance CCD camera (San Diego, California). Serial digital images were taken at 5 and 30 s intervals for a duration of several minutes at 0, 5, 10, 24, and 48 h after diluent, sense or anti-sense oligonucleotide treatment (see below). The 5 to 30 s intervals permitted study of bidirectional melanosome migration in melanocytes and evaluation of displacement of phagocytosed melanosomes in keratinocyte cytoplasm. Video frames were captured with an LG-3 scientific frame grabber card (Scion Corp. Frederick, Maryland) and analyzed for melanosome distribution using IP lab spectrum software (Scanalytics, Inc., Fairfax, Virginia).

Western blot analysis Total cellular proteins were harvested from either human newborn foreskin epidermal sheets or isolated keratinocytes or melanocytes in a lysis buffer containing 250 mM Tris–HCl (pH 7.5), 0.750 M NaCl, 2.5% NaDOC, 1% Triton X-100, 100 μg aprotinin per mL, and 1 mM phenylmethylsulfonyl fluoride. Twenty-four to 108 μg protein were loaded per lane, separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane at 320 mA for 4 h. After overnight blocking with 5% nonfat dry milk in Tween phosphate-buffered saline (PBS) (0.5% Tween-20 in PBS) at 4°C, the membranes were incubated overnight with a monoclonal antibody against the cytoplasmic dynein 74 kDa intermediate chain (monoclonal 74.1 Chemicon, Temecula, California) (Dillman and Pfister, 1994) at a dilution of 1:1000. After incubation, the membrane was washed four times in Tween-PBS, followed by incubation with an horseradish peroxidase-coupled secondary goat-anti-mouse antibody at a dilution of 1:1800 (Bio-Rad, Hercules, California) in 1% milk for 1 h. After Tween-PBS washing, the membrane was incubated for 1 min with the ECL Western Blotting Detection Reagent (Amersham, Biosciences, Piscataway, NJ) and bands were detected on preflashed Kodak X-Omat film and developed after several seconds of exposure.

Immunofluorescent staining and confocal laser scanning microscopy Keratinocytes cultured for several days in eight-chamber slides (described above) were fixed with 10% formalin for 15 min at room temperature, permeabilized for 5 min with 0.1% Triton X-100 on ice and then incubated with 5% normal goat serum for 30 min to block nonspecific binding sites. The cells were incubated for 1 h at 37°C with the monoclonal antibody against the intermediate chain (74 kDa; see above) at a dilution of 1:200 in PBS with 0.5% bovine serum albumin and rabbit polyclonal anti-tubulin antibody at 1:10 in PBS with 0.5% bovine serum albumin. After several rinses with PBS, fluorescein isothiocyanate (FITC) labeled goat anti-mouse (Sigma) and rhodamine-labeled goat anti-rabbit (Molecular Probes, Eugene, Oregon) were added at a dilution of 1:100 in PBS and 0.5% bovine serum albumin for 1 h at 37°C. Control preparations included the FITC conjugated goat anti-mouse IgG and rhodamine conjugated goat anti-rabbit IgG alone without the primary antibodies or isotype matched unrelated primary antibody (CD5, Sigma). We also used a polyclonal antibody to cytoplasmic dynein (gift of C. Collins) (Lin and Collins, 1992; Lin and Collins, 1993; Lin *et al*, 1994) and confocal immunofluorescence microscopy to assess the effect of anti-sense and sense DNA on cytoplasmic dynein expression. Finally, we tested all three commercially available cytoplasmic dynein heavy chain antibodies, including two polyclonal antibodies (R-325, C-18, Santa Cruz, Biotechnology, Inc., Santa Cruz, California) and one monoclonal antibody (Clone 440.4, Sigma-Aldrich, St Louis, Missouri) on experiments with sense and antisense treated keratinocytes and applied confocal quantitative

immunofluorescence microscopy to determine percent inhibition of cytoplasmic dynein heavy chain expression. After several rinses with PBS, the appropriate FITC-labeled goat anti-mouse (Sigma), FITC-labeled goat anti-rabbit or FITC-labeled chicken anti-goat (Molecular Probes) antibodies were added at a dilution of 1:100 in PBS and 0.5% bovine serum albumin for 1 h at 37°C. Control preparations included the FITC-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rabbit IgG or FITC-conjugated chicken anti-rabbit IgG alone without the primary antibody. After washing, the cells were mounted with coverslips using the slow-fade kit (Molecular Probes). The preparations were examined with a Leica confocal laser scanning microscope equipped with an argon ion laser with an output power of 2 to 50 mW and a 25 × (n.a. = 0.75) objective. Digitized images were stored on an optical disc and processed using IPlab spectrum software described above. Pseudo-color images were generated assigning the FITC signal for cytoplasmic dynein green, the rhodamine signal for microtubules red and the overlap of green and red signals yellow.

