ORIGINAL ARTICLE

Requirement of Dynactin p150^Glued Subunit for the Functional Integrity of the Keratinocyte Microparasol

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The keratinocyte microparasol, composed of a perinuclear microtubular/melano–phagolysosomal complex, protects the nucleus from UV-induced DNA damage. We have previously demonstrated that cytoplasmic dynein is the motor involved in the perinuclear-directed aggregation of phagocytosed melanosomes. Dynactin, of which p150^Glued is the major subunit, can link directly to microtubules and links organelles to dynein at different domains. To further define the mechanism of the microparasol, we transfected siRNA targeted against p150^Glued into human keratinocytes cultured with 0.5 mm fluorescent microspheres and performed time-lapse analysis, confocal immunolocalization, and Western immunoblotting after 24 and 48 hours. Western blots revealed a significant knockdown of the p150^Glued subunit. The knockdown decreased p150^Glued colocalization with microtubules and decreased perinuclear positioning of the convergent microtubular framework. It also inhibited perinuclear aggregation of phagocytosed fluorescent microspheres and reduced mean centripetal microsphere displacement. The findings provide evidence that dynactin p150^Glued plays an important role in the functional integrity of the keratinocyte microparasol.

INTRODUCTION

Melano–phagolysosomes act as an internal sunscreen against UV-induced DNA damage in human epidermis (Kobayashi *et al.*, 1998). The keratinocyte microparasol defines the functional photoprotective structure comprising the active aggregation of phagocytosed melanosomes on a radial microtubular array focused adjacent to the nucleus (Byers *et al.*, 2003; Scott, 2003). The microparasol is both perinuclear and supranuclear in keratinocytes, most prominent at the basal layer and less so in the suprabasal layer, and composes in part, the earlier recognized ''melanin cap'' observed *in situ* (Gates and Zimmermann, 1953) and *in vitro* (Archambault *et al.*, 1995; Gibbs *et al.*, 2000). Knockdown and immunofluorescence evidence indicates that the microtubule-based motor dynein plays a major role in the mechanism of the aggregation of melano–phagolysosomes around the keratinocyte nucleus (Byers *et al.*, 2003).

Dynactin, a multisubunit complex that includes the subunit p150^Glued, is essential for the dynein motor to transport membranous cargoes over long distances along microtubules (Schroer and Sheetz, 1991; for review see Schroer, 2004). Dynactin links membranous cargoes to dynein and disruption of its two structural domains results in major derangement of intracellular motility with loss of subcellular organization (Holleran *et al.*, 1998). Dynactin concentrates in mitotic spindle poles during mitosis and the centrosome during interphase, wherever microtubule minus-ends are focused (Gill *et al.*, 1991; Clark and Meyer, 1992; Paschal *et al.*, 1993; Kahana *et al.*, 1998) or anchored (Quintyne *et al.*, 1999). Dynactin is also found less abundantly at microtubule plus-ends (Valetti *et al.*, 1999; Vaughan *et al.*, 2002); its function there may control microtubule dynamics or capture endomembranes in the cell cortex for transport to the centrosome. Indeed, dynactin is found on endocytic organelles and membranes in the region of the Golgi apparatus (Habermann *et al.*, 2001). The p150^Glued subunit of dynactin plays a pivotal role in cargo transport as one of its domains binds the motor dynein (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995), whereas another domain directly binds microtubules (Waterman-Storer *et al.*, 1995; Vaughan *et al.*, 2002). This dual, alternative binding promotes dynein processivity along the microtubule (King and Schroer, 2000).

In this study, we test the hypothesis that dynactin p150^Glued subunit is required for the transport and maintenance of phagosomes in the perinuclear region of the keratinocyte. Phagocytosed melanosomes are the fabric of the keratinocyte microparasol whereas the radial microtubular array is the framework (Byers *et al.*, 2003). We provide evidence for an important role of dynactin p150^Glued in the microparasol mechanism using p150^Glued-specific siRNA for p150^Glued knockdown, Western immunoblotting, and time-lapse analysis of phagocytosed fluorescent microspheres.

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Abbreviations: Mw, molecular weight; std, standard

RESULTS

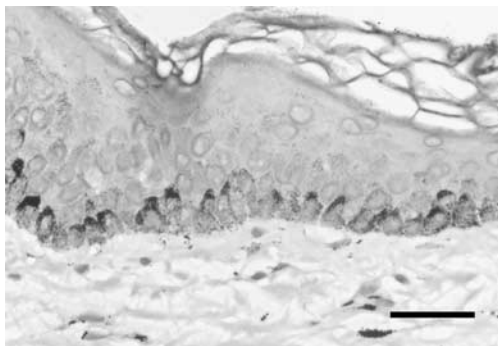### Expression of p150^{Glued} in human keratinocytes and knockdown of p150^{Glued} by siRNA targeting

The supranuclear melanin cap in human epidermis is well developed in tanned or intrinsically pigmented individuals (Gates and Zimmermann, 1953). The Fontana-Masson argentaffin reaction highlights melano–phagolysosomes in keratinocytes (Figure 1). Basal keratinocytes are more heavily pigmented than the supra-basal keratinocytes and show prominent supranuclear pigment aggregation or cap. By image analysis, the mean densitometry value of the supra-nuclear basal keratinocyte cytoplasm was significantly great-er than the value of the cytoplasm lateral to the nucleus in basal keratinocytes ($P < 0.001$). We have provided evidence that the mechanism of the microparasol or the motive force of perinuclear aggregation of melano–phagolysosomes is de-pendent on microtubules and dynein (Byers et al., 2003).

