Immunolocalization of cytoplasmic dynein and dynactin subunits in cultured macrophages: enrichment on early endocytic organelles

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SUMMARY

Cytoplasmic dyneins and their cofactor, dynactin, work together to mediate the movement of numerous cargo organelles toward the minus-ends of microtubules. In many cases, there is compelling evidence that dynactin functions in part to attach dyneins to cargo organelles, but this may not always be the case. We have localized three dynactin subunits (Arp1, p62 and p150Glued) and two subunits of conventional cytoplasmic dynein (dynein intermediate chain and dynein heavy chain 1) in murine macrophages using immunogold labeling of thawed cryosections. Using stereological techniques, we have quantified the relative distributions of each of these subunits on specific membrane organelles to generate a comprehensive analysis of the distribution of these proteins in a single cell type. Our results show that each of the subunits tested exhibits the same distribution with respect to different membrane organelles, with highest levels present on early endosomes, and lower levels present on later endocytic organelles, the mitochondrial outer membrane, the plasma membrane and vesicles in the Golgi region. An additional pool of punctate dynactin labeling was detected in the cell periphery, in the absence of dynein labeling. Even when examined closely, membrane organelles could not be detected in association with these dynactin-positive sites; however, double labeling with anti-tubulin antibody revealed that least some of these sites represent the ends of microtubules. The similarities among the labeling profiles with respect to membrane organelles suggest that dynein and dynactin bind to membrane organelles as an obligate unit. In contrast, our results show that dynactin can associate with microtubule ends in the absence of dynein, perhaps providing sites for subsequent organelle and dynein association to form a functional motility complex.

Key words: Electron microscopy, Microtubule, Motor protein, Organelle motility

INTRODUCTION

Cytoplasmic dyneins are a family of motor proteins that drive movement toward microtubule minus ends (for reviews, see Allan, 1996; Burkhardt, 1998; Goodson et al., 1997; Vallee and Sheetz, 1996). Genetic studies, in vitro motility assays and antibody-inhibition studies in intact and semi-intact cells have implicated cytoplasmic dyneins in numerous cellular processes. During mitosis, cytoplasmic dyneins participate in spindle organization and orientation, and poleward chromosomal movement (Clark and Meyer, 1999; Echeverri et al., 1996; Gonczy et al., 1999; Heald et al., 1996; Li et al., 1993; Starr et al., 1998; Vaisberg et al., 1993; Yeh et al., 1995).

In interphase cells, dynein activity maintains centrosome integrity and helps to organize the microtubule array (Clark and Meyer, 1999; Ma et al., 1999). In addition, it determines the intracellular distribution and movement of a variety of membrane organelles. In the endocytic pathway, dyneins have been shown to affect the intracellular distribution of early endosomes, lysosomes and phagosomes, and to facilitate fusion between these organelles (Aniento et al., 1993; Blocker et al., 1997; Burkhardt et al., 1997; Harada et al., 1998; Itin et al., 1999; Lin and Collins, 1993; Valetti et al., 1999). In the exocytic pathway, cytoplasmic dyneins have been shown to play a role in the movement of intermediates between the ER and the Golgi complex (Presley et al., 1997) and in maintaining the integrity and juxtanuclear location of the Golgi (Burkhardt et al., 1997; Corthesy Theulaz et al., 1992; Harada et al., 1998; Vaisberg et al., 1996). In neurons, dynein activity is required for axonal transport (Hirokawa et al., 1990; Kraemer et al., 1999; Schnapp and Reese, 1989; Waterman-Storer et al., 1997), and in epithelial cells, where microtubules are oriented with their minus ends facing toward the apical surface (Achler et al., 1989; Bacallao et al., 1989), it facilitates transport of vesicles to the apical cell surface (Lafont et al., 1994).

Genetic studies in budding yeast and filamentous fungi indicate that cytoplasmic dynein plays a role in nuclear orientation and movement in these systems (Eshel et al., 1993; Minke et al., 1999; Plamann et al., 1994; Xiang et al., 1994).