Immunofluorescence labeling of cytoplasmic dynein in keratinocytes *in situ* was carried out on frozen sections of normal adult forearm skin after placement on plus-coated slides (Fischer, Pittsburgh, Pennsylvania) and cold acetone (–20°C) fixation for 2 min. The sections were air dried and rehydrated in PBS. The sections were then incubated with anti-dynein antibodies and FITC-labeled secondary antibodies according to the same procedure described above for cultured keratinocytes. Sections were then washed with Tris buffer and counterstained by TO-PRO3-iodide for visualization of the nuclei (Molecular Probes) (Suzuki *et al*, 1997). After rinsing, sections were mounted using the slow-fade kit (Molecular Probes) and confocal microscopy and image processing as described above. Pseudo-color images were generated assigning cytoplasmic dynein staining green, bright field visualized melano-phagolysosomes red and green–red overlap signal yellow.

Preparation of sense and anti-sense oligonucleotides Oligonucleotides stabilized by sulfur-modified phosphorothioate linkages were obtained from Quality Controlled Biochemicals, Inc. (Hopkinton, Massachusetts). The oligonucleotides were based within a human fetal brain cytoplasmic heavy chain sequence (GenBank accession no. T05469 or NCBI GI: 316619) and nearly identical 392 bp human melanocyte cytoplasmic dynein heavy chain sequence (GenBank accession no.: AF234785 or NCBI GI: 7025518). This 392 bp sequence is located on chromosome 14 between bases 102647812 and 102648687 in the human genome. Two anti-sense sequences were 5′-TTTCAGCTTGCATAT CCCATAA-3′, corresponding to the complement of nucleotides 149 to 170 and 5′-TGCATTGTCAAAGGTTCCTCT-3′, corresponding to the complement of nucleotides 239 to 259 of the 392 bp sequence. These anti-sense nucleotide positions correspond to nucleotides 4512 to 4533 and 4602 and 4622, respectively, of the fully sequenced 15,500 nucleotides coding for the rat brain cytoplasmic dynein heavy chain (Zhang *et al*, 1993). Sense oligonucleotides of the same regions were also synthesized representing the exact RNA sequence.

Delivery of oligonucleotides in culture and functional analysis Twelve to 24 h after plating on to 35 mm dishes, keratinocytes and melanocyte cocultures were given serum-free KBM-2 medium supplemented with 2 μg per mL sense or anti-sense oligonucleotides taken from a 50 μM stock solution. Every 12 h an additional 1.7 μg per mL of oligonucleotide was added to the medium. Time-lapse studies on melanosome phagocytosis and melano-phagolysosome distribution were recorded within keratinocytes from 5 to 30 s intervals with duration of observation for several minutes at 0, 5, 24, and 48 h. Distribution of melanosomes and melano-phagolysosomes were recorded in digital images and the keratinocyte peripheral cytoplasms were analyzed for pigment by densitometry in multiple cells (50 cells treated with diluent, sense or anti-sense oligonucleotides; see *Statistical Analysis* section below).

Statistical analysis The light microscopic densitometry and confocal immunofluorescent data were subjected to statistical analysis using Statview (SAS Institute, Inc., Cary, North Carolina). Fifty mean fluorescent intensity (MFI) and densitometry measurements each 4 μm^2 in area were taken of the supranuclear and subnuclear keratinocyte cytoplasms in the *in situ* stained preparation. Cultured keratinocyte peripheral pigment distribution and cytoplasmic dynein heavy chain immunofluorescent staining was analyzed using Student's t test comparing diluent, sense and antisense oligonucleotide treated cells. The pigment densitometry distribution and MFI for cytoplasmic dynein was tested by regression analysis using Statview in both the *in vitro* and *in situ* preparations after image analysis with the IP lab spectrum software described above.

RESULTS

Cytoplasmic dynein intermediate chains colocalize with the supranuclear melanin cap in human keratinocytes *in situ* The supranuclear melanin cap in human epidermis is well developed in tanned or heavily pigmented individuals compared with lightly pigmented individuals (Gates and Zimmermann, 1953). The Fontana–Masson argentaffin reaction dramatically stains melanosomes in darkly pigmented individuals (**Fig 1**). Within the epidermis basally located melanocytes are readily identified with arborizations extending among the keratinocytes. The majority of the basal keratinocytes are more heavily pigmented than the suprabasal keratinocytes. Most striking, however, is the perinuclear melanosome distribution in basal keratinocytes and the distinct supranuclear melanosomal or melanin cap in suprabasal keratinocytes (**Fig 1**).