To test the hypothesis that p150^{Glued}-specific siRNA will disrupt the structural and functional integrity of the keratino-cyte microparasol, it is first necessary to determine whether p150^{Glued} is expressed in human keratinocytes and, second, whether its expression can be reliably knocked down. First, Figure 2a shows that a single band at the appropriate relative molecular mass at 150 kDa is detected in Western blots with antibodies against p150^{Glued} in four different donors. Second, knockdown of p150^{Glued} can be detected in these four different donor keratinocytes transfected with p150^{Glued}-specific siRNA compared with the mock controls (negative control RNA and lipofectamine or lipo-fectamine alone; see Materials and Methods) at both 24 and 48 hours after transfection. Densitometry revealed expression was reduced between 47 to 65% at 24 hours and 43 to 85% at 48 hours in the various donors relative to controls. Statistical densitometric analysis of the blots from the four donors reveals significant knockdown of expression of p150^{Glued} (Figure 2b, $P < 0.05$) in p150^{Glued}-specific siRNA-treated keratinocytes compared with negative control transfectants.

Dynactin p150^{Glued} knockdown markedly reduces its colocalization with microtubules and disrupts the perinuclear focusing of the microtubular framework of the keratinocyte microparasol

The dynactin p150^{Glued} subunit is known to localize to the centrosome/microtubule organizing center in interphase cells and can also be seen showing significant colocalization with microtubules (Gill et al., 1991; Clark and Meyer, 1992; Paschal et al., 1993; Kahana et al., 1998). Functional studies indicate that p150^{Glued} may serve as anchoring the minus-ends of microtubules at the centrosome (Quintyne et al., 1999). To test the hypothesis that p150^{Glued} plays an important role in organizing the radial microtubular array of the keratinocyte microparasol, confocal immunofluorescence
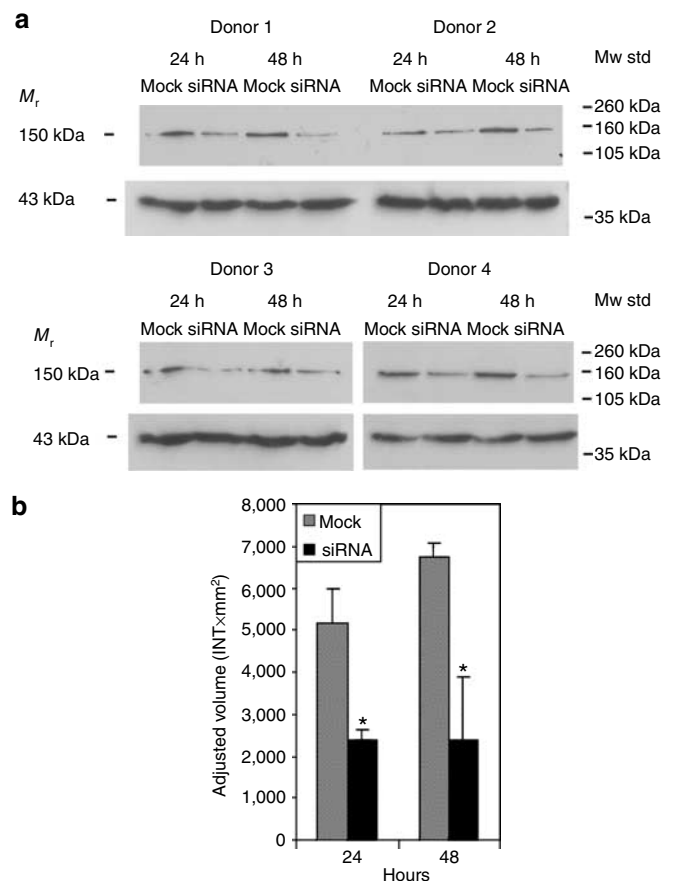
Figure 2. **Reduced p150^{Glued} expression by p150^{Glued}-specific siRNA knockdown.** (**a**) Western blot analysis of the p150^{Glued} subunit of the dynactin complex in negative control and p150^{Glued}-specific siRNA-transfected human keratinocytes. Four different experiments with four different donors are depicted. A single band at the appropriate relative mobility of 150 kDa is significantly reduced in keratinocytes transfected with p150^{Glued}-specific siRNA compared with control. Actin (43 kDa) immunoblot shown in lower panels; protein loading per lane is 50 μg. Mw std positions shown for assessment of relative molecular mass (M_r). (**b**) Densitometric analysis of Western blots of p150^{Glued} in negative control and p150^{Glued}-specific siRNA-treated cells. There is a significant decrease in p150^{Glued} expression in the p150^{Glued}-specific siRNA-targeted transfectants compared with negative control transfectants at 24 and 48 hours (*$P < 0.05$). Bars and error bars represent mean ± SD.
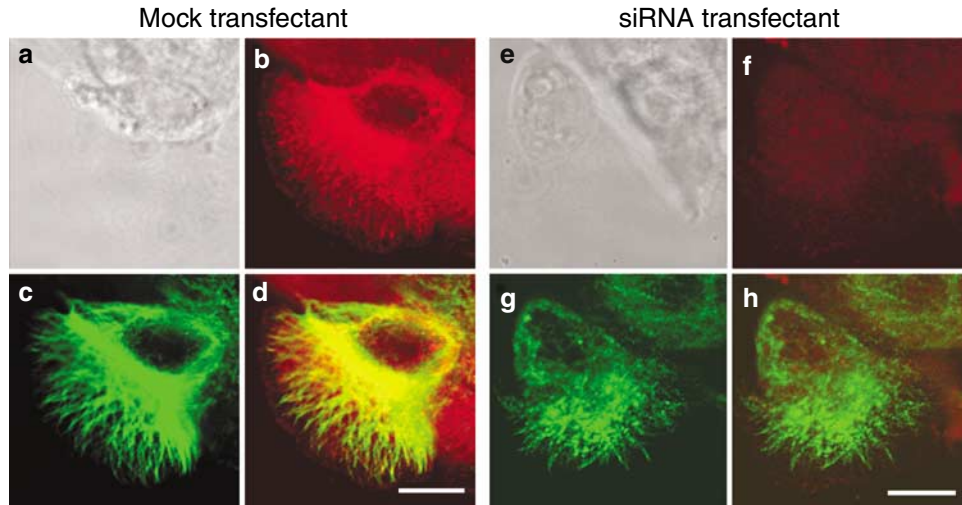

