Title

Reconstitution of microtubule-based organelle transport using factors purified from Dictyostelium

Author

Pollock, Nira,

Publication Date

1999

Peer reviewed|Thesis/dissertation

Reconstitution of Microtubule-based Organelle Transport Using Factors Purified from *Dictyostelium*

by

Nira Pollock

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

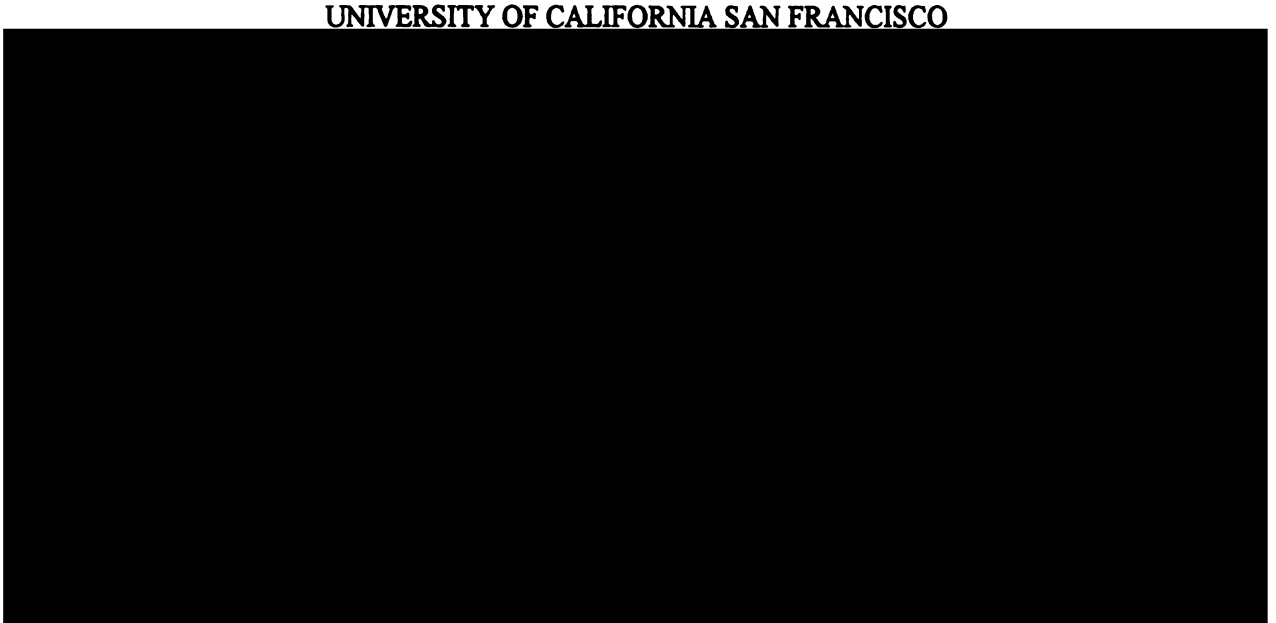
Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO



Date University Librarian

Degree Conferred: .....

To Mica Pollock, Estera Milman, and Sheldon Pollock

because all of their love and support

helped make this happen

Acknowledgments

My first thanks must go to my advisor, Ron Vale. Ron has been a major source of support over my years at UCSF. I first met Ron when I arrived as a first-year MSTP student, excited about starting graduate school and very uncertain about starting medical school right away. When I arranged to start graduate school first and then realized that I had inadvertently withdrawn myself permanently from the entire MSTP, I went in search of mentors who could help me right these wrongs with the medical school and M.D.-Ph.D. program. To my surprise, I faced one skeptic and unsupportive administrator or faculty member after another, each unwilling to provide any measure of support despite having strongly encouraged me to come to UCSF in the first place. When I finally came to Ron's office, I had little reason to believe that I was actually going to stay at UCSF at all. With great relief, I found that Ron was fully supportive of my decision to start graduate school first and was equally upset that I had been unwillingly locked out of the MSTP; moreover, he was willing to go to bat for me with the UCSF bureaucracy. He immediately wrote letters to administrators and faculty on my behalf, and engaged other graduate school faculty to help me, including Ira Herskowitz. Ron's willingness to stand up for me, in addition to being a pivotal factor in keeping me at UCSF, legitimized my personal need to structure my education in a way that was best for me. I remember him saying, "These are complicated decisions, and it makes sense that you are struggling with them--why should any twenty-two-year-old know what she wants to do for the rest of her life?" Months later, I was reaccepted into the MSTP (after having to go through almost the entire application process all over again) and proceeded on to medical school the following year. Six

years later, I have enjoyed my thesis work and am very excited about going back to complete the clinical portion of medical school, and I know that the decisions I made were the right ones. Without Ron's help, the situation might be quite different, and I am very grateful to him.

Throughout the years that I have worked in Ron's lab, his support of my science and of my activities outside of the lab have remained incredibly strong. Ron has had what seems like infinite enthusiasm for my project, perhaps in excess of my own enthusiasm at points--often I have come into his office tired of working on something and he has said, "Isn't this exciting? Isn't this FUN?" and I would try my best to absorb his energy. I came to Ron's lab because I wanted to solidify my ability to "think like a scientist," and this goal was fully met by working with Ron. He has been a huge amount of help in designing experiments and in writing up my work, and I have very much enjoyed learning how to construct scientific arguments from him. He has also been very supportive of the musical activities I have participated in outside of the lab, even when they were extremely time-consuming--he simply recognizes that there is much more to life than work.

A second big thanks for scientific and personal support goes to Geno de Hostos. I can't thank Geno enough for all of the technical help he gave me over the years, from showing me how to grow *Dictyostelium* to helping me finally get my unwilling DdUnc104 clone into an expression vector to making the DdUnc104 knockout. Geno was incredibly generous with his time and was a great teacher, and was always willing to help out with this project. He was also a great source of encouragement and support in the last months of this thesis. His sharp sense of humor is much appreciated.

I have really enjoyed working with members of the Vale lab over these years; they have proven to be both great scientists and great people.

Many thanks go to Nora Hom-Booher, who with great skill helped me enter the molecular biology world. The cloning of the DdUnc104 gene certainly owes a huge amount to her. I won't forget her dancing around the lab to her favorite CDs. Jim Wilhelm, Jim Hartman, and Josh Niclas were great sources of silliness and laughter to break up the day, in addition to being fountains of knowledge and technical advice. Josh was my predecessor in the organelle transport section of our lab, and his advice and support got me off to a good start. Jim Wilhelm's "pimp walk" and "shorty George" won't be forgotten, either. Ryan also has been enormously generous with his help and time in the last few months as I wrestled with various computer graphics programs, and I am very grateful. Jim W., Jim H., and Ryan have also greatly expanded my musical repertoire, albeit in very different ways. Cindy Hart was also a good friend in the lab, and it has been fun to share our various exploits over the years. Sally Cudmore was my partner in crime during the library screening for the DdUnc104 and DdKHC genes, and I am really glad I got a chance to work with her--we learned a lot together and had a good time in the process. Beth Holleran has picked up where Sally left off, and it has been great working with Beth in the lab for the last few months. She always has a smile to offer and somehow never seems to resent all those tiny little favors that add up. She also shares my frequent urge to run out of the lab and go shoe shopping, though it hasn't been realized often enough. Kurt Thorn and Sarah Rice have been really fun to have around in the lab. I've enjoyed talking to Kurt about music and drinking his beer. Sarah makes it certain that there is never an energy lull in our bay, and in the last months generously provided much-needed chocolate to get me through. (Similar thanks go to Paul Peluso, who more than once got me through a late-night purification experiment with Ben + Jerry's, in addition to providing lots of

help with the SMART system.) Pete Takizawa has provided a unique outlet for discussing two unrelated interests of mine, music and baseball. Brinda Govindan was an extremely friendly and enthusiastic labmate for a number of years, and it has been great to see her so excited about her new life as a teacher and a parent. Karen Dell has also been a warm, friendly, and helpful presence in the lab, and I wish her the best in her new editorial position. It has also been nice to overlap, though briefly, with Michio, Andrea, Yujia, and Hao. Thanks also to Phoebe, Arsie, Fausto, and Julianne for making things in the lab run smoothly, and to the other members of my thesis committee, Reg Kelly and Dave Morgan.

Many friends and family members are deserving of warm thanks. I feel extremely lucky to have been in graduate school with such a wonderful group of classmates. Dara Friedman has been a fantastic friend both in and out of the lab. We have shared a lot together; dancing and chocolate chip cookies and movies and performances and walks and bike rides and lots of talking and cooking and sometimes an experiment or two. She has always been willing to discuss just about anything at just about any time, whether it was personal or scientific. It has been wonderful to have such a good friend around at work every day. Emily Troemel has also been a really special friend over the last six years, and we have shared many great experiences and many memorable talks about our lives and ideas over dinners and cups of tea. I wish her the best in San Diego and look forward to hearing about her adventures there (and to visiting!). Amy Kistler and Sheila Jaswal have both made graduate school that much better. It has been great sharing food and books and the outdoors with Amy, and I look forward to more of the same. Sheila always brightens my day when I see her, and I appreciate all of her many attempts to entice me to go get a "snack." Outside of my graduate

school class, a number of other UCSF friends have made life good here. Mario de Bono has been a great friend, and we have shared a trip to New Mexico and salsa dancing and music and many things Italian. I look forward to seeing him in England. Katja Brose has also been a good friend and source of support. We shared our grief in losing our dear friend Christine Mirzayan last year. I thank Christine for all of her love and positive energy over the years that I knew her--I will always miss her. Dave Hackos has been an inspiration as he has coped with her loss. Micheal Galko shared a lot with me over the first years I was here, and I have many wonderful memories of time spent with him.

Outside of the UCSF community, thanks go to Josh White, who gave me so much love and support and perspective over the past year and a half. You just don't get any sweeter than Josh, and his excitement for learning about the world of cell biology made me remember that it was cool stuff all over again. Jon Blossom and Mike Matz also have been great friends who provided much fun distraction. A huge thanks goes to all the members of the San Francisco Bach Choir, the San Francisco Symphony Chorus, and the Philharmonia Baroque Chorale, who provided a hugely satisfying musical and social world outside of graduate school.

Finally, thanks go to the members of my family who have loved me and helped me through all of these years. My sister Mica and her soon-to-be husband Joe have been a mainstay of my life here in San Francisco. Being able to go over there and talk about or forget about everything, depending on the circumstance, has been really important for me. Mica is the one who always understands everything without me having to explain it, and the one who is always on my side. She and I have coincidentally come to the ends of our theses within weeks of each other, and her mantra "we've got to

feeeneesh" has given us a lot of laughs over this time. My parents, Sheldon Pollock and Estera Milman, have been unlimited sources of love and support--I only wish we lived in the same town so we could see each other more regularly. My grandmother, Elsie Pollock, has always been there with a hug or phone hug when I needed her, and her insistence that the institutions of medical school and graduate school were "being too hard on" me always reminded me to keep things in perspective. Thanks also to my grandmother, Sabina Milman, and my late grandfathers, Al Pollock and Elliot Milman, for unconditionally believing in me.

Reconstitution of Microtubule-based Organelle Transport Using Factors Purified from *Dictyostelium*

by

Nira Pollock

Abstract

Microtubule-based vesicle transport powered by motor proteins is a process of fundamental importance in eukaryotic cells, but the mechanism of this process is poorly understood. To gain insight into the molecular basis of organelle transport, I have reconstituted this process in vitro using defined components purified from *Dictyostelium discoideum*. First, I developed an in vitro, video microscopic motility assay using *Dictyostelium* extracts which supports high levels of bidirectional, microtubule-based vesicle transport. I then showed that robust bidirectional transport of KI-washed vesicles, immotile in the absence of soluble factors, could be reconstituted by adding back high-speed supernatants or microtubule-affinity-purified fractions.

Using a microtubule-affinity-purified fraction as a starting material, I performed a biochemical purification based on the video microscopic organelle transport assay to identify proteins responsible for plus-end-directed vesicle transport. I identified two polypeptides, of 245 kDa and 170 kDa, that are independently capable of supporting plus-end-directed motility of KI-washed vesicles at 2.6 μm/s and 1.9 μm/s, respectively. Both polypeptides were shown to be kinesin motors by peptide sequencing. I cloned the 245 kDa polypeptide in its entirety and showed it to be most closely related to *C. elegans* Unc104 and mouse KIF1A, neuron-specific

monomeric kinesins which transport synaptic vesicle precursors. Hydrodynamic analysis of the native 245 kDa protein, henceforth known as DdUnc104, demonstrated that it is a novel dimeric member of the Unc104/KIF1A class. Disruption of the DdUnc104 gene by homologous recombination, despite having no effect on general morphology or growth rate, produced a marked defect in overall organelle transport in vivo. However, no defect in the movement of mitochondria in vivo was observed, showing that the defect is specific for a currently unidentified organelle. This defect was shown to be specific for plus-end-directed transport using the reconstituted assay. These results indicate that DdUnc104 is responsible for the majority of plus-end-directed vesicle transport in *Dictyostelium*. Though DdUnc104 and CeUnc104/MmKIF1A must carry different cargoes, non-motor domain homology suggests that the mechanisms by which they bind their cargo may be closely related.

Reg B. Kelly

Dr. Regis Kelly, Committee Chair

Ron Vale

Dr. Ron Vale, Thesis Advisor

Table of Contents

List of Tables

List of Figures

Chapter 2

Appendix 1

Appendix 2

John Wiley & Sons, Inc.
Publishers Since 1807

PERMISSIONS DEPARTMENT
605 Third Avenue
New York, NY 10158-0012
WILEY
TEL 212 850.6011
FAX 212.850.6008

Ref. # pm914diss1

September 14, 1999

Nira Pollock
University of California at San Francisco
VIA FAX ONLY: 415-476-5233

Dear Ms. Pollock:

RE: Your September 10, 1999 request for permission to republish pages 304-314 from CELL MOTILITY & THE CYTOSKELETON (40:1998). This material will appear in your forthcoming dissertation, to be published by the University of California at San Francisco and University Microfilms, Inc. in 1999.

1. Permission is granted for this use, except that if the material appears in our work with credit to another source, you must also obtain permission from the original source cited in our work.
2. Permitted use is limited to your edition described above, and does not include the right to grant others permission to photocopy or otherwise reproduce this material except for versions made by non-profit organizations for use by visually or physically handicapped persons, and up to five copies of the published thesis may be photocopied by a microfilm company.
3. Appropriate credit to our publication must appear on every copy of your work, either on the first page of the quoted text, in a separate acknowledgment page, or figure legend. The following components must be included: Title, author(s) and /or editor(s), journal title (if applicable), Copyright © (year and owner). Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.
4. This permission is for non-exclusive print rights and microfilm storage rights by the University of California at San Francisco and University Microfilms, Inc., for the English language only, throughout the world. For translation rights, please contact our Subsidiary Rights Department.

Sincerely,

Patrick Murphy
Senior Permissions Assistant
212-850-6014 / Fax: 212-850-6008

pmdiss.doc

Introduction

Microtubule-based organelle movement plays a central role in cell organization and in the directed transport of materials within cells (reviewed in Bloom and Endow, 1995; Goodson et al., 1997; Lane and Allan, 1998; Allan, 1995). The sub-cellular localizations of a number of cellular compartments, including the ER (Feiguin et al., 1994; Lee et al., 1989) and Golgi (Corthesy-Theulaz et al., 1992; Feiguin et al., 1994; Holleran et al., 1996; Rogalski and Singer, 1984), and of certain populations of vesicles, including lysosomes (Hollenbeck and Swanson, 1990; Matteoni and Kreis, 1987; Swanson et al., 1987; Tanaka et al., 1998), are maintained by microtubule-based movement. Microtubules are also required for the movement of small vesicles between compartments, as in the processes of endocytosis (Aniento et al., 1993; Bomsel et al., 1990; Herman and Albertini, 1984; Matteoni and Kreis, 1987) and secretion (Gilbert et al., 1991; Lafont et al., 1994; Parczyk et al., 1989), and for the movement of mitochondria (Baumann and Murphy, 1995; Morris and Hollenbeck, 1995; Van Blerkom, 1991) and specialized organelle populations such as pigment granules in melanophores (McNiven and Porter, 1986; Rogers et al., 1997). The organization of the microtubules within most eukaryotic cells, with minus ends centrally anchored at the centrosome and plus ends extending out towards the plasma membrane, provides a structural framework for these processes.

All of these essential microtubule-based organelle movements require the activity of motor proteins, ATP-hydrolyzing protein machines that are capable of carrying cargo in a single direction along microtubule tracks. Since cytoplasmic dynein (Paschal et al., 1987) and kinesin (Vale et al., 1985) were discovered on the basis of their ability to move microtubules in vitro, there has been an explosion of discoveries of related motors and investigations into their cellular functions. It has been well established that cytoplasmic

dynein functions as a minus-end-directed organelle transport motor (Harada et al., 1998; Schnapp and Reese, 1989; Schroer et al., 1989). In addition, three classes of plus-end-directed motors have been shown to be involved in organelle movement along microtubules: conventional kinesins, heteromeric kinesins (KRP 85/95, KIF3A/B) and the "monomeric" kinesins (Unc104, KIF1A,B,C).

Because it was the first plus-end-directed microtubule motor to be discovered (Vale et al., 1985), conventional kinesin has been the subject of the majority of the studies of plus-end-directed organelle transport to date. A role for kinesin in lysosomal dispersion has been demonstrated by a number of techniques, including overexpression of mutant forms (Nakata and Hirokawa, 1995), antibody inhibition in vivo (Hollenbeck and Swanson, 1990), and a mouse knockout (Tanaka et al., 1998). Gene disruption of conventional kinesin (KIF5B) in mouse also confirmed a role for the protein in mitochondrial dispersion (Tanaka et al., 1998), while disruption of the kinesin gene in *Neurospora* demonstrated its importance for cell morphogenesis and polarized secretion (Seiler et al., 1997). Kinesin heavy chain mutations in *Drosophila* are lethal and mutant larvae exhibit a variety of neuromuscular defects, implicating kinesin in axonal transport (Saxton et al., 1991). Additionally, colocalization (Marks et al., 1994) and antisense (Feiguin et al., 1994) studies have suggested that kinesin plays a role in maintaining the morphology of the ER and Golgi.

Roles for the heteromeric kinesins in various types of intracellular transport have also been established. Originally purified from sea urchin using anti-kinesin peptide antibodies (Cole et al., 1992), KRP 85/95 (also called kinesin II) has since been shown by antibody microinjection studies to be required for the assembly of ciliary axonemes on sea urchin embryos

(Morris and Scholey, 1997), suggesting that it functions to deliver crucial ciliary components during ciliogenesis. Gene disruption of the homologous gene in mouse (KIF3A/B) confirms a role for the protein in ciliogenesis (Marszalek et al., 1999; Nonaka et al., 1998). In melanophores, dominant negative studies have established that KRP 85/95 is responsible for pigment granule dispersion (Tuma et al., 1998).

The third class of kinesin-related-proteins that has been implicated in organelle transport is the Unc104/KIF1A family. Unc104 was originally identified in a large genetic screen for neuromuscular defects in *C. elegans;* null cell lines have a clear defect in the plus-end-directed transport of synaptic vesicle precursors (Hall and Hedgecock, 1991; Otsuka et al., 1991). Two related proteins, mouse (Mm) KIF1A and B, were identified in a series of PCR screens to identify kinesin-related proteins in mouse (Aizawa et al., 1992). Colocalization experiments suggested that KIF1A, which is specifically expressed in neurons, was also localized to synaptic vesicle precursors (Okada et al., 1995); a role for KIF1A in synaptic vesicle precursor transport was subsequently confirmed by a mouse knockout (Yonekawa et al., 1998). KIF1B, in contrast, is ubiquitously expressed and was shown to colocalize with mitochondria. Recombinant KIF1B was shown to support limited motility of mitochondria (Nangaku et al., 1994), though the low frequency and velocity of movements raise some concern as to the physiologic validity of this finding. A fourth member of this family implicated in organelle transport is human KIF1C, a Golgi-associated kinesin which was recently identified through its binding to the protein-tyrosine phosphatase PTPD1 in a yeast two-hybrid screen (Dorner et al., 1998). Interestingly, overexpression of a mutant KIF1C incapable of binding ATP abolishes brefeldin-A-induced

Golgi to ER redistribution, suggesting that KIF1C is normally responsible for this transport pathway.

Two additional motor proteins have recently been identified as putative organelle transport motors. First, Rabkinesin-6 (Echard et al., 1998) is a Golgi-associated kinesin-related protein identified by a yeast two-hybrid screen that may act as a cellular effector of Rab6 activity. Second, KIFC2 is a new mouse kinesin-related protein, identified by PCR screen, which is localized to dendrites and may be involved in organelle transport (Hanlon and Goldstein, 1997; Saito et al., 1997). KIFC2 is unique among the kinesin-related putative organelle motors in that its motor domain is located C-terminally; however, its directionality has not yet been determined.

Though these studies have provided much information concerning which motors seem to be responsible for moving which organelles, there is currently relatively little insight into how motors actually bind to and transport organelles. Three main categories of mechanistic questions require attention. First, with the exception of dynein (see below) it is still unknown if motors, in the absence of additional soluble factors, are sufficient to move an organelle. Second, we would like to know how organelle transport is regulated. For instance, though it is known that kinesins are phosphorylated in vivo (Hollenbeck, 1993), the mechanistic significance of this phosphorylation is unclear; conflicting reports have suggested that phosphorylation of kinesin either enhances (Lee and Hollenbeck, 1995) or reduces (Sato-Yoshitake et al., 1992) its binding to membranes. Third, we need to know how motors are tethered to their cargo; specifically, which parts of the motor are required for binding to vesicles, and, in turn, which components on the vesicle surface are required for binding to the motor.

One approach to answering these questions is to reconstitute organelle motility in vitro using defined components. Such an approach avoids a number of the potential caveats associated with indirect approaches such as antibody inhibition (i.e. cross-reactivity with related motors) and co-localization of motors with various organelle populations (i.e. cross reactivity in addition to the fact that the location of a motor does not necessarily predict its function (Lin et al., 1996)). The potential for artifact remains in a reconstitution approach, given that motors can bind non-specifically to objects like carboxylated beads and move them along microtubules. However, if one purifies the components from an extract that demonstrates qualitatively similar motility to that seen in vivo, one can be hopeful that the activity is physiologically relevant.

This approach has been partially successful in the case of cytoplasmic dynein. In vitro organelle transport studies using chick brain extracts (Gill et al., 1991; Schroer and Sheetz, 1991) have shown that dynein alone is incapable of moving organelles and that it requires a cytosolic complex of proteins called dynactin as an activator. The relevance of dynactin for organelle transport was later confirmed by genetic and dominant negative studies (Burkhardt et al., 1997; McGrail et al., 1995; Tinsley et al., 1996). However, the mechanistic function of dynactin is still unclear. Affinity chromatography studies have demonstrated that the p150^{Glued} component of dynactin can bind to the intermediate chain of dynein (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995), and, in turn, that the ARP1 component of dynactin can bind to a spectrin isoform on Golgi membranes (Holleran et al., 1996). Together, these data suggest a model in which dynactin would function as a linker to tether dynein to membranes (Figure 1). However, the complexity of both the dynein and dynactin complexes have made it difficult

to dissect these processes more finely, and in vitro evidence of a functional role for these individual components of the complexes in minus-end-directed motility is still lacking.

In contrast to the work on dynein, less progress has been made towards an understanding of the mechanism of plus-end-directed transport (Figure 1). In vitro binding studies have shown that the tail of conventional kinesin heavy chain is capable of saturable binding to organelles (Skoufias et al., 1994). The light chains of conventional kinesins may also play a role in membrane binding, as evidenced by the fact that anti-light chain antibodies can release conventional kinesin from purified vesicles (Stenoien and Brady, 1997). With regards to the organelle surface, a putative receptor for conventional kinesin, kinectin, has been identified by affinity chromatography (Toyoshima et al., 1992). Antibodies against kinectin inhibit motility in vitro (Kumar et al., 1995), but the relevance of kinectin in vivo is not yet known. As kinectin has only been localized to the ER, one would expect additional family members to be present on other organelles, but these have not been discovered.

In vitro movement of organelles in the plus-end direction has been accomplished in a number of extract systems, including squid (Schnapp et al., 1992; Schroer et al., 1988; Vale et al., 1985), chick (Dabora and Sheetz, 1988; Gill et al., 1991; Schroer and Sheetz, 1991), frog egg (Allan and Vale, 1991; Niclas et al., 1996), melanophores (Rogers et al., 1997), macrophages (Blocker et al., 1997), and cytotoxic T-cells (Burkhardt et al., 1993). However, attempts to reconstitute plus-end-directed transport with defined components purifed from extracts have generally been unsuccessful, limited by either a lack of robust activity in the extract (e.g. frog) or by the inability to generate material on a scale for biochemical purification (e.g. squid). Experiments designed to

dissect the mechanism of plus-end-directed motility in complex extract systems have generated controversy as to whether soluble factors in addition to the motor are required (Schnapp et al., 1992; Schroer et al., 1988; Schroer and Sheetz, 1991), and, importantly, the actual identity of the motor responsible for the motility was uncertain in all cases. Early work by Schroer and Sheetz (1991) in chick brain extracts seemed very promising; reasonable levels of plus-end-directed transport activity were obtained and initial biochemical fractionations suggested the existence of an activator for plus-end motility which may well have been an Unc104/KIF1A-type motor. However, the activity was not purified further.

Only two attempts at in vitro reconstitution with purified components have generated motility. In one case, recombinant KIF1B, a kinesin-related protein cloned by homology, was shown to produce very limited mitochondrial motility in vitro (Nangaku et al., 1994). However, the frequency of movement was not reported, and the calculated velocities of a number of the vesicle movements presented as evidence of motility do not approach either the reported maximum velocity or the velocity seen in vivo. In the other example, kinesin purified from chromaffin cells was reproducibly shown to move chromaffin granule "ghosts" (Urrutia et al., 1991), though again at a rate well below most organelle transport rates in vivo or in extracts (Bloom and Endow, 1995). Additionally, it was later shown that little or no kinesin is present on mature chromaffin granules in vivo (Schmitz et al., 1994).

****

At the time that I entered the lab in 1994, Dr. Geno L. de Hostos was cloning kinesins from the cellular slime mold *Dictyostelium discoideum* by homology. We reasoned that an in vitro organelle transport assay using *Dictyostelium* extracts would be valuable for characterizing the kinesin knockout mutants generated through those studies. I set out to develop a video microscopy-based vesicle transport assay, using microtubules polymerized off of sea urchin axonemes to determine the directionality of movement. This in vitro assay turned out to be extremely robust, particularly for minus-end-directed movement. I then showed that the motility of KI-washed vesicles, which did not move on their own, could be restored by the addition of a high-speed supernatant (HSS). As I was doing this work, I was able to use the assay to characterize a *Dictyostelium* cell line overexpressing a truncated form of the dynein heavy chain (DHC) and to show that this mutant form of the DHC (380 kDa DHC; (Koonce and Samso, 1996)) functioned in a dominant negative fashion to inhibit minus-end-directed vesicle transport specifically. I also developed an assay for endocytic vesicle motility and described the speed and direction of these movements (Pollock et al., 1998; Chapter 1).

A distinct advantage of *Dictyostelium* is the ability to easily grow cells on a scale suitable for biochemical fractionation. An important advance in the project was the discovery that a microtubule-affinity-purified fraction prepared from the HSS was capable of supporting robust bidirectional movement of the KI-washed organelles (Pollock et al., 1998). This procedure demonstrated that we could substantially enrich for plus-end-directed motility and made it reasonable to attempt to purify the plus-end-directed motility factors (subject of Chapter 2). Using the assay, I was able to purify two plus-end-directed transport factors, of 245 kDa and 170 kDa, which were

independently capable of supporting plus-end-directed vesicle movements. Peptide sequence suggested that the 245 kDa polypeptide was a member of the Unc104/KIF1A family and that the 170 kDa polypeptide was a conventional kinesin homologue. Using a combination of degenerate PCR, library screen, and rapid amplification of cDNA ends (RACE), I obtained the complete clone of the 245 kDa kinesin and showed that it was most closely related to CeUnc104 and MmKIF1A. Hydrodynamic studies showed that DdUnc104 is dimeric, in contrast to the other members of this family which have been shown to be monomers in solution. A knockout of the DdUnc104 gene (made in collaboration with Geno), despite having no effects on cell morphology or growth rate, produced a dramatic decrease in the total amount of vesicle transport in vivo. In contrast, the movements of mitochondria in vivo were not affected, suggesting that the defect is not a generalized reduction in the transport of all organelles but rather a specific defect in the transport of an as yet unidentified organelle. In vitro assays using the knockout cell line confirmed that the defect was specific for plus-end-directed transport and that the DdUnc104 motor is responsible for the majority of the plus-end-directed movements in the extracts (Pollock et al, 1999, submitted; Chapter 2).

A major goal of this project has been to reconstitute transport using KI-washed vesicles and a recombinant motor purified in a different manner than the procedure used to isolate the endogenous plus-end-directed transport factors (i.e. via a His-tag). A successful reconstitution would demonstrate that the motor itself was sufficient to move organelles; if the motor proved to be insufficient for transport, it would be possible to purify additional required factors using the motility assay. A functional recombinant motor, in turn, would allow us to investigate which regions of

the motor were required for binding to the vesicle and/or for motility; additionally, we could use the motor as a tool with which to look for a receptor on the vesicle surface. Preliminary work on expression of recombinant DdUnc104 in *Dictyostelium* and attempts to reconstitute transport with the recombinant protein are described in Appendix 1.

A number of interesting additional observations have emerged from work on this system over the past five years and are detailed in Appendix 2. One area for future exploration involves dynein-based organelle transport. Throughout our purification of the plus-end-directed transport factors, we often observed abundant amounts of the dynein heavy chain in various fractions, but these fractions did not support minus-end-directed transport. A potential explanation for this finding is that an additional factor required for motility was lost or inactivated in the purification. Given the abundant levels of minus-end-directed transport in the extracts, it may be possible to purify this factor(s); information pertaining to the behavior of the minus-end-directed transport activity under various conditions is presented in Appendix 2. This section also details additional experiments pertaining to both plus-and minus-end-directed transport. Finally, Appendix 2 includes a discussion of relevant biochemical characteristics of the system which should prove useful for future work on organelle transport in *Dictyostelium*.

Figure 1. Model of dynein- and kinesin-based organelle transport mechanism. The current model for the mechanism by which dynein binds to and moves an organelle involves dynein binding to membranes indirectly via its activator, dynactin (Gill et al., 1991; Schroer and Sheetz, 1991). In this model, the ARP1 component of dynactin binds to a spectrin isoform on the membrane (Holleran et al., 1996). In turn, the p150^{Glued} component of dynactin can bind to the intermediate chain of dynein (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995), thereby tethering dynein to the membrane. The role, if any, for the demonstrated capability of dynein to directly bind membrane phospholipids (Lacey and Haimo, 1994; Muresan et al., 1996) is unclear. (Figure adapted from Holleran et al, 1998.)

In contrast, the current model for the mechanism by which kinesins bind to and transport membranes is far less complete. The kinesin tail is thought to be responsible for cargo binding, based on studies showing that the tail of conventional kinesin heavy chain is capable of binding saturably to organelles (Skoufias et al., 1994), but little additional evidence for such a role for the tail has emerged. The fact that anti-light chain antibodies release conventional kinesin from purified vesicles (Stenoien and Brady, 1997) suggests that conventional kinesin light chains may play a role in membrane binding. However, many of the plus-end-directed motors implicated in organelle transport do not have associated light chains. Whether additional soluble factors are required for binding to membranes/motility is controversial (Schnapp et al., 1992; Schroer et al., 1988; Schroer and Sheetz, 1991). Kinectin is a putative receptor for conventional kinesin that has been identified by affinity chromatography (Toyoshima et al., 1992), but a functional role for kinectin in vivo has not yet been established. (Kinesin figure adapted from D. Friedman, 1999 Ph.D. thesis.)