By image analysis the mean densitometry value of the supranuclear cytoplasm was significantly greater than the value of the infranuclear cytoplasm in the suprabasal keratinocytes ($p < 0.001$). To determine whether cytoplasmic dynein colocalizes with the supranuclear melanin cap, we prepared frozen sections of human skin from a moderately pigmented individual and incubated them with monoclonal antibodies against the cytoplasmic dynein intermediate chain (**Fig 2**). Phase contrast microscopy reveals melanosomes/melano-phagolysosomes in keratinocytes as focal supranuclear aggregates or caps (**Fig 2*a***, *arrows*). The same field examined with confocal fluorescence microscopy for cytoplasmic dynein intermediate chain shows multiple areas with increased fluorescent labeling (**Fig 2*b***). Double fluorescent labeling of cytoplasmic dynein intermediate chain and nuclei exhibits a striking supranuclear pattern (**Fig 2*c***, *arrows*), which coincides with the supranuclear melanosomes/melano-phagolysosomes (**Fig 2*a***, *arrows*). Pseudo-color overlay of the bright field imaging of pigment (red) with the cytoplasmic dynein intermediate chain (green) confirms the supranuclear colocalization (yellow; **Fig 2*d***). Control preparations without the primary antibody or with an isotype matched unrelated antibody (anti-CD5) revealed no epidermal staining (not shown). The colocalization of cytoplasmic dynein intermediate chain and cytoplasmic pigment distribution *in situ* was also confirmed using MFI, pigment densitometry, and regression analysis ($p < 0.001$; coefficient of correlation 0.7; **Fig 3**).

Cytoplasmic dynein intermediate chains are expressed in human keratinocytes Three cytoplasmic dynein intermediate chains are linked to the dynactin complex that is attached to the

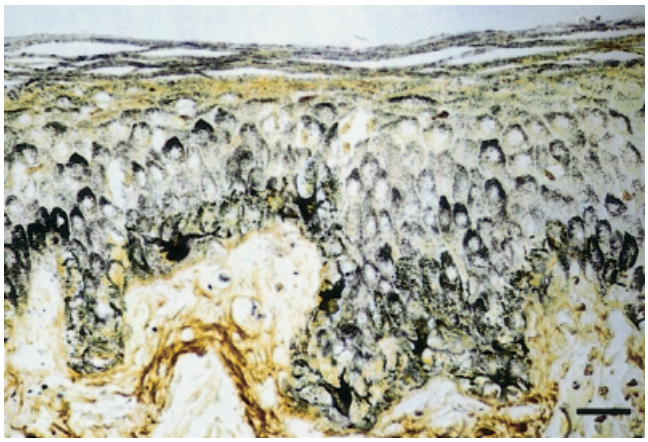

Figure 1. Melano-phagolysosomes are organized into supranuclear "caps" within keratinocytes. Note melanized dendritic melanocytes and adjacent keratinocytes with the supranuclear "caps". Melanin silver stained (Fontana–Masson) section of heavily melanized human epidermis. *Bar*: 50 μm.

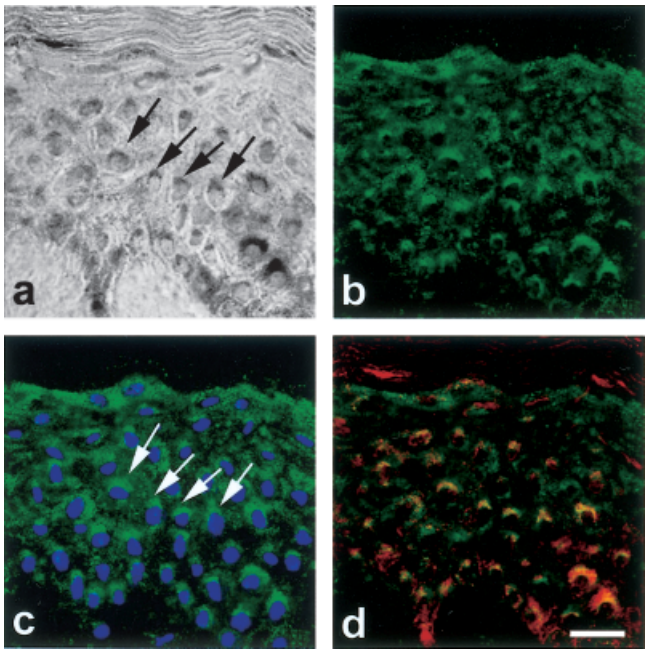

Figure 2. Intermediate chain of cytoplasmic dynein in keratinocytes is concentrated in a supranuclear pattern in human epidermis. (*a*) Phase contrast image of frozen section of human epidermis showing focal supranuclear aggregates or "caps" of melano-phagolysosomes (*arrows*). (*b*) Confocal fluorescence image showing focal increased cytoplasmic staining of the intermediate chain of cytoplasmic dynein in keratinocytes. (*c*) Double fluorescent confocal image with nuclear staining (blue) shows increased supranuclear localization of intermediate chain of cytoplasmic dynein over the nucleus (*arrows*) corresponding to "caps" in a. (*d*) Colocalization of melano-phagolysosomes (red) and cytoplasmic dynein intermediate chain (green) by computer generated pseudo-color overlay (yellow). *Bar*: 50 μm.