Figure 1. **Melano–phagolysosomes are organized into supranuclear "melanin caps" within basal keratinocytes.** Pigmented keratinocytes show prominent supranuclear aggregation of pigment compared with cytoplasm lateral to basal keratinocyte nuclei. Silver-stained (Fontana-Masson) section of skin type II–III human epidermis. Bar = 50 μm.

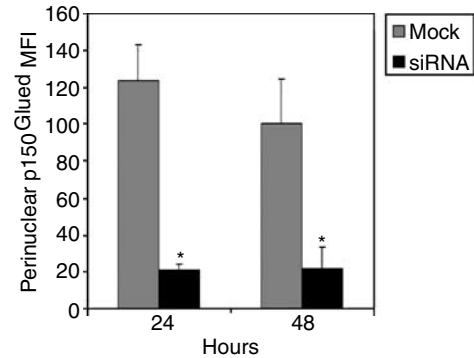Mock transfectant   siRNA transfectant

Figure 3. Double immunofluorescent localization of p150^{Glued} and microtubules in negative control and in siRNA p150^{Glued}-targeted human keratinocyte transfectants. (a) Phase contrast image of mock transfectant. **(b)** The p150^{Glued} subunit shows prominent perinuclear staining with subtle radial pattern identified in the periphery. **(c)** Anti-tubulin staining highlights a radial microtubular array with prominent concentration in the perinuclear cytoplasm. **(d)** Overlap pseudocolor image reveals colocalization of microtubules and p150^{Glued}. **(e)** Phase contrast image of siRNA p150^{Glued}-targeted transfectant. **(f)** There is marked reduction of perinuclear p150^{Glued} staining following p150^{Glued}-specific siRNA compared with negative control transfectants **b**. **(g)** Anti-tubulin staining reveals decreased perinuclear fluorescence of microtubules compared with negative control **c**. **(h)** Overlap pseudocolor image reveals decreased yellow colocalization of microtubules and p150^{Glued} compared with negative control **d**. Bar = 10 μm.

examined the distribution of microtubules and p150^{Glued} in cultured human keratinocytes after negative control and p150^{Glued}-specific siRNA transfection. Normal- and negative control-treated cells exhibit intense perinuclear staining for both p150^{Glued} and microtubules (Figure 3a–c). Peripheral to the intense centrosomal staining, the p150^{Glued} (Figure 3b) and the microtubule staining (Figure 3c) reveal a radial pattern, although the p150^{Glued} radial pattern is less distinct than the microtubular framework. This intense perinuclear and radial colocalization is confirmed by pseudocolor overlay analysis (Figure 3d). In contrast, p150^{Glued}-specific siRNA transfectants exhibited marked reduction of perinuclear p150^{Glued} fluorescence (Figure 3f) with loss of the radial colocalization pattern with microtubules (Figure 3h). Interestingly, there was also a marked reduction in perinuclear microtubular fluorescence or focusing (Figure 3g).

Quantitative image analysis of multiple cell perinuclear cytoplasms (see Materials and Methods) demonstrated significant reduction at 24 and 48 hours in mean fluorescence intensity (MFI) of p150^{Glued} labeling (Figure 4; $P<0.05$) in p150^{Glued}-specific siRNA-treated cells compared with negative control transfectants (mock).