To drive the movement of some, and perhaps all, of its cargo organelles, cytoplasmic dynein requires the accessory factor dynactin (Gill et al., 1991; Schroer and Sheetz, 1991). Like cytoplasmic dynein, dynactin is a large, multisubunit protein complex (reviewed in Holleran et al., 1998; Schroer, 1996).
The most abundant subunit of dynactin, the actin-related protein Arp1, forms a short filament capped on one end by the actin binding protein cap Z and on the other by a heterotetrameric complex containing p62 (Eckley et al., 1999; Schafer et al., 1994). The interaction of dynactin with cytoplasmic dynein is mediated, at least in part, by p150Glued, which binds to the intermediate chain of cytoplasmic dynein (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). Dynactin functions to anchor cytoplasmic dynein to at least some cargo organelles (Echeverri et al., 1996; Roghi and Allan, 1999), and has been shown to increase the processivity of dynein motor activity (King and Schroer, 2000). It has been proposed that the dynein-dynactin interaction provides a mechanism for regulating organelle motility. However, there is no direct evidence that dynein and dynactin always function together in vivo. Cytoplasmic dynein can bind directly to phospholipids (Lacey and Haimo, 1994), and direct interaction between the dynein light chain Tctex-1 and a transmembrane cargo protein has been demonstrated (Tai et al., 1999), raising the possibility that dynactin may not be required as a dynein cofactor for all membrane organelles. Conversely, if dynactin functions as a dynein attachment factor, a pool of organelles may exist that have bound dynactin, but not cytoplasmic dynein. This may be true in the case of interphase centrosomes, where dynactin is proposed to serve a dynein-independent function (Quintyne et al., 1999). Similar issues arise regarding the cytosolic pools of dynein and dynactin, where even less is known about interactions in intact cells. Dynactin has been shown to colocalize with CLIP-170 at microtubule plus ends (Valetti et al., 1999; Vaughan et al., 1999), and there is evidence that dynactin is recruited to these sites only under certain conditions (Vaughan et al., 1999). Whether these sites represent dynactin bound directly to microtubules or to membrane organelles has not been determined. The questions regarding dynein-dynactin interactions are further complicated if subcomplexes of these larger protein complexes exist in cells (Tai et al., 1998), especially if these can interact, as has been described for the interaction of dynactin with the dynein intermediate chain in the absence of heavy chain (Berrueta et al., 1999).

Although several studies have addressed the intracellular distribution of cytoplasmic dynein and dynactin, the interpretation of these studies has been limited by several factors. Previous light microscopy studies have yielded conflicting results (Blangy et al., 1995; Clark and Meyer, 1992; Echeverri et al., 1996; Gill et al., 1991; Karki et al., 1998; Lin and Collins, 1992; Marples et al., 1998; Pfarr et al., 1990; Roghi and Allan, 1999; Steuer et al., 1990; Tai et al., 1998; Vaisberg et al., 1996), probably because of differences in cell type, specific subunits and isoforms tested, fixation conditions, antibodies, and the inherently low resolution of light microscopy. Subcellular fractionation has been used to show attachment of dynein and dynactin to specific membrane organelles (Allan, 1995; Blocker et al., 1997; Fath et al., 1994; Fath et al., 1997; Kraemer et al., 1999; Niclas et al., 1996), but a comprehensive survey of organelles has not been performed. Moreover, this approach carries the risk that dynein and dynactin may dissociate from membranes during fractionation. Similarly, a few purified organelles have been shown to use dynein and dynactin to move along microtubules in vitro (Blocker et al., 1997; Corthesy Theulaz et al., 1992), but the finding that an organelle uses dynein and dynactin in vitro does not prove that it does so in vivo, and negative results in vitro are difficult to interpret. Finally, studies using microinjection of function-blocking antibodies, cells from knockout mice or overexpression of dominant-negative constructs have provided evidence that dynein and dynactin control the movement of some organelles in vivo (Burkhardt et al., 1997; Harada et al., 1998; Presley et al., 1997; Valetti et al., 1999), but these too give conclusive results only where perturbation of movement is observable. Effects on organelles that move infrequently, or only in response to specific signals, may be overlooked.

To address these issues, we have performed a comprehensive study of the membrane distribution of several dynein and dynactin subunits in a single interphase cell type using quantitative immunoelectron microscopy. This technique has the advantage that it allows unequivocal identification of specific membrane organelles. Our results show that two cytoplasmic dynein subunits (the 440 kDa heavy chain and the 70 kDa intermediate chain) and three dynactin subunits (p150Glued, p62 and Arp1) all have a similar membrane organelle distribution, consistent with the view that dynein and dynactin are normally associated as a complex on membranes, with neither protein complex bound at appreciable levels in the absence of the other. In contrast to these findings for membrane organelles, we find that the subunits of dynactin, but not dynein, are also abundant at punctate peripheral sites where microtubules apparently end. No membrane organelles can be detected at these sites.