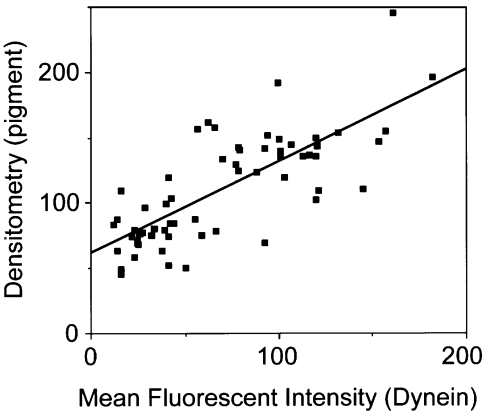

Figure 3. Cytoplasmic dynein intermediate chain MFI correlates with pigment distribution (densitometry). Computer-assisted regression analysis of the intermediate chain MFI pixel values with pigment densitometric values. Fifty MFI and densitometry measurements each 4 μm^2 in area were taken of the supranuclear and subnuclear keratinocyte cytoplasms. Pigment expression units are densitometric mean pixel intensity values. Significance $p < 0.001$ and coefficient of correlation 0.7.

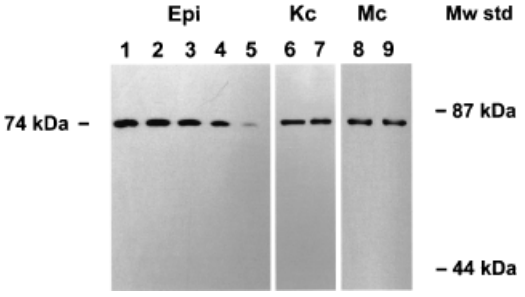

Figure 4. Western blot analysis identifies intermediate chain of cytoplasmic dynein in extracts from isolated whole human foreskin epidermis (Epi), isolated keratinocytes (Kc), and isolated melanocytes (Mc). A single band at the appropriate relative mobility of 74 kDa is detected in isolated human epidermis, keratinocytes, and melanocytes. *Lanes 1 to 5*: 108, 96, 84, 72, and 60 μg protein loading, respectively; *lanes 6 to 9*: 24 μg protein loading. Molecular weight standard (Mw std) positions shown for assessment of relative mobility.

membrane of the organelle undergoing transport (Hirokawa, 1998). We previously identified this intermediate chain of cytoplasmic dynein in isolated human melanocytes by western blot analysis (Byers *et al*, 2000). To identify this 74 kDa protein in human keratinocytes, we performed immunoblots on lysates of human epidermis and isolated keratinocytes using a monoclonal antibody (Pfister *et al*, 1996a, b). A single band at approximately 74 kDa is identified in both lysates (**Fig 4**), confirming that keratinocytes express cytoplasmic dynein intermediate chain. Control isolated melanocytes also exhibit the 74 kDa band as reported previously (Byers *et al*, 2000). Cytoplasmic dynein is expressed at similar levels relative to total lysate protein loading in melanocytes and keratinocytes but significantly less than levels identified in the epidermal lysates. Keratinocytes compose over 90% of the epidermis therefore the abundant amount of cytoplasmic dynein intermediate chain seen in lysates of human epidermis indicates a major contribution by the keratinocytes.

Cytoplasmic dynein plays a part in directed melanosomal/melano-phagolysosomal perinuclear aggregation Time-lapse analysis of human melanocyte and keratinocyte cocultures identified bidirectional melanosomal transport in melanocyte dendrites. Melanosomal transfer occurred by distal pinching off of the dendrite or dendrite fragmentation accompanied by keratinocyte phagocytosis of the pigment containing dendrite fragments (**Fig 5**). Gradual accumulation of perinuclear pigmenta-tion in keratinocytes occurred within a few days and, although occasional eccentricity is noted in the perinuclear accumulation, the majority of monolayer cultured keratinocytes exhibit perinuclear melanosome/melano-phagolysosome aggregates. To determine the distribution of cytoplasmic dynein in human keratinocytes relative to the perinuclear melano-phagolysosomes and the microtubule framework, we performed double immuno-fluorescent confocal scanning microscopy. Monoclonal antibodies to the 74 kDa intermediate cytoplasmic dynein chain revealed a punctate distribution that primarily colocalized with the perinuclear melano-phagolysosomes (**Fig 6*a*,*c***). Microtubules were identified emanating from the perinuclear microtubule organizing centers (**Fig 6*b***). Pseudo-color overlap images produced by image processing revealed partial colocalization of the punctate cytoplasmic dynein staining pattern with the converging perinuclear microtubules (**Fig 6*d***). Control isotype matched antibody (anti-CD5) for cytoplasmic dynein and secondary antibody showed no perinuclear staining (not shown). To test the role of cytoplasmic dynein in retrograde (minus-directed) microtubular melano-phagolysosome transport, cocultured melanocytes and keratinocytes were maintained and recorded by time-lapse microscopy at 5 and 30 s intervals for several minutes at 0, 5, 10, 24, and 48 h after treatment with diluent (control), sense (control) or the two different anti-sense cytoplasmic dynein heavy chain oligonucleotides. Cell morphology and distribution of melanosomes were analyzed by time-lapse microscopy. High-resolution time-lapse microscopy permitted visualization of the movement of individual melanosomes/melano-phagolysosomes. No significant