Knockdown of dynactin p150^{Glued} by p150^{Glued}-specific siRNA inhibits perinuclear migration and perinuclear aggregation of phagocytosed microspheres

Melanosomes are phagocytosed by keratinocytes and these melano–phagosomes are then transported to the perinuclear cytoplasmic region (Mottaz and Zelickson, 1967; Cohen and Szabo, 1968; Klaus, 1969; Wolff and Konrad, 1971; Wolff et al., 1974). We tested whether fluorescent polystyrene microspheres of the approximate size of melanosomes can be
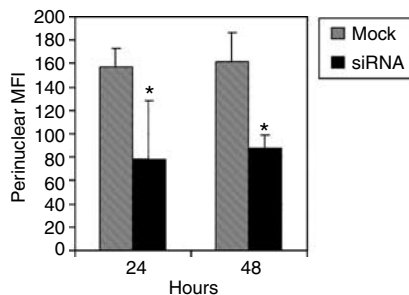

Figure 4. Perinuclear MFI of labeled p150^{Glued} in negative control and p150^{Glued}-specific siRNA transfectants. The fluorescence intensity of 50 perinuclear cytoplasms of cultured human keratinocytes was quantified by image analysis. There is significant knockdown in p150^{Glued} compared with negative control transfectants at both 24 and 48 hours (*$P<0.05$). Bars and error bars represent mean $\pm$ SD.

used to study the capture and transport of phagosomes along the microtubular framework of the keratinocyte. To provide functional evidence of the effect of knockdown of p150^{Glued} by siRNA targeting, we designed experiments to visualize the behavior of these phagocytosed fluorescent microspheres. These experiments utilize time-lapse microscopy of phago-cytosed fluorescently labeled 0.5 μm polystyrene micro-spheres in cultured human keratinocytes previously treated only 24 and 48 hours earlier with negative control or with siRNA targeted against p150^{Glued} (see Materials and Meth-ods). Control cells exhibit marked peripheral phagocytosis and transport of the microspheres into the perinuclear region
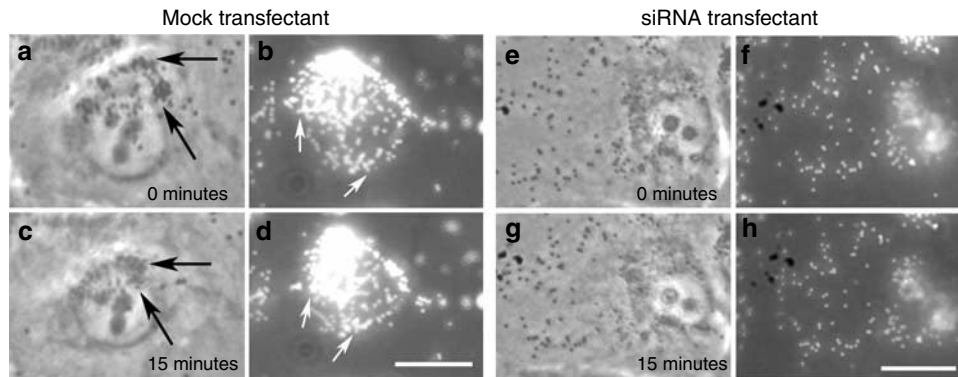
Figure 5. **Time-lapse analysis of phagocytosed fluorescently labeled 0.5 μm polystyrene microspheres in cultured human keratinocytes treated with negative control or siRNA-targeted against p150^{Glued}**. Two different mock transfectants are shown, one showing the microspheres using phase contrast (**a** and **c**) and another cell demonstrating the microspheres with fluorescence (**b** and **d**). Perinuclear aggregation of fluorescent microspheres is detected; arrows show inward displacement of microspheres from 0 minute (**a** and **b**) to 15 minutes (**c** and **d**). In contrast, p150^{Glued}-specific siRNA-transfected cells show reduced perinuclear aggregation of microspheres from 0 minute (**e** and **f**) to 15 minutes (**g** and **h**) compared with negative control transfectants (**a–d**). Phase contrast **e** and **g** and fluorescence **f** and **h**. Bar = 10 μm.

Figure 6. **siRNA targeting p150^{Glued} reduces perinuclear aggregation of phagocytic organelles compared with negative controls.** Perinuclear MFI of phagocytosed fluorescently labeled microspheres in 50 cell cytoplasms is significantly reduced in p150^{Glued}-specific siRNA-treated cells compared with negative control transfectants at both 24 and 48 hours (*$P < 0.05$). Bars and error bars represent mean ± SD.

where they form tightly aggregated clusters (Figure 5a–d, arrows). In contrast, p150^{Glued}-specific siRNA-transfected cells show decreased phagocytosis and transport of microspheres into the perinuclear region with numerous peripheral cytoplasmic microspheres (Figure 5e–h) compared with negative control transfectants. Quantitative analysis was performed by measuring the MFI of the perinuclear region at 24 and 48 hours in 50 cells in negative control and p150^{Glued}-specific siRNA-treated cells as outlined in Materials and Methods. The p150^{Glued}-specific siRNA-transfected cells exhibit a significant reduction in perinuclear MFI compared with negative control transfectants (Figure 6; $P < 0.05$), indicating reduced transport of microspheres to the perinuclear region.