**MATERIALS AND METHODS**

**Cell culture and antibodies**

The macrophages obtained from ATCC were maintained as described previously (Blocker et al., 1996). The chicken macrophage cell line HD-11, obtained from Dr T. Graf (EMBL, Heidelberg, Germany), was maintained as described in (Beug et al., 1979). Mouse monoclonal anti-dynactin antibodies 150.1 and 150B (anti-p150Glued), 45A (anti-Arp1) and 62B (anti-p62) were affinity-purified on protein A-Sepharose, and have been described previously (Schafer et al., 1994). Anti-dynein heavy chain 440.2 (Steuer et al., 1990) was a gift of Dr M. Sheetz, Duke University, and anti-dynein intermediate chain 70.1 (Steuer et al., 1990) was purchased from Sigma (St Louis, MO, USA). Affinity-purified rabbit anti-tubulin was a gift from Dr E. Karsenti (EMBL, Heidelberg, Germany). Rabbit anti-mouse Ig was from Organon Teknika/Cappel (Durham, NC, USA). Colloidal gold conjugates were purchased from the Department of Cell Biology, University of Utrecht, The Netherlands.

**Endocytic tracer studies**

To distinguish among endocytic organelles, cells were loaded with endocytic tracers. BSA-collodial gold particles of various sizes were prepared according to Slot and Geuze (Slot and Geuze, 1985). To load lysosomes and late endosomes, cells were pulsed with BSA-15 nm colloidal gold for 1 hour at 37°C, washed several times with PBS, and chased overnight in complete medium. To load early endosomes, cells were pulsed for 5 minutes at 37°C with BSA-5 nm colloidal gold particles, and fixed immediately after the pulse. For studies involving phagosomes, cells were allowed to take up 1 μm carboxylated polystyrene beads (Seradyn, Indianapolis, IN, USA), which had been coupled to fish skin gelatin as described in (Blocker et al., 1996). Beads were adjusted to 0.05% w/v in complete medium and incubated with cells for 1 hour at 37°C. Cells were then washed several times with PBS and chased for an additional hour in complete medium. This process was repeated.
procedure generates phagolysosomes, which are ‘late’ phagosomes containing lysosomal marker enzymes (Desjardins et al., 1994a; Desjardins et al., 1994b; Rabinowitz et al., 1992).

**Electron microscopy**

To each 10 cm dish of cells growing in 10 ml complete medium was added an equal volume of 2× fixative consisting of 0.4% EM Grade glutaraldehyde (Merck, Darmstadt, Germany) and 4% paraformaldehyde (Merck) in 0.4 M potassium phosphate, pH 7.4. After 10 minutes, the mixture was replaced by fresh 1× fixative and incubated for an additional 3 hours at room temperature. Cells were then washed with PBS, and excess aldehyde blocked by incubation for 10 minutes with 50 mM ammonium chloride (NH₄Cl) in PBS. After washing again in PBS, cells were scraped, pelleted, and the pellet embedded in 2% low melting point agarose in PBS. After the agarose was hardened by chilling, small blocks were cut, infiltrated overnight in 2.1 M sucrose in PBS as cryoprotectant, and frozen in liquid nitrogen. Ultrathin cryosections were cut at −100°C with a Leica FCS cryomicrotome using a Drukker Diamond knife. Sections were collected with 2.3 M sucrose/PBS and transferred to 100 mesh hexagonal grids coated with formvar and carbon, freshly glow discharged. Thawed sections were labeled as described previously (Blocker et al., 1997; Griffiths, 1993). Briefly, sections were blocked for 15 minutes with 0.5% fish skin gelatin (Sigma), 20 mM glycine in PBS, which was also used to dilute antibodies and to wash the grids after antibody incubations. Sections were labeled with the indicated primary antibodies, followed, when appropriate, with rabbit antimouse Ig, and detected with protein A-coated gold. Labeled grids were embedded in aqueous 2% methylcellulose (Sigma)/0.3% uranyl acetate (Serva, Heidelberg, Germany) to add contrast and prevent shrinkage upon drying. Grids were observed with a Zeiss EM-10 microscope at 80 kV.

**Stereological analysis**

Quantitation of the density of gold particles labeling each antigen was performed using standard stereological procedures. Since the cells were pelleted and embedded in agarose, which was randomly cut and mounted, the sections are considered to be isotropic, uniformly random sections. Grids were systematically screened, and approximately 30 photographs of each organelle of interest were taken, irrespective of their labeling. All organelles were photographed at a primary magnification of ×16,000 except for Golgi stacks and early endosomes, which were photographed at ×25,000. Negatives were enlarged on a projector as described by Griffiths and Hoppeler (Griffiths and Hoppeler, 1986) and overlaid with a square lattice grid (Weibel, 1979). Line spacing (d) of 40 mm was used to count intersections with the limiting membranes of phagosomes, mitochondria and late endosomes. A grid with a distance between test lines of 20 mm was used to count intersections with early endosomes and membranes of the Golgi stack. Points falling over the nucleus, mitochondria and cytoplasm were counted using a grid with d=20 mm.

