Biochemistry **1998,** *37,* 15033−15041

Cytoplasmic Dynein Contains a Family of Differentially Expressed Light Chains[†]

Stephen M. King,*[,‡] Elisa Barbarese,[§] James F. Dillman III,[||] Sharon E. Benashski,[‡] Khanh T. Do,[||]
Ramila S. Patel-King,[‡] and K. Kevin Pfister[||]

Departments of Biochemistry and Neurology and Center for Biomedical Imaging Technology, University of Connecticut Health Center, Farmington, Connecticut 06032-3305, and Department of Cell Biology, University of Virginia Health Science Center, Charlottesville, Virginia 22908-0439

Received May 11, 1998; Revised Manuscript Received August 19, 1998

ABSTRACT: Cytoplasmic dynein contains a series of accessory proteins associated with the motor containing heavy chains.[1] These include three distinct classes of light chains (M_r < ∼22 000). Here we demonstrate that a previously cloned protein termed rp3 is a bona fide M_r 14 000 light chain component of this microtubule motor complex. The rp3 polypeptide is ∼55% identical to the Tctex1 dynein light chain, and together, these two proteins define one branch of a diverse family of M_r 14 000 light chains associated with both cytoplasmic and flagellar dyneins. The Tctex1 and rp3 light chains are differentially expressed in various tissues: rp3 is most prevalent in liver and brain cytoplasmic dynein, whereas those tissues contain the least amounts of Tctex1. Immunofluorescence analysis was consistent with the tissue-specific distribution of these proteins and revealed that both rp3 and Tctex1 are present in multiple perinuclear punctate particles. Furthermore, in two cell lines, rp3 was found associated with an elongated structure located in the layer of cytoplasm above the nucleus. Electrophoretic/immunological analysis indicates that there are only single isoforms for these proteins in brain and PC-12 cells, suggesting that alterations in the M_r 14 000 light chains of dynein are achieved at the level of the individual proteins and not by posttranslational modification. Dissection of the cytoplasmic dynein complex revealed that Tctex1, an M_r 8000 LC dimer, and IC74 associate to define a basal-located intermediate chain/light chain complex analogous to that found in flagellar outer arm dynein.

Cytoplasmic dynein acts as a molecular motor and transports the cargo to which it is attached toward the minus end of microtubules. In addition to its several proposed functions in the movement of both spindles and chromosomes during mitosis (*1−4*), known cytoplasmic dynein cargoes include the Golgi apparatus and other membranous organelles (*5−8*). Clearly, in order to accomplish the directed movement of these diverse intracellular components, there must be regulatory mechanisms that impinge on cytoplasmic dynein to ensure the spatial and temporal control of motor activity as well as to determine to which cargo a particular motor unit should be attached.

The cytoplasmic dynein complex has a total mass of ∼1.25 MDa and consists of two heavy chains (HCs;[2] ∼530 kDa) that contain the motor and ATP hydrolytic sites (reviewed in ref *9*), several (probably two) copies of a 74 kDa intermediate chain (IC74) (*10*) that is a member of the WD (G_β) repeat family (*11*) and therefore likely forms a β-propeller structure (*12*), four light intermediate chains (LICs) of 50−60 kDa that are distantly related to ABC transporters (*13, 14*), and several light chains (LCs) with masses less than ∼22 kDa (*15, 16*).

Intriguingly, recent studies have uncovered considerable heterogeneity in the polypeptides that go to form this motor enzyme, which may well reflect specific requirements for specialized motor, cargo-binding, and/or regulatory activities within different cell and tissue types. The observed heterogeneity derives both from different gene products for a given component (e.g., there are two IC74 genes and three classes of cytoplasmic HC known at present; *17, 18*) and from the multiple variants generated by differential phosphorylation

---

[†] This study was supported by a New Investigator award from the Patrick and Catherine Weldon Donaghue Medical Research Foundation (to S.M.K.), by Grant RG 2843-A-2 from the National Multiple Sclerosis Society (to E.B.), and by Grants GM 51293 (to S.M.K.), NS 19943 (to E.B.), and NS 29996 (to K.K.P.) awarded by the National Institutes of Health.