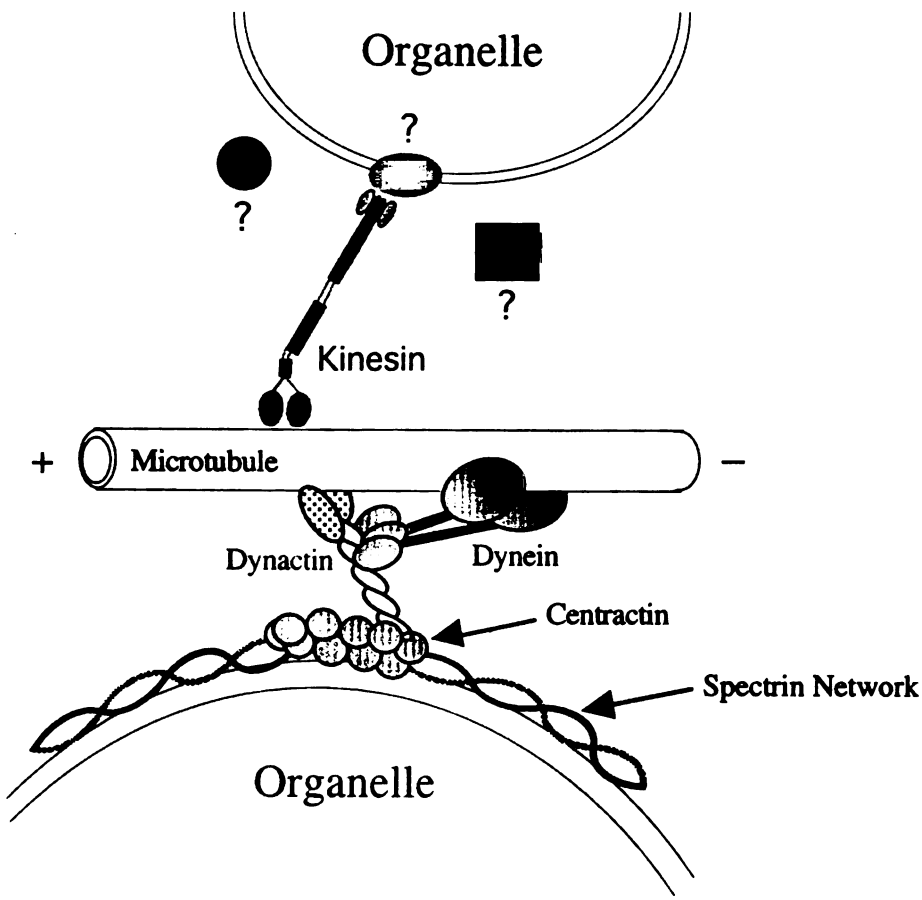
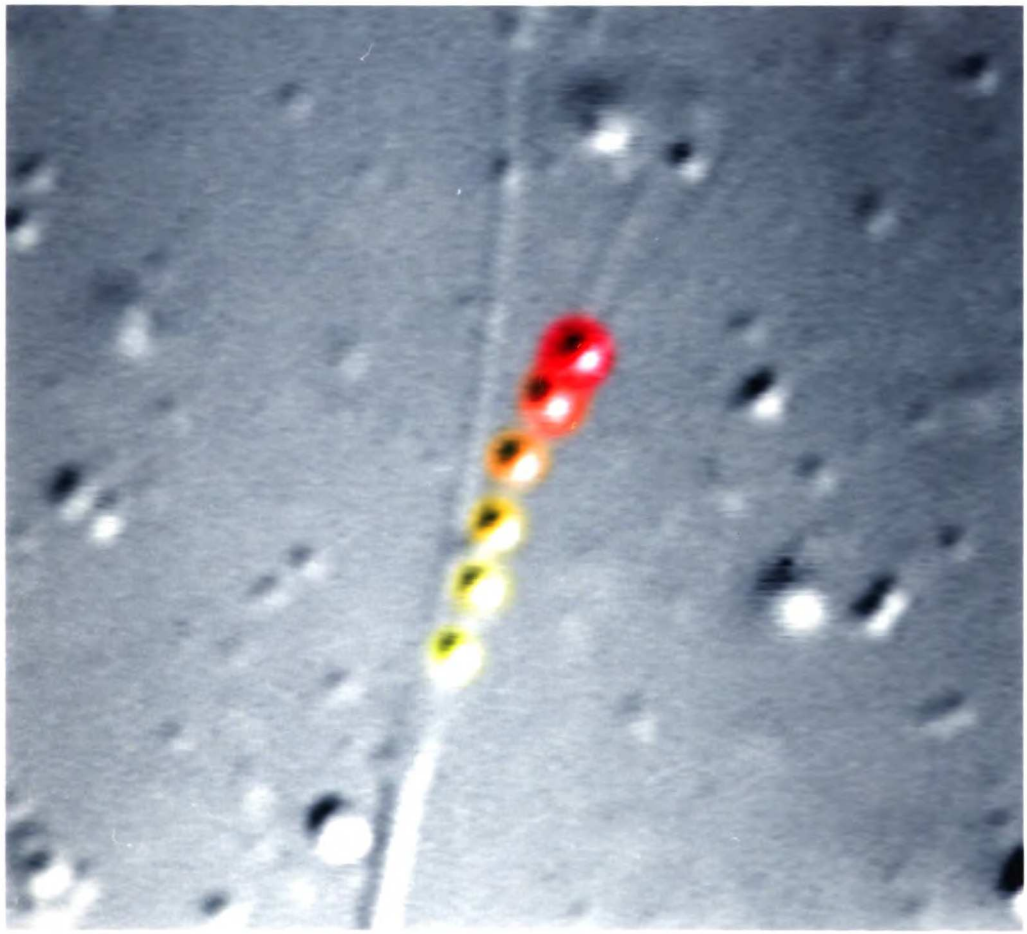

Organelle
?
?
?
Kinesin
+
Microtubule
−
Dynactin
Dynein
Centractin
Spectrin Network
Organelle

Figure 2. In vitro movement of a membrane vesicle (pseudo-colored) along an axoneme-nucleated microtubule track driven by a *Dictyostelium* kinesin motor. This plus-end-directed movement was viewed by video-enhanced differential interference contrast microscopy. Still images were captured at 0.5 second intervals and overlayed to create this composite image. The time sequence of motion is depicted as a color gradient (bottom to top).



Chapter 1

In Vitro Microtubule-Based Organelle Transport in Wild-Type *Dictyostelium* and Cells Overexpressing a Truncated Dynein Heavy Chain

Cell Motility and the Cytoskeleton 40:304–314 (1998)

In Vitro Microtubule-Based Organelle Transport in Wild-Type *Dictyostelium* and Cells Overexpressing a Truncated Dynein Heavy Chain

Nira Pollock,[1] Michael P. Koonce,[3] Eugenio L. de Hostos, [4] and Ronald D. Vale[1,2]*

[1]*Department of Pharmacology, University of California San Francisco, San Francisco, California*
[2]*Howard Hughes Medical Institute, University of California San Francisco, San Francisco, California*
[3]*Division of Molecular Medicine, Wadsworth Center, Empire State Plaza, Albany, New York*
[4]*Department of Biochemistry and Cell Biology, Rice University, Houston, Texas*

The transport of vesicular organelles along microtubules has been well documented in a variety of systems, but the molecular mechanisms underlying this process are not well understood. We have developed a method for preparing extracts from *Dictyostelium discoideum* which supports high levels of bidirectional, microtubule-based vesicle transport in vitro. This organelle transport assay was also adapted to observe specifically the motility of vesicles in the endocytic pathway. Vesicle transport can be reconstituted by recombining a high-speed supernatant with KI-washed organelles, which do not move in the absence of supernatant. Furthermore, a microtubule affinity-purified motor fraction supports robust bidirectional movement of the salt-washed organelles. The plus and minus end-directed transport activities can be separated by exploiting differences in their affinities for microtubules in the presence of 0.3 M KCl. We also used our assay to examine organelle transport in a strain of *Dictyostelium* overexpressing a 380-kDa C-terminal fragment of the cytoplasmic dynein heavy chain, which displays an altered microtubule pattern (380-kDa cells; [Koonce and Samso, Mol. Biol. Cell 7:935–948, 1996]). We have found that the frequency and velocity of minus end-directed membrane organelle movements were significantly reduced in 380-kDa cells relative to wild-type cells, while the frequency and velocity of plus end-directed movements were equivalent in the two cell types. The 380-kDa C-terminal fragment cosedimented with membrane organelles, although its affinity was significantly lower than that of native dynein. An impaired membrane-microtubule interaction may be responsible for the altered microtubule patterns in the 380-kDa cells. Cell Motil. Cytoskeleton 40:304–314; 1998.

Key words: vesicle transport; intracellular motility; microtubule-based motors

INTRODUCTION

The directed transport of vesicular organelles is an important component of intracellular sorting in eukaryotic cells. Cytoplasmic motor-driven movement of membrane organelles along microtubule or microfilament "tracks" has been documented in a wide variety of systems. In recent years, much progress has been made in

Contract grant sponsor: National Institutes of Health; Contract grant number: GM51532; Contract grant number: 38499; Contract grant sponsor: American Heart Association.

*Correspondence to: R.D. Vale, Department of Pharmacology, 513 Parnassus Ave., University of California San Francisco, San Francisco, CA 94143; E-mail: vale@phy.ucsf.edu

Received 13 February 1998; accepted 26 March 1998

assigning roles to motor proteins for specific cellular transport activities [Allan, 1995; Goodson et al., 1997]. However, the actual mechanisms by which motors bind to organelles and facilitate their movement are still largely a mystery.

To advance our understanding of microtubule-based organelle transport, it would be useful to study this process in a simple unicellular organism that offers the opportunity to combine biochemistry and genetics. Although the yeast *Saccharomyces cerevisiae* would appear to provide an attractive candidate for such a system, vesicle transport in this organism appears to be actin-based [Hill et al., 1996; Huffaker et al., 1988; Novick and Botstein, 1985]. In contrast, the cellular slime mold *Dictyostelium discoideum* has a radial array of microtubules emanating from a central microtubule organizing center or nucleus-associated body [Roos et al., 1984]; vesicles have been observed moving linearly along these microtubules, and motility was blocked by the microtubule depolymerizing drug nocodazole [Roos et al., 1987]. Intracellular vesicle sorting pathways in *Dictyostelium*, including endocytosis, phagocytosis, and secretion, have been well studied and appear similar to higher eukaryotes [Bacon et al., 1994; Lenhard et al., 1992; Ruscetti et al., 1994; Spudich, 1987]. *Dictyostelium* also has several experimental advantages, in that abundant starting material can be obtained for biochemical purifications and functions of particular genes can be tested by targeted disruption [De Lozanne and Spudich, 1987; Jung and Hammer, 1990; Kuspa and Loomis, 1994].

Studies of cytoplasmic microtubule-based motors in *Dictyostelium* have resulted in the purification of a 105-kDa kinesin-like protein [McCaffrey and Vale, 1989] and a cytoplasmic dynein [Koonce and McIntosh, 1990]. Like the dyneins of higher eukaryotes, *Dictyostelium* dynein is a complex composed of a 540-kDa heavy chain (DHC), an 85-kDa intermediate chain (IC), and at least two light chains (LC) of approximately 58 and 53 kDa [Koonce et al., 1994]. Because disruption of the DHC gene is lethal [Koonce and Knecht, 1998], Koonce and Samso [1996] constructed a series of *Dictyostelium* cell lines overexpressing various fragments of the dynein heavy chain (DHC) in order to define the function of cytoplasmic dynein in vivo. Unexpectedly, overexpression of a 380-kDa C-terminal heavy chain fragment encoding the dynein globular head (380-kDa cells; [Koonce and Samso, 1996]) generated a phenotype in which the interphase microtubule array lost its radial character and appeared collapsed toward the cell center. Koonce and Samso speculated that this phenotype could indicate a role for dynein in maintaining the spatial pattern of the interphase microtubule network. Like the full-length DHC, the 380-kDa fragment cosediments with microtubules in an ATP-dependent fashion and undergoes a UV-vanadate cleavage reaction. However, unlike the native DHC, it is unable to dimerize and does not bind either the 85-kDa IC or the 53-kDa LC, although it may weakly bind the 58-kDa LC. As these biochemical data alone do not explain the in vivo phenotype, additional functional studies are required. Clearly, an in vitro assay for organelle transport would be valuable for characterizing this and other microtubule-based motor mutants in *Dictyostelium*.

In this study, we report a method for preparing *Dictyostelium* extracts that support high levels of bidirectional microtubule-based vesicle motility in vitro and that can be used to study the motility of vesicles in the endocytic pathway. The velocities and qualitative aspects of these movements appear to replicate in vivo vesicle movements in *Dictyostelium* [Roos et al., 1987]. The combination of high-speed supernatants or fractions enriched in microtubule-based motor proteins with salt-washed vesicle fractions reconstitutes motility. Furthermore, factors involved in plus and minus end-directed motility can be separated based on differential microtubule affinity.

Our assay also has allowed us to take advantage of the first microtubule-based motor mutants created in *Dictyostelium* by examining the effects of the overexpression of the 380-kDa DHC fragment on organelle transport in vitro. We have found that the frequency and velocity of minus end-directed membrane organelle movements were specifically reduced in 380-kDa cells relative to wild-type cells, while the frequency and velocity of plus end-directed movements were equivalent in the two cell types. These results suggest that the in vivo phenotype of the 380-kDa cells may be linked to a defect in dynein-based organelle transport.

MATERIALS AND METHODS

Extract Preparation

Dictyostelium strain Ax-2 was grown in suspension at 22°C to a density of 4–8 × 10^6 cells/ml in HL-5 [Sussman, 1987] containing 100 μg/ml streptomycin and 100 U/ml penicillin. For the 380-kDa and 318-kDa cells, 10 μg/ml G418 (Geneticin; Gibco-BRL, Grand Island, NY) was also added. Approximately 6 × 10^8–1.2 × 10^9 cells were collected by centrifugation at 800*g* for 3 min in a clinical centrifuge at room temperature. The cell pellet was placed on ice, washed once with ice-cold Sorenson's phosphate buffer, pH 6.0 [Malchow et al., 1972], recentrifuged, and then resuspended in 1:1 w/v lysis buffer (LB: 30 mM Tris-HCl (pH 8), 4 mM EGTA, 3 mM DTT, 5 mM benzamidine, 10 μg/ml soybean trypsin inhibitor, 5 μg/ml TPCK/TAME, 10 μg/ml leupeptin, pepstatin A, and chymostatin, and 5 mM PMSF (phenylmethyl-sulfonyl fluoride) containing 30% (w/v) sucrose. Cells were lysed

by one passage through a Nuclepore polycarbonate filter (5-μm pore size, 25-mm diameter; Costar, Pleasanton, CA) using a 10-ml syringe. The lysate was centrifuged at 2,000*g* for 5 min at 4°C, and the resulting postnuclear supernatant (PNS) was layered over a 1-ml cushion of LB/25% sucrose and centrifuged in a Beckman TLA 100.4 rotor at 180,000*g* for 15 min at 4°C. The high-speed supernatant (HSS) was removed, and the vesicle pellet was resuspended in LB/15% sucrose (one-half volume of the original PNS). To prepare salt-washed vesicles, LB containing 1.2 M KI was added to the resuspended pellet to achieve a final concentration of 0.3 M KI. After incubation for 30 min on ice, membranes were diluted by adding 2 vol of LB/15% sucrose and centrifuged as before over a 1-ml cushion of LB/25% sucrose in a Beckman TLA 100.4 rotor. The cushion was aspirated, and the salt-washed membranes were resuspended in LB/30% sucrose (0.3 vol of the original PNS). 15-μl aliquots of the resuspended vesicles were frozen in liquid nitrogen and, upon thawing, 0.5 μl of fresh 1M PMSF was added.

Preparation of Extracts with Labeled Endocytic Vesicles

Approximately 8×10^8 cells were pelleted as described above and resuspended in 5 ml HL5 (final cell density 1.6×10^8 cells/ml) at room temperature (25°C). Cells were incubated for 10 min at 22°C while shaking at 180 rpm to recover endocytic function. To begin the pulse, 2.5 ml of HL5 containing 4 mg/ml rhodamine-conjugated dextran (tetramethyl rhodamine-conjugated dextran, 40,000 MW, neutral; Molecular Probes, Eugene OR) was added (final densities 1.1×10^8 cells/ml, 1.33 mg/ml dextran). Cells were incubated for 20 min while shaking at 180 rpm and then pelleted by centrifugation. Cells were washed twice with 50 ml Soerenson's phosphate buffer at 4°C and processed to a lysate as described above.

Preparation of ATP Releasate

Cells (1–3 l at a density of 4–8 × 10^6 cells/ml) were washed and lysed as described above, the only exception being that a 47-mm diameter filter and 30-ml syringe were used. A HSS was prepared as described above and incubated with 15 U/ml hexokinase, 3 mM glucose, 4 mM AMP-PNP/$MgCl_2$, and 20 μM taxol. Prior to preparation of the extract, bovine brain tubulin at approximately 2.5 mg/ml was polymerized with 10% DMSO and 1 mM GTP in 40 mM K-Pipes, pH 6.8, 0.5 mM EGTA, 2 mM $MgCl_2$ (0.5× BRB80) and 20 μM taxol by incubation for 10 min at 37°C. The microtubules were collected by centrifugation at 360,000*g* for 10 min in a Beckman TLA 120.1 rotor (22°C), and resuspended in the HSS (plus hexokinase, glucose, AMP-PNP, and taxol as above) for a final tubulin concentration of 0.5 mg/ml. The HSS/microtubule mix was incubated on ice for 20 min; microtubules and associated proteins were then centrifuged through a 1-ml cushion of LB/25% sucrose containing 20 μM taxol at 85,000*g* for 15 min in a Beckman TLA 100.4 rotor (4°C). The microtubule pellet was resuspended in LB/5% sucrose containing 5 mM ATP/$MgCl_2$ (1:20 vol of the original HSS volume) and incubated on ice for 15 min to release bound motor proteins. Microtubules were separated from the released proteins by centrifuging at 90,000*g* for 15 min in a Beckman TLA 120.1 rotor (4°C). The supernatant (ATP release) was collected and either frozen in liquid nitrogen or assayed directly (see below). To separate plus end- and minus end-directed transport activities, the microtubule pellet obtained after the initial incubation of microtubules with the HSS was resuspended in LB/5% sucrose containing 0.3 M KCl (1:20 vol of the original HSS volume) and immediately recentrifuged at 85,000*g* for 15 min in a Beckman TLA 100.4 rotor (4°C). The resulting supernatant ("salt wash") was dialyzed for 70 min against LB/5% sucrose using a microdialyzer system 100 (Pierce, Rockford, IL), exchanging the buffer in the chamber once after 30 min of dialysis. In parallel, the microtubule pellet was resuspended in LB/5% sucrose containing 5 mM ATP/$MgCl_2$ to elute the remaining motors as described above.

Organelle Transport Assays

Preparation of axoneme-nucleated MT structures. Flow cells (10 μl) were prepared by placing acetone-washed, 18 × 18 mm glass coverslips over two parallel strips of double-sided tape on a glass slide. Sea urchin sperm axonemes in 5 mM imidazole/NaOH (pH 7), 100 mM NaCl, 4 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1 mM EDTA, 0.1 mM ATP, 7 mM β-mercaptoethanol, and 50% glycerol were diluted 1:200 in 1 × BRB80, introduced into the flow cell, and incubated at room temperature for 4 min to allow axonemes to adhere to the glass surface. Next, approximately 1.5 mg/ml bovine brain tubulin in 10% DMSO, 1 mM GTP, and 0.5× BRB80 was introduced into the flow cell and the flow cell was placed on a 37°C heat block for 10 min to allow microtubules to polymerize off the ends of the axonemes. The tubulin concentration was titrated to generate axoneme–microtubule structures with much longer microtubules on one end than the other, and few microtubules free in solution. After the 10-min incubation, BRB80 containing 20 μM taxol at 37°C was introduced to stabilize the polymerized microtubules. Flow cells were stored for up to 2 days in a hydrated chamber.

Organelle transport assays. The flow cell was first washed with 10 μl of LB/15% sucrose to remove any free microtubules, followed by the introduction of 10 μl of assay mix consisting of, in the case of extracts, 2 μl PNS,

7.5 µl LB/15% sucrose, and 0.5 µl of a 20× ATP regenerating mix (20 mM ATP, 20 mM $MgCl_2$, 40 mM creatine phosphate, and 40 U/ml creatine kinase). For assessing motility in the presence of vanadate, aliquots of freshly made PNS were incubated with 2 mM ATP, 5 mM $MgCl_2$, 4 mM creatine phosphate, 4 U/ml creatine kinase, 2 mM DTT, and 40 µM sodium orthovanadate for at least 10 min on ice prior to beginning motility assays. Assay mixes for the HSS and ATP releasate fractions consisted of 5 µl HSS/ATP releasate, 3.5 µl LB/15% sucrose, 1 µl KI-washed vesicles (see above), and 0.5 µl ATP-regenerating mix. Assay mixes for more highly purified fractions such as the "salt wash" or post-salt wash ATP releasate consisted of 4 µl of the fraction, 4 µl LB/15% sucrose, 1 µl KI-washed vesicles, 0.5 µl of a 20-mg/ml casein stock, and 0.5 µl ATP-regenerating mix. If extracts with fluorescently labeled vesicles were to be observed, 0.5 µl of a 20× oxygen scavenger mix was added to the assay mix, maintaining a total final volume of 10 µl (20× oxygen scavenger mix: 20 mM DTT, 2 mg/ml glucose oxidase, 0.36 mg/ml catalase, and 0.24 M glucose; all stocks made separately in BRB80 and combined just before use). The movement of organelles in unlabeled extracts was observed using a Zeiss Axioplan microscope equipped with differential interference contrast (DIC) optics, a 50- or 100-W mercury arc lamp, and a 100×, 1.3 NA Plan-Neofluor objective. Images were detected using a camera (Newvicon; Hamamatsu Photonics, Bridgewater, NJ); contrast enhancement and background subtraction were performed with an image processor (Argus10; Hamamatsu Photonics), and recordings were made with a super VHS video tape recorder (AG-5700; Panasonic, Secaucus, NJ). Single-frame images were captured from video tape onto a Macintosh 8100/100, using VideoVision Frame Grabber and Photoshop software. To observe the movement of rhodamine-labeled organelles, the axoneme-nucleated microtubule array was first visualized by DIC as described above. The same field was then viewed using a 100-W tungsten lamp and a SIT camera (to confirm the location of the unlabeled axoneme), followed by observation of the fluorescent vesicles moving in the same field by fluorescence optics using the SIT camera.

Organelle motility was quantified by counting the number of vesicles moving per minute in each direction on a single axoneme/microtubule structure. Only axonemes with clearly defined polarity were used. Recordings of movement were performed on axonemes with between 6 and 12 microtubules (8–14 µm each in length) polymerized from the plus ends. If an organelle paused briefly and then continued in the same direction, it was scored as a single movement. Velocities of movements were quantified using custom software; only vesicles which moved smoothly over a distance of at least 1.5–2 µm were scored.

Dynein Membrane Association and Protein Analysis

PNS (320 µl prepared as described above) was made to 1.5 M sucrose by adding LB/2 M sucrose (680 µl) and layered above a 400-µl cushion of LB/2 M sucrose and below 400-µl and 500-µl layers of LB/1.4 M sucrose and LB/0.25 M sucrose, respectively. The membranes were then isolated by flotation to the 1.4 M/0.25 M sucrose interface by centrifugation at 200,000*g* for 30 min in a Beckman TLS 55 rotor (4°C). To assess the affinity of motor binding to the membranes, one-half of the floated membranes were diluted 1:5 in LB and repelleted by centrifugation at 175,000*g* for 15 min in a Beckman TLA 120.1 rotor (4°C).

Samples were separated on 4–12% or 5–15% gradient polyacrylamide gels under denaturing and reducing conditions. Gels were either stained with colloidal Coomassie or electroblotted to nitrocellulose membranes at 100 mA for 75 min. The blots were incubated with either a polyclonal antibody against the conserved kinesin LNLVDLAGSE domain (1 µg/ml) [Sawin et al., 1992] or an affinity-purified polyclonal antibody against the *Dictyostelium* dynein heavy chain (1:1,000) [Koonce and Samso, 1996] overnight at 4°C, and then incubated in HRP-conjugated secondary antibody (1:2,000; Amersham Life Sciences, Arlington Heights, IL) for 1 hr at room temperature. Blots were developed using a chemiluminescence kit (NEN Life Sciences, Beverly, MA) and exposed to Hyperfilm (Amersham).

RESULTS

An In Vitro Assay for Microtubule-Based Movements in *Dictyostelium* Cell Extracts

By refining buffer and protease inhibitor conditions, we developed a method for preparing *Dictyostelium* extracts that displayed robust bidirectional microtubule-based vesicle transport. The crude extract supported more than 30 vesicle movements/min per axoneme structure (Table I); this activity lasted for hours on ice and was stable to freeze-thaw in liquid nitrogen. The presence of fresh PMSF in the extract was found to be particularly important for maintaining the transport activity. In order to assess the directionality of movement of the *Dictyostelium* vesicles, a polarized array of microtubules was assembled by polymerizing bovine brain tubulin from the ends of sea urchin axonemes. The plus ends of microtubules could be identified by the significantly longer microtubules that grew off of one end of the axoneme [Allen and Borisy, 1974]. Vesicles moved bidirectionally on microtubules, with vesicles moving smoothly from axoneme to microtubule and vice-versa. Individual vesicles were often observed to change direction one or more times, and frequently paused midway through the

TABLE I. Frequencies and Velocities of Vesicle Movements in Wild-Type *Dictyostelium* Extracts*

	Frequency (movt/min/axoneme)		Velocity (μm/s)	
	Minus end	Plus end	Minus end	Plus end
A. PNS	26.7 ± 2.1	6.2 ± 0.7	2.74 ± 0.40	2.04 ± 0.30
B. HSS	15.3 ± 0.7	3.6 ± 1.1	2.75 ± 0.42	2.13 ± 0.38
C. ATP releasate	17.0 ± 2.8	5.9 ± 3.6	2.77 ± 0.49	2.11 ± 0.33

*Frequency of vesicle movements was quantified as movements per minute in each direction over a period of 4 min on microtubules nucleated off of a single axoneme (see under Materials and Methods). Frequencies represent the mean ± SD for the averages from n = 3, n = 2, and n = 3, separate preparations of PNS, HSS, and ATP releasate, respectively. For each preparation, one to four separate assays were performed and the results averaged. The velocities represent the mean ± SD for a total of n = 149, n = 35, and n = 93 minus end movements and n = 82, n = 16, and n = 51 plus end movements measured over all of the preparations of PNS, HSS, and ATP releasate, respectively.

length of their movement. Minus end-directed motility was approximately four times more frequent than plus end-directed motility, and the speeds of movement in the minus end direction were faster than in the plus end direction (Table I).

As *Dictyostelium* has been widely used as a model system for the study of endocytosis [e.g., Bacon et al., 1994; Bush et al., 1996; Padh et al., 1993], we also adapted our *Dictyostelium* assay to observe specifically the motility of endocytic vesicles in vitro. To achieve this, cells were incubated with rhodamine-conjugated dextran, a marker which is internalized by fluid-phase endocytosis. Wild-type cells incubated for 20 min with rhodamine-dextran at room temperature showed bright vesicular labeling when viewed by fluorescence microscopy, while cells that had been subjected to the same pulse at 4°C to inhibit endocytosis showed no labeling (data not shown). When extracts were prepared, fluorescent vesicles were observed moving along microtubules (Fig. 1). Most of the fluorescent vesicle movements were to the minus end of the microtubules/axoneme, although the ratios of minus to plus end-directed movements varied from preparation to preparation for reasons that are still unclear. The velocities of the endocytic vesicle movements were similar to those of the unlabeled vesicle population observed by DIC microscopy (minus end-directed: 2.2 ± 0.4 μm/sec, n = 58; plus end-directed: 1.8 ± 0.3 μm/sec, n = 27). Many of the labeled vesicles moved bidirectionally: over 80% (n = 49) of the endocytic vesicles that moved toward the plus end also exhibited at least one episode of minus end-directed movement. In contrast, only 60% (n = 76) of the total vesicle population (endocytic as well as others) in a typical crude extract observed by DIC microscopy exhibited such behavior.

The high level of minus end transport in the extracts suggests that cytoplasmic dynein may be the predominant motor functioning in this system. Dynein-generated motility has been shown to be more sensitive to inhibition by sodium orthovanadate than kinesin motility [Shimizu, 1995]; we therefore tested the effect of sodium orthovanadate on activity in the extracts. The addition of 40 μM vanadate to the PNS inhibited minus end-directed transport by approximately fourfold, reducing the number of minus end movements per minute per axoneme from 16.5 ± 4.7 to 4.1 ± 1.2 (n = 6 assays over two separate extracts). However, 40 μM vanadate did not inhibit the plus end-directed movements (2.4 ± 0.9 and 1.8 ± 0.3 movements/min/axoneme in the presence and absence of vanadate, respectively). These results are consistent with the involvement of cytoplasmic dynein in minus end-directed motility in this system.

Requirements for Vesicle Transport

To better define the protein requirements of the *Dictyostelium* vesicle transport assay, we examined whether soluble factors are necessary or whether all the factors required for transport are tightly bound to membranes as has been described in other studies [Muresan et al., 1996; Schnapp et al., 1992]. When vesicles were separated from soluble cytosolic components in the PNS by centrifugation through a sucrose cushion, the isolated vesicles exhibited full levels of motility in the plus end direction but no movement in the minus end direction (data not shown). When the vesicle pellet was washed in either buffer alone or buffer plus 0.3 M KI and then repelleted and resuspended, the vesicles did not move in the absence of cytosol. However, recombination of these "washed" vesicles with the HSS generated robust motility similar to that in the PNS (Table I). Therefore, the components necessary for plus end transport appear to be initially bound, albeit weakly, to the vesicle surface; in contrast, minus end motility is lost during vesicle isolation and requires the addition of soluble factors.

We next examined whether cytosolic factors that stimulate the transport of 0.3 M KI-washed organelles could be partially purified from the HSS by enriching for microtubule motor proteins, as has been successfully utilized in other reports [Schroer and Sheetz, 1991]. A microtubule motor-containing fraction was prepared by incubating the HSS with taxol-stabilized microtubules in the presence of hexokinase, glucose and AMP-PNP to cause tight binding of motors to microtubules, collecting the microtubules by centrifugation, and releasing motors from microtubules by the addition of 5 mM Mg^{2+}ATP. This "ATP releasate," in combination with 0.3 M KI-washed vesicles, supported robust bidirectional vesicle transport at levels similar to those in the PNS and HSS

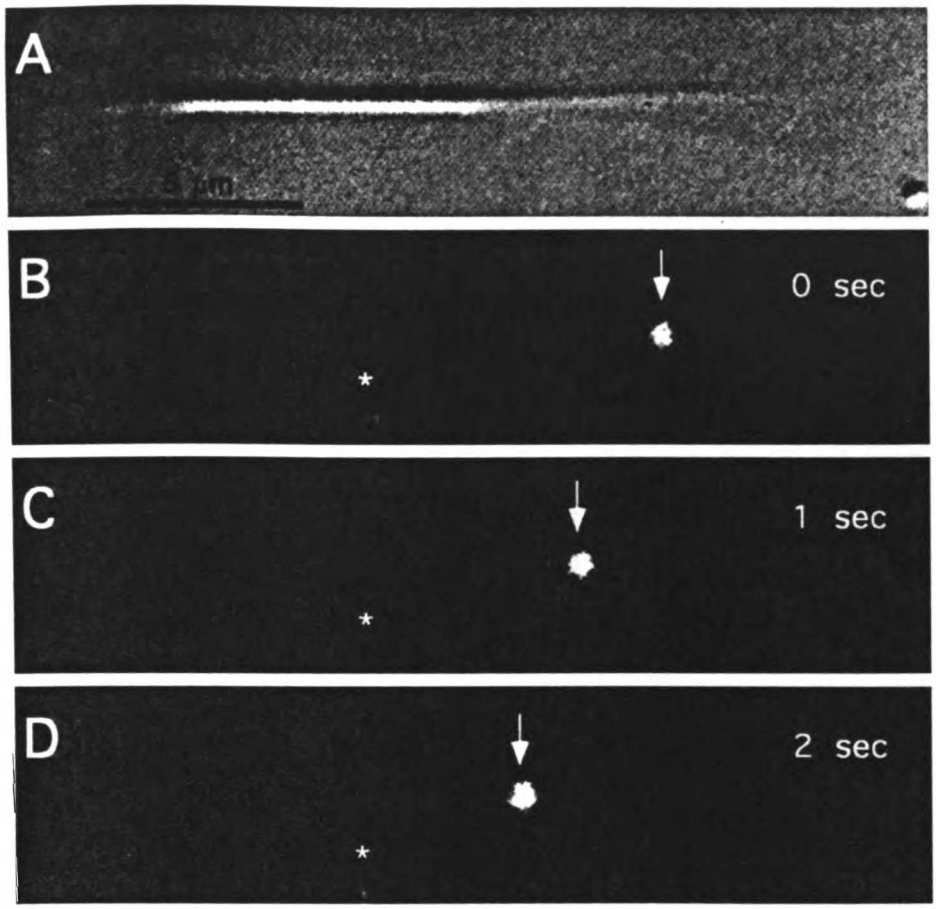


Fig. 1. Endocytic vescicles from *Dictyostelium* extracts move along microtubules in vitro. Endocytic vesicles were fluorescently labeled by incubating cells with rhodamine-conjugated dextran for 20 min to allow fluid-phase uptake of the marker, followed by preparation of extracts (see under Materials and Methods). **A:** DIC microscopic image of a sea urchin axoneme with nucleated bovine brain microtubules which was the substrate for the assay. The plus end is identified by the longer microtubules emerging from one end of the axoneme. **B–D:** Movement of an endocytic vesicle (*arrow*) in the same assay field visualized by fluorescence microscopy. This vesicle is moving toward the minus end of the microtubule. The star (★) indicates a stationary fluorescent vesicle, for comparison. Bar = 5 μm.