To determine the labeling of peripheral and juxtanuclear cytoplasm, a box corresponding to 10 points (d=20 mm) was positioned over randomly selected areas of cytoplasm near the plasma membrane or near the nuclear envelope, using only micrographs where these two structures were well separated. The defined areas of cytoplasm were randomly selected areas of cytoplasm near the plasma membrane, the nuclear inner and outer membranes, and the limiting membranes of phagosomes, mitochnondria and late endosomes. A grid with a distance between test lines of 20 mm was used to count intersections with early endosomes and membranes of the Golgi stack. Points falling over the nucleus, mitochondria and cytoplasm were counted using a grid with d=20 mm.

Quantitation of gold particles was performed as described by Griffiths (Griffiths, 1993), pp. 413-417. Au/membrane length: counted gold particles on the membranes + intersection of the membranes with the grid; Au/boundary length=Q (counted Au particles)/(π/4×intersections×d); Au/area: counted gold particles and points on the surface excluding the membranes; Au/area=Q (counted particles)/(points×d). Formuale are derived from (Griffiths and Hoppeler, 1986). Note that due to the size of the antibodies and protein A-gold particles, specific label may fall as far as 50 nm away from the membrane, and on either side of it (Griffiths, 1993). Where particles were present in aggregates, groups of two were counted as one particle, while groups greater than two were counted as two particles. Data represent the average of three (for dynactin antibodies) or two (for dynein antibodies) independent labeling experiments, ± standard error of the mean (s.e.m.).

**RESULTS**

**Dynactin subunits are enriched on endocytic organelles and mitochondria**

While it is known that approximately half the cellular complement of dynactin is found in association with membranes (Bingham et al., 1998), relatively little is known about the specific membrane organelles to which it binds. We therefore performed EM immunolocalization to ask where dynactin subunits are found within the cell. HD-11 chicken macrophages were used because of their reactivity with a panel of antibodies against different cytoplasmic dynein and dynactin subunits prepared from chicken tissue. Some organelles, including the inner and outer nuclear envelopes, the Golgi stack and mitochondria, were reliably identified on the basis of their morphology. To identify endocytic organelles, the cells were allowed to internalize BSA-coioidal gold particles (BSA-Au) prior to fixation. As described in Materials and Methods, lysosomes and late endosomes were loaded with 15 nm BSA-Au, while early endosomes were filled with 5 nm BSA-Au. Alternatively, cells were allowed to phagocytose 1 μm latex beads for 1 hour, followed by a 1 hour chase. We have demonstrated previously using murine macrophages that this procedure generates phagolysosomes bearing lysosomal markers (Desjardins et al., 1994a; Desjardins et al., 1994b; Rabinowitz et al., 1992), and that these organelles can move along microtubules in a dynein- and dynactin-dependent fashion (Blocker et al., 1997). Cells loaded in this way were fixed under conditions designed to preserve both cell structure and antigenicity, and prepared for immunoelectron microscopy of thawed cryosections (Griffiths, 1993). Since there is evidence that changes in cell growth conditions can affect dynein distribution (Lin and Collins, 1993; Lin et al., 1994), care was taken to avoid even brief periods of serum deprivation. The labeling density over the nuclear inner membrane, where dynactin is not expected to be present, represents background levels of label.

As shown in Fig. 1, labeling for p150Glu'd, the largest subunit of dynactin, was found at low levels over the limiting membranes of a variety of membrane organelles including early endosomes, late endosomes, phagosomes and mitochondria (Fig. 1A-I). Gold particles were also detected over the plasma membrane and the outer nuclear membrane (Fig. 1G and J, respectively). Although gold particles were rarely observed over the Golgi stack, label was frequently found over vesicular structures in the Golgi region (Fig. 1J,K). In all cases labeling was low, but the pattern of labeled organelles was reproducible.