* To whom correspondence should be addressed at the University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06032-3305. Tel: (860) 679-3347. Fax: (860) 679-3408. E-mail: king@panda.uchc.edu.

[‡] Department of Biochemistry, University of Connecticut Health Center.

[§] Department of Neurology and Center for Biomedical Imaging Technology, University of Connecticut Health Center.

[||] Department of Cell Biology, University of Virginia Health Science Center.

[1] Note on nomenclature: The cytoplasmic dynein polypeptide that is the main subject of this paper was first cloned as a candidate for causing X-linked retinitis pigmentosa type 3 in humans. For this reason, the protein was originally designated as "rp3". Since that report, the actual *RP3* gene has been located on the human X chromosome and found to encode a guanine nucleotide exchange factor. The current SWISSPROT database entry for rp3 (P51808) has been amended to simply note the homology of this protein with Tctex1. Here, we have retained the original designation of rp3 for the purpose of continuity and to allow this protein to be readily distinguished from the related Tctex1 LC. However, there will clearly be a need for a unified, internally consistent, nomenclature once all the components of this motor complex have been unambiguously identified.

[2] Abbreviations: HC, heavy chain; IC, intermediate chain; LC, light chain; LIC, light intermediate chain; MBP, maltose binding protein.

10.1021/bi9810813 CCC: $15.00

and/or alternative splicing (e.g., IC74 and the LICs) (*10*, *13*, *14*, *19*−*21*).

Recently, we identified LC components of M_r 8000, 14 000, and 22 000 associated with cytoplasmic dynein. The M_r 8000 protein is a highly conserved molecule that is common to both cytoplasmic and flagellar dyneins (*15*, *22*, *23*). This 89-residue polypeptide exists within the dynein complex as a dimeric structure (*24*). It also has been found associated with other enzyme systems including myosin V (*25*) and neuronal nitric oxide synthase (*26*); in the latter case the LC has been implicated in the regulation of enzyme activity both in vitro and in vivo, raising the possibility that it acts as a generalized regulatory element in a manner analogous to that of calmodulin. Moreover, biochemical fractionation strongly suggests that this small protein interacts with additional flagellar and cytoplasmic components that have yet to be identified.[3]

The M_r 14 000 LC was identified as the murine *t* complex-encoded protein Tctex1 (*16*) which is a candidate for one of the "distorter" products responsible for the extreme meiotic drive (transmission ratio distortion) exhibited by this complex genetic unit (see refs *27*−*29* for recent discussions). Examination of the GenBank database with the Tctex1 sequence identified another previously cloned protein (termed rp3) that is closely related to Tctex1; these molecules share ∼55% identity (*30*). The rp3 protein was initially of interest as it was a candidate for causing X-linked retinitis pigmentosa type 3; more recent studies have revealed that the actual *RP3* protein is a guanine nucleotide exchange factor (*31*). However, on the basis of the high degree of similarity between Tctex1 and rp3, we suggested previously that the rp3 protein also might represent a cytoplasmic dynein component (*16*).

In this report, we demonstrate that the rp3 protein is indeed dynein-associated and that it is most highly expressed in cells and tissues distinct from those which contain Tctex1. The data presented here support the hypothesis that multiple cell- and tissue-specific variants of cytoplasmic dynein exist which may be defined by their M_r 14 000 LC content. Furthermore, salt-induced dissociation of the cytoplasmic dynein particle has allowed us to determine the intradynein associations of the LC components and thus to further define the global organization of this microtubule motor complex.

EXPERIMENTAL PROCEDURES

Dynein Purification. Cytoplasmic dynein was purified from rat brain by the ATP-sensitive microtubule affinity procedure (*32*) using the modifications described previously (*15*). Subsequently, ATP-eluted dynein was further purified by sedimentation through a 5−20% sucrose density gradient. Alternatively, the enzyme was obtained directly from homogenates of rat brain, kidney, liver, spleen, and testis by immunoprecipitation with monoclonal antibody 74-1 that specifically recognizes IC74 (*19*).