(Table I). The velocities of the moving vesicles were equivalent in the PNS, HSS, and ATP releasate (Table I).

We then tested whether the plus end- and minus end-directed transport activities could be separated from each other on the basis of differences in their microtubule affinities. We found that washing the microtubule pellet with buffer containing 0.3 M KCl before incubating the microtubules with Mg/ATP generated a "salt wash" fraction with predominantly minus end activity and very little plus end activity. A subsequent incubation of the salt-washed microtubules with Mg/ATP resulted in an ATP releasate that was significantly depleted of minus end activity, but that contained abundant plus end activity (Fig. 2A). Combination of the salt wash and ATP releasate fractions did not produce levels of activity that were higher than the sum of the individual activities (data not shown). The two fractions were subjected to electrophoresis (Fig. 2B) and immunoblotting with antibodies against the dynein heavy chain and against a conserved sequence in the kinesin motor domain (LNLVDLAGSE) (Fig. 2C). The DHC is enriched in the salt-wash fraction relative to the ATP releasate, and the relative amounts in

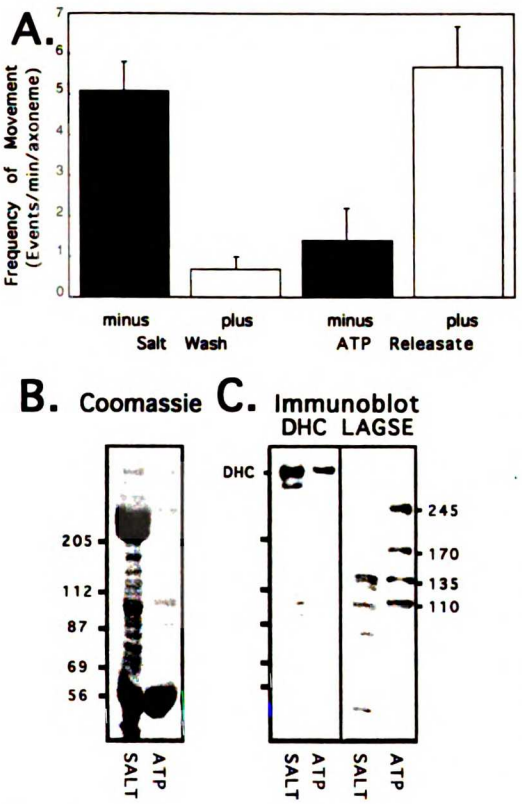


Fig. 2. Separation of minus end- and plus end-directed transport activities by differential microtubule affinity. High-speed supernatant (HSS) was incubated with microtubules, taxol, ATP-depleting reagents, and AMP-PNP. Microtubules were collected by centrifugation, resuspended in buffer containing 0.3 M KCl, and then re-sedimented. The supernatant ("salt wash") was dialyzed into assay buffer, while the pellet was further eluted with buffer containing Mg^{2+} ATP (see under Materials and Methods). **A:** Frequencies of plus end- and minus end-directed vesicle movements in the salt wash and the ATP releasate. Most of the plus end activity is retained in the ATP releasate, while the salt wash contains predominantly minus end activity. Frequencies were quantified as described in the legend to Table I. Values are averaged over three 4-min assays performed on each of three separate preparations of the two fractions. Error bars represent standard error of the mean (SEM) of the individual averages of the three preparations. **B:** Coomassie-stained gel (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) demonstrating the protein composition of the salt wash and ATP releasate. The positions of molecular weight standards are indicated on the left side of B. The thick band of approximately 245-kDa present in the salt wash is myosin, as evidenced by immunoblots using antibodies against *Dictyostelium* myosin II (not shown). **C:** Immunoblots of the two fractions using an antibody against the *Dictyostelium* dynein heavy chain and a pan-kinesin antibody (anti-LAGSE). *Tick marks (left)*, indicate the positions of the same five molecular weight standards labeled in B. The DHC is more prominent in the salt wash fraction, whereas four LAGSE-positive bands are enriched in the ATP releasate.

these two fractions correlate reasonably well with the distribution of minus end transport activity. These results further support the idea that cytoplasmic dynein is a minus end-directed organelle transport motor in this system. Conversely, four LAGSE-reactive polypeptides of approximately 245, 170, 135, and 110 kDa are selectively present in the ATP releasate compared to the salt wash, representing potential candidates for kinesin motors that may power plus end transport activity in the ATP releasate.

Overexpression of the Globular Head of Cytoplasmic Dynein Disrupts Minus End-Directed Transport

A *Dictyostelium* cell line overexpressing a C-terminal 380-kDa fragment (amino acids [aa] 1384–4725; Fig. 3A) comprising the globular head of cytoplasmic dynein (380-kDa cells; [Koonce and Samso, 1996]) displays an intriguing phenotype in which the interphase radial microtubule network can be collapsed in a whorl around the nucleus. It was suggested that this phenotype could be due to the overexpressed fragment interfering with an interaction of wild-type dynein with membranes or microtubules at the microtubule–plasma membrane interface [Koonce and Samso, 1996]. To test the possibility that the observed phenotype could be somehow correlated with a defect in dynein association with membranes and/or membrane transport, we examined vesicle motility in the 380-kDa cells using our in vitro assay. As shown in Figure 3B and C, the frequency of minus end-directed organelle movements was reduced in 380-kDa cells by 41% ($P < 0.002$) relative to the wild-type cells, while the frequency of plus end-directed movements was equivalent in the two cell types. Similarly, the velocity of minus end movements was reduced by 20% ($P < 0.001$) in the 380-kDa cell, relative to wild type, while the velocities of plus end-directed movements were equivalent.

The fact that the plus end-directed movements were unaffected in the 380-kDa cells shows that the observed defects in minus end transport are specific and are not likely to be attributable to a generalized defect in metabolic or cellular function. To further confirm this point, we also examined cells overexpressing a 318-kDa fragment of the DHC (deletion of a.a. 3105–3643 from the 380-kDa polypeptide) in our assay. The deletion in this construct is within the globular head domain, and it is unclear if this construct is folded in the same manner as the wild-type head domain; however, the 318-kDa fragment is expressed at the same levels as the 380-kDa fragment and is soluble. In contrast to the 380-kDa cells, we saw no defect in minus end transport in the 318-kDa cells relative to wild-type cells assayed in parallel (data not shown). Thus, protein overexpression alone is not

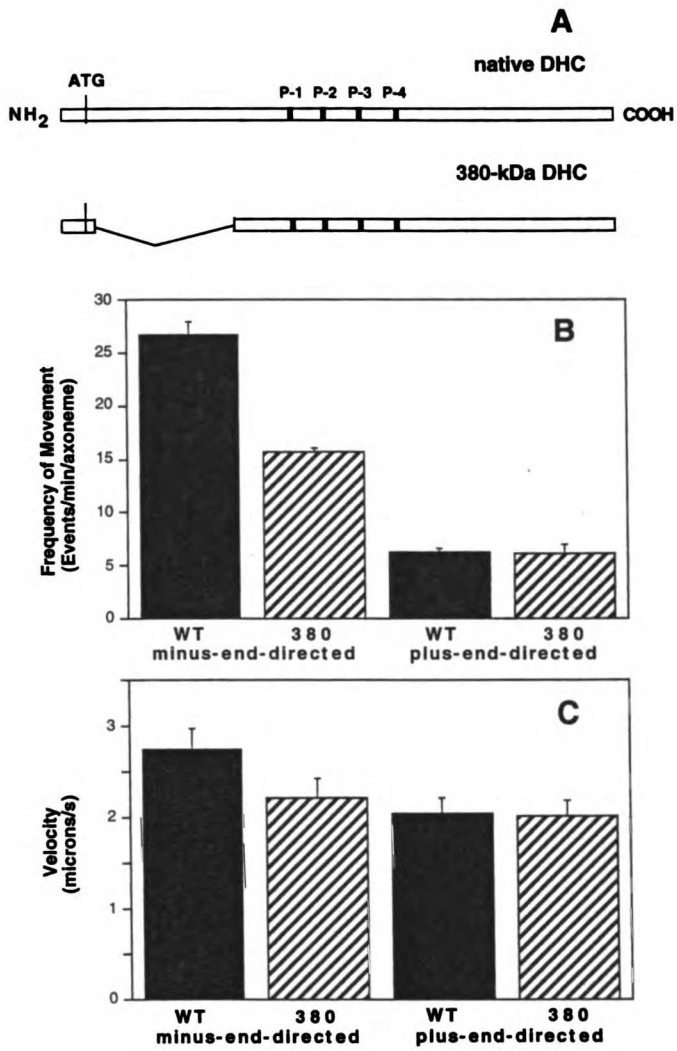


Fig. 3. The frequency and velocity of minus end-directed transport are reduced in extracts from the 380-kDa cells. **A:** The 380-kDa DHC construct as compared with the full-length DHC. The 380-kDa construct encodes the first 12 amino acids at the N-terminus, a linker encoding 3 a.a., and a.a. 1384–a.a. 4725. Closed boxes labeled P-1 through P-4 indicate P-loop motifs that form part of consensus sequences for ATP-binding domains. **B,C:** Frequencies (**B**) and velocities (**C**) of vesicle movements were quantified as described in the legend to Table I. Values are averaged over three 4-minute assays performed on each of three separate preparations of PNS from wild-type or 380-kDa cells. Error bars represent standard error of the mean (SEM) of the individual averages of the three preparations. *t*-test statistical analysis showed a statistically significant difference between the frequency and velocity of minus end-directed movements in WT vs. 380-kDa cells ($P < 0.002$), while the frequency and velocity of plus end-directed movements were equivalent within statistical limits in the two cell types. Total numbers of movements scored for velocity measurements were n = 149 and n = 133 minus end movements and n = 82 and n = 83 plus end movements for WT and 380-kDa cells, respectively.

responsible for the defect observed in minus end transport in the 380-kDa cells.

The 380-kDa Head Fragment Binds to Membranes In Vitro

The in vitro motility data suggests that the globular head is in some way able to interfere with minus end-directed vesicle transport. To examine whether this effect may be due to a dominant negative interaction that involves binding of the 380-kDa fragment to membranes, membranes from the PNS were isolated by flotation on a discontinuous sucrose gradient and the amount of DHC present was analyzed by immunoblotting. The 380-kDa fragment was associated with membranes prepared from the 380-kDa cells (Fig. 4B). However, the amount of full-length DHC bound to those membranes was similar to that in wild-type cells, suggesting that the fragment does not compete with full-length DHC for binding in vivo. When the membranes were diluted in buffer and then reisolated by sedimentation, the majority of the membrane-bound 380-kDa fragment dissociated while the full-length heavy chain was retained on the mem-

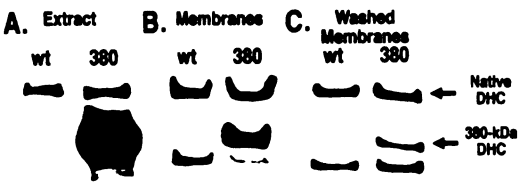


Fig. 4. Binding of the 380-kDa fragment to membranes. Membranes from extracts of wild-type (WT) and 380-kDa cells were isolated by flotation on a discontinuous sucrose gradient (see under Materials and Methods). **A:** Immunoblot of postnuclear supernatant (PNS) before centrifugation. *Arrows (right)*, indicate the location of the native DHC and the 380-kDa DHC fragment. **B:** Immunoblot of membranes collected from the 1.4 M/0.25 M sucrose interface after centrifugation. The 380-kDa fragment binds to membranes but does not reduce the amount of bound full-length DHC. The band below 380-kDa that appears in both cell types may be a degradation product recognized by the DHC antibody, as it increases with time of sample storage. **C:** Membranes isolated as in B, which were diluted 1:5 in lysis buffer and then collected by centrifugation. Comparison of B and C shows that most of the 380-kDa fragment dissociated from the membranes, whereas the full-length DHC remained bound.

branes (Fig. 4C). Thus, although the 380-kDa fragment can bind to membranes, its affinity is lower than that of the native DHC.

DISCUSSION

In this study, we have developed an in vitro assay for microtubule-based organelle transport in *Dictyostelium*, an organism tractable by both biochemical and molecular genetic approaches. Early studies reported occasional microtubule-based movements of vesicles in *Dictyostelium* extracts [McCaffrey and Vale, 1989], but such results were not consistently observed. By using an appropriate combination of buffer, protease inhibitor, and cell breakage conditions, we now can obtain robust movement in a reproducible manner. Vesicle transport can be reconstituted by recombining a high speed supernatant or a microtubule affinity-purified motor fraction with KI-washed organelles. We have also been able to separate the minus end and plus end transport activities by exploiting a difference in the salt sensitivity of their interactions with microtubules. The minus end activity can be eluted from microtubules by a wash in buffer containing 0.3 M KCl, while most of the plus end activity is retained on the microtubules during the wash and is only eluted by buffer containing 5 mM Mg/ATP. Immunoblots of the two fractions show that the DHC is more abundant in the salt wash than in the ATP releasate, correlating well with the distribution of minus end activity. The immunoblots further reveal four candidate kinesin motors in the ATP releaseate that may be involved in supporting some or all of the plus end-directed vesicle movements. This two-step elution protocol, which removes much of the dynein and myosin as well as many other polypeptides in the salt wash, provides a good starting point for further purification of the plus end transport activity.

Identifying all the components needed for organelle transport and defining their functions represent important goals for the organelle transport field. Whether components required for motility are tethered to membranes or are predominantly soluble remains controversial. In the *Dictyostelium* extracts, we have shown that the plus end transport activity is retained on vesicles upon initial isolation, though it can be subsequently removed by either a buffer or 0.3 M KI wash. By contrast, movement of vesicles in the minus end direction is dependent entirely on the addition of cytosol. This difference in the requirements for soluble factors for minus and plus end-directed vesicle transport has also been documented in squid axoplasm [Muresan et al., 1996; Schnapp et al., 1992]. However, in the squid system, components required for plus end transport remain tightly bound to vesicles even after stringent washes. It is unclear whether our results and those of other groups reflect a difference in the relative affinities of the plus end and minus end motors for membranes or, alternatively, a difference in the requirements for soluble accessory factors to activate membrane-bound motors, (e.g. dynactin for dynein-based motility; [Blocker et al., 1997; Gill et al., 1991; Schroer and Sheetz, 1991]). The assay system described here potentially can be exploited to address these issues by defining the complete set of factors necessary for the transport of an organelle.

Another approach to investigating the molecular mechanisms of organelle transport is to create mutants in known motor proteins or other factors and to examine both the in vivo and in vitro effects of the mutations on transport. While such studies are underway in *Drosophila* [Saxton et al., 1991] and have produced interesting in vivo phenotypes, in vitro organelle transport assays have not yet been developed for *Drosophila*. In *Dictyostelium*, a cell line overexpressing the globular head domain of cytoplasmic dynein was recently created (380-kDa cells; [Koonce and Samso, 1996]), representing the first available *Dictyostelium* mutant in a microtubule-based motor protein. These cells displayed a striking phenotype in which the normally radial array of interphase microtubules was collapsed toward the cell center. To examine whether this in vivo phenotype might be associated with a defect in membrane transport, we analyzed organelle transport in the 380-kDa cells using our in vitro assay. We have shown that the frequency and velocity of minus end transport were reduced in the 380-kDa cells by 40% and 20%, respectively, relative to wild-type cells. In contrast, the frequency and velocity of plus end-directed movements were equivalent in the two cell types. We have also

attempted to compare in vivo vesicle movements in the wild-type and 380-kDa cells, but these efforts were complicated by the difficulty in establishing the polarity of the in vivo movements, particularly in the 380-kDa cells which have a collapsed microtubule network.

Our in vitro organelle transport results are consistent with the suggestion by Koonce and Samso [1996] that the phenotype of the 380-kDa cells could represent a defect in dynein–membrane–microtubule interactions. The collapse of the microtubule network in the 380-kDa cells would seem to imply a specific defect in dynein-based interactions between the plasma membrane and the microtubule network that would normally serve to maintain the radial array of microtubules. Although we did not investigate the motility of plasma membrane vesicles specifically, the results of our examination of total vesicle motility are generally consistent with such a hypothesis.

Given our observation of a specific defect in minus end transport in the 380-kDa cells, what could be the molecular basis for such a defect? At the present time, the answer is unclear. One possibility is that the 380-kDa fragment could physically interfere with minus end transport by binding to microtubules and sterically blocking transport. However, such binding would most likely disrupt both plus end- and minus end-directed transport, which was not observed. Additionally, the 380-kDa fragment does not colocalize with microtubules by immunofluorescence in overexpressing cells [Koonce and Samso, 1996]. A second possibility is that the 380-kDa fragment could somehow be interfering with minus end transport by binding to membranes and exerting a dominant negative effect. In support of this idea, we have shown that the 380-kDa fragment is associated with endogenous membranes. However, its affinity for membranes is weaker than that of native dynein and it does not appear to compete with the native DHC for binding. Potentially, the membrane-bound 380-kDa fragment could exert a dominant negative effect if it interacts abnormally with microtubules and/or prevents native dynein interaction and motility. Although the precise mechanism cannot be further elucidated from the present data, the decrease in both frequency and velocity of organelle movement is consistent with this general hypothesis. An alternative possibility for a potential dominant negative action is that the 380-kDa fragment could titrate out a factor important for dynein function. One candidate for such an interaction is the 58-kDa dynein subunit, which has been shown to partially comigrate with the 380-kDa fragment upon centrifugation on a sucrose gradient [Koonce and Samso, 1996]. Alternatively, there may be small accessory light chains associated with *Dictyostelium* cytoplasmic dynein similar to those associated with mammalian brain dynein [King et al., 1996], any of which could potentially be titrated by the 380-kDa fragment.

Overall, the use of *Dictyostelium* for in vitro studies of organelle transport offers several advantages. First, our assay displays an extremely robust level of activity, supporting more movement than *Xenopus* egg extracts [e.g., Niclas et al., 1996] or mammalian extracts [e.g., Blocker et al., 1997; Schroer and Sheetz, 1991]. Second, it is possible to generate far larger quantities of material for biochemical experiments than can be obtained in other systems that display highly active organelle transport in vitro, such as squid axoplasm. Thus, the assay described here can be used to purify motors that stimulate organelle transport, as well as to identify accessory factors that may work in concert with these motors [Blocker et al., 1997; Gill et al., 1991; Schroer and Sheetz, 1991]. Finally, the effects of additional mutations in microtubule-based motors or accessory factors can be examined using this assay system.

ACKNOWLEDGMENTS

We thank members of the Vale laboratory for their helpful discussions. N.P. is a student in the Medical Scientist Training Program. M.K. is supported by NIH grant GM51532. E.L.de H. is supported by a Career Development Award from the American Heart Association. R.D.V. is supported in part by NIH grant 38499.

REFERENCES

Allan, V. (1995): Membrane traffic motors. FEBS Lett. 369:101–106.

Allen, C., and Borisy, G.G. (1974): Structural polarity and directional growth of microtubules of flagella. J. Mol. Biol. 90:381–402.

Bacon, R.A., Cohen, C.J., Lewin, D.A., and Mellman, I. (1994): *Dictyostelium discoideum* mutants with temperature-sensitive defects in endocytosis. J. Cell Biol. 127:387–399.

Blocker, A., Severin, F.F., Burkhardt, J.K., Bingham, J.B., Yu, H., Olivo, J.C., Schroer, T.A., Hyman, A.A., and Griffiths, G. (1997): Molecular requirements for bi-directional movement of phagosomes along microtubules. J. Cell Biol. 137:113–129.

Bush, J., Temesvari, L., Rodriguez-Paris, J., Buczynski, G., and Cardelli, J. (1996): A role for a rab4-like GTPase in endocytosis and in regulation of contractile vacuole structure and function in *Dictyostelium discoideum*. Mol. Biol. Cell 7:1623–1638.

De Lozanne, A., and Spudich, J.A. (1987): Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination. Science 236:1086–1091.

Gill, S.R., Schroer, T.A., Szilak, I., Steuer, E.R., Sheetz, M.P., and Cleveland, D.W. (1991): Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein. J. Cell Biol. 115:1639–1650.

Goodson, H.V., Valetti, C., and Kreis, T.E. (1997): Motors and membrane traffic. Curr. Opin. Cell Biol. 9:18–28.

Hill, K.L., Catlett, N.L., and Weisman, L.S. (1996): Actin and myosin function in directed vacuole movement during cell division in *Saccharomyces cerevisiae*. J. Cell Biol. 135:1535–1549.

Huffaker, T.C., Thomas, J.H., and Botstein, D. (1988): Diverse effects of B-tubulin mutations on microtubule formation and function. J. Cell Biol. 106:1997–2010.

Jung, G., and Hammer, J.A. III (1990): Generation and characterization of *Dictyostelium* cells deficient in a Myosin I heavy chain isoform. J. Cell Biol. 110:1955–1964.

King, S.M., Barbarese, E., Dillman, J.F.I., Patel-King, R.S., Carson, J.H., and Pfister, K.K. (1996): Brain cytoplasmic and flagellar outer arm dyneins share a highly conserved M, 8,000 light chain. J. Biol. Chem. 271:19358–19366.

Konnce, M.P. and Knecht, D.A. (1998): The cytoplasmic dynein heavy chain is an essential gene product in *Dictyostelium*. Cell Motil. Cytoskeleton 39:63-72.

Koonce, M.P., and McIntosh, J.R. (1990): Identification and immunolocalization of cytoplasmic dynein in *Dictyostelium*. Cell Motil. Cytoskeleton 15:51–62.

Koonce, M.P., and Samso, M. (1996): Overexpression of cytoplasmic dynein's globular head causes a collapse of the interphase microtubule network in *Dictyostelium*. Mol. Biol. Cell 7:935–948.

Koonce, M.P., Grissom, P.M., Lyon, M., Pope, T., and McIntosh, J.R. (1994): Molecular characterization of a cytoplasmic dynein from *Dictyostelium*. J. Eukaryote Microbiol. 41:645–651.

Kuspa, A., and Loomis, W.F. (1994): Transformation of *Dictyostelium*: Gene disruptions, insertional mutagenesis, and promoter traps. Methods Mol. Genet 3:3–21.

Lenhard, J.M., Mayorga, L., and Stahl, P.D. (1992): Characterization of endosome–endosome fusion in a cell-free system using *Dictyostelium discoideum*. J. Biol. Chem. 267:1896–1903.

Malchow, D., Naegele, B., Schwarz, H., and Gerisch, G. (1972): Membrane-bound cyclic AMP phosphodiesterase in chemotactically responding cells of *Dictyostelium discoideum*. Eur. J. Biochem. 28:136–142.

McCaffrey, G., and Vale, R.D. (1989): Identification of a kinesin-like microtubule-based motor protein in *Dictyostelium discoideum*. EMBO J.8:3229–34.

Muresan, V., Godek, C.P., Reese, T.S., and Schnapp, B.J. (1996): Plus end motors override minus-end motors during transport of squid axon vesicles on microtubules. J. Cell Biol. 135:383–397.

Niclas, J., Allan, V.J., and Vale, R.D. (1996): Cell cycle regulation of dynein association with membranes modulates microtubule-based organelle transport. J. Cell Biol. 133:585–593.

Novick, P., and Botstein, D. (1985): Phenotypic analysis of temperature-sensitive yeast actin mutants. Cell 40:405–416.

Padh, H., Ha, J., Lavasa, M., and Steck, T.L. (1993): A post-lysosomal compartment in *Dictyostelium discoideum*. J. Biol. Chem. 268:6742–6747.

Roos, U.-P., De Brabander, M., and De Mey, J. (1984): Indirect immunofluorescence of microtubules in *Dictyostelium discoideum*. A study with polyclonal and monoclonal antibodies to tubulins. Exp. Cell Res. 151:183–193.

Roos, U.-P., De Brabander, M., and Nuydens, R. (1987): Movements of intracellular particles in undifferentiated amebae of *Dictyostelium discoideum*. Cell Motil. Cytoskel. 7:258–271.

Ruscetti, T., Cardelli, J.M, Niswonger, M L., and O'Halloran, T.J. (1994): Clathrin heavy chain functions in sorting and secretion of lysosomal enzymes in *Dictyostelium discoideum*. J. Cell Biol. 126:343–352.

Sawin, K.E., Mitchison, T.J., and Wordeman, L.G. (1992): Evidence for kinesin-related proteins in the mitotic apparatus using peptide antibodies. J. Cell Sci. 101:303–313.

Saxton, W.M., Hicks, J., Goldstein, L.S., and Raff, E.C. (1991): Kinesin heavy chain is essential for viability and neuromuscular functions in *Drosophila*, but mutants show no defects in mitosis. Cell 64:1093–1102.

Schnapp, B.J., Reese, T.S., and Bechtold, R. (1992): Kinesin is bound with high affinity to squid axon organelles that move to the plus-end of microtubules. J. Cell Biol. 119:389–399.

Schroer, T.A., and Sheetz, M.P. (1991): Two activators of microtubule-based vesicle transport. J. Cell Biol. 115:1309–1318.

Shimizu, T. (1995): Inhibitors of the dynein ATPase and ciliary or flagellar motility. Methods Cell Biol. 47:497–501.

Spudich, J.A. (1987): Introductory remarks and some biochemical considerations. Methods Cell Biol. 28:3–8.

Sussman, M. (1987): Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. Methods Cell Biol. 28:9–29.

Chapter 2

Reconstitution of Membrane Transport Powered by a Novel Dimeric Kinesin Motor of the Unc104/KIF1A Family Purified from *Dictyostelium*

Abstract

Motor-powered movement along microtubule tracks is important for membrane organization and trafficking. However, the molecular basis for membrane transport is poorly understood, in part because of the difficulty in reconstituting this process from purified components. Using video microscopic observation of organelle transport in vitro as an assay, we have purified two polypeptides (245 kDa and 170 kDa) from *Dictyostelium* extracts that independently reconstitute plus-end-directed membrane movement at in vivo velocities. Both polypeptides were found to be kinesin motors, and the 245 kDa protein (called DdUnc104) is a close relative of *C. elegans* Unc104 and mouse KIF1A, neuron-specific motors that deliver synaptic vesicle precursors to nerve terminals. A knockout of the DdUnc104 gene produces a pronounced defect in organelle transport in vivo and in the reconstituted assay. Interestingly, DdUnc104 functions as a dimeric motor, in contrast to other members of this kinesin subfamily which are monomeric.

Introduction

Movement of membrane organelles along microtubules plays an important role in the organization of the cytoplasm and the delivery of macromolecules in most eukaryotes (reviewed in Goodson et al., 1997; Lane and Allan, 1998). Distributions of large intracellular membrane compartments, such as the perinuclear location of the Golgi and lysosomes and the extended reticular network of the endoplasmic reticulum, are achieved through microtubule-based movement. Transport between compartments in the secretory or endocytic pathways also relies upon active transport along a polarized microtubule network. In most cells, the microtubules extend from the centrosome, where their minus-ends are located, towards the plasma membrane. This organization provides a navigational system for membrane trafficking between the cell center and the periphery.

Membrane transport along microtubules is driven by several types of ATP-hydrolyzing molecular motors. Cytoplasmic dynein (minus-end-directed) (Harada et al., 1998; Schnapp and Reese, 1989; Schroer et al., 1989) and 3 classes of plus-end-directed kinesin proteins (the conventional kinesins (Hollenbeck and Swanson, 1990; Schroer et al., 1988; Tanaka et al., 1998), the heteromeric kinesins (e.g. KRP85/95, KIF3A/B) (Morris and Scholey, 1997; Nonaka et al., 1998; Tuma et al., 1998), and the 'monomeric' kinesins (e.g. Unc104, KIF1A, KIF1B) (Hall and Hedgecock, 1991; Nangaku et al., 1994; Yonekawa et al., 1998)) have all been shown to serve as motors for organelle transport. Rabkinesin-6 (Echard et al., 1998) and KIFC2 (Hanlon and Goldstein, 1997; Saito et al., 1997) are also candidate organelle transport motors. None of the above motors, however, was discovered based upon an ability to stimulate organelle movement. Rather, they were initially

identified through in vitro microtubule gliding assays (kinesin (Vale et al., 1985), cytoplasmic dynein (Paschal et al., 1987)), antibody (e.g. KRP85/95; Cole et al., 1992) and PCR (KIFs; Aizawa et al., 1992) screens designed to uncover new kinesin superfamily members, or genetic screens in *C. elegans* for muscular defects (Unc104 (Hall and Hedgecock, 1991)). The involvement of these motors in organelle transport was later established using approaches such as gene knockouts (Gindhart et al., 1998; Harada et al., 1998; Nonaka et al., 1998; Saxton et al., 1991; Seiler et al., 1997; Tanaka et al., 1998), in vivo antibody microinjections (Lippincott-Schwartz et al., 1995; Morris and Scholey, 1997; Rodionov et al., 1991; Tuma et al., 1998), co-localization with membranes (Okada et al., 1995; Pfister et al., 1989), and inhibition of organelle transport in vitro by antibodies, pharmacologic inhibitors, or immunodepletion (Blocker et al., 1997; Schnapp and Reese, 1989; Schroer et al., 1988; Schroer et al., 1989).

The above studies have provided valuable insight into both the biological roles of and types of subcellular membranes transported by these motors. Nevertheless, the mechanisms by which motors interact with and move their cargoes are poorly understood. In vitro organelle transport studies, however, have shown that cytoplasmic dynein is incapable of moving organelles by itself and requires dynactin as a cytosolic activator (Gill et al., 1991; Schroer and Sheetz, 1991). Dynactin's importance for dynein-based organelle transport in vivo was subsequently confirmed by genetic and dominant negative studies (Burkhardt et al., 1997; McGrail et al., 1995; Tinsley et al., 1996). Although dynactin's precise role is unclear, recent studies have shown that the Arp1 subunit of dynactin interacts with a spectrin isoform, which may provide a mechanism for docking dynein onto intracellular membranes (Holleran et al., 1996). Another study, however,

has reported that rhodopsin acts as a direct dynein receptor in the absence of dynactin (Tai et al., 1999).

Less is known about the mechanism of organelle motility driven by plus-end-directed motors. Whether soluble activators are required for kinesin-based in vitro organelle motility has been controversial (Schnapp et al., 1992; Schroer et al., 1988; Schroer and Sheetz 1991; Urrutia et al., 1991). A potential receptor for conventional kinesin (kinectin) has been identified through affinity chromatography (Kumar et al., 1995; Toyoshima et al., 1992), but its role in vivo has not been established. Furthermore, unlike the in vitro assays for cytoplasmic dynein described above, plus-end-directed organelle transport has not been faithfully reconstituted using a biochemically defined system. Highly purified or recombinant conventional kinesin has been shown to bind to membranes (Skoufias et al., 1994), but not to reproducibly elicit their movement on microtubules (Schroer et al., 1988).

To make progress on understanding the molecular basis of plus-end-directed organelle transport, a system amenable to both in vitro biochemical reconstitution and genetic analysis is needed. Here, we have developed such a system using the cellular slime mold *Dictyostelium discoideum*. Using video microscopic observation of in vitro organelle movement as a biochemical fractionation assay, we have purified two polypeptides that independently reconstitute transport. When combined with salt-washed vesicles that do not move on their own, the purified proteins faithfully reconstitute plus-end-directed movement with the same properties observed in crude extracts and in vivo, suggesting that they are likely to be physiologically important for this process. One of the purified factors is a novel dimeric kinesin that is closely related to Unc104 and KIF1A, monomeric kinesin motors that transport synaptic vesicle precursors in

Materials and Methods

Purification of Plus-end-directed Transport Activity

Dictyostelium HSS was prepared as previously described (Pollock et al., 1998). Wild-type cells (5 l at a density of 4-8 x 10^6 cells/ml in HL-5 media (Sussman, 1987) containing 100 μg/ml streptomycin and 100 U/ml penicillin) were collected by centrifugation at 4,000g for 15 min at 4°C and washed in 1 l ice-cold Sorenson's phosphate buffer, pH 6.0 (Malchow et al., 1972). Cells were resuspended in 1:1 w/v lysis buffer (LB: 30 mM Tris-HCl (pH 8.0), 4 mM EGTA, 3 mM DTT, 5 mM benzamidine, 10 μg/ml soybean trypsin inhibitor, 5 μg/ml TPCK/TAME, 10 μg/ml leupeptin, pepstatin A, and chymostatin, and 5 mM PMSF (phenylmethyl-sulfonyl fluoride)) containing 30% (w/v) sucrose. The suspension was split into thirds and each third was lysed by one passage through a Nuclepore polycarbonate filter (5-μm pore size, 47-mm diameter; Costar, Pleasanton, CA) using a 10-ml syringe. The lysate was centrifuged at 2,000g for 5 min at 4°C. The resulting post-nuclear supernatant (PNS) was layered over a 1-ml cushion of LB/25% sucrose and centrifuged in a Beckman TLA 100.4 rotor at 180,000g for 15 min at 4°C to obtain a high speed supernatant (HSS). 5 l of cells generated approximately 20 ml of HSS.