To determine the density of p150Glu'd label associated with
each membrane organelle, stereological analysis of the labeled cryosections was performed and the number of gold particles per membrane length was determined. As shown in Fig. 2A, p150\(^{\text{Glued}}\) was most highly enriched on early endosomes and on the outer membrane of mitochondria, with significant levels also present on the plasma membrane and late endocytic organelles (late endosomes/lysosomes and phagolysosomes). Although the rough endoplasmic reticulum could not always be reliably identified in our cryosections, the finding that low or negligible levels of p150\(^{\text{Glued}}\) label could be detected over the outer nuclear membrane suggests that the rough ER binds little or no p150\(^{\text{Glued}}\). p150\(^{\text{Glued}}\) labeling was also absent from membranes of the Golgi stack. Label over vesicular profiles in the Golgi region was consistently present, but due to the small vesicular profiles found in this region, this label was prohibitively difficult to quantitate as a function of membrane length. The overall pattern of p150\(^{\text{Glued}}\) labeling of membrane organelles obtained with 150B was confirmed using 150.1, an independent antibody to the same dynactin subunit. Moreover, very similar results were obtained after labeling of murine J774 macrophages with 150B (data not shown).

Labeling with 45A, a monoclonal antibody reactive with Arp1, gave a pattern of labeling very similar to that obtained with the antibodies to p150\(^{\text{Glued}}\) (Fig. 2B). Comparable results were also obtained with an antibody that recognizes the p62 subunit of the Arp1 filament (Fig. 2C), with the exception that the amount of label on early endosomes was somewhat lower than with the other two dynactin subunits. By comparison with the dynactin antibodies, control labeling with irrelevant antibody or with secondary antibody alone gave low levels of label on all membrane compartments (Fig. 2D). Thus, the similar labeling patterns obtained with the three anti-dynactin antibodies are due to a similar distribution of their respective specific antigens. That the various subunits of dynactin have strikingly similar distributions on membrane organelles is consistent with the view that they associate with membranes as a unit.

**The distribution of cytoplasmic dynein on membrane organelles mirrors that of dynactin**

To determine whether dynein and dynactin might associate with membrane organelles independently of each other, we next analyzed the intracellular distribution of cytoplasmic dynein. Cytoplasmic dynein heavy chain was found in association with the same set of organelles that labeled for dynactin (Fig. 3). Stereological analysis showed that the pattern of labeling with the anti-dynein heavy chain antibody 440.2 mirrored the labeling with anti-dynactin antibodies (Fig. 4A). Similar results were obtained using 70.1, an antibody reactive with the 70 kDa dynein intermediate chain, with the only differences being the slightly

**Fig. 1.** Immunolocalization of p150\(^{\text{Glued}}\) on different membrane organelles. HD11 macrophages were allowed to take up endocytic tracers, fixed and cryosectioned, and thawed sections were labeled with anti-p150\(^{\text{Glued}}\) mAb 150B followed by 10 nm protein A colloidal gold. In all cases, arrowheads mark p150\(^{\text{Glued}}\)-positive organelles. (A-C) Early endosomes filled with 5 nm BSA-Au; (D-F) late endosomes/lysosomes filled with 15 nm BSA-Au; (G) plasma membrane (PM) and a phagolysosome containing a latex bead (P); (H,I) mitochondria; (J,K) unlabeled Golgi stacks (G) with label over adjacent membranes. J also shows a labeled mitochondrion (M), and a rare instance of the nuclear envelope label over adjacent membranes. N, nucleus. Bar, 200 nm.

**Fig. 2.** Stereological analysis of the density of dynactin label on membrane organelles. Randomly selected electron micrographs prepared as in Fig. 1 were analyzed using standard procedures (see Materials and Methods). The area of the indicated membrane organelles was determined, the number of colloidal gold particles associated with each was scored, and the density of gold label was calculated for each organelle. Results from three independent labelings for each antibody (150B, 45A (Arp1) and 62B) are expressed as the number of gold particles (Au) per linear \(\mu\)m of membrane (means \(\pm\) s.e.m.). (D, control) shows labeling with secondary antibody alone; similar results were obtained with an irrelevant primary antibody. NIM, nuclear inner membrane (represents background); NOM, nuclear outer membrane; PM, plasma membrane; Mito, mitochondrial outer membrane; Phag, phagosome outer membrane; LE, late endosomes/lysosomes; EE, early endosomes; GS, Golgi stack.
lower labeling of early endosomes and mitochondria (Fig. 4B). Thus, we find that three subunits of dynactin and two subunits of cytoplasmic dynein have very similar distributions on cellular membranes. These results suggest that dynein and dynactin are bound together on membrane surfaces.