Targeting dynactin p150^{glued} decreases centripetal displacement of phagosomes

Individual cytoplasmic microsphere motility was assessed by time-lapse image analysis using fluorescently labeled phagosomes as outlined in Material and Methods. Phagosome displacement was evaluated by placing different-colored

overlay tags to each independently resolved fluorescent particle or edge of a particle in a cluster using signal analytics software described in the Material and Methods section. Fifteen minutes later, a second identical-colored tag was placed over the phagosome. The displacement distance was measured and the absolute and centripetal displacement vectors of the particles were calculated. Average velocity and centripetal average velocity over 15 minutes were then calculated and plotted on histograms as described in Materials and Methods. Interestingly, there was no significant difference in average velocity in negative control and p150^{Glued}-specific siRNA-treated cells (Figure 7a). In contrast, centripetal average velocity was significantly decreased in the p150^{Glued}-specific siRNA-transfected cells compared with negative control transfectants (Figure 7b; $P < 0.001$), and the biphasic centripetal velocity pattern seen in mock transfectants was abrogated by the p150^{Glued}-specific siRNA.

DISCUSSION

In this study, we provide evidence that dynactin p150^{Glued} plays a role in phagosome centripetal migration and perinuclear accumulation in human keratinocytes. Transfection with siRNA targeted against the p150^{Glued} subunit of dynactin decreased expression of p150^{Glued}, while reducing the perinuclear accumulation of phagocytosed polystyrene microspheres. This knockdown of p150^{Glued} resulted in the maintenance of overall cytoplasmic microsphere transport but dramatically reduced centripetal (microtubule minus-directed) transport. Knockdown of the dynactin p150^{Glued} subunit, similar to numerous other studies on protein complexes, probably renders the remaining dynactin protein complex unstable or reduced because of stoichiometric constraints. Melano–phagosomes are the fabric of the microparasol in the perinuclear and supranuclear caps and our data provide evidence that the perinuclear targeting of phagosomes is independent of any particular melanosomal components or signals. The findings also provide further evidence that focusing of the microtubular framework at the
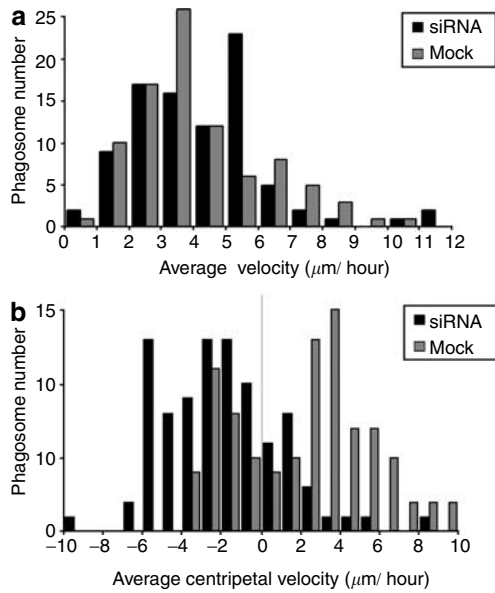
Figure 7. **Cytoplasmic motility analysis of phagocytosed fluorescently labeled 0.5 μm polystyrene microspheres.** Histograms illustrating microsphere average velocity over (**a**) 15 minutes and (**b**) average centripetal velocity in cells treated with negative control (gray bars) or with siRNA-targeted against p150[Glued] (black bars). There is no significant difference in microsphere average velocity in negative control and p150[Glued]-specific siRNA-transfected cells **a**. However, p150[Glued]-specific siRNA transfection significantly reduces average centripetal velocity ($P < 0.001$) and abrogates the biphasic velocity distribution.

perinuclear region also requires a functional dynactin complex. Taken together, the evidence indicates that the dynactin p150[Glued] subunit is required for the functional integrity the keratinocyte microparasol.

The p150[Glued] subunit of dynactin binds the intermediate chain of dynein (Vaughan and Vallee, 1995) and has another site capable of binding directly to microtubules (Vaughan et al., 2002). This dual binding with the motor and its structural framework provides for processivity of the dynein/dynactin/cargo along microtubules toward the minus-end. The p150[Glued] subunit localizes predominantly with the cell center where microtubules are focused and is also found distributed intermittently along peripheral microtubules (Waterman-Storer et al., 1995; Askham et al., 2002). In the periphery of the cell, the p150[Glued] dynactin subunit appears to be involved in a search-capture mechanism of phagosomes for minus-end-directed microtubule transport (Vaughan et al., 2002). How does dynactin thus move out to the periphery to capture cargo? Recent evidence indicates that cargo may have both membrane-bound minus-directed motors and plus-directed motors (such as kinesin motors) at the same time, thus explaining bidirectional movement of cargo with switching of the motors (Ma and Chisholm, 2002). Furthermore, nonconventional kinesins may also contribute to minus-directed transport (Case et al., 1997). Actin-based motility also permits polarized or directional cargo movement by myosin V, a member of a third family of motors that may take turns at being alternatively switched on (Mehta et al., 1999).