A microtubule-affinity-purified fraction (ATP releasate) was then prepared from the HSS as previously described (Pollock et al., 1998). Briefly, HSS was incubated with 15 U/ml hexokinase, 3 mM glucose, 4 mM AMP-PNP/$MgCl_2$, 20 μM taxol, and 0.5 mg/ml taxol-stabilized microtubules for 20 min on ice. Microtubules and associated proteins were centrifuged through a 1 ml cushion of LB/25% sucrose containing 20 μM taxol at 85,000g for 15 min in a Beckman TLA 100.4 rotor (4°C). The microtubule pellet was

resuspended in LB/5% sucrose containing 0.3 M KCl (1:12 vol of the original HSS volume) and immediately recentrifuged at 85,000g for 15 min in a Beckman TLA 100.4 rotor (4°C). The resulting supernatant ("salt wash"), containing predominantly minus-end-directed transport activity, was removed. The microtubule pellet was resuspended in phosphate buffer (10 mM potassium phosphate (pH 6.8), 1 mM EGTA, 3 mM DTT, 5 mM benzamidine, 10 μg/ml soybean trypsin inhibitor, 5 μg/ml TPCK/TAME, 10 μg/ml leupeptin, pepstatin A, and chymostatin, and 5 mM PMSF)/5% sucrose containing 5 mM ATP/$MgCl_2$ (1:20 vol of the original HSS volume) to elute the plus-end-directed transport activity. After incubation for 15 min on ice, microtubules were separated from the released proteins by centrifuging at 90,000g for 15 min in a Beckman TLA 120.1 rotor (4°C). The supernatant (ATP releasate) was collected and either assayed directly (see below) or frozen in liquid nitrogen.

1.5-2 ml of ATP releasate in phosphate buffer/5% sucrose was diluted to 2.26 ml in phosphate buffer/5% sucrose and $CaCl_2$ was added to 1.2 mM. 2.2 ml of the releasate was then loaded onto a 1.8 ml hydroxyapatite column (20 μm ceramic hydroxyapatite; American International Chemical, Natick, MA) equilibrated in phosphate buffer/5% sucrose (for hydroxyapatite and Mono Q chromatography buffers, protease inhibitors included only benzamidine (5 mM), TPCK/TAME (5 μg/ml), and PMSF (1 mM)). The column was run on the SMART chromatography system (Amersham Pharmacia Biotech, Piscataway, NJ). Sample was loaded at a flow rate of 250 μl/min and 600 μl fractions were eluted at 500 μl/min in two steps of 200 mM and 300 mM potassium phosphate (3.6 ml each). For motility assays, 45 μl aliquots of all fractions were dialyzed for 2 hr against LB pH 8.0/5% sucrose using a microdialyzer system 100 (Pierce, Rockford, IL), exchanging

the buffer in the chamber once after 30 min of dialysis. For further chromatography, the 200 mM potassium phosphate peak fractions were pooled and dialyzed against LB pH 9.0/5% sucrose using a microdialyzer system 500 (Pierce Chem. Co.).

The dialysate of the pooled hydroxyapatite fractions was diluted to 2.26 ml in LB pH 9.0/5% sucrose and 2.2 ml was loaded onto a 100 µl SMART system Mono Q column (MonoQ PC 1.6/5, Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in LB pH 9.0/5% sucrose. The column was loaded at 50 µl/min, and 100 µl fractions were eluted at 100 µl/min with a 115 mM NaCl step elution (500 µl) followed by a 170-300 mM NaCl gradient (1 ml). 45 µl aliquots of fractions were dialyzed against LB pH 8.0/5% sucrose as above for use in motility assays.

In Vitro Organelle Transport Assays

Sea urchin sperm axoneme-nucleated microtubule structures were prepared in flow cells (10 µl) as described previously (Pollock et al., 1998). KI vesicles were also prepared as described previously (Pollock et al., 1998); these vesicles, which are a mixed population isolated from the crude extract, were isolated by sedimentation through a sucrose cushion, treated with 0.3 M KI to dissociate weakly bound proteins, and then re-sedimented through a sucrose cushion. The flow cells were first washed with 10 µl of LB pH 8.0 (without PMSF)/15% sucrose to remove any free microtubules, followed by the introduction of 10 µl of assay mix consisting of, in the case of HSS, 5 µl of HSS, 3.5 µl LB/15% sucrose, 1 µl KI-washed vesicles, and 0.5 µl ATP-regenerating mix (Pollock et al., 1998). Assay mixes for the ATP releasate consisted of 5 µl of ATP releasate, 3.0 µl LB/15% sucrose, 1 µl KI-washed vesicles, 0.5 µl of a 20 mg/ml casein stock, and 0.5 µl ATP-regenerating mix.

Assay mixes for fractions from the hydroxyapatite and Mono Q columns (including the hydroxyapatite load) consisted of 8 µl of the fraction, 1 µl KI vesicles, 0.5 µl casein, and 0.5 µl ATP regenerating mix. The movement of organelles was observed using a Zeiss Axioplan microscope equipped with differential interference contrast (DIC) optics, a 50- or 100-W mercury arc lamp, and a 63x, 1.4 NA Plan-Neofluor objective. Images were detected using a camera (Newvicon; Hamamatsu Photonics, Bridgewater, NJ); contrast enhancement and background subtraction were performed with an image processor (Argus10; Hamamatsu Photonics), and recordings were made with a super VHS video tape recorder (AG-5700; Panasonic, Secaucus, NJ).

Organelle motility was quantified by counting the number of movements in each direction on a single axoneme/microtubule structure. Only axonemes with clearly defined polarity were used. Recordings were performed on axonemes with between 6 and 12 microtubules (8-14 µm each in length) polymerized from the plus ends. If an organelle paused briefly and then continued in the same direction, it was scored as a single movement. Velocities of movements were measured using an NIH-IMAGE-based measuring program developed by J. Hartman; only vesicles which moved smoothly over a distance of at least 1.5-2 µm were scored.

In Vivo Transport Assays

To observe linear organelle movements in live cells, 20 µl of null or control cells (grown to 3-7x10^6 cells/ml in HL-5 media containing 100 µg/ml streptomycin, 100 U/ml penicillin and 5 µg/ml blasticidin (ICN, Costa Mesa, CA)) were introduced into flow cells (20 µl) made with coverslips that had been incubated overnight in 1M HCl and then washed extensively with

water. The flow cells were inverted for 2 min and then viewed by DIC optics as described above. Each cell was observed for 4 min, and the slide was discarded after 10 min of observation. Only linear and continuous movements >1 μm in length were scored.

To observe the movement of mitochondria in live cells, $1x10^8$ cells were collected by centrifugation at 800g for 3 min at room temperature (25°C). Cells were resuspended in 10 ml HL5 containing P/S/blasticidin and 50 nM Mitotracker (Molecular Probes, Eugene, OR). Cells were incubated for 15 min at 22°C while shaking at 180 rpm. The cells were repelleted and washed 1x in 10 ml Sorenson's phosphate buffer at room temperature (25°C) and resuspended in 5 ml HL5 plus P/S/blasticidin. Cells were incubated for 30 min at 22°C while shaking at 180 rpm. Cells were then diluted 1:5 in phosphate buffer and flowed into flow cells (20 μl) prepared with acid-washed coverslips as described above. The flow cell was inverted for 2 min and then viewed by fluorescent optics using a 63x, 1.4 NA objective and a SIT camera (Hamamatsu Photonics, Bridgewater, NJ). Each cell was viewed for 1 min and the slide was discarded after a maximum of 15 min of observation. Only linear and continuous movements >1 μm in length were scored.

Hydrodynamic Analysis

Sucrose density gradients. 200 μl of ATP releasate in LB pH 8.0/5% sucrose/0.15M NaCl was loaded onto a 2.2 ml 10-25% continuous sucrose gradient (in LB/0.15M NaCl) and spun for 5 hr at 200,000g in a Beckman TLS-55 rotor (4°C). In parallel, a 200 μl mix of calibration standards (BSA, 4.3S; aldolase, 7.4S; catalase, 11.3S; ferritin, 17.6S; and thyroglobulin, 19.4S) at 1 mg/ml each in LB/5% sucrose/0.15 M NaCl was run on a separate gradient. 200 μl fractions were collected from each gradient. The sedimentation

profiles of the calibration proteins were monitored by Coomassie staining, while the profile of DdUnc104 was monitored by immunoblotting with an affinity-purified DdUnc104 peptide antibody (see below). The S value of DdUnc104 was determined by comparing its position in the gradient to the positions of the standards plotted against their S values.

Gel Filtration. 50 µl of the DdUnc104 peak from the sucrose gradient was loaded onto a 2.4 ml gel filtration column (SMART system: Superose 6 PC 3.2/30, Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in 30 mM Tris-HCl (pH 8.0), 4 mM EGTA, 3 mM DTT, 5 mM benzamidine, 5 µg/ml TPCK/TAME, and 0.15 M NaCl. The column was run at 30 µl/min and 50 µl fractions were collected. The elution profile of DdUnc104 was again monitored by immunoblotting. The elution profiles of calibration standards with known Stokes radii (ovalbumin, 3.1 nm; aldolase, 4.8 nm; catalase, 5.2 nm; ferritin, 6.1 nm; and thyroglobulin, 8.5 nm) prepared in the same buffer were assessed by Coomassie stain. Linear plots of the $(-\log K_{av})^{1/2}$ versus Stokes radius for each standard were used to determine the Stokes radius of DdUnc104 from its K_{av} ($K_{av} = V_e-V_o/V_t-V_o$, where V_e = elution volume, V_o = void volume as determined by the elution volume of blue dextran, and V_t = total column volume or the total accessible volume as assessed by the elution profile of 1 M NaCl).

Partial specific volume and axial ratio calculations were made using the Sednterp program developed by John Philo. Coiled-coil predictions were made using the worldwide web version of the program COILS (Lupas et al., 1991) available at http://www.isrec.isb-sib.ch/software/COILS.

Peptide sequencing

Mono Q fractions containing the 245 kDa and 170 kDa polypeptides were pooled, TCA precipitated, and separated on 4-12% gradient polyacrylamide gels (Novex, San Diego, CA) under denaturing and reducing conditions followed by staining with Coomassie Blue. After cutting out the protein bands, in-gel digestion with Endoproteinase Lys- C (Boehringer Mannheim, Indianapolis, IN) was carried out as described (Hellman et al., 1995). Gel-extracted peptides were then fractionated using a Vydac microbore C8 column (The Separations Group, Hesperia, CA) and individual peptides subjected to Edman degradation with a Protein Sequencer, Model 492 (PerkinElmer, Applied Biosystems Division, Foster City, CA).

Peptide Antibody Production and Immunoblotting

A peptide based upon DdUnc104 amino acids 359-373 was synthesized with a C-terminal cysteine and used for immunization of a rabbit (QCB, Inc., Hopkinton, MA). Peak bleeds were pooled, and the antibody was purified against the synthetic peptide coupled to a thiol coupling resin using the QCB standard antibody purification protocol. The purified antibody was dialyzed into 80 mM Tris-HCl pH 8.0, 4 mM EGTA for storage.

For immunoblotting, samples were separated on 4-12% gradient polyacrylamide gels (Novex, San Diego, CA) and electroblotted to nitrocellulose membranes at 100 mA for 75 min. The blots were incubated with the DdUnc104 peptide antibody (1:500) overnight at 4°C, and then incubated in HRP-conjugated secondary antibody (1:2000; Amersham Life Sciences, Arlington Heights, IL) for 1 hr at room temperature. Blots were developed using a chemiluminescence kit (NEN Life Sciences, Beverly, MA) and exposed to Hyperfilm (Amersham).

Cloning of the DdUnc104 Gene

One of the peptide sequences of the 245 kDa protein was class-conserved among members of the Unc104/KIF1A subfamily. Therefore, fully degenerate oligonucleotides corresponding to the VVNEDAQ peptide (a.a. 360-366) obtained from peptide sequencing and to a sequence highly conserved in the Unc104/KIF1A family, KSYTMMG, were used to prime a PCR reaction using *Dictyostelium* oligo (dT)-primed 1st strand cDNA as a template (Superscript Preamplification System; Gibco BRL, Grand Island, NY). The expected ~780 bp PCR product was cloned and sequenced. Primers made to this PCR product were used to generate a 470 bp probe which was used to screen a *Dictyostelium* vegetative cDNA library (λZAP; Stratagene, La Jolla, CA; kindly provided by Dr. Rick Firtel, UCSD). A ~2 kb DdUnc104 cDNA clone containing the starting methionine and an upstream stop codon was obtained from the screen; however, this cDNA terminated prematurely. To obtain further downstream sequence, total RNA was extracted from vegetative cells (Trizol, Gibco BRL) and poly A^+ RNA was further purified using an mRNA separator kit (Clontech, Palo Alto, CA). The poly A^+ RNA was used as a template to generate cDNA which was then used for a 3'RACE reaction (Rapid Amplification of cDNA Ends; Marathon cDNA Amplification Kit, Clontech) using a primer based on the 3' end of the λZAP fragment. This method yielded a 1.8 kb DdUnc104 fragment that was continuous with the λZAP fragment but which still terminated prematurely. Primers based on the 3' end of this new fragment were used in a new 3' RACE reaction, using the same cDNA pool, to obtain a 3 kb fragment containing the remainder of the DdUnc104 gene. (Note: the DdUnc104 gene is also known as *ksnA* in *Dictyostelium* nomenclature.) To confirm the sequences obtained from the RACE products, two additional independent

PCR reactions were performed for each segment and the resulting clones sequenced. Percent identity between protein sequences was calculated using the worldwide web version of the program Blast available at http://www.ncbi.nlm.nih.gov/BLAST. Multiple sequence alignment was done with the program Pileup (Genetics Computer Group) and the output was shaded using MACBOXSHADE.

Disruption of the DdUnc104 gene

A 1.4 kb cassette conferring blasticidin resistance (Adachi et al., 1994) was cloned between the adjacent BglII sites (nt #2115-2134) in the DdUnc104 sequence. The 3.2 kb construct was released from the plasmid by digestion with EcoRV (nt 1484) and Xba I (nt 3306), and transformed into cells by electroporation as described (de Hostos et al., 1993). Transformants were selected on Petri plates containing liquid DD-broth20 media (Manstein et al., 1995) supplemented with 5 μg/ml of blasticidin (ICN, Costa Mesa, CA). Isolated colonies were picked and transferred to 24-well plates. After further growth, the cells were collected for screening by PCR. Approximately 10^6 cells were resuspended in 50 μl lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 0.3% Tween-20, 60 μg/ml proteinase K) and incubated for 45 min at 56°C and 10 min at 94°C. One μl of the crude lysate was used as a template for 25 μl PCR reactions set up using standard protocols. Pairs of a primer specific to the blasticidin resistance cassette and a DdUnc104-specific primer flanking the disruption site were used for the PCR. Two independent null cell lines were obtained and were shown to behave identically. Genomic DNA was prepared from selected transformant strains (Qiagen, Valencia, CA) and analyzed by Southern blotting as described previously (de Hostos et al., 1998) using the EcoRV-XbaI fragment of the DdUnc104 cDNA as a probe. For

Northern analysis, RNA was prepared using Trizol (Gibco BRL) and the EcoRV-XbaI fragment was used as a probe. For Western analysis, 5 µl of ATP releasates prepared from null and control cells (see below) were immunoblotted using the affinity-purified DdUnc104 antibody.

Preparation of ATP releasates from DdUnc104 null and control cells

For analysis of in vitro motility, ATP releasates were prepared from null and control cells (500-800 ml at a density of 0.6-1x10^7 cells/ml in HL5 containing P/S/blasticidin) as described above, with two modifications. First, the 0.3 M KCl wash prior to ATP elution of the microtubule pellet was omitted to preserve minus-end motility in the ATP releasate. Second, the buffer for the ATP release was LB pH 8.0/5% sucrose containing 5 mM ATP/$MgCl_2$.

Results

Purification of Proteins that Stimulate Plus-End-Directed Organelle Transport from Dictyostelium Extracts

Previously, we developed a method of preparing extracts from *Dictyostelium discoideum* that supported abundant bidirectional microtubule-based vesicle transport; the velocities and travel distances of moving organelles were similar to those displayed in vivo (Pollock et al., 1998; Roos et al., 1987). We also showed that motion of KI-washed organelles, which did not move on their own, could be reconstituted by the addition of a high-speed supernatant (HSS) (Pollock et al., 1998). Here, we wished to purify the factor(s) responsible for plus-end-directed vesicle transport using a biochemical fractionation scheme. Microtubules grown in a polarized manner from salt-extracted axonemes (Allen and Borisy, 1974) were used to determine the direction of vesicle movement in these experiments. Microtubule affinity was used as a first purification step, since both plus- and minus-end vesicle transport activities cosediment with exogenously added microtubules in the absence of ATP and the presence of AMPPNP and can be subsequently released from the microtubules by ATP (Pollock et al., 1998). Such procedures are known to enrich for microtubule motors and associated proteins. Furthermore, the plus-end-directed organelle transport activity could be partially separated from the more abundant minus-end-directed activity by incubating the microtubules with 0.3 M KCl (to extract minus-end-directed activity selectively) prior to the ATP release step. This microtubule affinity procedure resulted in a 80-fold increase in specific activity for plus-end-directed organelle transport relative to the HSS (Table I).

To rule out the possibility that the vesicle transport observed was due to nonspecific interactions with membrane lipids, we tested whether treatment of KI vesicles with trypsin (100 μg/ml for 30 min on ice followed by soybean trypsin inhibitor (SBTI) at 4 mg/ml for 10 min) reduced their movement in the presence of the ATP release fraction. Plus-end-directed vesicle motility was decreased 3-fold by this treatment compared to a control in which trypsin was preblocked with SBTI prior to addition to the vesicles. Thus, proteins on the vesicle surface play an important role in motility.

Since the ATP release fraction contained numerous polypeptides (Fig. 1A load), we further purified the plus-end-directed vesicle transport activity using hydroxyapatite chromatography (Fig. 1A). The activity bound to the column and could be eluted by potassium phosphate. Three prominent polypeptides appeared in the activity-containing fractions: the ~540 kDa dynein heavy chain and two polypeptides of 245 kDa and 170 kDa. Even though dynein was prominent in the hydroxyapatite eluate, very little (<2 movements/axoneme) or no minus-end-directed transport was observed in several independent purifications.

The peak fractions of the hydroxyapatite column were pooled, applied to a Mono Q column and eluted with a shallow NaCl gradient, which proved necessary to separate the 245 kDa and 170 kDa polypeptides from one another (Fig. 1B). The organelle transport activity peaked consistently with the 245 kDa polypeptide in six purification efforts, but activity was also detected in the later-eluting fractions that contained both the 170 kDa and 245 kDa polypeptides. In the Mono Q column, cytoplasmic dynein eluted earlier than the peak of plus-end-directed vesicle transport (Fig. 1B). Again, little or no minus-end transport activity was observed in the peak dynein fractions.

The co-fractionation of activity with the 245 kDa polypeptide in the Mono Q column strongly implicated it as a factor that can stimulate plus-end motility. However, in the hydroxyapatite column, an early-eluting fraction containing the 170 kDa but not the 245 kDa polypeptide (fraction 16 in Fig. 1A) also stimulated transport. This finding raised the possibility that the 170 kDa polypeptide was also capable of promoting plus-end-directed organelle movement. To address whether both polypeptides were truly independent transport-stimulating factors, we performed two additional experiments. First, we diluted a peak 245 kDa Mono Q fraction (containing nominal 170 kDa polypeptide) to a level of activity which was 25% of that in the 170 kDa peak fraction. By silver staining, the quantity of the 245 kDa polypeptide in this diluted fraction was greater than that found in an equal volume of the four-fold more active 170 kDa peak fraction. This result indicates that organelle transport in the 170 kDa peak fraction could not be stimulated solely by the 245 kDa polypeptide contaminating this fraction.

Next, we measured the velocities of plus-end-directed movements from column fractions that had minimal overlap in their content of 170 and 245 kDa polypeptides (Fig. 2). Movement velocities from the 245 kDa- or 170 kDa-enriched fractions were 2.62 ± 0.50 ($n = 27$) and 1.86 ± 0.74 μm/sec ($n = 23$) respectively (mean ± SD; $p < 0.001$). The velocity histogram of movements generated by the 170 kDa fraction appeared bimodal. The majority of movements (65%) were between 1-2 μm/sec; in contrast, this velocity range represented only 11% of the movements stimulated by the 245 kDa fraction. The 170 kDa fraction also contained a subset of fast movements between 2-3.5 μm/sec, which corresponded to the average velocity produced by the 245 kDa fraction. Since the 170 kDa fraction contained some contaminating 245 kDa even in our purest fractions (Fig. 2), it seems likely

that these few fast movements were due to the 245 kDa polypeptide. Thus, we conclude that the 245 kDa and 170 kDa polypeptides both stimulate plus-end-directed organelle transport, but at different velocities.

Our hydroxyapatite and Mono Q fractionation experiments showed that the amount of organelle transport in our assay was always in quantitative agreement with the amount of the 245 and 170 kDa polypeptides in the fraction. From examination of silver stained gels from many columns, we could not identify any other polypeptide that clearly and consistently copurified with either the 245 kDa or 170 kDa polypeptides (though our gel system would not have clearly resolved polypeptides <25 kDa). From this data, we believe that that the 245 kDa and 170 kDa polypeptides can stimulate transport in the absence of associated polypeptides, although a low molecular weight factor or a catalytic activity present in low stoichiometry could have been present and not detected in this analysis.

Identification of the 245 kDa and 170 kDa Polypeptides as Kinesin-Related Proteins

To establish the molecular identity of the 245 kDa and 170 kDa polypeptides, we obtained peptide sequence from tryptic fragments. Both polypeptides contained peptide sequences that implicated them as members of the kinesin superfamily. A peptide from the 170 kDa protein contained the sequence LYLVDLAGSEK, which includes the highly conserved DLAGSE motif of the switch II region of the kinesin nucleotide binding site (Sablin et al., 1996). The 245 kDa polypeptide yielded a tryptic fragment containing the conserved VIxAL motif found in the α4 helix of kinesin motors. Since the 245 kDa polypeptide was the more robust motor in our in vitro organelle

transport assay, we chose this kinesin for further cloning and characterization.

The 245 kDa polypeptide was cloned using a combination of degenerate PCR, library screening, and rapid amplification of cDNA ends (RACE) (see Materials and Methods for details). The complete open reading frame predicts a protein of 2205 amino acids and a molecular weight of 248 kDa and contains all five peptides identified by direct sequencing (Fig. 3A). We are therefore confident that we cloned the correct polypeptide identified in our biochemical purification. Sequence alignments revealed a clear homology of the 245 kDa polypeptide to the Unc104/KIF1A class of kinesin motors (Fig. 3A). Two founding members of this class, mouse (Mm) KIF1A (190 kDa) and the *C. elegans* (Ce) ortholog Unc104 (174 kDa), are monomeric kinesin motors that have been implicated in the transport of synaptic vesicle precursors in neurons (Hall and Hedgecock, 1991; Okada et al., 1995; Yonekawa et al., 1998). The Unc104/KIF1A class also includes motors implicated in other functions such as mitochondrial transport (KIF1B; Nangaku et al, 1994), Golgi-ER transport (KIF1C; Dorner et al, 1998), and cytokinesis (Klp38B; Ohkura et al, 1997).

The sequence of the 245 kDa kinesin reveals the following domain structure. The first 353 residues show high amino acid identity to the conserved catalytic core that defines the kinesin superfamily (Vale and Fletterick, 1997). Within the kinesin superfamily, the catalytic core of the 245 kDa kinesin is most similar to the Unc104/KIF1A class (40-60% identical residues). All members of the Unc104/KIF1A class also contain a highly conserved extension in loop 3 (L3), which is unique among kinesins (R. Case and R.Vale, unpublished observations). This L3 extension is also found in the 245 kDa kinesin (Fig. 3A). Several, but not all, members of the

Unc104/KIF1A class contain several lysine residues in the microtubule-binding loop (L12) (Okada and Hirokawa, 1999) (Fig. 3A). In the 245 kDa kinesin, this loop only contains two lysines and thus is much less charged.

For approximately 250 a.a. following the catalytic core, the 245 kDa kinesin shows significant identity to all kinesins in the Unc104/KIF1A class (Fig. 3A, B). The latter half of this class-conserved region contains a domain that is also found in non-motor proteins such as human AF-6 and *Drosophila* cno (Ponting, 1995), but whose function is poorly understood. Beyond the AF-6/cno domain, the 245 kDa kinesin continues to exhibit strong homology to CeUnc104 and MmKIF1A for an additional ~370 a.a.; however, sequence identity to other Unc104/KIF1A class kinesins (e.g. KIF1B) does not continue far past the AF-6/cno domain (Fig. 3B). The sequence in the middle portion of the 245 kDa kinesin (residues 973-1522) is novel, and contains repeats of glutamine or serine which are often found in *Dictyostelium* proteins (de Hostos et al., 1998; Shaw et al., 1989). Residues 1523-1616 are predicted to form a pleckstrin homology (PH) domain, whose greatest similarity in blast searches is to a C-terminal PH domain in MmKIF1A and CeUnc104 (Fig. 3A). This PH domain is not found in other motors belonging to this kinesin class (Fig. 3B). The sequences of MmKIF1A and CeUnc104 end shortly after the PH domain, but the 245 kDa kinesin extends for another 589 amino acids. This region does not exhibit significant sequence identity to any other protein in the data base. In conclusion, the 245 kDa kinesin shows more identity to the two ortholog motors CeUnc104 and MmKIF1A than to other members of the Unc104/KIF1A class. Since there is somewhat more non-motor domain homology to CeUnc104 than to MmKIF1A, we term the 245 kDa kinesin *Dictyostelium* (Dd) Unc104.

We also examined DdUnc104 for possible coiled-coil domains using a sequence prediction program (Lupas et al., 1991). This analysis revealed that both MmKIF1A and DdUnc104 contain two regions in the N-terminal half of the molecule (~a.a. 359-448 and 652-679 in DdUnc104), one of which is also present in CeUnc104, that have a propensity towards coiled-coil formation (Fig. 3C). However, hydrodynamic analyses of MmKIF1A (Okada et al., 1995) and CeUnc104 (Pierce et al., 1999) indicated that these regions do not cause dimerization. Interestingly, the majority of the unique ~600 a.a. extension of DdUnc104 was also predicted to have a high probability of coiled-coil formation (Fig. 3C), raising the possibility that DdUnc104, in contrast to MmKIF1A and CeUnc104, is dimeric.

DdUnc104 is a Homodimer

To determine the quaternary structure of DdUnc104, we analyzed its hydrodynamic behavior using velocity sedimentation through sucrose density gradients and gel filtration (see Materials and Methods). In two separate experiments, the S value was 10.9 and 10.2 S (avg. 10.6). The sucrose gradient peak was then subjected to gel filtration, and a Stokes radius of 10.5 nm was measured in both experiments. Together with a partial specific volume of 0.73 cm^3/g estimated from the DdUnc104 amino acid sequence, the native molecular weight was determined to be 480 kDa. Given the polypeptide's molecular weight of 248 kDa, this result indicates that DdUnc104 is a dimer and also argues against the presence of a stoichiometrically-associated polypeptide of significant mass. These hydrodynamic data also suggest that DdUnc104 has an extended shape with an axial ratio of 15.1. Thus, DdUnc104 is the first dimeric motor described for the Unc104/KIF1A class of kinesin motors.

A Knockout of DdUnc104 Causes In Vitro and In Vivo Defects in Organelle Transport

To learn more about the function of DdUnc104 in vivo, the DdUnc104 gene was disrupted by homologous recombination. A gene disruption construct was made by inserting a blasticidin resistance cassette into a DdUnc104 cDNA fragment. The linearized construct was transformed into wild-type cells by electroporation and transformants were screened by PCR (See Materials and Methods). A cell line was identified that gave the PCR products expected from a gene disruption event. Disruption of the DdUnc104 gene was established by Southern analysis, and absence of the DdUnc104 RNA and protein was confirmed by Northern and Western blotting (Fig. 4). A blasticidin-resistant cell line in which the construct had integrated elsewhere in the genome was used as a control (Fig. 4A).

The DdUnc104 null cells grew normally and showed no gross morphological defects. In addition, after starvation, the KO cells aggregated and differentiated normally and at a rate like that of wild-type cells. Thus, this motor is not essential for viability, cell division, and differentiation.

The DdUnc104 knockout provided an opportunity to assess whether DdUnc104 was a bona fide organelle transport motor in vivo. To address this question, linear (>1 μm) movements of organelles in control and DdUnc104 null cells were observed by video DIC microscopy; earlier studies have shown that these movements are predominately microtubule-based (Roos et al., 1987). Quantitative measurements revealed that the mutant cells exhibited a 62% reduction in overall movements (Figure 5A). In *Dictyostelium*, organelle movement is not perfectly radial from the center to periphery, and hence it is very difficult to score the polarity of movement in

vivo. Nevertheless, this result indicates that DdUnc104 drives organelle transport in vivo. Interestingly, when we examined the movement of mitochondria by labeling cells with a mitochondrial dye (Mitotracker, (Poot et al., 1996)), no difference in the frequency of mitochondrial movements in null and control cells was observed (Fig. 5B). This result indicates that DdUnc104 is not required for the movement of mitochondria. Thus, the transport defect in the KIF1A null cells is not due to a generalized reduction in the movement of all organelles, but rather reflects a specific defect in the transport of an as yet unidentified organelle population(s).

Since the polarity of organelle movement could not be determined in vivo, we analyzed whether the organelle motility defect in the DdUnc104 null cells was specific for the plus-end direction using our in vitro assay. An ATP release fraction prepared from either the control or null cells was incubated with KI-washed vesicles and scored for motility on our polarity-defined microtubule substrate. Minus-end-directed motility driven by dynein (Pollock et al., 1998) was nearly identical for the fractions obtained from control and null cells. However, dramatically, plus-end motility was decreased by 90% in the null cells (Fig. 5C). This result confirms that the DdUnc104 polypeptide is responsible for the majority of the plus-end-directed motor activity seen in our biochemical assay.

Discussion

Purification of Motors Involved in Organelle Transport

Microtubule-based motor proteins have been purified using in vitro microtubule gliding activity (Paschal et al., 1987; Vale et al., 1985) or reactivity with pan-kinesin antibodies (Cole et al., 1992). However, these highly purified motors have not efficiently transported organelles. Here, we have approached the problem differently by directly purifying soluble factors needed for plus-end-directed movement of organelles. This strategy led to the discovery of two kinesin motors that drive organelle movement.

Several findings argue that the purified motors are the ones that power plus-end-directed organelle transport in *Dictyostelium*. First, the specific activity of plus-end-directed organelle transport increased throughout the purification, indicating that the relevant activities from the starting extract were being enriched at each step. Second, the speed and quality of movement (continuous motion for several microns) produced by the two purified motors was virtually identical to that observed in extracts (Pollock et al., 1998) and in living cells (Roos et al., 1987). The role of the 245 kDa DdUnc104 motor in organelle transport was further confirmed by a gene knockout. Although several other kinesin genes have been identified in *Dictyostelium* (de Hostos et al., 1998), the 245 kDa DdUnc104 and 170 kDa kinesin are likely to represent the major organelle transport motors, since the DdUnc104 knockout results in the loss of the vast majority of plus-end-directed organelle movement in vitro. It is likely that the remainder of the movement is produced by the 170 kDa motor, and this can be established in the future by analyzing cells that have knockouts in both the DdUnc104 and 170 kDa kinesin genes.

In principle, our approach could have purified a soluble activator of an organelle-bound motor or a combination of an activator and a motor. Although these possibilities cannot be ruled out and further studies are required, our data does not favor these scenarios. First, there is no polypeptide that co-purifies stoichiometrically with DdUnc104. This data, along with the hydrodynamic analysis, argues that this motor does not have an associated light chain, although it is possible that a small subunit (<25 kDa) eluded detection in our SDS-PAGE analysis. Furthermore, based upon many purification trials, we have not detected any substoichiometric polypeptide that consistently co-fractionates with DdUnc104. We also believe that DdUnc104 is not activated by a protein that overlaps but does not co-purify with the DdUnc104 fractions, since the relative amounts of organelle transport activity mirror the concentrations of DdUnc104. With regard to the membranes, all components required for transport are vesicle-associated after 0.3 M KI treatment but are partially destroyed by trypsin. From these studies, we hypothesize that plus-end-directed organelle transport is driven by a direct interaction of DdUnc104 with a tightly associated, and possibly integral, membrane protein(s).