Dynactin is present in punctate structures not associated with membranes or with cytoplasmic dynein

In the course of our analysis of the membrane-associated pool of dynactin, we observed significant levels of label over regions of the cytoplasm where no membrane organelles could be detected. Frequently, 2-7 gold particles were arranged in clusters (Fig. 5, arrowheads), or in short linear arrays (Fig. 5, arrows), a pattern that was apparent for each of the dynactin antibodies. These aggregates were not observed with the control antibodies, nor with the dynactin antibodies labeling membranes, arguing that they do not result from antibody or protein A polyvalence (signal amplification), but rather correspond to aggregates of antigen. Although electron-dense structures were sometimes observed under the labeled areas at high magnification (e.g. see clusters in Fig. 7B), no lipid bilayers could be found, even after extraction of cytoplasm with streptolysin-O, a procedure that can facilitate the detection of otherwise obscure membrane organelles (not shown).

This non-membrane-associated dynactin label was not homogeneously distributed throughout the cell, but was considerably more abundant in the periphery. Fig. 5A and B show representative peripheral and juxtanuclear regions, respectively, from the same antibody labeling. This difference was quantified by determining how many gold particles that were not clearly membrane-associated fell within 1 μm of the cell surface or the nucleus. Although this division is, by nature, imprecise, our results clearly showed that these areas are different with respect to dynactin labeling. Over three times more label for p150Glued was found over the cell periphery than over the perinuclear cytoplasm, where near-background levels of label were observed (Fig. 6A). Similar results were obtained for Arp1 and p62 subunits of dynactin (Fig. 6B,C). By contrast, little dynein label was observed over the cytoplasm, and there was no corresponding enrichment in the cell periphery (Fig. 6D,E). Control labeling without primary antibody showed only background levels of label over both peripheral and juxtanuclear cytoplasm (Fig. 6F).

Given that a significant portion (in brain extracts, approximately 50% of dynactin is soluble rather than membrane-associated, as determined by subcellular fractionation; Bingham et al., 1998), it seemed likely that this peripheral labeling might represent a cytosolic pool of dynactin. Yet, the presence of dynactin aggregates suggested that this pool might be associated with structures other than membranes. Based upon immunofluorescence studies, dynactin has been reported to associate, together with the microtubule binding protein CLIP-170, with the distal ends of microtubules.
EM localization of dynein and dynactin (Valetti et al., 1999; Vaughan et al., 1999). We therefore asked if the peripheral aggregates of dynactin observed in our cryosections might be associated with microtubules by performing double labeling for Arp1 and tubulin. Many of the Arp1-positive punctae in the periphery also labeled with anti-tubulin antibody (Fig. 7, small arrows). Omission of either primary antibody yielded no label for that antigen (data not shown), verifying that the colocalization was antigen-specific. Colocalization of the two proteins, however, was not complete. Occasionally, aggregates of dynactin label were seen in the absence of tubulin label. It is unclear whether this labeling pattern actually represents free dynactin or arises due to an inability to detect cross-sectioned microtubules by labeling with anti-tubulin. However, when microtubules were sectioned longitudinally, we readily observed tubulin labeling over considerable lengths in the absence of Arp1 label (Fig. 7C,D, arrowheads). In many cases, Arp1 label was detected at the ends of the tubulin tracts (Fig. 7C,D, large arrows). This, together with the finding that dynactin label is enriched in the periphery, whereas microtubules are most concentrated in the cell center, supports the view that dynactin is associated with specific regions of microtubules, most likely their plus-ends.

**DISCUSSION**

Although it is widely assumed that dynactin is required for all dynein-mediated motility of membrane organelles, there is relatively little direct evidence that dynein and dynactin necessarily associate with the same organelles in vivo. Moreover, rigorous study of the stoichiometry of membrane-attached dynactin has not been conducted, leaving open the possibility that some dynactin subunits associate with membranes in the absence of others. We have conducted a detailed analysis of the distribution of two subunits of
cytoplasmic dynein and three subunits of the dynactin complex in a single macrophage cell line. The use of immunoelectron microscopy for these studies permitted the unequivocal identification of membrane organelles, and by performing stereological analysis we were able to determine for each antigen-antibody pair the relative enrichment on different organelles, calculated as a function of the length of organelle membrane (Griffiths, 1993). Our results show that the distributions of three different dynactin subunits on membrane organelles follow similar profiles. While the sensitivity of immunoelectron microscopy is too low to permit colocalization of individual molecules, it can be argued on a population basis that dynactin is most likely bound to membrane organelles as an intact complex. Furthermore, since we found that two subunits of cytoplasmic dynein also follow the same pattern, it seems likely that conventional cytoplasmic dynein does not interact with membranes in the absence of dynactin. Thus, while it is formally possible that dynein and dynactin bind the same membranes via independent mechanisms, the simplest interpretation of our results is that the dynein-dynactin complex forms in the cytosol and binds to membranes as a unit.