For dynein dissection experiments, sucrose gradient-purified dynein was dissociated by the addition of 3 M KI to a final concentration of 0.6 M (*13*, *33*). Dissociated dynein subcomplexes subsequently were separated by sedimentation through a second 5−20% sucrose gradient containing 0.6 M KI.

Fusion Protein and Antibody Preparation. Oligonucleotides encoding a 20-residue segment of the rp3 protein (and an in-frame stop codon) were synthesized directly, annealed, and subcloned across the *Xmn*I/*Xba*I sites of pMal-c2 (New England Biolabs, Beverly, MA). The resulting C-terminal fusion of the 20-residue section of rp3 to maltose binding protein (MBP) was purified by amylose affinity chromatography and used for the immunization of rabbit R5270. The entire human rp3 coding region was obtained from IMAGE consortium clone no. 291896 using the polymerase chain reaction. Sequencing revealed that this clone contained a single base pair change compared to the previously published coding sequence (U02556; *30*). This C to T transition occurred in the third position of an Ala codon and did not affect the primary structure of the encoded protein. The rp3 coding region was subcloned into the pMAL-c2 vector as described above. The fusion protein was subsequently digested with factor Xa to separate rp3 from MBP, and the electrophoretically isolated rp3 protein was used to obtain a highly specific antibody fraction by blot purification.

A rabbit polyclonal antibody that specifically reacts with Tctex1 (R5205) was described previously (*16*). Characterization of monoclonal antibodies that recognize IC74 of cytoplasmic dynein (74-1), kinesin heavy chain (H-2), and the p50 component of dynactin (50−1) has been reported by Dillman and Pfister (*19*), Pfister et al. (*34*), and Paschal et al. (*35*), respectively.

Gel Electrophoresis and Immunoblotting. Samples were routinely separated by electrophoresis in 5−15% SDS−polyacrylamide gels as described previously (*36*). Gels were either stained with Coomassie blue or were blotted to nitrocellulose in 10 mM $NaHCO_3$, 3 mM Na_2CO_3, 0.01% SDS, and 20% methanol. All solutions used for probing the blots contained 5% dry milk and 0.1% Tween-20 in Tris-buffered saline except for the final washes with 0.5% Triton X-100 and 50 mM Tris-HCl, pH 6.8. Antibody reactivity was observed using a peroxidase-conjugated secondary antibody combined with chemiluminescent detection (ECL, Amersham) and Fuji RX film.

Two-dimensional electrophoresis of dynein immunoprecipitates was performed as described by Dillman and Pfister (*19*). Following electrophoresis the gels were either dried for autoradiography or blotted to PVDF membrane and probed sequentially with antibodies against Tctex1 and rp3 as described above.

Computational Methods. Searches of the Genbank and Expressed Sequence Tag databases were performed with BLAST (*37*). Pairwise sequence comparisons were made using GAP (*38*).

Cell Culture and Immunofluoresence Microscopy. Primary cultures of rat brain oligodendrocytes were fixed with formaldehyde and treated for immunocytochemistry as detailed by Ainger et al. (*39*) and Barbarese et al. (*40*). Normal rat kidney fibroblast (NRK) cells were fixed with methanol (−20 °C) for 10 min followed by 1 min in acetone at 0 °C. Rat B104 CNS-derived neuronal cells were prepared using both fixation procedures. Tctex1, rp3, and IC74 of cytoplasmic dynein were detected using rabbit polyclonal antibodies R5205 (*16*) and R5270 and monoclonal antibody