Our results differ somewhat from in vitro studies of plus-end-directed organelle transport using other systems. A recombinant KIF1B motor was reported to transport mitochondria in vitro (Nangaku et al., 1994), although the average frequency and velocity of movements were not reported, and several examples of moving mitochondria shown in the paper reveal transport rates (<0.1 μm/sec) slower than those seen in vivo. In contrast, vesicle movements produced by the two motors purified in our study occur over long distances and match in vivo velocities. For conventional kinesin, fractionation experiments by Schroer and Sheetz (1991) indicated that

kinesin requires an activator for transporting organelles, although its identity has not been established through further biochemical purification. Interestingly, many of the plus-end-directed organelle movements in this activator fraction were fast (> 2 μm/sec), comparable in speed to the movements observed in our study. It is possible that the movement observed in this fraction was due not to a kinesin activator but to an Unc104/KIF1A-type motor.

DdUnc104: A Relative of the Synaptic Vesicle Precursor Transporter Unc104/KIF1A

The sequence of the 245 kDa kinesin reveals that it belongs to the Unc104/KIF1A class of motors, since its amino acid identity extends well past the superfamily-conserved catalytic core. Based upon phylogenetic trees of kinesin sequences from the data base, the Unc104/KIF1A class encompasses approximately 16 members from various organisms. Of these various members, DdUnc104 appears to be most similar to *C. elegans* Unc104 and mouse KIF1A. Most notably, its homology to CeUnc104/MmKIF1A uniquely extends well past the AF-6/cno domain found in many family members, and these three motors are also the only ones that contain a conserved PH domain at or near their C-termini. However, DdUnc104 and CeUnc104/MmKIF1A must carry different types of cargo. MmKIF1A and CeUnc104 are expressed only in the nervous system, where they specifically transport synaptic vesicle precursors (Hall and Hedgecock, 1991; Yonekawa et al., 1998). Thus, a DdUnc104-like motor from unicellular organisms most likely evolved to acquire a more specialized function in the nervous system of higher eukaryotes. However, the conservation of non-motor domains in

DdUnc104 and CeUnc104/MmKIF1A suggests that the mechanisms by which they bind their cargo may be closely related.

DdUnc104 is the first member of the Unc104/KIF1A kinesin subfamily identified and fully sequenced from a unicellular organism. A partial clone of a KIF1B homolog, TLKIF1, has recently been cloned from the thermophilic fungus *Thermomyces lanuginosus* (Sakowicz et al, 1999), suggesting that members of this kinesin class may be common in lower eukaryotes. Since DdUnc104 is an evolutionarily distant member of the Unc104/KIF1A kinesin class, sequences that are uniquely conserved between it and other family members are likely to define regions that serve important functions for this class of motors. One such conserved motif is an unique extension in loop 3. Based upon the location of this loop in the conventional kinesin and Ncd crystal structures, these extra amino acids would be expected to extend towards the nucleotide and hence may be involved in modulating enzyme kinetics. The AF-6/cno homology domain is also highly conserved between DdUnc104 and its higher eukaryotic relatives; its position adjacent to the catalytic core suggests that it may be involved in motor mechanics or regulation. In the C-terminal tail domain, the PH domains of DdUnc104, CeUnc104 and MmKIF1A are more similar to one another than to the PH domains from other proteins. PH domains are thought to be involved in membrane interactions (Rebecchi and Scarlata, 1998; Saraste and Hyvonen, 1995), and the similar PH domains of these motors may reflect a conserved function in cargo binding.

A surprising difference between DdUnc104 and other members of this class concerns their oligomeric state. KIF1A (Okada et al., 1995), KIF1B (Nangaku et al., 1994), and CeUnc104 (the N-terminal half) (Pierce et al., 1999) are monomeric, and sequence analysis of other members suggests that

they also function using a single motor domain. Hence, this class has been referred to as the 'monomeric kinesins' (Hirokawa, 1998; Vale and Fletterick, 1997). However, the dimeric nature of DdUnc104 raises the question of whether other motors in this class can function as dimers under some circumstances. For DdUnc104, dimerization is constitutive due to the long coiled-coil C-terminal to its PH domain. However, KIF1A and its higher eukaryotic relatives also contain sequences that are predicted to form coiled-coils (Fig. 3B), and perhaps binding of the motor tail to receptors on the membrane can trigger dimerization in a functionally equivalent manner.

An alternative model is that higher eukaryotic Unc104/KIF1A family members have evolved to have efficient motor function as monomers. Consistent with this idea, some of the Unc104/KIF1A family members from higher eukaryotes have an insertion of several lysines in the microtubule binding loop. For MmKIF1A, these lysines have been shown to be important in enabling this monomeric motor to move processively along microtubules in vitro (Okada and Hirokawa, 1999). In contrast, the equivalent loop in DdUnc104 has fewer lysines, perhaps because they are not needed for motility by this dimeric motor.

Remarkably, although the knockout of the DdUnc104 motor dramatically decreases organelle transport, it has little effect on the morphology, division, and differentiation of these cells. This result suggests either that membrane trafficking can be achieved by vesicle diffusion, that DdUnc104 activity is redundant with another activity (perhaps with the 170 kDa kinesin), or that other mechanisms act to compensate for the loss of this motor. However, with regard to this latter point, it is interesting that minus-end-directed motility is identical in extracts from the null and wildtype cells,

indicating that the decrease in plus-end-directed motility does not elicit any compensatory change in the level of dynein-based movement.

An Assay for Identifying Additional Organelle Transport Factors

Our reconstituted assay system provides a starting point for identifying additional factors involved in organelle transport and for understanding their function. As documented in earlier work (Schroer and Sheetz, 1991), we find that *Dictyostelium* cytoplasmic dynein loses its ability to transport organelles during purification, suggesting that it becomes separated from a required cytosolic factor. It will be interesting to determine if this factor is dynactin (Gill et al., 1991) or whether another type of activator is utilized by *Dictyostelium*. In addition, this assay provides an opportunity for identifying the membrane receptors that interact with the DdUnc104 and 170 kDa kinesin motors so that movement can eventually be reconstituted in a completely defined system. The molecular genetics offered by *Dictyostelium* also provides a testing ground for determining whether membrane proteins that bind kinesins in vitro indeed serve as bona fide receptors in vivo.

TABLE I. Purification of organelle transport activity

	Protein Conc. (mg/ml)	Specific Activity (U/mg protein)		Fold Enrichment (relative to HSS)	
		minus-end	plus-end	minus-end	plus-end
A. HSS	23.8	0.13	0.03	1	1
B. Salt Wash	3.1	0.41	0.06	3	2
C. ATP Releasate	0.6	0.56	2.30	4	77
D. Hydroxyapatite Peak	0.16	--	3.75	--	125
E. Mono Q Peak	0.03	--	11.67	--	389

Protein concentrations were measured by Bradford assay for the HSS, salt wash, and ATP releasate fractions (A, B, and C). Measurements were obtained for 2-3 separate preparations and the results averaged. Because the hydroxyapatite and Mono Q peaks (D and E) were too dilute to obtain protein concentrations by Bradford, concentrations were measured by comparative densitometry of Coomassie-stained fractions relative to BSA standards. Values reported for D and E correspond to hydroxyapatite fraction 17 and Mono Q fraction 13 in Figure 1. For specific activity calculations, one unit of activity in the organelle transport assay was defined as one movement per minute on a single axoneme/microtubule structure of defined polarity. Multiple assays were performed for each fraction and the results averaged. The average number of movements per minute supported by the fraction over a four minute assay was divided by the number of microliters of the fraction used in the total 10 ml assay volume to obtain the activity concentration in movements/min/ml. This value was then divided by the protein concentration of the fraction to calculate specific activity in U/mg protein. Little or no minus-end-directed movement was observed in the last two columns (--).

Figure 1. Purification of two factors supporting plus-end-directed vesicle transport activity. Panel A. A microtubule affinity-purified fraction was prepared from HSS (see Materials and Methods) and loaded onto a hydroxyapatite column (load). Three sets of fractions (flow-through and 200 and 300 mM potassium phosphate step elutions) were collected, and each fraction was independently assayed for its ability to transport KI-washed organelles in the plus-end direction. Activity in each fraction was quantified as plus-end-directed movements per axoneme-microtubule complex over a four-minute assay. Two assays were performed for each fraction, and the results averaged. Activity was spread over three fractions (16, 17, and 18) in the 200 mM potassium phosphate eluate and consistently copeaked with the 170 kDa and 245 kDa polypeptides. Although there was a significant amount of dynein heavy chain (DHC) that was eluted by 200 mM potassium phosphate, virtually no minus-end-directed transport activity was observed. Myosin, which is an abundant contaminant in the ATP releasate and also migrates at 245 kDa, eluted at higher potassium phosphate (300 mM; lane 22) and could therefore be separated from the other 245 kDa polypeptide; the myosin fraction displayed no transport activity. The fractions with peak activity from the hydroxyapatite column were pooled, dialyzed, and applied to a Mono Q column (Panel B). The column was eluted with a step of 115 mM NaCl followed by a 170-275 mM NaCl gradient; fractions were collected and assayed as above. The activity again co-eluted with the 245- and 170 kDa polypeptides (fractions 13 and 14). Although in this column, polypeptides in the 60-80 kDa range copeaked with activity, they were not observed to copeak in other purifications.

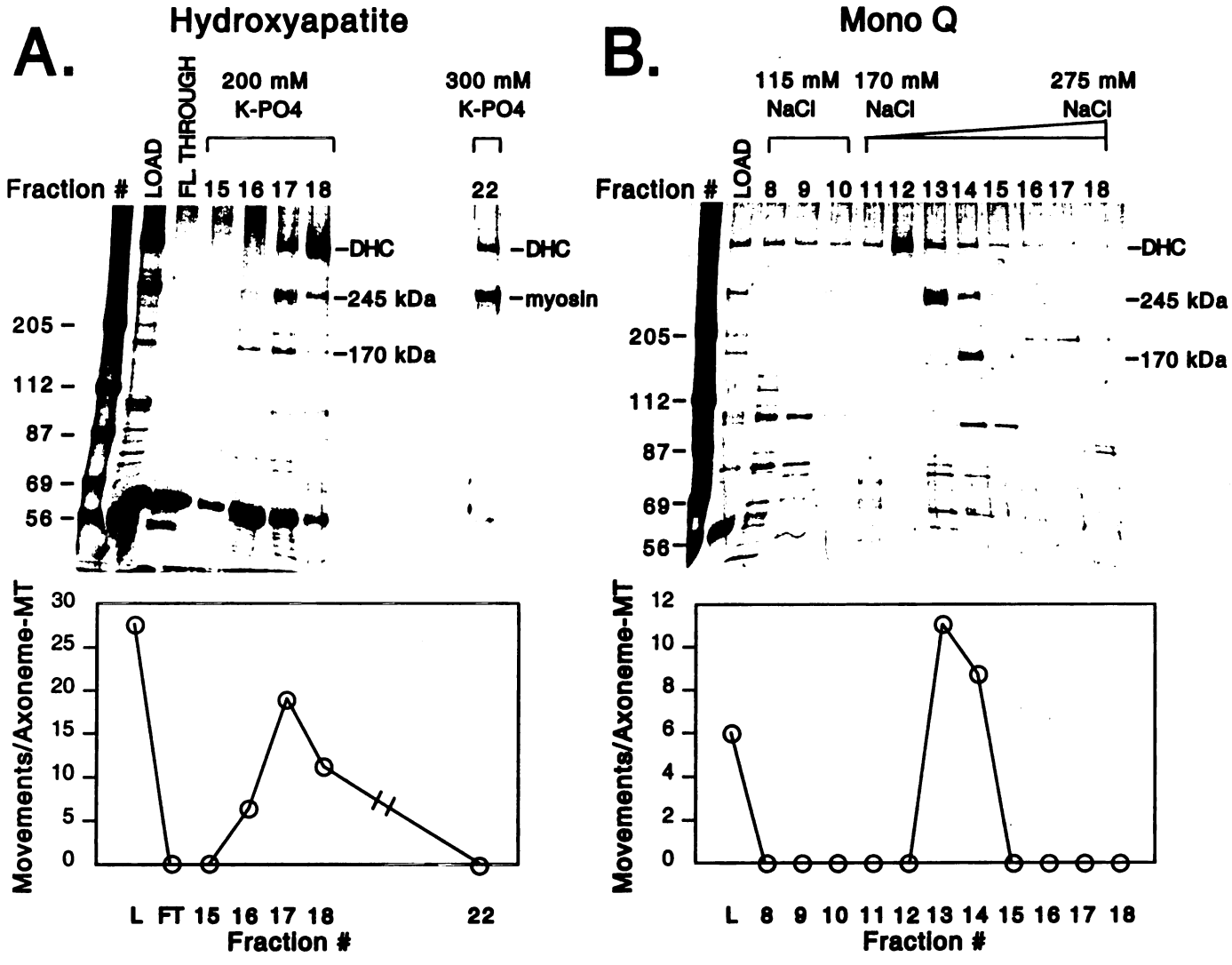

A.
Hydroxyapatite
200 mM K-PO4
300 mM K-PO4
LOAD
FL THROUGH
Fraction #
15 16 17 18
22
-DHC
-245 kDa
-170 kDa
-myosin
205
112
87
69
56
Movements/Axoneme-MT
30
25
20
15
10
5
0
L FT 15 16 17 18
22
Fraction #
B.
Mono Q
115 mM NaCl
170 mM NaCl
275 mM NaCl
LOAD
Fraction #
8 9 10 11 12 13 14 15 16 17 18
-DHC
-245 kDa
-170 kDa
205
112
87
69
56
Movements/Axoneme-MT
12
10
8
6
4
2
0
L 8 9 10 11 12 13 14 15 16 17 18
Fraction #

Figure 2. Velocities of plus-end-directed vesicle movements stimulated by the 245 kDa- and 170 kDa-containing fractions. Velocities of plus-end-directed vesicle movements were measured in Mono Q fractions that had minimal overlap between the 245 kDa and 170 kDa polypeptides by silver stain (see inset for each histogram). The 245 kDa polypeptide (top panel) and the 170 kDa polypeptide (bottom panel) are indicated by the arrows. The presence of a small amount of contaminating 245 kDa polypeptide in the 170 kDa fraction (bottom panel) is indicated by an asterisk. Dynein heavy chain (DHC) was present in both fractions, but minus-end-directed motion was not observed. Histograms demonstrate that the average velocity of vesicle movements generated by the 245 kDa polypeptide is significantly higher than that of the 170 kDa polypeptide. The small number of fast movements generating the apparent bimodal distribution of the 170 kDa histogram is likely due to the small amount of 245 kDa polypeptide present in the fraction.

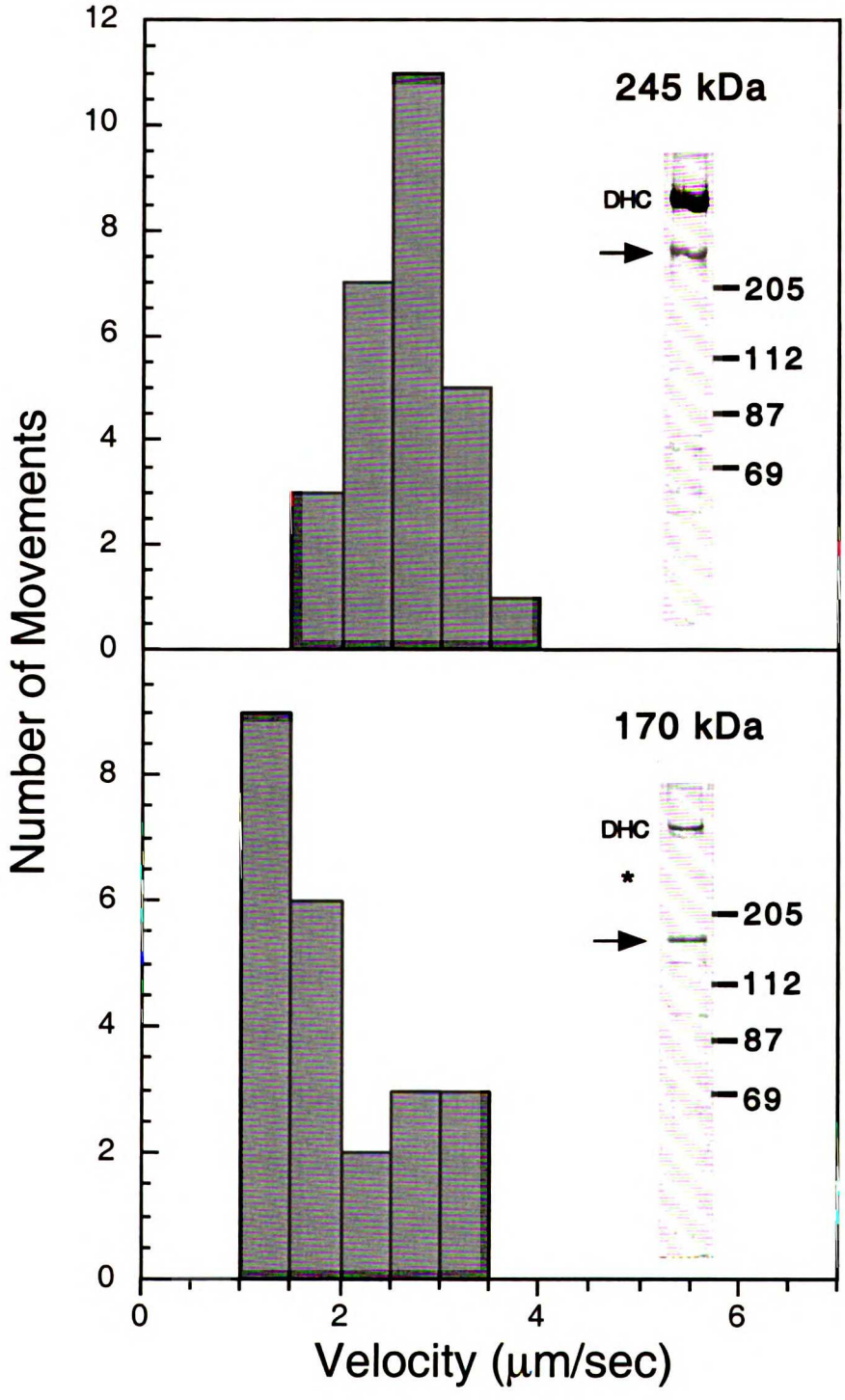
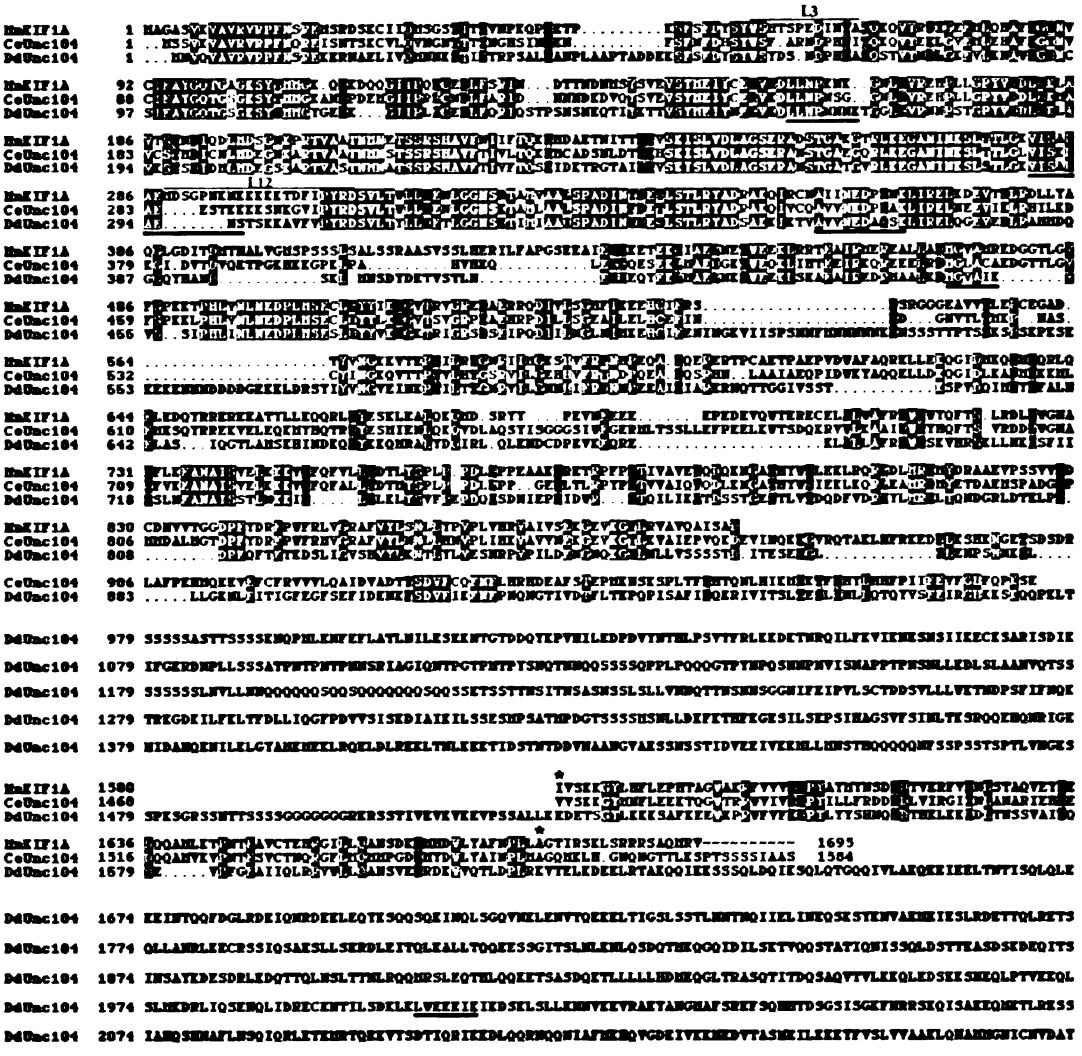

245 kDa
DHC
205
112
87
69
170 kDa
DHC
*
205
112
87
69
Number of Movements
Velocity (μm/sec)
0
2
4
6
8
10
12

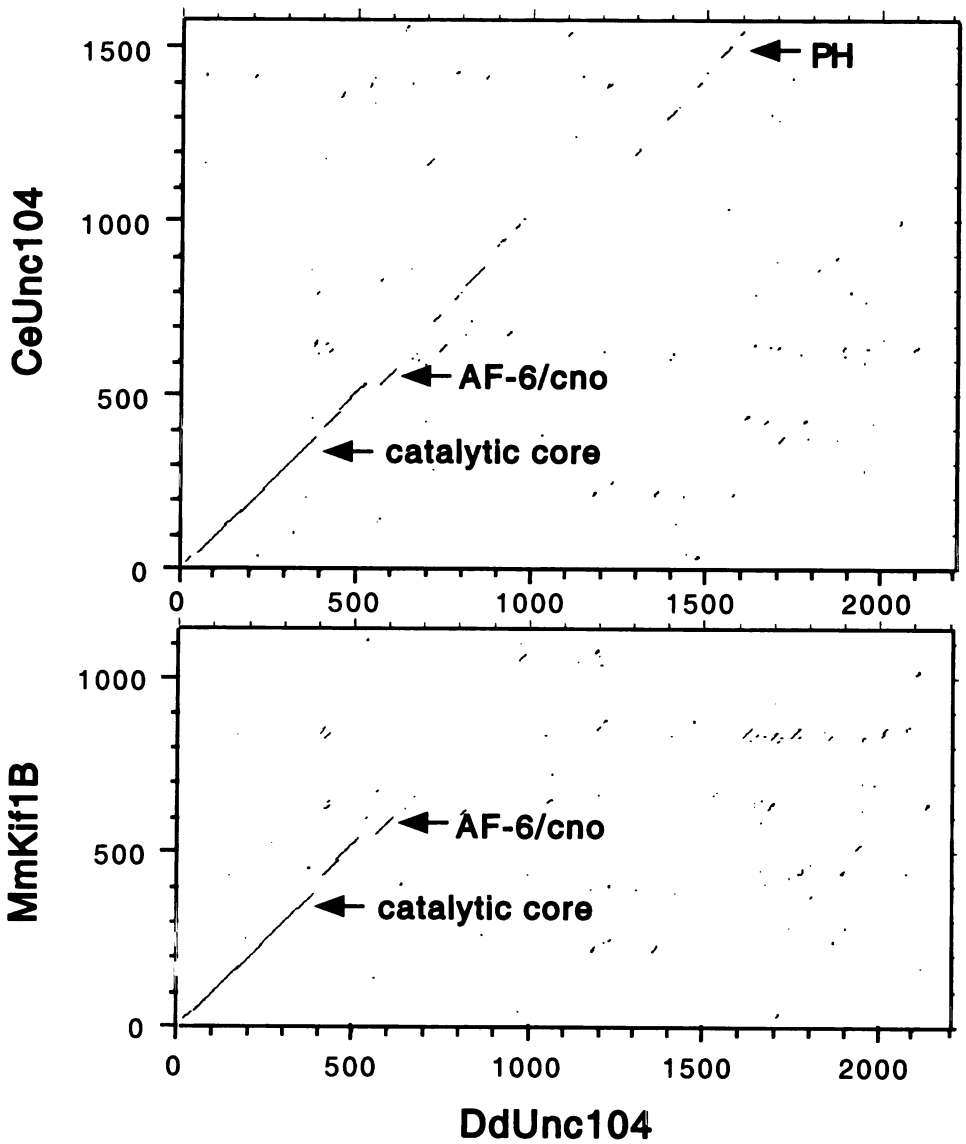
Figure 3. The 245 kDa protein is a member of the Unc104/KIF1A family. The complete 2205 amino acid sequence of the 245 kDa protein, which we hereafter refer to as DdUnc104, is indicated in Panel A. The amino acid sequences of homologous regions of mouse KIF1A and *C. elegans* Unc104 are displayed; regions of MmKIF1A and CeUnc104 which are not homologous are omitted. Amino acid identities between all three motors are indicated in black shading, while similar amino acids shared by all three are shaded gray. Peptide sequences obtained from tryptic fragments (see Materials and Methods) are underscored with black lines. The predicted positions of loop 3 (L3: DdUnc104 a.a. 63-73) and loop 12 (L12: DdUnc104 a.a. 296-309) are also indicated (see text for discussion of these loops). Amino acids 1523-1616 of DdUnc104 comprise a pleckstrin homology domain. This region, the boundaries of which are indicated by asterisks, is homologous to a similar region in MmKIF1A and CeUnc104. Both MmKIF1A and CeUnc104 end just after the PH domain, while DdUnc104 continues for an additional 589 amino acids. Panel B shows dot matrix plot comparisons of DdUnc104 with CeUnc104 (top) and MmKIF1B (bottom). The locations of the catalytic core, AF-6/cno, and PH domains are indicated (arrows for the core and AF-6/cno domains mark the C-terminal boundaries of these domains). The homology of DdUnc104 to CeUnc104 extends well past the AF-6/cno domain, and additional homology (including the PH domain) is evident in the C-terminal portions of the two motors. In contrast, the homology of DdUnc104 to MmKIF1B ends shortly after the AF-6/cno domain, and no C-terminal homology is apparent. Dot matrix plots comparing DdUnc104 to human (Hs) KIF1C and *Drosophila* (Dm) Klp38B (not shown) looked similar to the plot for MmKIF1B. Panel C shows the probability of coiled-coil formation along the lengths of the MmKIF1A, CeUnc104, and DdUnc104 proteins.

MmKIF1A and DdUnc104 share two regions of high probability of coiled-coil formation in the N-terminal half of the molecule, one of which is also present in CeUnc104. Uniquely, the large C-terminal region of DdUnc104 also has a high probability of coiled-coil formation.

A.



B.


1500
1000
500
0
CeUnc104
PH
AF-6/cno
catalytic core
0
500
1000
1500
2000
1000
500
0
MmKif1B
AF-6/cno
catalytic core
0
500
1000
1500
2000
DdUnc104

C.

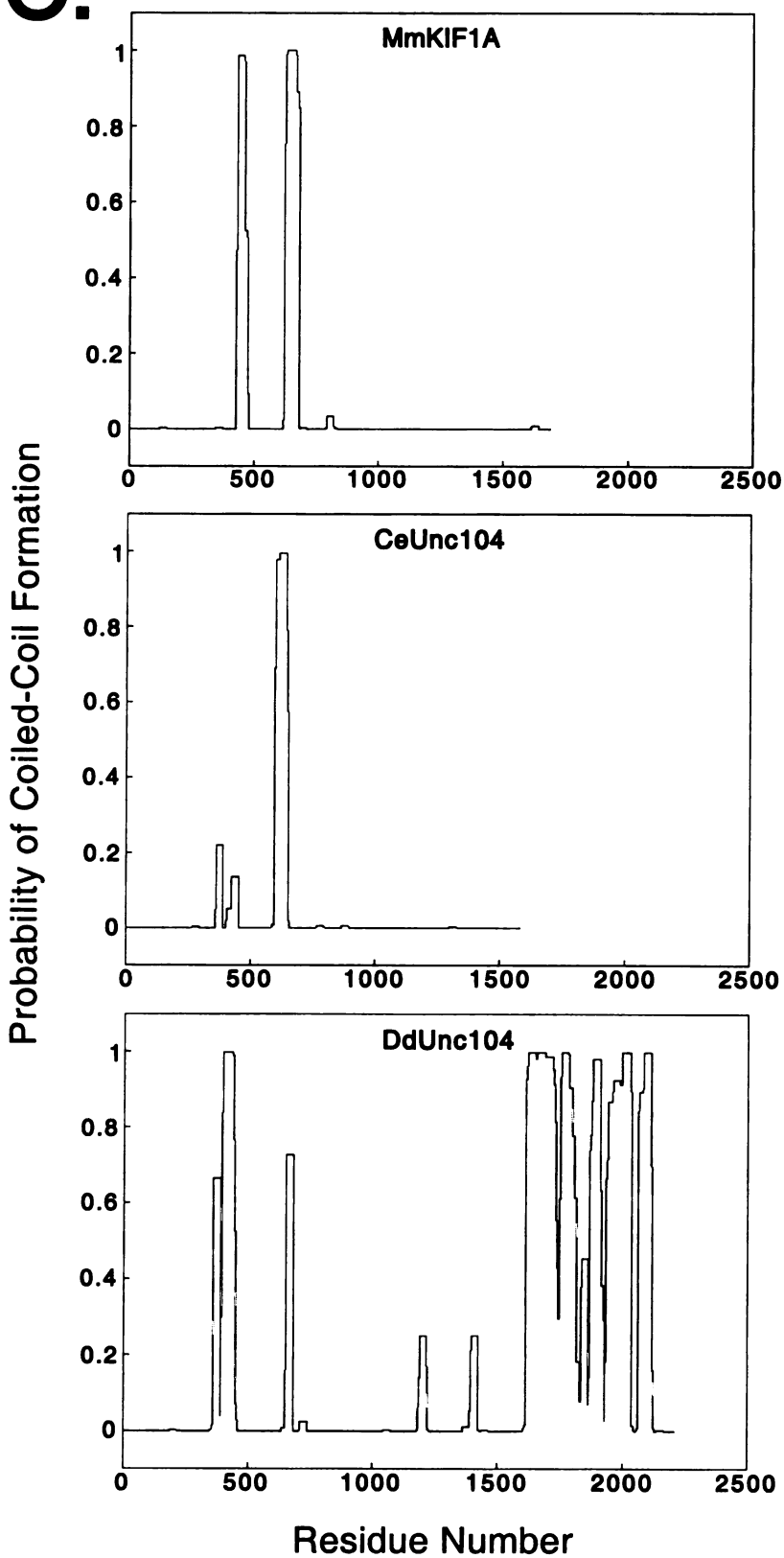

MmKIF1A
CeUnc104
DdUnc104
Probability of Coiled-Coil Formation
Residue Number
0
0.2
0.4
0.6
0.8
1
0
500
1000
1500
2000
2500

Figure 4. Disruption of the DdUnc104 gene (*ksnA* in *Dictyostelium* nomenclature) by homologous recombination. A blasticidin resistance cassette was inserted into a DdUnc104 cDNA fragment (see Materials and Methods) and transformed into wild-type *Dictyostelium*. Blasticidin-resistant transformants were screened by PCR (see Materials and Methods). Disruption of the gene was confirmed by Southern blot (Panel A), using the DdUnc104 cDNA fragment as a probe. Wildtype cells, in lane 1, display the 1.8 kb fragment expected for the undisrupted DdUnc104 gene. In contrast, the null cells (lane 2) have the 3.2 kb fragment expected if the disruption construct integrated uniquely and properly into the DdUnc104 gene. A blasticidin-resistant control cell line (lane 3) displays both the wildtype 1.8 kb fragment, indicating that the DdUnc104 gene is intact, and the gene-disruption construct fragment of 3.2 kb, indicating that the construct integrated elsewhere in the genome. The fainter high molecular weight band in lane 3 is most likely due to a small amount of partial digestion. The absence of production of DdUnc104 RNA in the null cells was confirmed by Northern blot (Panel B) using the DdUnc104 cDNA fragment as a probe. A single $\geq$ 6 kb DdUnc104 transcript (arrow) is present in control cells (lane 1) but not null cells (lane 2). The absence of the DdUnc104 protein in the null cells was confirmed by immunoblot (Panel C). ATP release fractions were prepared from control and null cells and immunoblotted using an affinity-purified peptide antibody (see Materials and Methods). The 245 kDa DdUnc104 protein (arrow) is present in fractions from control cells (lane 1) but not null cells (lane 2).