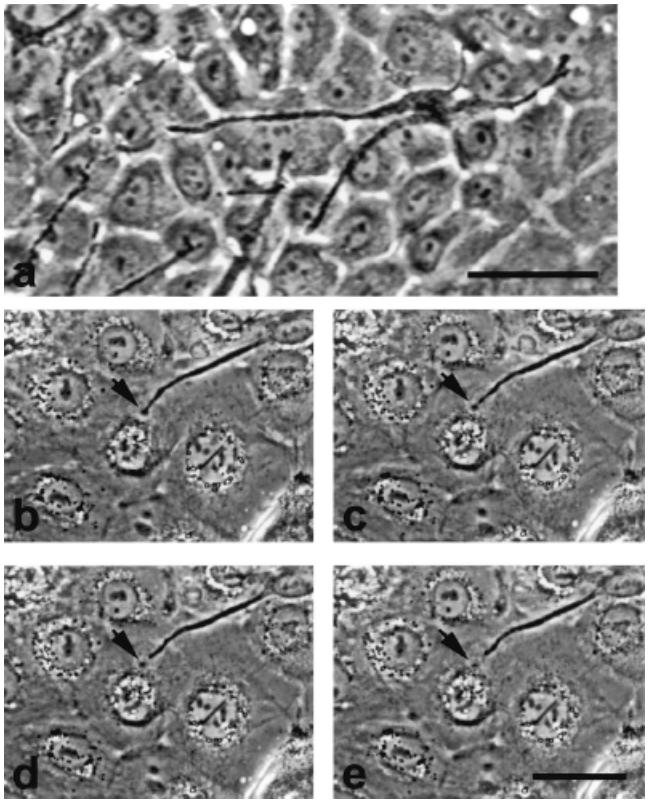

Figure 5. Time-lapse analysis of coculture indicates transferred melanosomes accumulate as perinuclear melano-phagolysosomes in cultured human keratinocytes. (*a*) Phase contrast photomicrograph of human melanocyte/keratinocyte coculture. Heavily melanized dendritic melanocytes and keratinocytes with perinuclear melano-phagolysosomes are seen. *Bar.* 50 μm. (*b–e*) Time-lapse series with 10 s intervals showing distal fragmentation of melanocyte dendrite (see *arrows*), phagocytosis, melano-phagosome formation and perinuclear aggregation of melano-phagolysosomes within keratinocytes. *Bar.* 20 μm.