Dynein and dynactin distribution on membrane organelles

Although we found the ratio of dynein and dynactin subunits on membrane organelles to be quite constant, we observed significant differences in the amount of label associated with different organelles. In keeping with functional studies showing that cytoplasmic dynein and dynactin facilitate endocytic traffic (Aniento et al., 1993; Bomsel et al., 1990; Valetti et al., 1999) and drive centripetal movement of endosomes and lysosomes (Burkhardt et al., 1997; Harada et al., 1998; Valetti et al., 1999), we detected dynein and dynactin in association with endocytic organelles. Within the endocytic pathway, the highest levels of label were detected over early endosomes, with later endocytic organelles labeled at lower levels. The finding of high levels of dynein and dynactin in association with early endosomes may indicate that these organelles are especially motile, in keeping with their highly dynamic and fusogenic nature (Aniento et al., 1993; Bomsel et al., 1990; Kreis et al., 1989; Valetti et al., 1999). However, the relationship between dynein/dynactin binding to membranes and the motility of these membranes is not yet clear. In a previous study, we showed that dynein/dynactin driven motility of phagosomes in vitro increases with their intracellular maturation between 20 minutes and 12 hours of uptake (Blocker et al., 1997), but when we quantified the levels of dynein and dynactin associated with maturing phagosomes by immuno-EM, we observed no significant changes over the same time course (data not shown). This suggests that at least for these later endocytic organelles, the observed changes in motility are not due to levels of bound dynein/dynactin, but rather to changes in motor activity. In this respect, our findings for phagosomes appear to be similar to those of Roghi and Allan (Roghi and Allan, 1999), who showed for membranes cycling between the ER and the Golgi that regulation occurs at the level of motor activity rather than membrane binding.

Though we could not quantify their relative abundance, we also observed dynein and dynactin label over vesicular profiles closely associated with the Golgi stack. There is a large body of evidence that dynein and dynactin are important for mediating trafficking into the Golgi and maintaining Golgi

Fig. 6. Stereological analysis of the density of cytoplasmic dynein and dynactin label. Random micrographs from cells labeled as described for Figs 1 and 3 were analyzed by counting the number of gold particles not clearly associated with membrane organelles, and falling within 1 μm of the plasma membrane (PCyt) or 1 μm of the nucleus (Ncyt; see Materials and Methods for details). To control for non-specific antibody binding, the number of gold particles falling over mitochondria (Mito) and nuclei (Nuc) were also scored; this level of label represents background labeling for each antibody. Data from three (A-C,F) or two (D,E) independent labelings are expressed as number of gold particles per μm² of cytoplasm (mean ± s.e.m.).
structure and location. However, attempts to analyze the distribution of dynein and dynactin subunits on the Golgi complex have given mixed results. At the immunofluorescence level, DHC2, but not DHC1 or DHC 3, was found to localize to Golgi membranes in NRK cells (Vaisberg et al., 1996). DHC1 was shown to localize to the Golgi in Xenopus fibroblasts and human A431 cells, though a vesicular pattern was labeled by the same antibody in other mammalian cell lines (Roghi and Allan, 1999). Similar cell-specific patterns were reported for dynein intermediate chain and for TcTex-1, a cytoplasmic dynein light chain (Tai et al., 1998). In each of these studies, colocalization was performed with markers for Golgi subdomains; however, the distribution of dynein within the Golgi complex could not be definitively determined at the resolution of light microscopy. To date, little analysis has been performed at the EM level. In one study, the Arp1 subunit of dynactin in mammalian spermatids was found to localize to the cis-Golgi network (CGN) and the cis-medial cisternae of the Golgi stack (Fouquet et al., 2000). Our results in macrophages indicate that dynein and dynactin are associated not with the Golgi stack, but with vesicles in the Golgi region. A similar conclusion was reached based upon biochemical studies in epithelial cells, where dynein was absent from Golgi stack fractions, but present in vesicular Golgi fractions and budding membranes (Fath et al., 1994; Fath et al., 1997). Based upon the work of Fouquet and coworkers (Fouquet et al., 2000) and on the abundance of evidence showing that dynein and dynactin facilitate traffic between ER and Golgi, it seems likely that, in our cells, many of the labeled Golgi vesicles are derived from the ERGIC/CGN. In epithelial cells, where microtubule polarity is different, the vesicles are more likely to derive from the trans-portion of the Golgi complex. While at first it might seem surprising that dynein and dynactin associated with peripheral Golgi vesicles could function to maintain the localization and integrity of the Golgi stack, this must be considered in light of the dynamic nature of this organelle. Previous studies have shown that microtubule depolymerizing agents can induce fragmentation and dispersal of the stack (Cole et al., 1996; Yang and Storrie, 1998), with reassembly of small stacks at ‘ER exit sites’. When depolymerizing agents are removed, the ‘stacklets’ are able to move along microtubules toward the MTOC (Ho et al., 1989). In this context, it is reasonable that the location of the stack under steady-state conditions could be determined by the minus-end movement of ER-Golgi intermediates, with subsequent assembly of larger stack structures at the site where these intermediates coalesce.