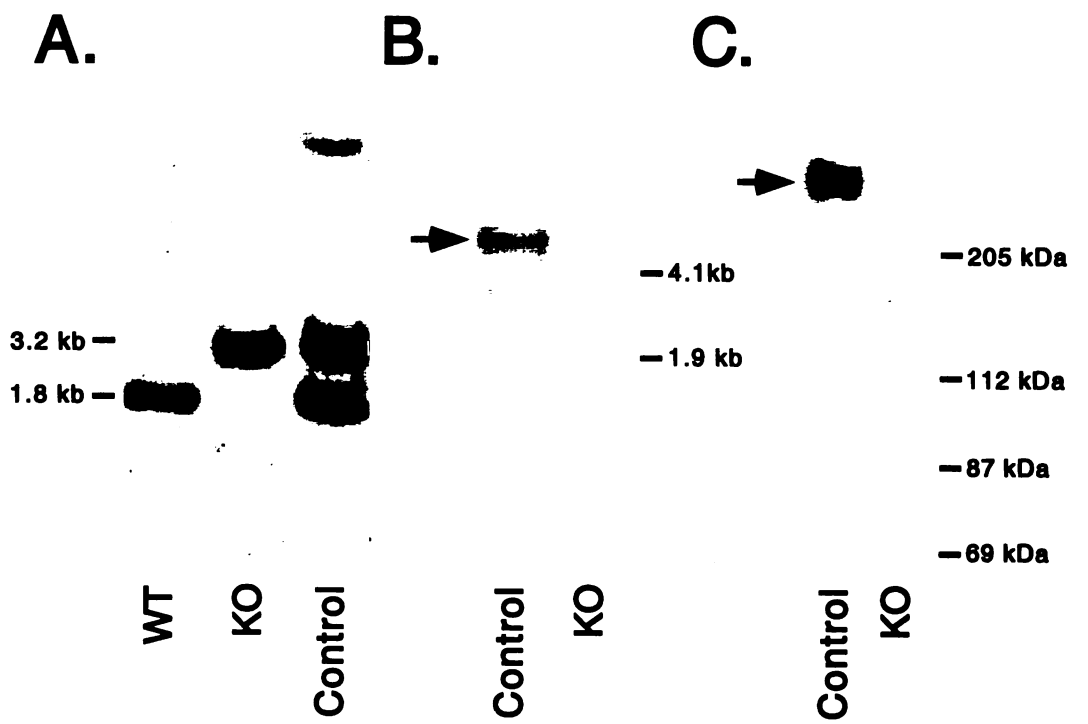
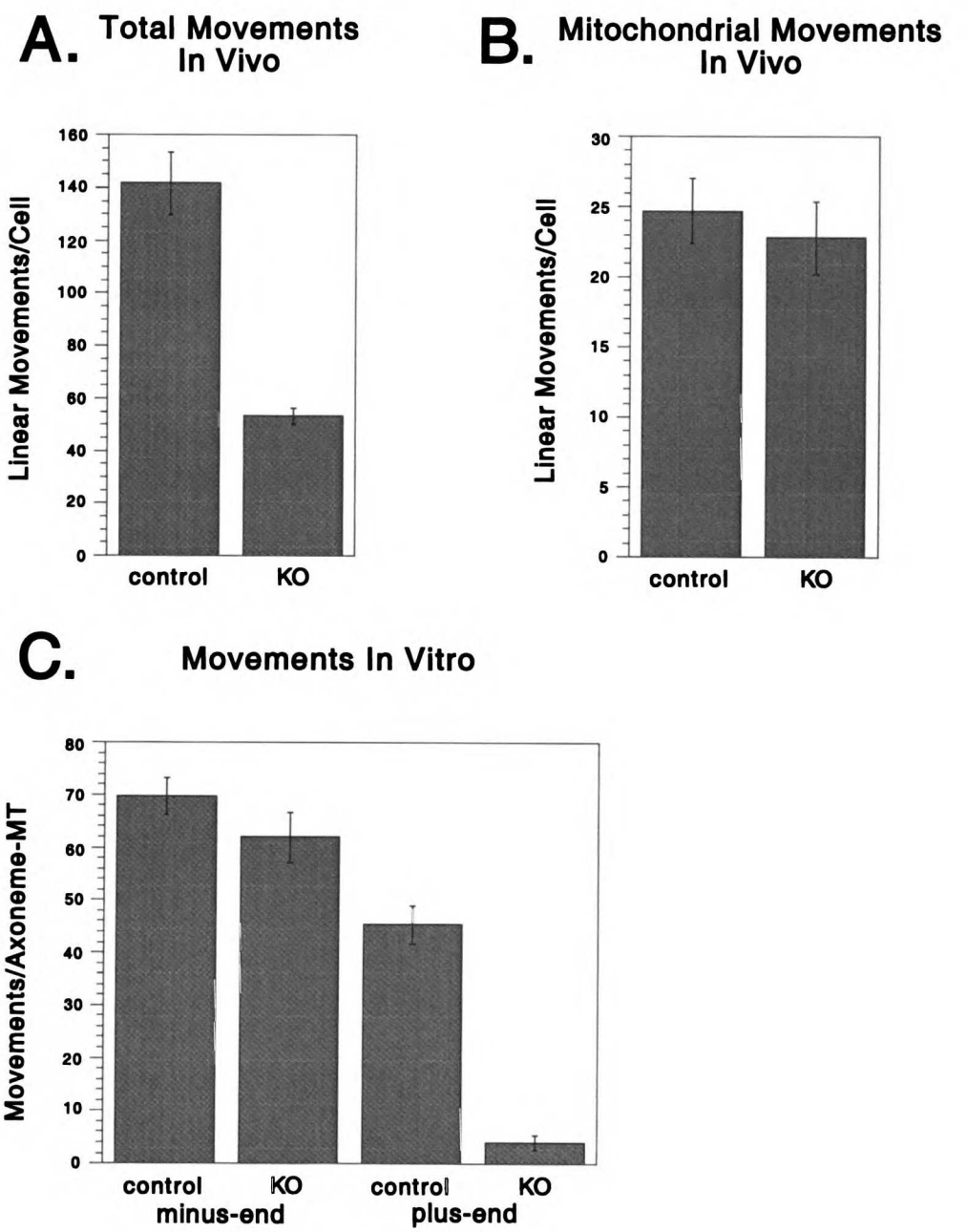

A.
B.
C.
3.2 kb
1.8 kb
4.1kb
1.9 kb
205 kDa
112 kDa
87 kDa
69 kDa
WT
KO
Control
Control
KO
Control
KO

Figure 5. The DdUnc104 null cell line has in vivo and in vitro defects in organelle transport. In Panel A, in vivo movements in control and null cells (those from Figure 4) were observed by differential interference contrast microscopy. Only linear and continuous movements greater than one micron in length were scored, and each cell was observed for four minutes. The total number of movements per cell was averaged over all cells observed (values shown are the mean ± SEM; n=10 cells for each cell type). The number of movements was significantly reduced in the null cells relative to the control cells (t-test; $p<0.001$). In Panel B, in vivo movements of mitochondria labeled with a mitochondrial dye (Mitotracker) were observed by fluorescence microscopy. Only linear and continuous movements of > 1 μm were scored, and each cell was observed for one minute. The total number of movements per cell was averaged over all cells observed (values shown are the mean ± SEM; control, n=27 cells; null, n=22 cells). There was no statistically significant difference in the control and null means by t-test. In Panel C, ATP releasates were prepared from the control and null cells and combined with KI vesicles in our in vitro assay (see Materials and Methods), and minus-end- and plus-end-directed vesicle movements were scored. Multiple four-minute assays were performed on each of two separate ATP release preparations for each cell line and the results for all assays averaged (values shown are the mean ± SEM; control, n= 6 assays; null, n=8 assays). The number of plus-end-directed movements was significantly reduced in the null cells relative to the control (t-test; $p<0.001$), while the levels of minus-end-directed movement were statistically equivalent by t-test.


A.
Total Movements
In Vivo
Linear Movements/Cell
160
140
120
100
80
60
40
20
0
control
KO
B.
Mitochondrial Movements
In Vivo
Linear Movements/Cell
30
25
20
15
10
5
0
control
KO
C.
Movements In Vitro
Movements/Axoneme-MT
80
70
60
50
40
30
20
10
0
control
KO
minus-end
control
KO
plus-end

Conclusion

Summary of findings

To advance our understanding of microtubule-based organelle transport, I have developed a new system for studying organelle movement using the cellular slime mold *Dictyostelium*. First, I developed a method for preparing *Dictyostelium* extracts that supported high levels of bidirectional microtubule-based vesicle motility in vitro, as detailed in Chapter 1. This assay was a differential interference contrast (DIC)-based video microscopic assay using microtubules polymerized off of sea urchin axonemes to determine the polarity of movements. The velocities and qualitative aspects of these movements appeared to reproduce vesicle movements seen in vivo in *Dictyostelium* (Roos et al., 1987). When vesicles were separated from soluble cytosolic components in the crude extract by centrifugation, the isolated vesicles were fully motile in the plus-end direction but exhibited no movement in the minus-end direction. The plus-end-directed activity could be removed by washing the vesicles with either buffer or buffer plus 0.3 M KI. However, bidirectional vesicle transport could be fully reconstituted by recombining a high-speed supernatant (HSS) with the KI-washed organelles.

I took advantage of the new assay to examine organelle transport in a strain of *Dictyostelium* overexpressing a 380-kDa C-terminal globular head fragment of the cytoplasmic dynein heavy chain (380 kDa cells; (Koonce and Samso, 1996)). These cells had been previously shown to display an altered microtubule pattern in which the interphase microtubule array, normally radial in character, appeared collapsed towards the cell center (Koonce and Samso, 1996). I found that the frequency and velocity of minus-end-directed vesicle movements were significantly reduced in 380-kDa cells relative to wild-type cells, while the frequency and velocity of plus-end-directed movements remained equivalent in the two cell types. Because this result

suggested that the 380 kDa globular head was somehow able to interfere with minus-end-directed vesicle transport, I examined whether this effect could be due to a dominant negative interaction that involved binding of the 380 kDa fragment to membranes. I found that the 380 kDa fragment was indeed associated with membranes, but that it did not compete with the full-length DHC for binding in vivo, and its affinity was significantly lower than that of the native DHC. We hypothesized that the altered microtubule patterns in the 380 kDa cells might be due to impaired membrane-microtubule interactions (Chapter 1).

In addition to the DIC-based assay described above, I also developed a fluorescence-based assay to observe specifically the movement of endocytic vesicles (Chapter 1). Endocytic vesicles labeled by uptake of rhodamine-dextran were observed to move predominantly in the minus-end-direction; relative to the total vesicle population observed by DIC, a greater proportion of endocytic vesicles moved bidirectionally. The velocities of endocytic vesicle movements were similar to those measured for the total vesicle population observed by DIC (Chapter 1).

An important advance in the project was the discovery that minus-end- and plus-end-directed transport activities could be partially purified from the HSS by microtubule affinity (Chapter 1). When exogenous taxol-stabilized microtubules were incubated with the HSS in the presence of hexokinase, glucose, and AMPPNP to cause tight binding of motors to the microtubules, both minus-end- and plus-end-directed activities cosedimented with the microtubules and could be released from the microtubules by ATP. Additionally, the minus-end-directed transport activity, which represented approximately 80% of the movements in the HSS, could be partially separated from the plus-end-directed activity by

incubating the microtubules in 0.3 M KCl (to selectively elute minus-end-directed activity) before the ATP release step. This step resulted in an 80-fold enrichment for plus-end-directed transport specific activity in the post-salt-wash-ATP releasate relative to the HSS, and made it possible to purify the factors responsible for plus-end-directed transport from the ATP releasate (Chapter 2).

The work detailed in Chapter 2 represents the first purification of a motor protein on the basis of its ability to move an organelle. Additionally, this is the first in vitro reconstitution of plus-end-directed vesicle transport with biochemically defined components that convincingly recapitulates in vivo motility. I used the DIC-based assay to purify two polypeptides (245 kDa and 170 kDa) from the ATP releasate which were independently capable of powering plus-end-directed organelle transport. The 245 kDa and 170 kDa polypeptides moved vesicles at different velocities (2.6 μm/s and 1.9 μm/s, respectively). By peptide sequence, both polypeptides were identified as kinesins. Partial cloning of the 170 kDa polypeptide (Nora Hom-Booher, Sally Cudmore, and Beth Holleran) indicates that it is the *Dictyostelium* homologue of conventional kinesin, a small piece of which had been previously identified by PCR screen (K3; (de Hostos et al., 1998). Peptide sequence of the 245 kDa polypeptide clearly identified it as a member of the Unc104/KIF1A family, a group of monomeric kinesins that have been shown to be involved in vesicle transport in higher eukaryotes (Hall and Hedgecock, 1991; Okada et al., 1995; Yonekawa et al., 1998; Nangaku et al, 1994; Dorner et al, 1998; Ohkura et al, 1997).

Careful inspection of silver-stained gels from the final purification fractions confirmed that there were no additional polypeptides that cofractionated stoichiometrically with either the 245 or 170 kDa polypeptides,

indicating that neither kinesin has an associated light chain. This concurs with observations that other members of the Unc104/KIF1A family do not appear to have associated light chains (Nangaku et al., 1994; Okada et al., 1995); similarly, a precedent for a conventional kinesin with no co-purifying light chains exists in the case of *Neurospora* kinesin (Steinberg and Schliwa, 1995). However, our gel system would not have resolved proteins <25 kDa, and it is possible that a low-molecular weight co-peaking polypeptide may have been present and not detected in this analysis.

Because the 245 kDa kinesin was the dominant activity in the motility assay, I chose it for further characterization. I cloned the complete gene for the 245 kDa kinesin by a combination of degenerate PCR, library screen, and rapid amplification of cDNA ends (RACE). The complete 6.6 kb open reading frame predicts a protein of 2205 amino acids with a molecular weight of 248 kDa. For the first ~600 amino acids, the 245 kDa kinesin shows significant homology to all of the kinesins in the Unc104/KIF1A class. The latter half of this class-conserved region contains a domain of unknown function which is also found in non-motor proteins such as human AF-6 and *Drosophila* cno (Ponting, 1995). The 245 kDa kinesin continues to exhibit strong homology to CeUnc104 and MmKIF1A, neuron-specific motors which transport synaptic vesicle precursors, for an additional ~370 a.a. beyond the AF-6/cno domain. In contrast, the homology to other Unc104/KIF1A class members (i.e. KIF1B, C, Klp38B) does not continue far past the AF-6/cno domain. This region of extended homology to CeUnc104 and MmKIF1A is followed by a ~550 a.a. novel region (residues 973-1522). Residues 1523-1616 form a pleckstrin homology domain, which in blast searches shows greatest similarity to PH domains in MmKIF1A and CeUnc104; PH domains are not found in any of the other motors in this class. Pleckstrin homology domains

have been implicated in membrane binding (Rebecchi and Scarlata, 1998; Saraste and Hyvonen, 1995), and it is possible that the PH domains of these motors represent cargo-binding domains. Though CeUnc104 and MmKIF1A terminate soon after their PH domains, the 245 kDa kinesin extends for a further ~590 residues; by blast search, this region is not homologous to any known protein. In summary, the 245 kDa kinesin is more homologous to CeUnc104 and MmKIF1A than to other members of the Unc104/KIF1A class. Because there is somewhat more extensive non-motor domain homology to CeUnc104 than to MmKIF1A, we have named the 245 kDa kinesin *Dictyostelium* (Dd) Unc104.

Examination of DdUnc104 using a coiled-coil prediction program revealed that both it and MmKIF1A contain two regions in the N-terminal half, one of which is also conserved in CeUnc104, which have a high probability of coiled-coil formation. Despite the presence of these regions, MmKIF1A and CeUnc104 do not seem to form dimers in solution (Okada et al., 1995; Pierce et al., 1999). Uniquely, the majority of the 600 a.a. C-terminal extension of DdUnc104 has a high propensity towards coiled-coil formation. Hydrodynamic analysis using gel filtration and velocity sedimentation through sucrose gradients showed that DdUnc104 is in fact dimeric, making it the first dimer within this class of motors. That the native molecular weight of DdUnc104 as determined by hydrodynamic studies (480 kDa) is almost exactly double the molecular weight predicted from its amino acid sequence (248 kDa) lends support to our preliminary conclusions from the purification that no additional polypeptides of significant mass are associated with DdUnc104.

It is interesting to consider why members of this family would have different oligomeric states. As discussed in Chapter 2, one possibility is that

higher eukaryotic Unc104/KIF1A family members actually do have efficient processivity as monomers, perhaps due to an insertion of several extra lysines in the microtubule-binding loop (Okada and Hirokawa, 1999). The fact that these lysines are not conserved in DdUnc104 might support this hypothesis, as this dimeric motor may not need them to enhance its processivity. An attractive alternative model is that the "monomeric" motors actually act as functional dimers, potentially dimerizing on the vesicle surface with the help of a receptor.

The relevance of DdUnc104 in organelle transport in *Dictyostelium* was confirmed by a knockout, made in collaboration with Geno de Hostos. The null cells, while having no apparent defects in growth rate, morphology, or development, exhibited a clear defect in overall vesicle movements in vivo. Interestingly, the defect seen in overall transport in vivo was not observed when specifically looking at mitochondrial motility, demonstrating that the defect was specific for a currently unidentified organelle population. In vitro assays using ATP releasates prepared from KO and control cells demonstrated clearly that the defect was specific for plus-end-directed transport and confirmed that this motor is the dominant plus-end-directed organelle motor in *Dictyostelium*.

The finding that the DdUnc104 null cells have no apparent defect in morphology, growth rate, or development despite having clear defects in organelle transport could be explained in a number of ways. Diffusion could potentially compensate for the lack of directed transport activity, or another mechanism could be compensatorily upregulated (however, it is noteworthy that minus-end-directed transport in the null cells is equal to that in control cells). Alternatively, the functions of DdUnc104 could be partially redundant with those of another motor; potentially, the 170 kDa KHC homologue. The

role of the DdKHC can potentially be elucidated by double knockouts of the DdUnc104 and DdKHC genes.

Future Directions

An immediate goal for this project is to reconstitute plus-end-directed organelle transport in vitro using recombinant DdUnc104 and KI-washed vesicles. The initial efforts to express full length, His-tagged DdUnc104 in *Dictyostelium* are detailed in Appendix 1. If the reconstitution is successful, a number of interesting experiments are immediately possible. First, structure-function analysis can be used to define the regions of the motor responsible for binding to membranes as well as those regions that are necessary for motility. One obvious candidate for a cargo binding region is the PH domain. Additionally, the recombinant motor can be used as a tool with which to identify the receptor on the organelle. The existence of a protein receptor for the plus-end-directed motors in this system is implied by the result that trypsinization of the KI vesicles reduced their plus-end-directed motility in the presence of the ATP releasate by three-fold compared to control vesicles (Chapter 2). To identify a receptor, detergent-solubilized membrane fractions could be loaded onto an affinity column prepared with the recombinant motor. Ideally, a motor lacking the membrane-binding domain could be used as a negative control to ensure the identification of functionally relevant binding partners. The relevance of the receptor could be confirmed by knockout as per the DdUnc104 protein.

If reconstitution with the purified recombinant DdUnc104 is unsuccessful (and it can be established that the lack of activity is not due to mutations, insufficient protein quantities, or the presence of the His tag; see Appendix 2), it is possible that the motor itself is insufficient for transport.

Though in the purification there were no additional polypeptides that clearly and consistently cofractionated with DdUnc104, the possibility remains that there is a catalytic or otherwise sub-stoichiometric factor required for plus-end-directed transport that co-purified with DdUnc104 but was missed in our analysis. It is also possible that these proteins would not co-purify with the recombinant motor, since it would be purified by a different method than that used for the endogenous protein. If the recombinant motor is functional in the presence of HSS or ATP releasate prepared from the DdUnc104 null cell lines, this would argue that a required accessory factor indeed exists. It would then be worthwhile to attempt to purify the factor(s) from the HSS or ATP releasate.

An additional area for investigation involves the question of what membranes are being moved by DdUnc104. The membrane substrate used in the purification was a mixed population of organelles isolated from the crude extract by sedimentation through a sucrose cushion. We know that DdUnc104 does not move mitochondria, given the result that mitochondria move normally in the DdUnc104 null cells, but the identity of the DdUnc104 cargo is still in question. Ideally, this question can be addressed with a fluorescence-based reconstituted assay similar to that developed for the observation of endosome motility, using recombinant DdUnc104 in combination with vesicles from cell lines in which specific vesicle markers have been GFP-tagged (Weber et al., 1999).

Finally, it would be interesting to return to a study of minus-end-directed transport using this assay system. Interestingly, as detailed in Appendix 2, the cytoplasmic dynein heavy chain (DHC) was present in a number of fractions throughout the purification of the plus-end-directed transport factors, but minus-end-motility was not observed. These results

imply that there is an additional factor required for motility that had been lost or inactivated in the purification, an observation that is consistent with previous work demonstrating a requirement for dynactin in dynein-based motility (Gill et al., 1991; Schroer and Sheetz, 1991). Given that minus-end motility is extremely robust in the extracts, and that the activity can be purified by microtubule affinity as described above, it may be possible to purify the additional factor. This factor may well be the *Dictyostelium* homologue of dynactin, but it may also be a novel factor. Even if it is dynactin, the ability to reconstitute minus-end-directed transport in vitro with relatively robust levels of activity using purified components would be a substantial advance in the field. Despite the fact that the available in vitro assays utilizing purified dynein and dynactin (Gill et al., 1991; Schroer and Sheetz, 1991) represent an important milestone in the dissection of the mechanism of minus-end-directed transport, the low levels of activity in these assays appear to have limited further progress towards an understanding of the specific mechanistic roles played by the various subunits of dynein and dynactin.

With an accessory factor in hand, a number of interesting experiments to investigate the mechanistic role of the accessory factor could be performed. Depending on the complexity of the accessory factor, it may be possible to reconstitute the active factor using entirely recombinant polypeptides. If the accessory factor is composed of multiple subunits, as is dynactin, mutant individual recombinant subunits could be substituted into the accessory factor complex and their effects on motility assessed in the reconstituted assay. Alternatively, the individual wild-type or mutant subunits could be added one at a time to the dynein complex in order to address what role each plays in the transport process. By using the motility assay in combination

with membrane binding assays, parameters such as membrane binding and organelle transport processivity and velocity could be examined.

In summary, it is my hope that the system developed in this work for studying organelle transport in *Dictyostelium* will provide insights into the mechanism of organelle transport that are generally applicable to membrane transport in higher organisms. The sequence conservation between DdUnc104 and Unc104/KIF1A family members from higher eukaryotes suggests that this might indeed be the case. Though DdUnc104 and CeUnc104 certainly transport different types of cargo, it is quite possible that their means of transporting cargo, i.e. the membrane-binding domains of the motors themselves as well as vesicle-associated receptor molecules, are in fact conserved. I look forward to future advances in our understanding of how a motor moves an organelle.

Appendix 1

Expression of Recombinant DdUnc104 in *Dictyostelium*

A key goal of this project since its conception has been to reconstitute vesicle transport using completely defined components: specifically, recombinant transport factors and KI-washed vesicles. A functional recombinant protein would allow us to perform structure-function analysis on the motor to look for regions responsible for membrane-binding and transport, as well as being a valuable tool with which to look for a receptor on the membrane surface.

We decided to express the DdUnc104 protein in *Dictyostelium* for a number of reasons. First, codon bias in *Dictyostelium* genes poses a potential problem for gene expression in other systems, especially given the length of the DdUnc104 gene product. Second, expression in *Dictyostelium* assures that post-translational modifications that are potentially important for transport activity will in fact be made. The fact that endogenous DdUnc104 binding partners will be available in the native host has both advantages and disadvantages. Though it did not appear from the purification of DdUnc104 that additional factors were necessary for transport, the potential role of a catalytic or otherwise sub-stoichiometric accessory factor in transport activity cannot be excluded. Expression in *Dictyostelium* would ensure that these factors were present to activate the recombinant DdUnc104. On the other hand, the presence of such accessory factors and the possibility that they could co-purify with the recombinant motor could make it difficult to conclude whether or not the motor is sufficient for transport if it successfully reconstitutes. Nonetheless, purification of the recombinant motor by a different method than that used to purify the endogenous protein should eliminate all but relevant co-fractionating proteins.

Because the DdUnc104 gene was cloned by a combination of different methods (see Chapter 2) that generated fragments with very little overlap, it

was necessary to PCR the full-length clone (Expand, Boehringer Mannheim) using 1st-strand *Dictyostelium* cDNA (prepared from poly A^+ mRNA using the Gibco Superscript kit) as a template. Because of the potential for PCR error, clones were obtained from two separate PCR reactions. Restriction sites on the PCR primers allowed the cloning of each 6.6 kb fragment into the *Dictyostelium* expression vector pDXA-3H (which contains a 3' 8-His tag) (Manstein et al, 1995), generating the constructs B-43, 5S3S9, and 5S3S10. The 3' primers used to PCR the complete gene also included a Tobacco Etch Virus (TEV) protease site which in the final construct lies just upstream of the 3' His tag; this site allows the His tag to be cleaved off the recombinant protein using TEV protease. The three constructs were sequenced and each was determined to have a 6-7 independent single base-pair mutations that had been introduced by the PCR, 4-5 of which produced point mutations. Most critically, one of the constructs (5S3S9) had a mutation in the primer itself, likely introduced during synthesis of the primer, that caused a frame-shift just upstream of the 3' His tag.

The three expression constructs were transformed into wild-type cells. Low levels of transforming DNA in combination with the pREP plasmid generated transiently-expressing cell lines, while transformation with larger amounts of DNA generated permanently expressing lines in which the expression construct was integrated into the genome. Extracts and high-speed supernatants (HSS) of all six cell lines were prepared (see Chapter 1 and 2 Materials and Methods) and expression levels in the HSS were evaluated by Coomassie staining and immunoblot using the anti-DdUnc104 peptide antibody. Western blots of HSS show that the His-tagged proteins are expressed at higher levels than the endogenous protein by comparison with wild-type HSS (Fig. 1A; 5/31/99 experiment) and are the same size (245 kDa)

as the native DdUnc104 protein (Fig. 1B; 5/15/99 experiment). However, expression levels were not high enough to be able to distinguish the expression products by Coomassie, especially given the co-migration of myosin with DdUnc104 (Fig. 1C; 5/31/99 experiment). The His-tagged proteins were purified from the HSS using cobalt affinity chromatography (Talon resin; Clontech, Palo Alto, CA) and eluted with 100 mM imidazole. As expected, the 5S3S9 construct, which should not have a 3' His tag, did not bind to the cobalt resin (Fig. 1D; 6/5/99 experiment). The imidazole eluates were dialyzed into LB pH 8.0 (see Chapter 1, Materials and Methods) for 2 h using a microdialyzer system 100 (Pierce, Rockford, IL), exchanging the buffer once after 30 min of dialysis.

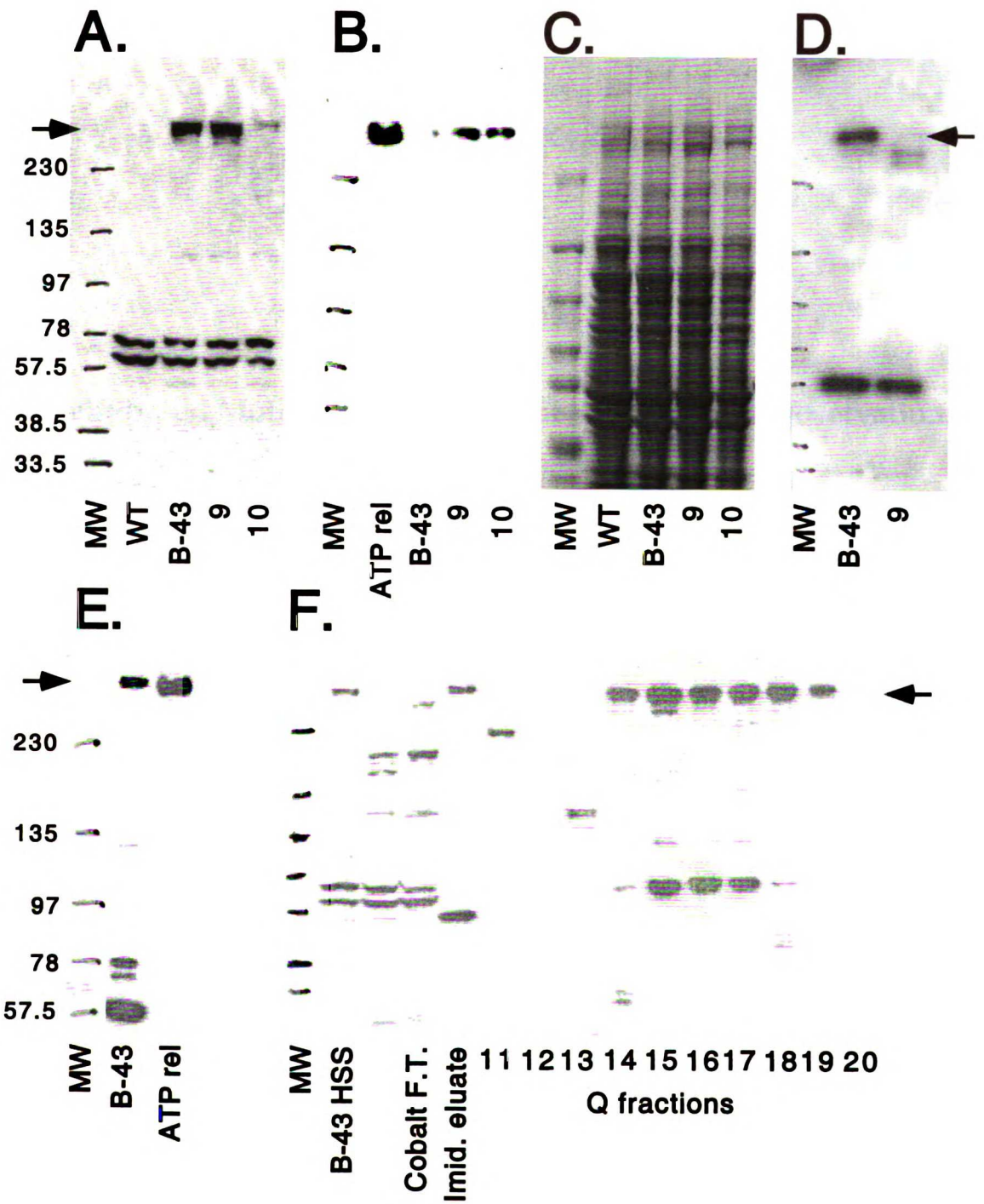
Attempts to reconstitute transport using KI-washed organelles and the cobalt-purified His-tagged B-43 or 5S3S10 constructs have not yet been successful, even when the 3'His tag was cleaved off of the protein using TEV protease. Barring the need for additional soluble factors to promote transport, two potential causes for the lack of activity need to be addressed. First, we must fix the PCR mutations in the constructs; Nora Hom-Booher is currently working to splice together a full-length error-free construct. It could potentially be useful to develop a gliding assay for DdUnc104 to confirm that the recombinant protein has active motor function, though only one of the mutations in the constructs is actually in the motor domain (5S3S10 construct). In preliminary attempts to perform gliding assays (6/9/99) with cobalt column eluate fractions containing the recombinant proteins, microtubules stuck to the glass but did not move. One preliminary result arguing that the recombinant protein is at least partially functional is that an ATP releasate prepared from a B-43 imidazole eluate fraction (6/3/99) contained the recombinant DdUnc104 protein, suggesting that the protein

can bind microtubules and be released by ATP. Second, we need to perform larger-scale preps of the recombinant protein under the assumption that we are working with insufficient levels of protein. (Note: it may also be prudent to consider baculovirus expression, given the relatively low levels of expression of these constructs in our current *Dictyostelium* strains). By immunoblot, imidazole eluates prepared from the B-43 integrated cell line (which has the highest expression levels among the three constructs) have lower concentrations of DdUnc104 protein than does the active ATP releasate (Fig. 1E; see also 6/24/99 experiment). Though Mono Q chromatography of the eluates concentrated the protein to levels comparable to those seen in the ATP releasates (Fig. 1F; see also 8/24/99 experiment), no activity was observed in the Mono Q fractions. It may be worthwhile to attempt to remove the His tag from these concentrated products in case it is in fact inhibitory.