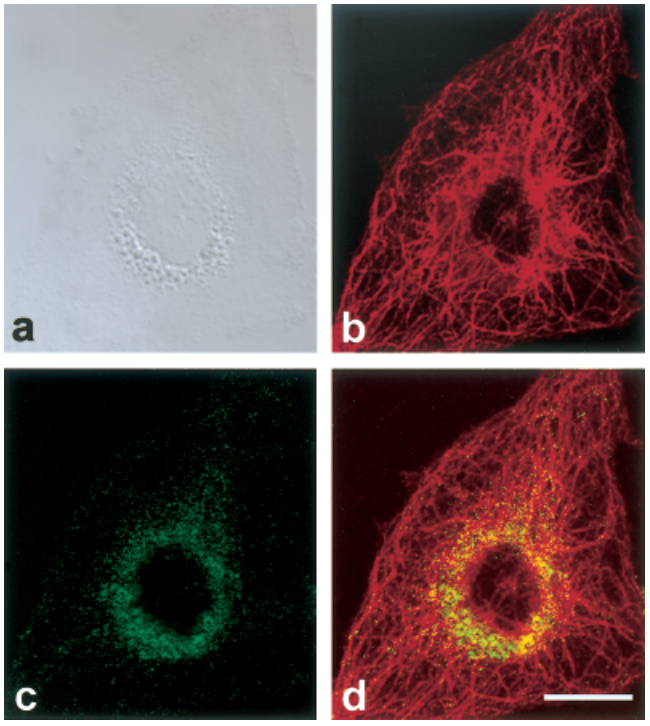

Figure 6. Intermediate chain of cytoplasmic dynein colocalizes with perinuclear melano-phagolysosomes and perinuclear microtubules in cultured human keratinocytes. (*a*) Differential interference contrast image showing perinuclear aggregation of melano-phagolysosomes and other large vesicular organelles compared with small peripheral cytoplasmic vesicular organelles. (*b*) Anti-tubulin staining showing radial arrangement of microtubules with convergence into perinuclear microtubule organizing centers. (*c*) Distribution of the intermediate chain of cytoplasmic dynein is concentrated in the perinuclear region corresponding to the melano-phagolysosomes and other large vesicular organelles with scant, scattered small aggregates of peripheral cytoplasmic staining. (*d*) Overlap pseudo-color image of microtubules and cytoplasmic dynein. *Bar.* 10 μm.

differences among the test cultures were seen at 5 and 10 h after treatment with the oligonucleotides. At 24 and 48 h, diluent-treated and sense-treated cells exhibited normal perinuclear aggregation of melanosomes/melano-phagolysosomes, whereas antisense treated cells with either of the two anti-sense sequences demonstrated progressive dispersion of the keratinocyte perinuclear aggregates throughout the cytoplasm (**Fig 7**; sense control and one antisense oligonucleotide data are shown). Densitometric analysis of bright field digitized images quantified the pigment distribution in the peripheral cytoplasm. Fifty cells in each treatment category were analyzed. Both anti-sense treatments reduced significantly perinuclear pigment aggregates and caused a significant dispersal of pigment into the peripheral cytoplasm compared with diluent or sense treated controls ($p < 0.001$) (**Fig 8**; sense control and one anti-sense oligonucleotide data are shown).

Immunofluorescent labeling of cytoplasmic dynein and image analysis of MFI revealed anti-sense treated cells exhibited a significant reduction in cytoplasmic dynein expression compared with controls. Experiments with sense and antisense treated keratinocytes and quantitative immunofluorescent confocal microscopy examined the expression of the cytoplasmic dynein heavy chain using three commercially available antibodies described in the *Materials and Methods* section. Very weak signals were detected using these antibodies for immunocytochemistry on human keratinocytes except with antibody C-18. Using this antibody, we detected a significant reduction in confocal cytoplasmic dynein heavy chain MFI between sense and antisense treated keratinocytes after 48 h of treatment (147 ± 10 *vs* 123 ± 16; $p < 0.001$). The findings indicate a 16% mean reduction of cytoplasmic dynein heavy chain fluorescence; however, reduction was variable in individual keratinocytes with a MFI range of 132 to 161 in the sense treated cells and 90 to 149 in the antisense treated cells, indicating variable transfection and up to 38% reduction in heavy chain fluorescence with anti-sense treatment.

DISCUSSION

The pigmentation of human epidermis is the result of transport of melanosomes within melanocytes from the centrosomal region to the peripheral dendrites and transfer into keratinocytes by phagocytosis (Mottaz and Zelickson, 1967; Cohen and Szabo, 1968; Klaus, 1969; Wolff and Konrad, 1971; Wolff *et al*, 1974). The molecular mechanisms that are required for these multiple steps are under intense investigation. Much evidence suggest that melanosomes, as all membrane bound organelles, are transported within the cytoplasm by actin and microtubule based molecular motors (Lambert *et al*, 1999; Vancoillie *et al*, 2000b; Smith and Simmons, 2001). Indeed, the bidirectional transport of melanosomes within human melanocytes appears to be the balance of cell center directed cytoplasmic dynein–dynactin microtubule based transport (Byers *et al*, 2000; Vancoillie *et al*, 2000a, b), the peripheral-directed myosin V actin based transport (Provance *et al*, 1996; Wu *et al*, 1998), and the kinesin microtubule based