---

[3] S. E. Benashski and S. M. King, unpublished results.

```
                    .         .         .         .         .
   rp3   1 MEEYHRHCDEVGFNAEEAHNIVKECVDGVLGGEDYNHNNINQWTASIVEQ 50
             ||:| .  :|  |  :|  ||||| ::  :||  | |. :|||| .:|||
Tctex1   1 MEDY.QAAEETAFVVDEVSNIVKEAIESAIGGNAYQHSKVNQWTTNVVEQ 49

                      .         .         .         .         .
   rp3  51 SLTHLVKLGKAYKYIVTCAVVQKSAYGFHTASSCFWDTTSDGTCTVRWEN 100
           .|. | |||| :|||||| :.||.  | |||||||||...||.|||||||
Tctex1  50 TLSQLTKLGKPFKYIVTCVIMQKNGAGLHTASSCFWDSSTDGSCTVRWEN 99

                      .
   rp3 101 RTMNCIVNVFAIAIVL 116
           :|| |||. | :.|
Tctex1 100 KTMYCIVSAFGLSI.. 113
```

FIGURE 1: Sequence comparison of rp3 and Tctex1. This comparison between human rp3 (U02556; *30*) and human Tctex1 (H11202; *58*) was generated by GAP using the default parameters. The symbols (|, :, .) represent identity and conservative and semiconservative substitutions, respectively. The 20-residue segment of rp3 outlined in bold type was fused to MBP for polyclonal antibody production.

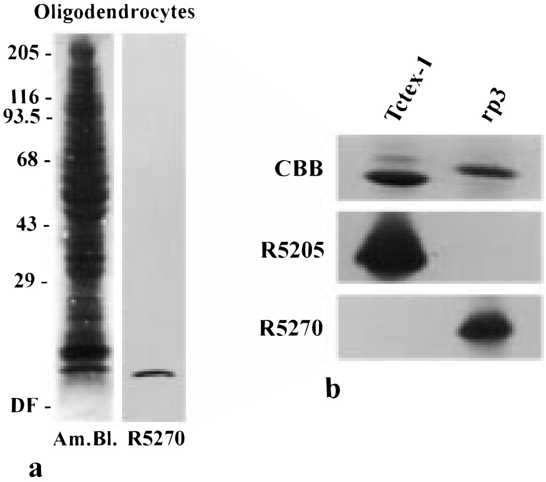

FIGURE 2: Specificity of the R5270 antibody. (a) Total cellular protein from ~10^7 rat brain oligodendrocytes was blotted to nitrocellulose and probed with blot-purified R5270 antibody (R5270). A single band of M_r ~14 000 was observed. Subsequently, the blot was stained with Amido Black (Am.Bl.) to reveal total protein. (b) Recombinant Tctex1 and rp3 were separated from MBP by factor Xa digestion, electrophoresed, and stained with Coomassie blue (upper panel). Identical samples were blotted to nitrocellulose and probed with the R5205 and R5270 antibodies (lower panels). Both antibodies are highly specific for their respective antigens.

74-1 (*19*), respectively. Microtubules were stained with a commercial anti-tubulin monoclonal antibody. Fluorescein-, Cy3- and Texas red-conjugated secondary antibodies were used. The Golgi apparatus was detected using Texas red-conjugated wheat germ agglutinin and membrane-bounded acidic compartments stained with the dye LysoSensor (Molecular Probes, Eugene, OR). B104 cells and oligodendrocytes were imaged using a Zeiss Axioskop microscope coupled to a Bio-Rad MRC 600 laser scanning confocal imaging system or a Zeiss Axiovert 135 with an LSM confocal system. Both microscopes were equipped with a 63 × 1.40 na Plan Apochromat objective. NRK cells were observed using a Nikon Diaphot microscope with epifluorescence illumination and a 60× objective.

Rat adrenal pheochromocytoma (PC-12) cells were grown in DMEM medium containing 5% fetal calf serum and 10% equine serum. These cells were radiolabeled with ^{35}S in vivo by overnight growth in medium containing [^{35}S]methionine (*20*).