Additionally, we have transformed the expression constructs into the DdUnc104 null cell lines (See Chapter 2) in order to see if the recombinant protein, in the context of an ATP release fraction, can function to complement the in vitro plus-end-directed organelle transport defect in the null cells. If the constructs are functional in that context, it could then be determined whether the lack of in vitro activity thus far observed with the purified recombinant protein is due to insufficient quantities of purified protein, a loss of activity during the affinity purification, or a requirement for an additional transport factor present in the ATP release that does not copurify with the recombinant protein.

Figure 1. Expression and purification of DdUnc104 constructs in *Dictyostelium*. In Panel A, HSS was prepared from the three integrated expression strains (B-43, 5S3S9, and 5S3S10; lanes 3-5) as well as from wild-type cells (lane 2) and immunoblotted using an affinity-purified peptide antibody against DdUnc104 (Chapter 2). The expression strains clearly produce more DdUnc104 protein (arrow) than is present in wild-type cells (note: the anti-DdUnc104 peptide antibody does not give a signal on immunoblots of wild-type HSS due to low DdUnc104 concentration). The expressed constructs are the same size (245 kDa) as the endogenous protein, as shown in Panel B. HSS were prepared from the three pREP expression strains (lanes 3-5); ATP releasate prepared from wild-type cells served as a control (lane 2). Panel C is a Coomassie-stained gel of the same HSS used for the immunoblot in Panel A. Despite being elevated relative to wild-type, DdUnc104 expression levels in the integrated cell lines are not high enough to be able to clearly distinguish the expression products by Coomassie, especially given the co-migration of myosin with DdUnc104. The His-tagged proteins were purified from the HSS using cobalt affinity chromatography and eluted with 100 mM imidazole. Immunoblots of the imidazole eluates prepared from the B-43 and 5S3S9 integrated strains are shown in Panel D. While the B-43 DdUnc104 construct clearly binds cobalt and elutes with imidazole, the 5S3S9 construct (which should not have a 3' His tag) is appropriately absent in the eluate, indicating that it did not bind to the cobalt column. Panel E shows immunoblots of equal volumes of the dialyzed B-43 (integrated) imidazole eluate, which did not support motility, and the wild-type ATP releasate, which is very active for plus-end-directed transport. The imidazole eluate clearly has lower concentrations of DdUnc104 than the ATP releasate. When the imidazole eluate was concentrated by Mono Q

chromatography (Panel F), the concentrations of DdUnc104 in some of the Mono Q eluate fractions (14-18) were comparable to those in the ATP release. However, no activity was observed in these fractions.


A.
B.
C.
D.
230
135
97
78
57.5
38.5
33.5
MW
WT
B-43
9
10
MW
ATP rel
B-43
9
10
MW
WT
B-43
9
10
MW
B-43
9
E.
F.
230
135
97
78
57.5
MW
B-43
ATP rel
MW
B-43 HSS
Cobalt F.T.
Imid. eluate
11 12 13 14 15 16 17 18 19 20
Q fractions

Appendix 2

Dynein-based Motility and Additional Experimental Observations

A. Dynein-based motility

Though the majority of the work presented here has focused on plus-end-directed organelle transport, the *Dictyostelium* system established in this work also offers the opportunity to study minus-end-directed transport. Minus-end-directed transport activity is extremely robust in the crude extract and HSS, and can be enriched by a microtubule affinity step (ATP releasate; Chapter 1). The minus- and plus-end-directed activities also can be partially separated from each other by exploiting the fact that the minus-end-directed activity has a comparatively lower microtubule affinity in the presence of 0.3 M KCl (Chapter 1; see also below and section B for additional information on the behavior of the minus-end-directed activity in different preparations of microtubule-affinity fractions).

Throughout our purification of the plus-end-directed transport factors, we often observed abundant amounts of the dynein heavy chain (DHC) in various fractions, but these fractions did not support minus-end-directed transport. Indeed, the ATP release fraction that is the starting material for the purification, having been prepared by eluting microtubules with 0.3 M KCl prior to eluting with ATP, supports very little minus-end-directed transport despite containing abundant DHC. In cases where the ATP release did contain significant amounts of minus-end-directed transport activity, the activity was lost over the subsequent chromatographic steps, despite the recovery of much of the DHC itself (see below). It is possible that this absence of motility reflects a loss of the intrinsic activity of the dynein heavy chain or the disassembly of the dynein complex over the course of the experimental manipulations. However, an intriguing alternative possibility is that an additional factor required for motility may have been lost or inactivated in the purification. Such a requirement has been documented in work by

Schroer and Sheetz (1991), who showed that dynactin was required to activate dynein-based organelle movements. The additional factor could well be *Dictyostelium* dynactin, which has not yet been discovered, but it could also be a novel factor. Given the abundant levels of minus-end-directed transport in the extracts, it might be possible to purify these factors from either an ATP release fraction (prepared without a 0.3 M KCl wash to preserve minus-end-motility) or from the 0.3 M KCl wash fraction itself.

Though, as mentioned, there was little or no minus-end-directed activity observed under the final chromatographic conditions used to purify the plus-end-directed transport factors (hydroxyapatite chromatography followed by Mono Q, using the post-salt-wash ATP release as a starting material; see Chapter 2), a number of experiments were performed over the course of developing the plus-end-directed purification which provide useful information pertaining to the minus-end-directed activity. Specifically, careful attention has been given to the correlation between the fractionation profiles of minus-end-directed activity and the dynein heavy chain (DHC), as well as to whether any other polypeptides stoichiometrically co-fractionate with the DHC. Additionally, as an anti Arp-1 antibody (UP454, provided by the Holzbaur lab, University of Pennsylvania) recognizes an appropriately-sized band in *Dictyostelium* which may indicate the presence of dynactin in this organism (Figure 1; 9/17/96 experiment), I have also taken note of the fractionation profile of the anti-Arp1-reactive band.

1. Microtubule affinity

See section B.2 below.

2. Sucrose gradients

A number of experiments were performed in which ATP releases with high levels of bidirectional transport activity were loaded onto 10-25%

sucrose gradients (spun for 3 hr, 55K rpm, 4°C, TLS-55 rotor); after centrifugation, fractions were collected and assayed. Though plus-end-directed activity was recovered from these gradients in multiple experiments, minus-end-directed activity was never recovered from a sucrose gradient fraction. Under these centrifugation conditions much of the DHC often pelleted to the bottom of the tube, but even in those cases the amounts of dynein remaining in upper fractions as assessed by Coomassie stain still represented enough of a percentage of the DHC in the active load that one would expect to see motility (I should note, however, that I did not perform a dilution curve of minus-end-directed motility to determine the true minimum amount of dynein necessary to produce motility). As a control, I tested whether the concentration of sucrose in the dynein peak fractions was itself enough to inhibit motility, and it was not. In one experiment (4/21/98), I tested whether adding the peak DHC fractions to the plus-end-directed activity-containing fractions would stimulate motility, and it did not. Interestingly, in a separate experiment (11/1/96), I observed that by immunoblot, the anti-Arp1-reactive band (which peaked in fraction 8) did not co-peak with the DHC, which pelleted to the bottom of the tube.

3. Mono Q chromatography

In Mono Q columns (100 µl Mono Q PC 1.6/5, SMART system) in which ATP releasates prepared without a salt wash (and which therefore had robust minus-end-directed activity) were used as the load (e.g. 6/27/97; buffers were LB pH 8.0 +5% sucrose, ± 1M NaCl), little or no minus-end-directed activity was recovered from the column (no more than one or two movements per assay). However, a strong DHC band was visible by Coomassie, eluting at 250-300 mM NaCl.

Minus-end-directed activity was also not recovered off of Mono Q columns run in Hepes pH 7.4 (3/7/98), Pipes pH 6.8 (3/16/98), or LB pH 9.0 (4/8/98, 4/15/98) when post-salt-wash ATP releasates were used as the load, though clear DHC peaks were observed in all cases. In some cases, the amount of DHC in the inactive elution fractions actually exceeded the amount of DHC in the active load (e.g. 4/15/98).

4. Mono S chromatography

When an ATP releasate prepared without a salt wash was applied to a Mono S column, robust minus-end-directed activity was recovered (Fig. 2; 7/18/97). 200 µl of an ATP releasate prepared in 25 mM Pipes pH 6.8, 1 mM EGTA, and 5% sucrose buffer was loaded onto a Mono S column (100 µl Mono S PC 1.6/5, SMART system) at 50 µl/min. Fractions were eluted at 100 µl/min with a 1 ml 0-0.5 M NaCl gradient. Fractions were dialyzed against LB pH 8.0 (see Chapter 2, Materials and Methods) and assayed. A strong peak of minus-end-directed activity (25-26 movements per 4 min assay) eluted at approximately 200-250 mM NaCl and clearly peaked with the DHC; though there were some other co-peaking bands, none were clearly stoichiometric (Fig. 2). Estimates of protein concentration indicated a 10.5 fold purification of specific activity in the minus-end peak fraction relative to the load.

A different result was obtained when the Mono S column was preceded by a Mono Q column. In this experiment (9/27/97), 1 ml of a post-salt-wash ATP release was loaded onto a Mono Q column and the peak plus-end-directed activity fractions (eluting at 200-400 mM NaCl) from this column were combined. The pooled fractions were diluted to 1 ml in Pipes buffer and loaded onto the Mono S column, which was run under the same conditions described above. Interestingly, the peak of minus-end-directed activity in this column (5 movements per assay) eluted at approximately 0-

100 mM NaCl, while the majority of the DHC eluted at 150-300 mM NaCl. The recovery of minus-end-directed activity in this experiment is surprising, given that minus-end-directed activity was not recovered off of other Mono Q columns (see section 3, above). If reproducible, interpretations of this experiment could include either an overlap between a small amount of dynein and an activator in the active fractions, or the existence of an second minus-end-directed motor. In a single attempt to reproduce this experiment (2/26/98), the peak activity fractions from the Mono Q column were dialyzed into Pipes buffer before loading onto the Mono S column. No activity was recovered off of this Mono S column, but this may have been because this particular run included an unnecessarily long wash before beginning the gradient.

5. Hydroxyapatite chromatography

Little or no (≤ 2 movements per assay) minus-end-directed activity was ever recovered off of a hydroxyapatite column, though significant amounts of the DHC were present in fractions which contained robust plus-end-directed activity (Chapter 2). However, the load for all of these columns was the post-salt-wash ATP releasate, which itself had little minus-end-directed activity.

6. Trypsinization experiments

Whether a protein component on the vesicle surface plays a role in minus-end-directed transport has been a controversial subject in the organelle transport field for some time. Despite evidence that protein components such as spectrin participate in the binding of dynein to vesicles (Holleran et al., 1996), there is also experimental evidence for the direct binding of dynein to phospholipids (Lacey and Haimo, 1994). Moreover, trypsinized squid vesicles as well as liposomes have been shown to move in

the minus-end direction in the presence of squid cytosol (Muresan et al., 1996). In preliminary experiments to determine if a membrane-associated protein(s) plays a role in minus-end-motility in the *Dictyostelium* system (11/2/96), membranes (non-KI-washed) were trypsinized with 50 μg/ml trypsin and the trypsin was subsequently blocked with 2 mg/ml STI. Minus-end-directed motility of the trypsinized vesicles in the presence of high-speed supernatant dropped to 1/5-1/10 of control (no trypsin) levels, suggesting that a protein component on the vesicle surface is indeed important. However, this result was not repeated. Later, I worked out a reproducible and controlled trypsinization protocol (see 12/10/98 experiment and Chapter 2) and clearly showed the importance of a membrane-associated protein component for plus-end-directed transport (Chapter 2). However, these experiments did not provide insight into whether such a component is important for minus-end-directed transport because they were done with a post-salt wash ATP release fraction which contained little intrinsic minus-end-directed activity. It would be worthwhile to repeat these experiments using the newly developed protocol and an ATP releasate prepared to preserve minus-end-directed motility.

B. Additional experiments pertaining to both plus- and minus-end-directed transport

1. Heterologous experiments

In collaboration with Mike Jaffe (UCSD), I tried various combinations of *Dictyostelium* vesicles/cytosol with *S. cerevisiae* vesicles/cytosol in my motility assay (2/8/95). No microtubule-based motility was observed in a variety of buffers. We also tried to set up an actin-based motility assay using

actin polymerized off of acrosomes, but again observed no motility in a variety of different buffers.

In a separate set of experiments, canine pancreas microsomes obtained from Manu Hegde (Lingappa lab, UCSF) were combined with *Dictyostelium* HSS. A few minus-end-directed movements were observed (5-7 in a single 4 min assay), but it should be noted that these movements may have actually been *Dictyostelium* vesicles in the HSS itself.

Finally, Dr. Karen Dell and I added purified 9-kDa dynein light chain (provided by Dr. Steve King) to a HSS to test the hypothesis that it might inhibit transport through competition with the native dynein complex for membrane binding. We did not see inhibition of minus-end-directed motility. We also tested the effect of GRK2 (tubulin kinase) on activity in the crude extract, but no change in activity was observed.

2. Microtubule-affinity fractions

a. General considerations

A good deal of myosin is present in the ATP releasates prepared by the methods presented in Chapters 1 and 2. The overall abundance of myosin in *Dictyostelium* makes contamination almost impossible to avoid, but the large quantities of myosin present in the ATP releasate are presumably due to actin-myosin complexes that form during the ATP depletion conditions in the ATP release prep (i.e. hexokinase/glucose) and then pellet with the microtubules upon centrifugation. Numerous attempts were made to eliminate the myosin from the preparation, including (1) adding apyrase during the extract preparation followed by a pre-spin to pellet actin-myosin complexes and (2) adding cytochalasin to the extract. Neither of these conditions reduced the amount of myosin in the ATP release. The time of addition of the cytochalasin (it was added to the HSS prior to the microtubule

binding step) was probably inappropriate; it would have been more logical to add it during the preparation of the extract itself. Potentially, leaving hexokinase/glucose out of the ATP release prep would have helped, though some reduction in minus-end-directed transport recovery would have ensued (see below, **b**).

b. Microtubule binding conditions

Four different conditions known to cause binding of motors to microtubules were examined while developing the ATP release protocol: hexokinase/glucose, hexokinase/glucose plus AMP-PNP, AMP-PNP alone, and AMP-PNP plus apyrase. Rough estimates of the recovery of minus-end-directed and plus-end-directed activity under different conditions are presented in Table 1A (12/17/96 experiment). The combination of hexokinase/glucose and AMP-PNP was chosen for the final protocol, as it allowed good recovery of both minus-end- and plus-end-directed activities.

c. Microtubule wash conditions

Because ATP release fractions prepared by simply binding motors to microtubules by the above methods, spinning out the microtubules, and releasing motors with ATP were extremely complex as assessed by Coomassie stain, three conditions for washing the microtubules prior to releasing motors with ATP were investigated: wash with buffer only, wash with buffer plus 0.1 M KCl, and wash with buffer plus 0.3 M KCl. Rough estimates of the activity remaining in the ATP releasates after these washes are presented in Table 1B (1/14/97 experiment). The majority of the minus-end-directed activity was eluted in the 0.3 M KCl wash and robust minus-end-directed transport was observed in this wash fraction after dialysis (Chapter 1).

d. Microtubule release conditions

Four different conditions for releasing motors from microtubules were tested to see how each would elute minus-end-directed vs. plus-end-directed activity from microtubules (1/20/97 experiment). These conditions included ATP/$MgCl_2$, ATP/EDTA, and ADP/$MgCl_2$. Following release with each of these sets of reagents, the microtubules were released a second time with ATP/$MgCl_2$. Rough estimates of the activity obtained in each releasate are presented in Table IC. Perhaps the most informative part of this experiment was the observation that, after eluting with ADP/$MgCl_2$, eluting the microtubules a second time with ATP/$MgCl_2$ released a significant amount of additional activity. The sum of the activities in the two releasates is greater than that in the single release with ATP/$MgCl_2$, suggesting that the single ATP/$MgCl_2$ release is not releasing all of the available activity from the microtubules. It may therefore be useful to include a second ATP release step in each ATP release preparation when maximal recovery is desired.

One additional interesting observation from this experiment is that the relative levels of DHC in the various fractions did not exactly match the minus-end-directed activity distribution. Specifically, there was more minus-end-directed motility present in the first release of condition 3 relative to the second release of condition 2, but more DHC was present in the latter release fraction. The same discrepancy was observed for the Arp-1-reactive band.

3. UV-vanadate experiments

Perhaps the most widely accepted way to show that dynein is responsible for a motility process in vitro is to create a biochemical knockout of dynein by cleaving the heavy chain through UV irradiation/sodium-ortho-vanadate treatment (e.g. Niclas et al, 1996). Towards this goal, many attempts were made to find combinations of UV irradiation and vanadate

concentration which together would cleave the dynein heavy chain but which individually did not perturb transport. I found that 100 μM vanadate was necessary to efficiently cleave the DHC, but that this concentration inhibited motility even in the absence of UV treatment. I attempted to remove the vanadate following the cleavage event by dialysis or spin columns but was unsuccessful. Even more problematic was the fact that control crude extracts lost activity over the time course required for UV/vanadate treatment and dialysis. Finally, we were able to show that minus-end-directed transport in the assay was specifically inhibited by low concentrations of vanadate (Chapter 1).

4. Membrane flotation experiments

To assess the binding of dynein to membranes, I performed a number of membrane flotation experiments; the final protocol is presented in Chapter 1. However, it is worth noting some of the key factors that I discovered were necessary for these experiments to work reproducibly. The main problem stemmed from the loss of membrane-bound dynein over the long time course of experiments which involved preparing extracts, isolating vesicles by sedimentation, resuspending them in buffer with the appropriate concentration of sucrose, and then floating the membranes in discontinuous sucrose gradients. I solved this problem by simply preparing the extract in the appropriate amount of sucrose and then floating the membranes directly from the extract. I also discovered that using fresh extracts (as opposed to frozen) was necessary both for recovering bound dynein and for obtaining reproducible results. A single experiment blotting with the LAGSE pan-kinesin antibody suggests that both DdUnc104 and DdKHC are also present on floated membranes, as would be expected (see 9/20/96).

5. *Dictyostelium* centrosomes

I tried making one preparation of *Dictyostelium* centrosomes using a method modifed from Sellitto et al (1992) to use as a substrate for polymerizing endogenous *Dictyostelium* or bovine brain microtubules (see 8/24/94). I was able to polymerize some asters using bovine brain tubulin, but the asters contained only a few microtubules each.

6. Endocytic vesicle assays

When the assay for observing specifically the transport of endocytic vesicles was first developed (see Chapter 1), we hoped that we could use the assay to investigate the motility of vesicles in different compartments within the endocytic pathway. Therefore, pulse-chase experiments were performed to label vesicles at various stages of the endocytic cycle. *Dictyostelium* were incubated with rhodamine-dextran for pulses of 5-10 min, and then chased in dextran-free media for 0-60'. Extracts were then prepared from these cells and the motility of the fluorescently labeled vesicles was observed. Unfortunately, the results from these experiments were extremely inconsistent, in that extracts prepared from duplicates of specific pulse-chase time points demonstrated dissimilar levels of movement and ratios of plus- to minus-end-directed movement. This inconsistency made it impossible to draw any conclusions about the levels/directionalities of transport for any particular population of vesicles along the endocytic pathway.

Because changes in cellular pH have been shown to affect the localization and morphology of endocytic compartments (Heuser, 1989; Parton et al., 1991), we examined the effects of reagents that increase the pH of the normally acidic endocytic compartments on endocytic vesicle motility in our assay. These reagents included a weak base, NH_4Cl, and the carboxylic ionophores monensin and nigericin, which raise the pH inside vesicles by collapsing Na^+/H^+ or K^+/H^+ gradients across membranes (Mollenhauer et

al., 1990). These results were also inconsistent, with the reagents having an inhibitory effect on motility in some experiments and not in others. NH_4Cl reduced minus-end-directed motility specifically to 1/5-1/2 of control levels, but this effect was also seen in the presence of equimolar NaCl. The extreme sensitivity of minus-end-directed motility to salt was observed in other experiments as well (see section B.7).

Finally, attempts were made to feed *Dictyostelium* magnetic beads in the hopes of isolating pure populations of endocytic vesicles using a magnetic apparatus (Rodriguez-Paris et al., 1993). However, the vesicles isolated in this manner did not move. Collaborations with Juan Rodriguez-Paris and James Cardelli (Louisiana State University) in which magnetically-isolated endocytic vesicles prepared in their laboratory were sent to us for motility assays also failed, perhaps because the vesicles were always frozen.

7. Salt sensitivities

The effects of various concentrations of NaCl on motility were assessed (1/24/97). Minus-end-directed motility was more salt-sensitive, being completely inhibited when NaCl was added to 50 mM NaCl in the assay. Plus-end-directed motility was still strong at this concentration, but increasing the salt to 75 mM eliminated activity.

8. Other Fluorescently-tagged vesicles:

PNS was prepared from cell lines prepared by Dr. Geno de Hostos (UCSF) expressing an ER-localized GFP construct and motility was examined using the fluorescent vesicle assay (Chapter 1). Only green haze was observed using normal fluorescent optics. However, when the same extracts were observed using the evanescent wave scope and Cy-5 labeled axonemes, a few movements of GFP-labeled ER vesicles were observed.

C. Additional information/troubleshooting the system

A number of small but important observations about the *Dictyostelium* organelle transport system are worth reporting here, for the benefit of those who are carrying on this work.

1. Taking care of cells

--I have found that if wild-type cells are passed for many months (i.e. 4-5 months), activity of extracts made from the cells is reduced. If activity starts to fall, start up new cells from spores.

--Media should not be more than a few months old, or the cells will not grow efficiently.

--Cells should not sit on the bench without shaking if at all possible. Over time they will die due to lack of oxygen, though I have not determined exactly how long this takes.

--Cells should not be allowed to get too dense--this also reduces the levels of activity. If they overgrow ($>1x10^7$ for two days), start up new cells. If they have only been overgrown for a day, split them back at low dilution to let them recover.

--Regarding cell lines expressing recombinant motors--integrated cell lines should be started up every few months from spores, as the levels of expression fall over time (this was definitely true for Mike Koonce's 380 kDa cells (Chapter 1)).

2. Preparing extracts

--As mentioned above, to obtain maximal activity, extracts should not be made from cultures that are $>1x10^7$ cells/ml.

--Cell pellets cannot be frozen prior to making extracts; pellets of cells which were frozen in LB/30% sucrose and to which PMSF was added upon thawing generated extracts with no activity.

--When lysing cells with Nuclepore filters, the filter should be wet in ddH_20 and lightly blotted on a paper towel before assembling the apparatus.

--For lysis, the Nuclepore filter works well because it does not shear small protease-filled vesicles. However, I have not tried other methods of lysing the cells, and alternative methods may be available that would be more convenient for large scale extract preparation.

--4.7 ml of cell suspension is the minimum volume for use of the large (47 μm) filter sets. Smaller samples will get lost in the filter and no lysate will be recovered.

--PMSF is a key reagent for recovering robust activity in extracts. It should be made fresh every two months using fresh ETOH (i.e. an unopened bottle), and stored in small aliquots with dessicator to prevent water absorption. Additionally, when working with extracts, the PMSF should always be added to buffers/suspensions at the last minute to avoid excess hydrolysis. I tried using Pefablock instead of PMSF to avoid the hydrolysis problems associated with PMSF, but the crude extracts lost activity much faster than with PMSF.

--Chymostatin is also important for recovery of robust activity, and should not be omitted.

--Both I and Sally Cudmore, who worked with the system for a few months in the lab as a post-doc, have noticed increased levels of activity when certain buffers are used to make the ATP releasate instead of LB (LB: 30 mM Tris-HCl (pH 8.0), 4 mM EGTA). Specifically, minus-end-directed activity seems to be enhanced in Pipes buffer (25 mM Pipes pH 6.8, 1 mM

EGTA, 5% sucrose), and plus-end-directed activity (minus-end-activity was not tested) is enhanced in K-P04 buffer (10 mM K-P04 (pH 6.8), 1 mM EGTA).

--The HSS tends to lose activity on ice over many hours. Additionally, the HSS does not freeze well; in contrast, the ATP releasate is stable indefinitely in liquid nitrogen.

--When doing vesicle preps, take care to resuspend the vesicles as little as possible to avoid breakage. Vesicles should be stored in liquid nitrogen and used for $\leq$ 1 week for optimal activity; it may be possible to use them for a bit longer but I generally try not to.

--In general, I find that over the past few years I rarely see strong plus-end-directed motility in the HSS, even though ATP releasates made from these HSS obviously have robust plus-end-directed activity. In contrast, minus-end-directed activity in the HSS is always strong. After doing a KI vesicle prep, I always test it for activity using the HSS prepared during the prep; two or three plus-end-directed movements in a brief assay is usually enough evidence that the vesicles will move well in the plus-end-direction in the presence of ATP releasate.

--The ATP releasate actually has some small vesicles in it which are bidirectionally motile (see 7/11/99 experiment). These vesicles are not observed in fractions from columns in which the ATP releasate has been used as the load (e.g. 7/20/98 experiment).

3. Transport assays

--When titrating the tubulin for making axoneme-microtubule structures, the tubulin should be thawed, re-frozen, and thawed before titrating it; new large aliquots of tubulin can then be aliquoted in appropriate small volumes and polymerization upon a second thaw will match the titration values.

--Primarily out of habit, I do not add PMSF to the LB/15% sucrose that I use as the buffer in the motility assay. I do add all of the other standard protease inhibitors.

--When doing transport assays, use a new 15 µl tube of frozen KI vesicles every hour. Thaw the tube and add 0.5 µl PMSF.

--Casein should be included in assays of any samples which are more dilute than the HSS (i.e. ATP releasates and column fractions, etc.).

--Use only axonemes which have microtubules of clearly distinguishable lengths polymerized from both ends. Often if microtubules can only be seen on one end, those on the other end have actually broken off and the polarity will be misinterpreted.

--For fluorescent endocytic vesicle assays, I tried using rhodamine microtubules polymerized off of axonemes and FITC-labeled vesicles, but the polarity of the microtubules was unclear and switching back and forth between the filters was problematic. If both the vesicles and the microtubules were labeled with rhodamine, it was impossible to distinguish the vesicles from the microtubules. Additionally, fluorescent extracts tend to lose activity on ice more quickly than regular extracts, so assays have to be done quickly. Finally, care should be taken to be certain that the cameras for DIC and fluorescence are oriented in the same direction, or the polarity of the fluorescent vesicle movements will be misinterpreted.

4. Antibodies

a. Dynein antibodies:

695, 697: anti-DHC polyclonals, gift of Mike Koonce (Wadsworth Center, New Jersey). Both antibodies work for Westerns at 1:1500; 697 is cleaner.

Orion, Triton: polyclonals against the N-terminus of DHC; also gifts of Mike Koonce.

An antibody against the DHC ATPase domain, again a gift from Mike Koonce, was tested to see if it would inhibit minus-end-motility in the crude extract; it did not (7/27/95).

b. Dynactin antibodies:

UP454: antibody against centractin (Arp1): recognizes a band in crude *Dictyostelium* extract that is approximately the same size as the 45 kDa Arp1 band in rat (see Fig. 1 and 9/16/96).

Dart, Port: antibodies against rat p150^{Glued}. Did not seem to recognize any equivalently-sized protein in *Dictyostelium* extracts (9/16/96).

c. DdUnc104 antibodies:

We generated a polyclonal antibody against a synthetic peptide (AVVNEDAQGKLIRELC-amide) based on DdUnc104 amino acids 359-373. The sequence AVVNEDAQGK was obtained from peptide sequencing (note: the glycine was eventually shown to be incorrect; the amino acid at this position based on the actual clone is S). The sequence LIREL was a guess based on homology between Unc104/KIF1A family members at this postition; the sequence of the DdUnc104 clone confirms that these amino acids are accurate for the DdUnc104 protein. The antibody was affinity purified using the standard QCB protocol; it works cleanly at 1:500 on immunoblots of ATP releasate (Chapter 2, Appendix 1) but does not give a signal on immunoblots of HSS (note: use antibody from Rabbit #2 ONLY; Rabbit #1 did not work at all). This antibody also immunoprecipitates DdUnc104, but it does not seem to deplete DdUnc104 from ATP releasates (e.g. 12/8/98).

d. DdKHC antibodies:

Two of the four peptide antibodies made against the human KHC in our lab recognize the 170 kDa DdKHC protein in crude extracts: LTA (against the neck; 1:2500) and CKVS (against the switch 2 loop; 1:1000) (see 10/2/96). In an ATP release, the CGST antibody (against the insert 2 loop; 1:1000) also recognizes the protein (see 4/2/98).

Interestingly, a retrospective look at early blots of ATP releasates and hydroxyapatite fractions using Gretchen McCaffrey's "Floppy" antibody, an antibody made against the 105 kDa kinesin that she purified by microtubule gliding, shows that this antibody recognizes the 170 kDa DdKHC (see 11/21/97 and 11/26/97) in addition to a 105 kDa band.

As expected, both the 245 kDa and 170 kDa kinesins are recognized by both the LAGSE and HYPR pan-kinesin antibodies (Sawin et al, 1992).

e. His antibodies:

The Clontech mouse anti-6-His antibody (1:2500) recognizes the His-tagged DdUnc104 recombinant protein weakly, with quite a bit of background (6/8/99).

5. Cloning/sequence analysis

--The DdUnc104 full-length clone is hard to work with. It took several weeks to successfully clone the full-length (6.6 kb) insert into the pDXA-3H vector, and it has proven difficult to clone it out of the pDXA-3H vector and into a Bluescript vector. This may be due to the size of the transcript. In general, electroporating and transformation into XL10 Gold cells (Stratagene) were the most effective.

--Despite the fact that the DdUnc104 protein is clearly cytoplasmic, given the method of its purification, Prosite scans of the DdUnc104 peptide sequence suggest that the protein has a bipartite nuclear localization signal

Table I. Recovery of organelle transport activities in microtubule-affinity fractions prepared under various conditions

A. Microtubule binding conditions	Movements/assay	
	Minus-end	Plus-end
1. Hexokinase/glucose	82	5
2. Hexokinase/glucose + AMP-PNP	77	15
3. AMP-PNP only	65	15
4. AMP-PNP + apyrase	55	15

B. Microtubule wash conditions	Movements/assay	
	Minus-end	Plus-end
1. No wash	57	16
2. Buffer wash	24	9
3. Buffer + 0.1 M KCl	8	7
4. Buffer + 0.3 M KCl	1	13

C. Microtubule release conditions (1st release)	Movements/assay			
	1st release		2nd release	
	Minus	Plus	Minus	Plus
1. 5 mM ATP/$MgCl_2$	70	38	N.D.	N.D.
2. 5 mM ATP/ 10 mM EDTA	59	4	16	14
3. 5 mM ADP/$MgCl_2$	50	29	36	21

In section A, different reagents were added to high speed supernatants to cause motors to bind to microtubules. The microtubules were then pelleted and eluted with 5 mM ATP. The final concentrations used were 15 U/ml hexokinase, 3 mM glucose, 4 mM AMP-PNP, and 2 U/ml apyrase. Plus-end-directed transport was equivalent in all conditions which included AMP-PNP (2,3,4). Immunoblot analysis of the four ATP releasates indicated that the 170 kDa DdKHC was present in equal amounts in releasates 2, 3, and 4. In section B, different conditions were used to wash the microtubules (to which motors had been bound using hexokinase/glucose and AMP-PNP) prior to releasing with 5 mM ATP. The buffer used in conditions 2, 3 and 4 was LB + 5% sucrose. Almost all of the minus-end-directed activity was removed by the 0.3 M KCl wash, while the majority of the plus-end-directed activity was retained. The higher levels of plus-end-directed activity in condition 4 relative to 2 and 3 are presumably due to experimental error. In section C, different conditions were used to elute motors from microtubules as a first release step, followed by eluting a second time with ATP/$MgCl_2$

(except when the first release was itself in ATP/$MgCl_2$; N.D.=not done). ATP/EDTA produced a somewhat selective elution of minus-end-directed activity in the first release step. ADP/$MgCl_2$ eluted both minus-end- and plus-end-directed activities.

Figure 1. The UP454 anti-Arp-1 antibody recognizes a putative Arp-1 homologue in *Dictyostelium*. ~50 μg of rat low speed supernatant (LSS) and *Dictyostelium* crude extract (post-nuclear supernatant; PNS) were immunoblotted using the UP454 antibody. The antibody recognizes a band in *Dictyostelium* (arrow) that is roughly the same size as the 45 kDa Arp-1 protein in rat.