In keratinocytes, to construct an effective microparasol to protect the nucleus from damaging UV light, there is a bias of inward movement of melano–phagosomes. Evidence suggests that in keratinocytes, as in melanocytes, microtubules are oriented with the plus-ends in the periphery and minus-ends in the perinuclear centrosome (Byers et al., 2000, 2003). Although human melanocytes demonstrate melanosome bidirectional movement via microtubule and actin-based motility by myosin V (Lambert et al., 1998), kinesin (Hara et al., 2000; Vancoillie et al., 2000c), and dynein (Byers et al., 2000; Vancoillie et al., 2000b) with dynactin linkage (Vancoillie et al., 2000a), there is a slight bias toward the periphery in the process of melanosomal transfer to keratinocytes. In contrast to melanocytes, the net inward force on melano–phagosomes in keratinocytes overrides the net outward "pull" of myosin V and kinesin (Byers et al., 2003) and thus allows for the focusing of the melano–phagolysomal ''fabric'' of the microparasol into the perinuclear microtubule organizing center in a perinuclear position. The dynein/dynactin/cargo complex is not only transported centripetally, but it may then be anchored at the centrosome by either the p150[glued] microtubule-binding site (with EB1) (Askham et al., 2002), dynein anchoring to microtubules (Delanoue and Davis, 2005), or both. The functional assays in our study provide evidence that the p150[Glued] subunit and hence dynactin is involved in a capture-transport mechanism in the assembly of the keratinocyte microparasol. This capture-transport is not dependent on any melanocyte or melanosomal protein or signal as polystyrene microsphere phagosomes may replace melano–phagosomes in a melanocyte-free keratinocyte culture system. Keratinocyte proteins recruited during the phagocytic process are sufficient for proper targeting of the cargo. Dynactin at the plus-ends of microtubules in the periphery of the cell probably plays a role in this recruitment as dynein can then be targeted to this dynactin in the periphery and the entire membrane-bound cargo/dynactin/dynein can then be transported along the microtubules to the cell center. Disruption of available dynactin would thus explain the dramatic reduction in centrosomal transport and centrosomal MFI in our functional experiments.

The marked reduction in centrosomal or perinuclear MFI following only a 50% decrease in p150[Glued] on Western blotting may be due in part to a contribution of at least four effects. First, the non-dynein motors are probably dispersing all organelles and the attached p150[Glued] throughout the cytoplasm away from the perinuclear area; therefore, the density of fluorochrome of the secondary antibody is reduced to a much greater extent than a 50% decrease. In other words, only a 50% knockdown of p150[Glued] appears to be sufficient to tip the balance toward the competing motors kinesin and myosin V in the tug of war on the phagosomes to nearly abrogate perinuclear accumulation. Second, the microtubules are also less focused because of the reduction in p150[Glued], further contributing to the loss in perinuclear fluorescence. Third, a 50% decrease of a low-concentration protein results in a precipitous drop in the confocal fluorescence signal detected as may fall on the lower end

of an S-shaped protein concentration to fluorescent signal curve. Fourth, variable transfection efficiency may give this result; indeed, numerous keratinocytes at the periphery and edge of the culture dish were confluent and just beginning to stratify. These may not have experienced as efficient a transfection and less of a knockdown, thus contributing disproportionately more p150[Glued] in the Western blot compared with those visualized with fluorescence in the center of the chamber slide.

Velocity of dynein-dependent centripetal motility of cytoplasmic organelles depends on cell type. Highly specialized pigment cells responsible for rapid camouflage color change in certain fish demonstrate pigment centripetal aggregation velocities up to 20 μm/second (Byers and Porter, 1977). In other fish that change color more slowly, melanosomes in the pigment cells move centripetally more than 20 times more slowly at 0.7 μm/second (Clark and Rosenbaum, 1982). In contrast, human melanocytes show centripetal melanosome migration rates nearly 100 times slower at 0.2–0.6 μm/minute (Byers et al., 2000; Hara et al., 2000). Our data indicates that centripetal migration rates in human keratinocytes at 0.07 μm/minute are again nearly 10 times slower than human melanocytes. However, it must be noted that these latter measurements are based on net displacement over a 15-minute interval and do not reflect the saltatory or intermittent velocities that are usually much higher (0.2 μm/second) when melanosomes are observed at short time intervals in amphibian melanophores, which are specialized pigment cells for color change (Deacon et al., 2003). The cell-type differences in centripetal velocities are probably dependent in part on number of microtubule motors per organelle (Levi et al., 2006), myosin V contribution (Wu et al., 1997), other differences such as microtubule polymerization, cytoplasmic viscosity and size of particle, and intermediate filament *versus* microtubule density. Keratinocytes have a high density of intermediate filaments that may contribute to cytoplasmic drag on transport of phagosomes. The velocities given in this study may also be relatively low as they are not velocities of short duration such as during brief saltatory displacements occurring over a few seconds, but are average velocities measured over a 15-minute interval that include pauses and positive and negative saltatory displacements. Nevertheless, they may be used for functional comparative assays between the controls *versus* siRNA-treated keratinocytes.

The distribution of phagosome velocity in siRNA transfectants is not perfectly Gaussian with an additional peak at 5 μm/hour. The phagosome velocity in the mock transfectants was essentially Gaussian with a peak at 3 μm/hour. However, the difference was not statistically significant. Nonetheless, the slight increase in velocity with decreased p150[glued] may be due to reduction of opposing bound dynein motors via disruption of the dynactin/dynein complex on the same phagosome with bound kinesin (Ma and Chisholm, 2002). Indeed, the presence of a biphasic centripetal velocity in the mock transfectants, one positive and the other negative, probably reflects at least two motors operating on the phagosomes, one set acting retrograde, the other orthograde

along microtubules and probably represents the two major opposing microtubule motors, dynein and kinesin, respectively. Two opposing motors may act on the same cargo (Ma and Chisholm, 2002); however, when the p150 glued is knocked down, the centripetal velocity is largely abrogated, whereas the orthograde motor is not. The slightly greater negative centripetal velocities (orthograde) in the siRNA-treated cells may again reflect loss of binding to microtubules by the reduced p150 glued and/or reduction of intermittent centripetal saltatory movements mediated by reduced availability of dynactin/dynein complexes. The precise mechanism is unknown; the relative contribution of a variety of motors including actin-based myosin V and tethering proteins in the face of disruption of the dynactin/dynein complex on phagosomes will require further investigation.