Considering that the distribution of mitochondria is not perturbed by procedures that block dynein or dynactin function (Burkhardt et al., 1997), we were surprised at the relatively high levels of dynein and dynactin label decorating mitochondrial outer membranes. However, mitochondria move bidirectionally along microtubules in vivo (Baumann and Murphy, 1995; Morris and Hollenbeck, 1995), and the minus-end motor involved has yet to be identified. Given our new results, it seems likely that dynein and dynactin are responsible for minus-end directed mitochondrial movement, and that functional assays have failed to show this because mitochondria move only infrequently and appear to remain anchored to actin or other cytoskeletal elements much of the time.

Levels of label at the plasma membrane were much lower, but still above background. Binding of dynein and dynactin at specialized domains of the plasma membrane has been observed during mitosis in mammalian epithelial cells and in early C. elegans embryos (Busson et al., 1998; Skop and White, 1998), where it is thought that they function to facilitate spindle orientation and partitioning into daughter cells. It is possible that we observed only low levels of label at the cell surface because our studies were restricted to interphase cells where spindle movements are not relevant. However, we have also failed to detect plasma membrane accumulation of either dynein or dynactin during MTOC reorientation in T cell:antigen-presenting cell conjugates (C. Mattis and J. K. Burkhardt, unpublished results), another process in which these proteins might be expected to play a role.

**Dynein and dynactin distribution in the cell periphery**

A significant portion of the dynactin labeling we observed was found not over membrane organelles, but in aggregates and short linear arrays in the cell periphery. In many cases, the peripheral dynactin labeling colocalized with the ends of microtubules. These results confirm at EM resolution the light-level observations that dynactin colocalizes with CLIP-170 at

![Fig. 7. Dynactin subunits are associated with microtubule ends.](image)
a subset of microtubule ends (Valetti et al., 1999; Vaughan et al., 1999). By analogy with CLIP-170, which binds to early endocytic organelles (Pierre et al., 1992), it has been proposed that the dynactin present at microtubule ends associates with membrane organelles. Deletion of the metal binding motif of CLIP-170, which abolishes its ability to bind endosomes (Pierre et al., 1994), also abolishes its ability to anchor dynactin at microtubule ends (Valetti et al., 1999), raising the possibility that the two proteins colocalize by interacting with the same membrane organelle. Despite our concerted efforts, we were unable to detect membrane structures underlying the peripheral punctate dynactin labeling or the regions where dynactin colocalized with tubulin. Moreover, the clustered pattern of dynactin labeling at these peripheral sites was clearly distinct from that associated with early endosomes, where individual gold particles were predominant. Our results thus suggest that dynactin can associate with microtubule ends in the absence of membranes.

While we could readily detect dynactin at microtubule ends, we found little or no cytoplasmic dynein at these sites. Vaughan et al. (1999) also found no significant colocalization of dynein with dynactin at microtubule ends under physiological conditions. These investigators found that dynein accumulates at these sites if cells are incubated even briefly at reduced temperature, and that acidification of the growth medium releases dynactin from microtubule ends. Under the conditions we used (growth medium was buffered to neutral pH and fixative was added directly to the warm medium), the two studies are in good agreement. We conclude that at steady state, cells contain at least two distinct pools of dynactin, one associated with dynein and membranes, and one at microtubule ends. This distribution must be dynamic, since it is readily altered by changes in temperature or pH. It has been proposed that the dynactin docked at microtubule ends represents a "premotile state" that is poised to interact with membrane organelles and then dynein to allow movement. Our findings suggest that the ternary associations of membrane organelles, dynactin and microtubule ends is transient, and that once they have formed, the assembled organelle motility-complexes move quickly away from the peripheral sites of dynactin aggregation.

REFERENCES


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