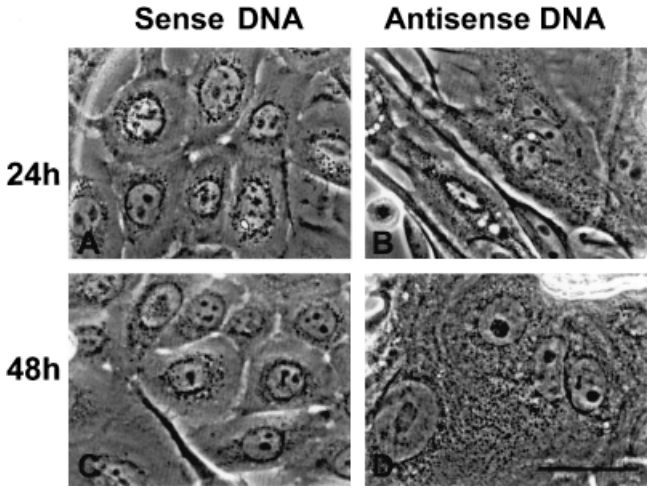

Figure 7. Anti-sense DNA complementary to cytoplasmic dynein sequence disperses perinuclear melano-phagolysosomes in keratinocytes at 24 and 48 h compared with sense DNA treated controls. Co-cultures of human neonatal melanocytes and keratinocytes (*A–C*). (*A*) Sense DNA treated cells at 24 h show tight perinuclear aggregates of melano-phagolysosomes whereas anti-sense DNA treated cells at 24 h (*B*) reveal perinuclear and numerous dispersed melano-phagolysosomes. (*C*) Sense DNA treated cells at 48 h still show tight perinuclear aggregates, whereas anti-sense DNA treated cells at 48 h (*D*) exhibit numerous dispersed melano-phagolysosomes. *Bar.* 10 μm.

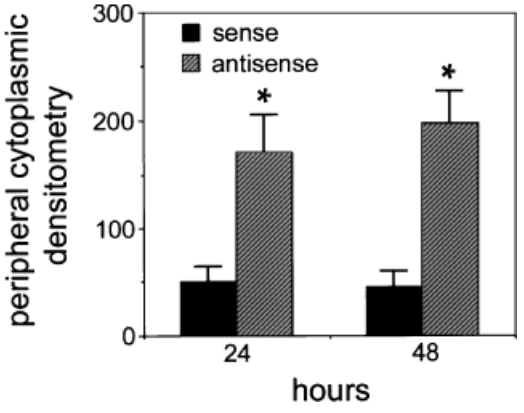

Figure 8. Anti-sense DNA complimentary to cytoplasmic dynein distributes perinuclear pigment in cultured keratinocytes to the peripheral cytoplasm. Densitometric analysis of 200 peripheral cell cytoplasms at each time point and treatment condition shows significant increase in the peripheral pigment in anti-sense (striped bars) compared with sense treated control keratinocytes (black bars). Bars and error bars represent mean, SD, respectively. $^{*}p < 0.001$.

transport (Hara *et al*, 2000; Vancoillie *et al*, 2000c). Melanosomes or melanosome aggregates that are phagocytosed by adjacent keratinocytes, however, do not exhibit bidirectional centripetal and centrifugal transport but similar to lysosomes, are tightly maintained in a perinuclear location. Our identification of the cytoplasmic dynein intermediate chain in isolated human keratinocytes and the inhibition of the tight perinuclear aggregation by antisense oligonucleotides to the cytoplasmic dynein heavy chain provides evidence that cytoplasmic dynein plays an important part in the cell center directed transport of melano-phagolysosomes. This finding is analogous to its role in the maintenance of the centrosomal distribution of lysosomes as shown by lysosome dispersion in cultured mouse blastocyst cells with cytoplasmic dynein knockout (Harada *et al*, 1998).

It is unknown whether the microtubule polarities in keratinocytes resemble melanocytes. Our recent antisense studies on human melanocytes (Byers *et al*, 2000) provide evidence that the melanocyte dendrite microtubules are oriented similar to nerve axons (Baas *et al*, 1988) and fish melanophores (McNiven and Porter, 1986) with the plus ends directed towards the periphery as opposed to nerve dendrites with mixed polarity (Baas *et al*, 1988). Likewise, our antisense experiments in this study suggest that the microtubules are oriented with the plus ends in the periphery and minus ends in the perinuclear centrosome in keratinocytes; however, in keratinocytes, as opposed to the bidirectional motility of melanosomes in melanocytes, it is apparent that the force acting on melano-phagolysosomes by the cytoplasmic dynein–dynactin complex overrides the peripherally directed forces of myosin V or kinesin. Nonetheless, despite cytoplasmic dynein perinuclear concentration, we also identified scattered peripheral punctate staining in both the *in vitro* and *in situ* keratinocyte cytoplasms. This supports recent observations on the bidirectional movement of cytoplasmic dynein associated structures and switching activity of both plus and minus-directed motors associated with particular membrane bound cargoes (Ma and Chisholm, 2002). Indeed, cytoplasmic dynein is also peripherally associated at adherens junctions with β-catenin and E-cadherin in epithelia (Ligon *et al*, 2001) albeit much less than observed in the perinuclear region. Similarly, we observed faint focal staining of the cell–cell contacts in keratinocytes (data not shown). Cytoplasmic dynein has also been associated with breakdown of the nuclear envelope during prophase (Salina *et al*, 2002). Specificity of cellular function within cellular compartments and among different cell types likely reflects in part differential expression of members of the dynein superfamily.