There was no total abrogation of phagocytosis and transport, indicating inefficiency of transfection or redundancy of functional proteins for phagocytosis and transport. Indeed, the capture and transport mechanism is highly complicated and involves many other proteins. For example, cytoplasmic dynein is targeted at the peripheral membrane in association with adherens junctions containing β-catenin and E-cadherin in epithelia (Ligon et al., 2001). Likewise, the binding of p150[Glued] to dynein, microtubules, and other docking- or motility-associated proteins is complex and involves many protein–protein contacts. The p150[Glued] subunit has a cytoskeletal-associated protein, glycine-rich domain that binds EB1 and CLIP-170, the two latter proteins themselves are also capable of binding microtubules (Berrueta et al., 1999; Askham et al., 2002; Bu and Su, 2003; Ligon et al., 2003). The p150[Glued] subunit also has binding domains for the kinesin family motor Eg5 (Blangy et al., 1997) and kinesin II (Deacon et al., 2003), indicating a possible capacity to regulate these other microtubule-based motors or to structurally tag along with these plus-based motors to reach the periphery where p150[Glued] may again participate in the capture and transport of a phagosome.

An intact dynactin complex is essential for metazoan organisms (McGrail et al., 1995), and despite the complexity of its role in cytoplasmic motility, cell mitosis, and nuclear envelope breakdown, most of its functions relate to its linkage of membrane-bound organelles by direct binding to the microtubular framework or via the motors dynein and a subset of kinesins (Schroer, 2004). Knockdown of the p150[Glued] subunit probably disrupts the functional complex of dynactin because of stoichiometric constraints. In the interphase keratinocyte, we provide evidence that when dynactin p150[Glued] is knocked down the microtubule framework is less focused on the perinuclear region, findings that would also contribute to decreased dynactin-mediated phagosome perinuclear accumulation. Indeed, dynactin appears important in anchoring microtubules at the centrosome as disruption of the dynactin complex results in loss of the focused radial array in interphase as well as loss of centripetal migration of endosomes (Quintyne et al., 1999). However, the focusing capacity appears to be due to direct microtubular-binding of dynactin p150[Glued] (with other possible docking proteins), as a focused microtubular frame-

work persists even when dynein is absent (Quintyne and Schroer, 2002). Likewise, our data suggest the focused microparasol's microtubular framework is dependent in part on dynactin.

The keratinocyte microparasol envelopes the keratinocyte nucleus and is composed of melano–phagolysosomes (the fabric), a microtubular framework, and a force mechanism that pulls and holds the fabric in place (Byers et al., 2003; Scott, 2003; Byers, 2006). This unique structure is ideally situated to protect the keratinocyte nucleus from UV-induced damage (Kobayashi et al., 1998). The organization of the melanin around and over the nucleus has long been recognized (Gates and Zimmermann, 1953), but only recently, evidence for the motive force maintaining the position of the granules indicates an important role for dynein (Byers et al., 2003). In this study, we have further dissected the functional mechanism of the microparasol by providing evidence for a role of the p150[Glued] subunit of dynactin in the capture and centripetal transport of phagosomes to the perinuclear area and the maintenance of a focused microtubular framework.

MATERIALS AND METHODS

Melano–phagolysosome visualization

In situ melano–phagolysosomes were identified by staining with the Fontana-Masson silver method. Briefly, skin from the sun-exposed area of an adult pigmented individual (type II–III) was routinely fixed and embedded. Sections were deparaffinized, treated with 10% silver nitrate at 56°C for 1 hour and 0.2% gold chloride for 1 minute and mounted. Densitometry was performed comparing supranuclear cytoplasm with lateral cytoplasm of adjacent 50 cells (see Digital Time-Lapse and Image Analysis and Statistical Analysis sections).

Cell culture

Keratinocytes 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 minutes 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% DMEM (Gibco BRL, Grand island, NY), 20% F12 nutrient mix (Sigma Chemical Co., St Louis, MO; 10.7 g mix and 2.2 g NaHCO$_3$/l), 0.18 mM adenine (Sigma), 10% fetal bovine serum, 1.4×10^{-6} M hydrocortisone (Calbiochem-Behring Corp., La Jolla, CA), and 10 μg/ml epidermal growth factor (Calbiochem-Behring Corp). Isolated human keratinocytes without melanocytes were obtained at second passage in secondary keratinocyte medium KBM-2 with supplied growth factors (Clonetics, San Diego, CA). This protocol was approved by the Boston University Internal Review Board and conducted according to the Declaration of Helsinki Principles.

p150[Glued]-specific siRNA-induced p150[Glued] knockdown and loading of fluorescent phagosomes into keratinocyte cultures

Human keratinocytes were transfected using the lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) with either 250 pmol siRNA (anti-p150[Glued] DNCT1, prevalidated from Ambion, Austin, TX), 250 pmol negative control RNA (cat. no. 4611, Ambion) or diluent alone.