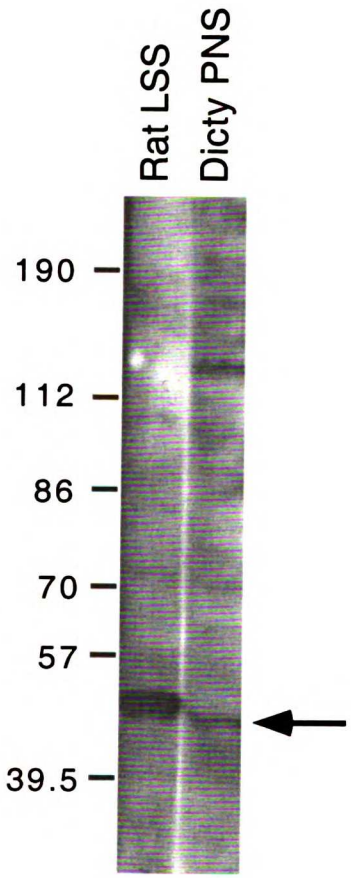
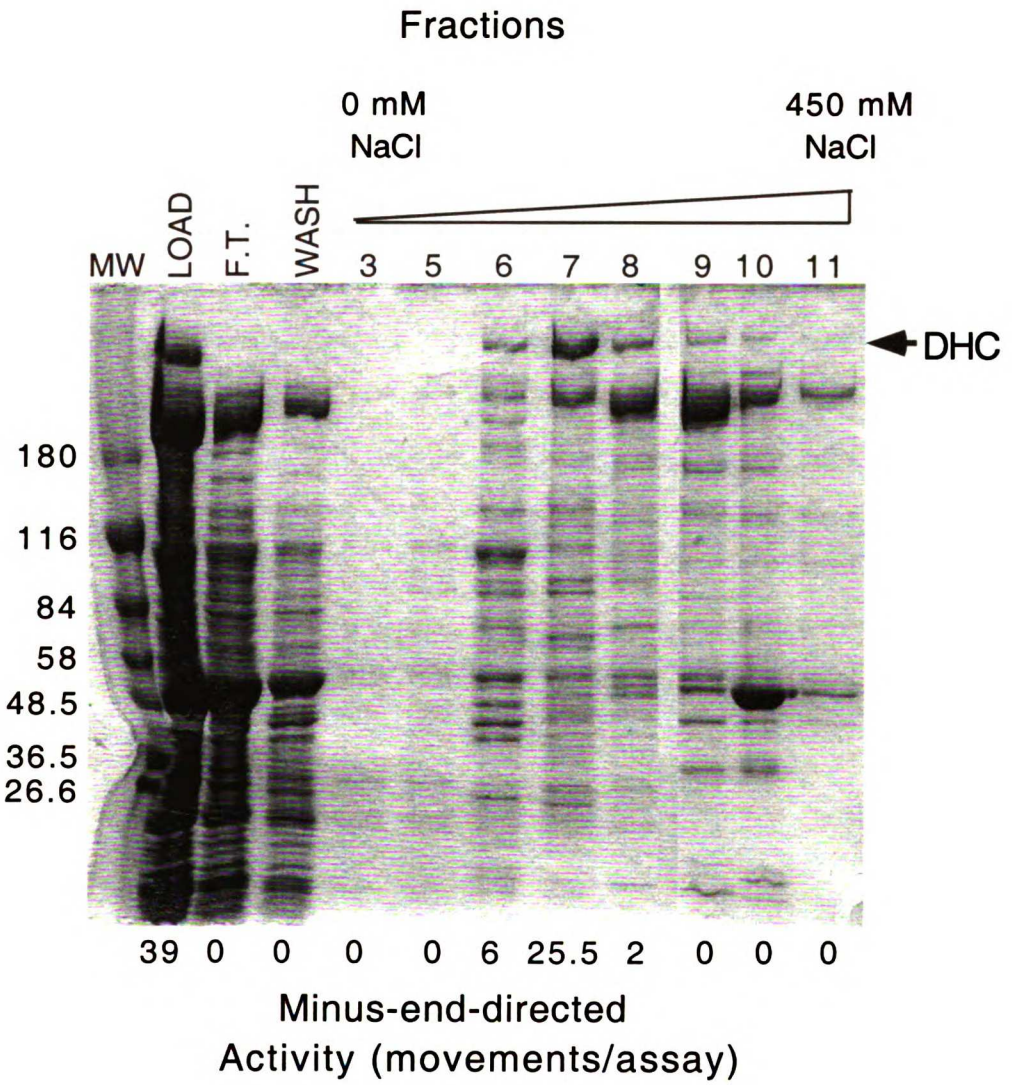

Rat LSS
Dicty PNS
190
112
86
70
57
39.5

Figure 2. Purification of minus-end-directed organelle transport activity by Mono S chromatography. 200 µl of ATP releasate prepared in Pipes buffer (see text) was loaded onto a 100 µl Mono S column and eluted with a 0-500 mM NaCl gradient (fractions 3-12; fractions 3-11 are shown in this Coomassie-stained gel). Minus-end-directed activity co-peaked strongly with the DHC (arrow).


Fractions
0 mM NaCl
450 mM NaCl
MW
LOAD
F.T.
WASH
3
5
6
7
8
9
10
11
DHC
180
116
84
58
48.5
36.5
26.6
39
0
0
0
0
6
25.5
2
0
0
0
Minus-end-directed
Activity (movements/assay)

References

Adachi, H., T. Hasebe, K. Yoshinaga, T. Ohta and K. Sutoh. 1994. Isolation of *Dictyostelium discoideum* cytokinesis mutants by restriction enzyme-mediated integration of the blasticidin S resistance marker. *Biochem. Biophys. Res. Commun.* 205:1808-1814.

Aizawa, H., Y. Sekine, R. Takemura, Z. Zhang, M. Nangaku and N. Hirokawa. 1992. Kinesin family in murine central nervous system. *J. Cell Biol.* 119:1287-96.

Allan, V. 1995. Membrane traffic motors. *FEBS Letters.* 369:101-106.

Allan, V. J. and R. D. Vale. 1991. Cell cycle control of microtubule-based membrane transport and tubule formation in vitro. *J. Cell Biol.* 113:347-359.

Allen, C. and G. G. Borisy. 1974. Structural polarity and directional growth of microtubules of Chlamydomonas flagella. *J. Mol. Biol.* 90:381-402.

Aniento, F., N. Emans, G. Griffiths and J. Gruenberg. 1993. Cytoplasmic dynein-dependent vesicular transport from early to late endosomes. *J. Cell Biol.* 123:1373-1387.

Baumann, O. and D. B. Murphy. 1995. Microtubule-associated movement of mitochondria and small particles in *Acanthamoeba castellanii. Cell Motil. Cytoskel.* 32:305-17.

Blocker, A., F. F. Severin, J. K. Burkhardt, J. B. Bingham, H. Yu, J. C. Olivo, T. A. Schroer, A. A. Hyman and G. Griffiths. 1997. Molecular requirements for

bi-directional movement of phagosomes along microtubules. *J. Cell Biol.* 137:113-129.

Bloom, G. and S. Endow. 1995. Motor Proteins 1: kinesin. *Protein Profile.* 2:1112-1138.

Bomsel, M., R. Parton, S. A. Kuznetsov, T. A. Schroer and J. Gruenberg. 1990. Microtubule- and motor-dependent fusion in vitro between apical and basolateral endocytic vesicles from MDCK cells. *Cell.* 62:719-31.

Burkhardt, J. K., C. J. Echeverri, T. Nilsson and R. B. Vallee. 1997. Overexpression of the dynamitin (p50) subunit of dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. *J. Cell Biol.* 139:469-484.

Burkhardt, J. K., J. J. McIlvain, M. P. Sheetz and Y. Argon. 1993. Lytic granules from cytotoxic T cells exhibit kinesin-dependent motility on microtubules in vitro. *J. Cell Sci.* 104:151-162.

Cole, D. G., W. Z. Cande, R. J. Baskin, D. A. Skoufias, C. J. Hogan and J. M. Scholey. 1992. Isolation of a sea urchin egg kinesin-related protein using peptide antibodies. *J. Cell Sci.* 101:291-301.

Corthesy-Theulaz, I., A. Pauloin and S. R. Pfeffer. 1992. Cytoplasmic dynein participates in the centrosomal localization of the Golgi complex. *J. Cell Biol.* 118:1333-1345.

Dabora, S. L. and M. P. Sheetz. 1988. Cultured cell extracts support organelle movement on microtubules in vitro. *Cell Motil. Cytoskeleton.* 10:482-95.

de Hostos, E. L., G. McCaffrey, R. Sucgang, D. W. Pierce and R. D. Vale. 1998. A developmentally regulated kinesin-related motor protein from *Dictyostelium discoideum. Mol. Biol. Cell.* 9:2093-2106.

de Hostos, E. L., C. Rehfuess, B. Bradtke, D. R. Waddell, R. Albrecht, J. Murphy and G. Gerisch. 1993. *Dictyostelium* mutants lacking the cytoskeletal protein coronin are defective in cytokinesis and cell motility. *J. Cell Biol.* 120:163-173.

Dorner, C., T. Ciossek, S. Muller, N. P. H. Moller, A. Ullrich and R. Lammers. 1998. Characterization of KIF1C, a new kinesin-like protein involved in vesicle transport from the Golgi apparatus to the endoplasmic reticulum. *J. Biol. Chem.* 273:20267-20275.

Echard, A., F. Jollivet, O. Martinez, J. J. Lacapere, A. Rousselet, I. Janoueix-Lerosey and B. Goud. 1998. Interaction of a golgi-associated kinesin-like protein with Rab6. *Science.* 279:580-585.

Feiguin, F., A. Ferreira, K. S. Kosik and A. Caceres. 1994. Kinesin-mediated organelle translocation revealed by specific cellular manipulations. *J. Cell Biol.* 127:1021-1039.

Gilbert, T., A. Le Bivic, A. Quaroni and E. Rodriguez-Boulan. 1991. Microtubular organization and its involvement in the biogenetic pathways

of plasma membrane proteins in Caco-2 intestinal epithelial cells. *J. Cell Biol.* 113:275-88.

Gill, S. R., T. A. Schroer, I. Szilak, E. R. Steuer, M. P. Sheetz and D. W. Cleveland. 1991. Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein. *J. Cell Biol.* 115:1639-1650.

Gindhart, J. G., C. J. Desai, S. Beushausen, K. Zinn and L. S. Goldstein. 1998. Kinesin light chains are essential for axonal transport in *Drosophila*. *J. Cell Biol.* 141:443-454.

Goodson, H. V., C. Valetti and T. Kreis. 1997. Motors and membrane traffic. *Curr. Opin. Cell Biol.* 9:18-28.

Hall, D. H. and E. M. Hedgecock. 1991. Kinesin-related gene *unc-104* is required for axonal transport of synaptic vesicles in *C. elegans* . *Cell.* 65:837-847.

Hanlon, D. W. and L. S. B. Goldstein. 1997. Characterization of KIFC2, a neuronal kinesin superfamily member in mouse. *Neuron.* 18:439-51.

Harada, A., Y. Takei, Y. Kanai, Y. Tanaka, S. Nonaka and N. Hirokawa. 1998. Golgi vesiculation and lysosome dispersion in cells lacking cytoplasmic dynein. *J. Cell Biol.* 141:51-59.

Hellman, U., C. Wernstedt, J. Gonez and C. H. Heldin. 1995. Improvement of an in-gel digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. *Anal. Biochem.* 224:451-455.

Herman, B. and D. F. Albertini. 1984. A time-lapse video image intensification analysis of cytoplasmic organelle movements during endosome translocation. *J. Cell Biol.* 98:565-576.

Heuser, J. 1989. Changes in lysosome shape and distribution correlated with changes in cytoplasmic pH. *J. Cell Biol.* 108:855-864.

Hirokawa, N. 1998. Kinesin and dynein superfamily proteins and the mechanism of organelle transport. *Science.* 279:519-526.

Hollenbeck, P. J. 1993. Phosphorylation of neuronal kinesin heavy and light chains in vivo. *J Neurochem.* 60:2265-75.

Hollenbeck, P. J. and J. A. Swanson. 1990. Radial extension of macrophage tubular lysosomes supported by kinesin. *Nature.* 346:864-866.

Holleran, E. A., M. K. Tokito, S. Karki and E. L. F. Holzbaur. 1996. Centractin (ARP1) associates with spectrin revealing a potential mechanism to link dynactin to intracellular organelles. *J. Cell Biol.* 135:1815-1830.

Holleran, E.A., S. Karki, and E.L.F. Holzbaur. 1998. The role of the dynactin complex in intracellular motility. *Int. Rev. Cytol.* 182:69-109.

Karki, S. and E. L. F. Holzbaur. 1995. Affinity chromatography demonstrates a direct binding between cytoplasmic dynein and the dynactin complex. *J. Biol. Chem.* 270:28806-28811.

Koonce, M. P. and M. Samso. 1996. Overexpression of cytoplasmic dynein's globular head causes a collapse of the interphase microtubule network in *Dictyostelium*. *Mol. Biol. Cell.* 7:935-948.

Kumar, J., H. Yu and M. P. Sheetz. 1995. Kinectin, an essential anchor for kinesin-driven vesicle motility. *Science.* 267:1834-1837.

Lacey, M. L. and L. T. Haimo. 1994. Cytoplasmic dynein binds to phospholipid vesicles. *Cell Motil. Cytoskel.* 28:205-212.

Lafont, F., J. K. Burkhardt and K. Simons. 1994. Involvement of microtubule motors in basolateral and apical transport in kidney cells. *Nature.* 372:801-803.

Lane, J. and V. Allan. 1998. Microtubule-based membrane movement. *Biochimica et Biophysica Acta.* 1376:27-55.

Lee, C., M. Ferguson and L. B. Chen. 1989. Construction of the endoplasmic reticulum. *J. Cell Biol.* 109:2045-2055.

Lee, K. D. and P. J. Hollenbeck. 1995. Phosphorylation of kinesin in vivo correlates with organelle association and neurite outgrowth. *J. Biol. Chem.* 270:5600-5605.

Lin, S. X., K. K. Pfister and C. A. Collins. 1996. Comparison of the intracellular distribution of cytoplasmic dynein and kinesin in cultured cells: motor protein location does not reliably predict function. *Cell Motil. Cytoskeleton*. 34:299-312.

Lippincott-Schwartz, J., N. B. Cole, A. Marotta, P. A. Conrad and G. S. Bloom. 1995. Kinesin is the motor for microtubule-mediated Golgi-to-ER membrane traffic. *J. Cell Biol.* 128:293-306.

Lupas, A., M. Van Dyke and J. Stock. 1991. Predicting coiled coils from protein sequences. *Science*. 252:1162-1164.

Malchow, D., B. Naegele, H. Schwarz and G. Gerisch. 1972. Membrane-bound cyclic AMP phosphodiesterase in chemotactically responding cells of *Dictyostelium discoideum*. *Eur. J. Biochem.* 28:136-142.

Manstein, D. J., H. P. Schuster, P. Morandini and D. M. Hunt. 1995. Cloning vectors for the production of proteins in *Dictyostelium discoideum*. *Gene*. 162:129-134.

Marks, D. L., J. M. Larkin and M. A. McNiven. 1994. Association of kinesin with the Golgi apparatus in rat hepatocytes. *J. Cell Sci.* 107:2417-2426.

Marszalek, J. R., P. Ruiz-Lozano, E. Roberts, K. R. Chien and L. S. Goldstein. 1999. Situs inversus and embryonic ciliary morphogenesis defects in mouse

mutants lacking the KIF3A subunit of kinesin-II. *Proc. Natl. Acad. Sci.* 96:5043-8.

Matteoni, R. and T. E. Kreis. 1987. Translocation and clustering of endosomes and lysosomes depends on microtubules. *J. Cell Biol.* 105:1253-1265.

McGrail, M., J. Gepner, A. Silvanovich, S. Ludmann, M. Serr and T. S. Hays. 1995. Regulation of cytoplasmic dynein function in vivo by the *Drosophila* Glued complex. *J. Cell Biol.* 131:411-425.

McNiven, M. A. and K. R. Porter. 1986. Microtubule polarity confers direction to pigment transport in chromatophores. *J. Cell Biol.* 103:1547-55.

Mollenhauer, H. H., D. J. Morre and L. D. Rowe. 1990. Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochimica et Biophysica Acta.* 1031:225-246.

Morris, R. L. and P. J. Hollenbeck. 1995. Axonal transport of mitochondria along microtubules and F-actin in living vertebrate neurons. *J. Cell Biol.* 131:1315-26.

Morris, R. L. and J. M. Scholey. 1997. Heterotrimeric kinesin-II is required for the assembly of motile 9+2 ciliary axonemes on sea urchin embryos. *J. Cell Biol.* 138:1009-1022.

Muresan, V., C. P. Godek, T. S. Reese and B. J. Schnapp. 1996. Plus-end motors override minus-end motors during transport of squid axon vesicles on microtubules. *J. Cell Biol.* 135:383-397.

Nakata, T. and N. Hirokawa. 1995. Point mutation of adenosine triphosphate-binding motif generated rigor kinesin that selectively blocks anterograde lysosome membrane transport. *J. Cell Biol.* 131:1039-1053.

Nangaku, M., R. Sato-Yoshitake, Y. Okada, Y. Noda, R. Takemura, H. Yamazaki and N. Hirokawa. 1994. KIF1B, a novel microtubule plus end-directed monomeric motor protein for transport of mitochondria. *Cell.* 79:1209-1220.

Niclas, J., V. J. Allan and R. D. Vale. 1996. Cell cycle regulation of dynein association with membranes modulates microtubule-based organelle transport. *J. Cell Biol.* 133:585-593.

Nonaka, S., Y. Tanaka, Y. Okada, S. Takeda, A. Harada, Y. Kanai, M. Kido and N. Hirokawa. 1998. Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. *Cell.* 95:829-837.

Ohkura, H., T. Torok, G.Tick, J. Hoheisel, I. Kiss, and D.M. Glover. 1997. Mutation of a gene for a Drosophila kinesin-like protein, Klp38B, leads to failure of cytokinesis. J. Cell Sci. 110:945-954.

Okada, Y. and N. Hirokawa. 1999. A processive single-headed motor: kinesin superfamily protein KIF1A. *Science*. 283:1152-1157.

Okada, Y., H. Yamazaki, Y. Sekine-Aizawa and N. Hirokawa. 1995. The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde axonal transport of synaptic vesicle precursors. *Cell*. 81:769-780.

Otsuka, A. J., A. Jeyaprakash, A. J. Garcia, L. Z. Tang, G. Fisk, T. Hartshorne, R. Franco and T. Born. 1991. The C. elegans unc-104 gene encodes a putative kinesin heavy chain-like protein. *Neuron*. 6:113-22.

Parczyk, K., W. Haase and C. Kondor-Koch. 1989. Microtubules are involved in the secretion of proteins at the apical cell surface of the polarized epithelial cell, Madin-Darby canine kidney. *J. Biol. Chem.* 264:16837-46.

Parton, R. G., C. G. Dotti, R. Bacallao, I. Kurtz, K. Simons and K. Prydz. 1991. pH-induced microtubule-dependent redistribution of late endosomes in neuronal and epithelial cells. *J. Cell Biol.* 113:261-274.

Paschal, B. M., H. S. Shpetner and R. B. Vallee. 1987. MAP 1C is a microtubule-activated ATPase which translocates microtubules in vitro and has dynein-like properties. *J. Cell Biol.* 105:1273-82.

Pfister, K. K., M. C. Wagner, D. L. Stenoien, S. T. Brady and G. S. Bloom. 1989. Monoclonal antibodies to kinesin heavy and light chains stain vesicle-like structures, but not microtubules, in cultured cells. *J. Cell. Biol.* 108:1453-1463.

Pierce, D. W., N. Hom-Booher, A. J. Otsuka and R. D. Vale. 1999. Single-molecule behavior of monomeric and heteromeric kinesins. *Biochemistry*. 38:5412-5421.

Pollock, N., M. P. Koonce, E. L. de Hostos and R. D. Vale. 1998. In vitro microtubule-based organelle transport in wild-type *Dictyostelium* and cells overexpressing a truncated dynein heavy chain. *Cell Motil. Cytoskel.* 40:304-314.

Ponting, C. P. 1995. AF-6/cno: neither a kinesin nor a myosin, but a bit of both. *TIBS*. 20:265-266.

Poot, M., Y. Z. Zhang, J. A. Kramer, K. S. Wells, L. J. Jones, D. K. Hanzel, A. G. Lugade, V. L. Singer and R. P. Haugland. 1996. Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. *J. Histochemistry and Cytochemistry*. 44:1363-72.

Rebecchi, M. J. and S. Scarlata. 1998. Pleckstrin homology domains: a common fold with diverse functions. *Annu. Rev. Biophys. Biomol. Struct.* 27:503-28.

Rodionov, V. I., F. K. Gyoeva and V. I. Gelfand. 1991. Kinesin is responsible for centrifugal movement of pigment granules in melanophores. *Proc Natl Acad Sci U S A*. 88:4956-60.

Rodriguez-Paris, J. M., K. V. Nolta and T. L. Steck. 1993. Characterization of lysosomes isolated from *Dictyostelium discoideum* by magnetic fractionation. *J. Biol. Chem.* 268:9110-9116.

Rogalski, A. A. and S. J. Singer. 1984. Associations of elements of the Golgi apparatus with microtubules. *J. Cell Biol.* 99:1092-100.

Rogers, S. L., I. S. Tint, P. C. Fanapour and V. I. Gelfand. 1997. Regulated bidirectional motility of melanophore pigment granules along microtubules in vitro. *Proc Natl Acad Sci.* 94:3720-3725.

Roos, U.-P., M. De Brabander and R. Nuydens. 1987. Movements of intracellular particles in undifferentiated amebae of *Dictyostelium discoideum*. *Cell Motil. Cytoskel.* 7:258-271.

Sablin, E. P., F. J. Kull, R. Cooke, R. D. Vale and R. J. Fletterick. 1996. Crystal structure of the motor domain of the kinesin-related motor ncd. *Nature.* 380:555-559.

Sakowicz, R., S. Farlow, and L.S.B. Goldstein. 1999. Cloning and expression of kinesins from the thermophilic fungus *Thermomyces lanuginosus*. Pro. Sci. In press.

Saito, N., Y. Okada, Y. Noda, Y. Kinoshita, S. Kondo and N. Hirokawa. 1997. KIFC2 is a novel neuron-specific C-terminal type kinesin superfamily motor for dendritic transport of multivesicular body-like organelles. *Neuron.* 18:425-38.

Saraste, M. and M. Hyvonen. 1995. Pleckstrin homology domains: a fact file. *Current Opinion Structural Biol.* 5:403-408.

Sato-Yoshitake, R., H. Yorifuji, M. Inagaki and N. Hirokawa. 1992. The phosphorylation of kinesin regulates its binding to synaptic vesicles. *J Biol Chem.* 267:23930-6.

Saxton, W. M., J. Hicks, L. S. Goldstein and E. C. Raff. 1991. Kinesin heavy chain is essential for viability and neuromuscular functions in *Drosophila,* but mutants show no defects in mitosis. *Cell.* 64:1093-102.

Sawin, K.E., T.J. Mitchison, and L.G. Wordeman. 1992. Evidence for kinesin-related proteins in the mitotic apparatus using peptide antibodies. *J. Cell Sci.* 101: 303-313.

Schmitz, F., K. T. Wallis, M. Rho, D. Drenckhahn and D. B. Murphy. 1994. Intracellular distribution of kinesin in chromaffin cells. *Eur. J. Cell Biol.* 63:77-83.

Schnapp, B. J. and T. S. Reese. 1989. Dynein is the motor for retrograde axonal transport of organelles . *Proc Natl Acad Sci U S A.* 86:1548-52.

Schnapp, B. J., T. S. Reese and R. Bechtold. 1992. Kinesin is bound with high affinity to squid axon organelles that move to the plus-end of microtubules . *J. Cell Biol.* 119:389-99.

Schroer, T. A., B. J. Schnapp, T. S. Reese and M. P. Sheetz. 1988. The role of kinesin and other soluble factors in organelle movement along microtubules . *J. Cell Biol.* 107:1785-92.

Schroer, T. A. and M. P. Sheetz. 1991. Two activators of microtubule-based vesicle transport. *J. Cell Biol.* 115:1309-1318.

Schroer, T. A., E. R. Steuer and M. P. Sheetz. 1989. Cytoplasmic dynein is a minus-end-directed motor for membranous organelles. *Cell.* 56:937-946.

Seiler, S., F. E. Nargang, G. Steinberg and M. Schliwa. 1997. Kinesin is essential for cell morphogenesis and polarized secretion in *Neurospora crassa. Embo J.* 16:3025-3034.

Sellitto, C., M. Kimble, and R. Kuriyama. 1992. Heterogeneity of microtubule organizing center components as revealed by monoclonal antibodies to mammalian centrosomes and to nucleus-associated bodies from *Dictyostelium. Cell Motil. Cytoskeleton* 22:7-24.

Shaw, D. R., H. Richter, R. Giorda, T. Ohmachi and H. L. Ennis. 1989. Nucleotide sequences of *Dictyostelium discoideum* developmentally regulated cDNAs rich in (AAC) imply proteins that contain clusters of asparagine, glutamine, or threonine. *Mol. Gen. Genet.* 218:453-459.

Skoufias, D., D. G. Cole, K. P. Wedaman and J. M. Scholey. 1994. The carboxyl-terminal domain of kinesin heavy chain is important for membrane binding. *J. Biol. Chem.* 269:1477-1485.

Steinberg, G. and M. Schliwa. 1995. The *Neurospora* organelle motor: a distant relative of conventional kinesin with unconventional properties. *Mol. Biol. Cell.* 6:1605-1618.

Stenoien, D. L. and S. T. Brady. 1997. Immunochemical analysis of kinesin light chain function. *Mol. Biol. Cell.* 8:675-689.

Sussman, M. 1987. Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods Cell Biol.* 28:9-29.

Swanson, J., A. Bushnell and S. C. Silverstein. 1987. Tubular lysosome morphology and distribution within macrophages depend on the integrity of cytoplasmic microtubules. *Proc. Natl. Acad. Sci. USA.* 84:1921-1925.

Tai, A. W., J.-Z. Chuang, C. Bode, U. Wolfrum and C.-H. Sung. 1999. Rhodopsin's carboxy-terminal cytoplasmic tail acts as a membrane receptor for cytoplasmic dynein by binding to the dynein light chain Tctex-1. *Cell.* 97:877-887.

Tanaka, Y., Y. Kanai, Y. Okada, S. Nonaka, S. Takeda, A. Harada and N. Hirokawa. 1998. Targeted disruption of mouse conventional kinesin heavy chain, kif5B, results in abnormal perinuclear clustering of mitochondria. *Cell.* 93:1147-1158.

Tinsley, J. H., P. F. Minke, K. S. Bruno and M. Plamann. 1996. P150Glued, the largest subunit of the dynactin complex, is nonessential in *Neurospora* but required for nuclear distribution. *Mol. Biol. Cell.* 7:731-742.

Toyoshima, I., H. Yu, E. R. Steuer and M. P. Sheetz. 1992. Kinectin, a major kinesin-binding protein on ER . *J. Cell Biol.* 118:1121-31.

Tuma, M. C., A. Zill, N. Le Bot, I. Vernos and V. Gelfand. 1998. Heterotrimeric kinesin II is the microtubule motor protein responsible for pigment dispersion in *Xenopus* melanophores. *J. Cell Biol.* 143:1547-1558.

Urrutia, R., M. A. McNiven, J. P. Albanesi, D. B. Murphy and B. Kachar. 1991. Purified kinesin promotes vesicle motility and induces active sliding between microtubules in vitro. *Proc Natl Acad Sci U S A.* 88:6701-5.

Vale, R. D. and R. J. Fletterick. 1997. The design plan of kinesin motors. *Annu. Rev. Cell Dev. Biol.* 13:745-777.

Vale, R. D., B. J. Schnapp, T. S. Reese and M. P. Sheetz. 1985. Organelle, bead and microtubule translocations promoted by soluble factors from the squid giant axon. *Cell.* 40:559-569.

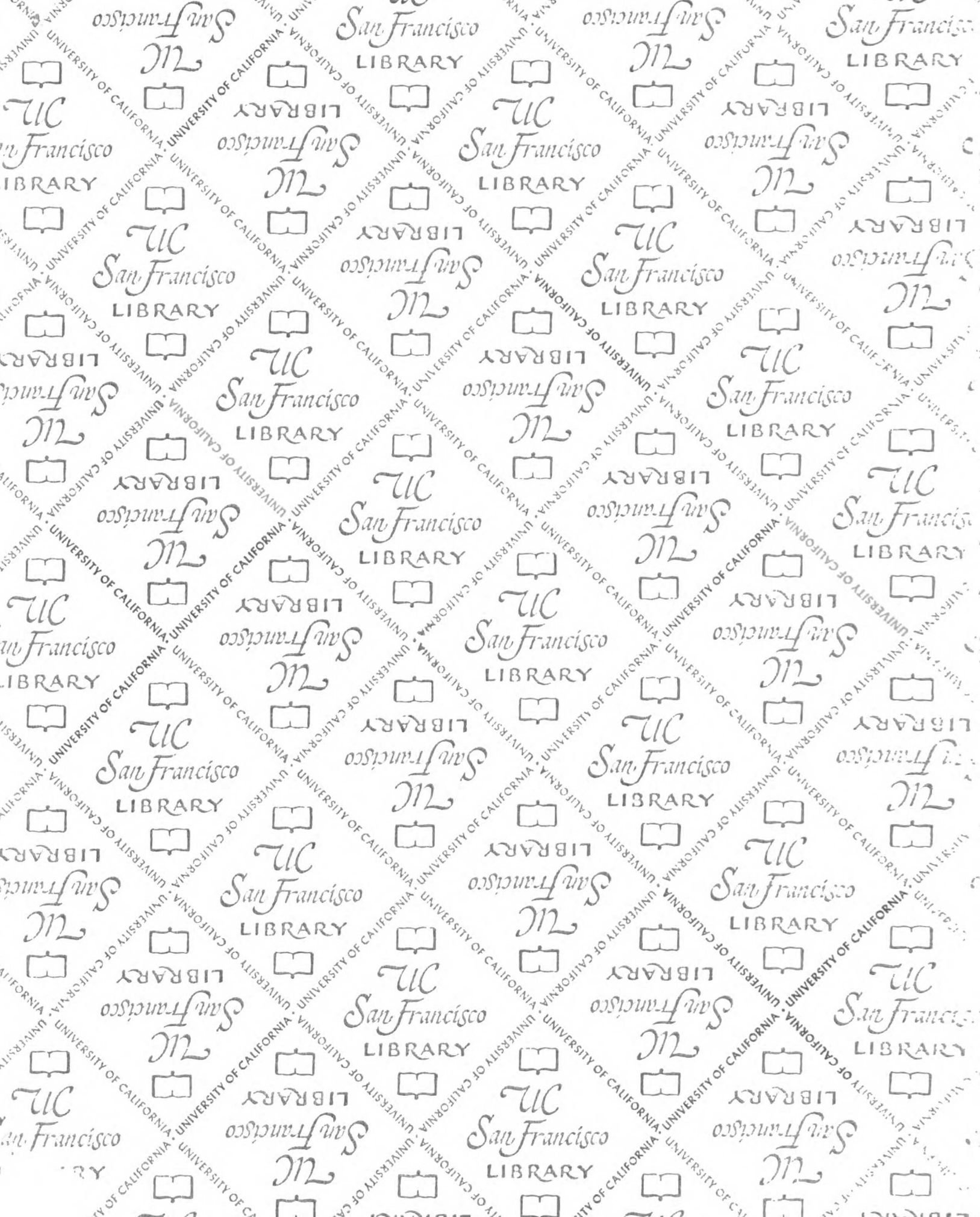
Vale, R. D., T. S. Reese and M. P. Sheetz. 1985. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell.* 42:39-50.

Van Blerkom, J. 1991. Microtubule mediation of cytoplasmic and nuclear maturation during the early stages of resumed meiosis in cultured mouse oocytes. *Proc. Natl. Acad. Sci. USA.* 88:5031-5.

Vaughan, K. T. and R. B. Vallee. 1995. Cytoplasmic dynein binds dynactin through a direct interaction between the intermediate chains and p150*Glued*. *J. Cell Biol.* 131:1507-1516.

Weber, I., G. Gerisch, C. Heizer, J. Murphy, K. Badelt, A. Stock, J. M. Schwartz and J. Faix. 1999. Cytokinesis mediated through the recruitment of cortexillins into the cleavage furrow. *EMBO J.* 18:586-94.

Yonekawa, Y., A. Harada, Y. Okada, T. Funakoshi, Y. Kanai, Y. Takei, S. Terada, T. Noda and N. Hirokawa. 1998. Defect in synaptic vesicle precursor transport and neuronal cell death in KIF1A motor protein-deficient mice. *J. Cell Biol.* 141:431-441.


UC
San Francisco
LIBRARY
UNIVERSITY OF CALIFORNIA · UNIVERSITY OF CALIFORNIA