The dynein superfamily includes at least 12 members of the axonemal dyneins involved in the wave generating shearing forces in cilia and flagella (Brokaw, 1994) and at least four cytoplasmic dyneins. The ubiquitous cytoplasmic dynein heavy chain Dyhl (MAPlc, DHcl, and DHCla; Koonce *et al*, 1992; Mikami *et al*, 1993; Vaisberg *et al*, 1993; Zhang *et al*, 1993) appears throughout phylogeny from amoeba to the classic microtubule-dependent fast axonal retrograde (minus end directed) organelle transport (Schroer *et al*, 1989; Muresan *et al*, 1996). Two cytoplasmic dynein family members have been identified in brain (Tanaka *et al*, 1995), three in fibroblasts and HeLa cells (Vaisberg *et al*, 1996) and possibly four in rat testis (Criswell and Asai, 1998). These different isoforms may be involved in different organelle transport, secretion, mitotic spindle organization, chromosome, or nuclear migration (Holzbaur and Vallee, 1994; Schroer, 1994; Tanaka *et al*, 1995; Vaisberg *et al*, 1996). Our cytoplasmic dynein anti-sense oligonucleotides were made to a short Dyh1 sequence that we recently cloned from melanocytes (Byers *et al*, 2000). The lack of complete peripheral dispersion of melano-phagolysosomes in our antisense studies on keratinocytes may be due to functional redundancy of cytoplasmic dynein heavy chain isoforms or other recently identified minus-directed microtubule motors, including kinesin-related motors (Case *et al*, 1997; Henningsen and Schliwa, 1997; Hirokawa, 1998; Sablin *et al*, 1998; Smirnova *et al*, 1998; Matuliene *et al*, 1999; Wade and Kozielski, 2000; Noda *et al*, 2001). In addition, the lack of complete dispersal in the antisense treated cells, may also be in part due to slow protein turnover and limited delivery of anti-sense. MFI of the cytoplasmic dynein heavy chain, however, was reduced significantly in the antisense treated cells compared with sense treated cells, indicating significant reduction in cytoplasmic dynein levels. This supports the disruption of cytoplasmic dynein synthesis and release of melano-phagolysosomes from the centrosome. The fluorescent signal was clearly not abrogated in the anti-sense treated cells, indicating the partial pigment dispersion is due to incomplete reduction of synthesis of the cytoplasmic dynein heavy chain in relation to protein turnover. Variable efficiency of transfection with a subset of cells receiving sufficient anti-sense to induce partial inhibition of the heavy chain expression was

confirmed by the large standard deviation of the immunocytochemical data of the 48 h antisense treatment. It is likely that only by direct injection of antibodies into individual cells (Nilsson and Wallin, 1997) or by knockout or gene silencing techniques (Harada *et al*, 1998; Harborth *et al*, 2001) may one achieve total release of pericentriolar organelles. Likewise, multiple isoforms or other minus-directed motors may prevent total release.

To provide additional evidence for a role of cytoplasmic dynein in the melano-phagolysosome perinuclear aggregation other than antisense experiments, we used antibodies against the cytoplasmic dynein intermediate chain (Pfister *et al*, 1996a, b) to determine whether it colocalizes with the melano-phagolysosomes and perinuclear microtubule organizing centers. The cytoplasmic dynein intermediate chain is the linkage protein that binds the entire motor complex to the $p150^{Glued}$ subunit of the dynactin complex and associated membrane bound organelles (Karki and Holzbaur, 1995, 1999; Vaughan and Vallee, 1995; Steffen *et al*, 1997). In further support of a functional role of cytoplasmic dynein in maintenance of the perinuclear melano-phagolysosomes, we found that the intermediate chain immunolocalization significantly colocalizes with the perinuclear distribution of melano-phagolysosomes and the convergent microtubules. These findings are consistent with the role of cytoplasmic dynein in maintaining the "inward" pull on melano-phagolysosomes towards the keratinocyte cell center. This force overrides the "outward" pull of the plus end directed motor proteins and actin based motor proteins in keratinocytes. Interestingly, there was incomplete overlap of cytoplasmic dynein and microtubules in the perinuclear region in the confocal overlay images. This may indicate a role of other target proteins in the perinuclear aggregation. Indeed, recent evidence indicates that cytoplasmic dynein light intermediate chain 1 binds to pericentrin, a molecule associated with centrosomes and takes part in the assembly of pericentrin and γ tubulin containing new microtubule initiation sites (Tynan *et al*, 2000; Young *et al*, 2000). In conclusion, we show that the intermediate chain of cytoplasmic dynein is expressed in human keratinocytes, is preferentially located in the perinuclear melano-phagolysosome region *in vitro* and colocalizes to the supranuclear melanin cap in keratinocytes *in situ*. We provide functional evidence that cytoplasmic dynein is involved in the maintenance of the perinuclear melano-phagolysosomal aggregates. Taken together, these findings support a role for cytoplasmic dynein in the formation of the supranuclear melanin cap or "microparasol" to better protect the nucleus from UV-induced DNA damage.

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