Twenty-four hours later, they were transfected again with a total of 11.5 μl of lipofectamine in 2.5-ml media with the p150[Glued]-specific siRNA, diluent, or negative control RNA. Twenty-four hours later, the designated 24-hour dishes were incubated for 2 hours with 30 μl of the 0.5 μm in diameter FITC-polystyrene microspheres (Polysciences, Warrington, PA), then recorded every 15 minutes for 1.5–2 hours using video time-lapse analysis, and then the 24-hour plates were harvested for Western blotting. Forty-eight hours after the last transfection, fluorescent microspheres were added to the remaining 35 mm dishes for 2 hours and then recorded similar to the 24-hour plates (see Digital Time-Lapse section). The keratinocyte perinuclear cytoplasms were analyzed for aggregation of the phagocytosed microspheres by MFI in multiple cells (50 cells each observed treated with p150[Glued]-specific siRNA, diluent, or negative control RNA; see Statistical analysis section).

Digital time-lapse and image analysis

Intracellular motility of the phagocytosed fluorescent microspheres was analyzed by time-lapse computerized microscopy as described previously with modifications (Byers et al., 2003) using the ×40 objective (NA = 0.55) with a Cohu high performance CCD camera (San Diego, CA). Alternating images between phase contrast and fluorescence were captured with an LG-3 scientific frame grabber card (Scion Corp., Frederick, MD) and analyzed for perinuclear microsphere distribution (MFI) using IP lab spectrum software (Scanalytics Inc., Fairfax, VA).

Western blot analysis

Total cellular proteins were harvested from isolated p150[Glued]-specific siRNA, diluent, or negative control RNA-transfected keratinocytes 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/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Fifty micrograms of total cellular protein were loaded per lane, separated on 10% SDS/PAGE and transferred to nitrocellulose membrane at 100 V for 1.5 hours. After 2 hours blocking with 5% non-fat dry milk in Tween-PBS (0.5% Tween-20 in PBS) at room temperature, the membranes were incubated overnight with a monoclonal antibody against the p150[Glued] subunit (BD Transduction Laboratories, Franklin Lakes, NJ) at a dilution of 1:1000. After four washes, the horse radish peroxidase-coupled secondary sheep anti-mouse antibody at a dilution of 1:1800 (GE Healthcare, formerly Amersham, Waucashaw, WI) in 1% milk was incubated for 1 hour. Loading control used horse radish peroxidase-coupled goat anti-actin at a dilution of 1:2000 (SC-1616, Santa Cruz, CA). The blots were developed with the ECL Western Blotting Detection Reagent (GE Healthcare, formerly Amersham).

Immunofluorescent staining and confocal laser scanning microscopy

Keratinocytes exposed to p150[Glued]-specific siRNA, diluent, or negative control RNA transfection was followed by no treatment or treatment with fluorescent microspheres in eight chamber culture slides (BD Falcon, Bedford, MA). They were then fixed at 24 and 48 hours with 3% paraformaldehyde in PHEM (60 mM PIPES, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 10 mM EGTA and 4 mM MgSO$_4$, pH 7.0) buffer for 10 minutes at room temperature, then 5 minutes with 0.1% Triton X-100 on ice and

incubated with 5% normal goat serum for 20 minutes. The cells were incubated for 45 minutes at 37°C with the monoclonal antibody against the p150^{Glued} subunit (BD Transduction Laboratories), at a dilution of 1:40 and rat monoclonal anti-tubulin antibody (YOL 1/34 Novus Biologicals, Littleton, CO) at 1:300. Rhodamine Red™ – X-conjugated goat anti-mouse and FITC-labeled goat anti-rat (both from Jackson Immunoresearch, West Grove, PA) were both added after washes at a dilution of 1:100 for 45 minutes at 37°C. Control preparations included the Rhodamine Red™ – X-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rat IgG alone without the primary antibodies or with isotype-matched unrelated primary antibody (anti-CD5; USBiological, Swampscott, MA). Cells were mounted using a photobleaching retardance kit (Vectashield, Burlingame, CA) and viewed with a Leica confocal laser scanning microscope with argon ion laser and output power of 2–50 mW and a ×25 objective (NA = 0.75). Digitized images were processed using IPlab spectrum software.

Statistical analysis

The cytoplasmic microsphere MFI, microsphere displacement distance over 15 minutes (average velocity) and confocal immuno-fluorescent data were subjected to statistical analysis using Statview (SAS Institute Inc., Cary, NC). Fifty perinuclear cytoplasms were analyzed using MFI values within a 16 μm^2 cytoplasmic area adjacent to each keratinocyte nuclear perimeter in both the double immunofluorescent-stained preparations and fluorescent phagocy-tosed microsphere preparations. Cultured keratinocyte microsphere cytoplasmic distributions, centripetal velocities, and the p150^{Glued} subunit staining were analyzed using Student's *t*-test comparing p150^{Glued}-specific siRNA, diluent, or negative control RNA-treated cells.

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