RESULTS

Previous analysis of an M_r 14 000 LC associated with cytoplasmic dynein found that it was identical to the murine *t* complex-encoded protein Tctex1 (*16*). Examination of the Genbank database revealed that Tctex1 is closely related to another previously cloned protein termed rp3 (*30*). A sequence comparison between these two molecules generated using GAP is shown in Figure 1. Tctex1 and rp3 share 55% sequence identity (74% similarity); the probability of this match occurring by chance is 6.0 × 10^{-44} (calculated by BLAST). These observations raised the possibility that the rp3 protein also might be a cytoplasmic dynein LC.

To test this hypothesis, it was necessary to generate an antiserum that specifically reacts with rp3 but not with Tctex1. Accordingly, the 20-residue segment of rp3 that is most dissimilar between these two molecules (outlined in bold type in Figure 1) was fused to MBP and used for polyclonal antibody production in rabbit R5270. Following blot affinity purification of the R5270 antiserum against the full-length recombinant rp3 protein, the antibody preparation was used to probe total protein derived from rat brain oligodendrocytes (the cell type previously used to characterize both Tctex1 and M_r 8000 LC antisera; *15, 16*) (Figure 2a). This antibody preparation was highly specific and recognized a single band migrating at M_r ~14 000. To further test the specificity of this preparation, recombinant Tctex1 and rp3 proteins were separated from the MBP fusion partner by factor Xa digestion and electrophoresed to resolve the LCs from MBP. When probed with the R5270 and R5205 (made against recombinant Tctex1; *16*) antibodies, it was found that both antisera were highly specific for their respective polypeptides and did not cross-react (Figure 2b). The recombinant rp3 protein migrated slightly more slowly in polyacrylamide gels than did Tctex1, which is consistent with their calculated molecular weights of 13 061 and 12 451, respectively.

To determine whether the rp3 protein was associated with cytoplasmic dynein, a rat brain homogenate was fractionated and probed with the R5270 antibody. This revealed that most (~70%) of total brain rp3 protein was microtubule-associated following the addition of taxol to the homogenate; a similar proportion of the IC74 component of cytoplasmic dynein also was observed in the microtubule pellet (Figure 3). Both IC74 and rp3 remained microtubule-associated through a buffer wash. A minor amount (<5%) of rp3 (but no detectable IC74) was released from microtubules upon treatment with 5 mM GTP. Elution with 5 mM ATP

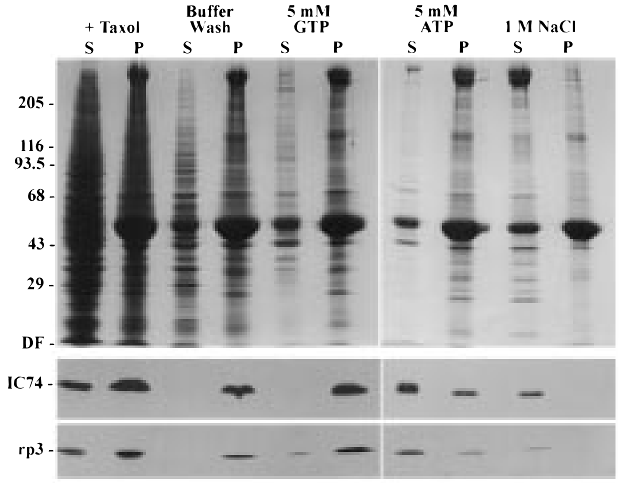

FIGURE 3: The rp3 protein copurifies with cytoplasmic dynein from rat brain extracts. Electrophoretic analysis of the fractionation of microtubule-associated proteins from a rat brain homogenate in a 5−15% acrylamide gradient gel. Equivalent samples of high-speed supernatants (S) and pellets (P) are shown following sequential incubations of the homogenate with taxol, buffer, 5 mM GTP, 5 mM ATP, and 1 M NaCl. The upper panel shows the Coomassie blue-stained gels [M_r markers and dye front (DF) are indicated at the left]. The lower panels are immunoblots of identical samples probed with 74-1 and R5270 to reveal IC74 of cytoplasmic dynein and the rp3 protein, respectively.

solubilized ∼75% of both rp3 and IC74; the remaining microtubule-bound fractions of both proteins were completely released with 1 M NaCl. Thus, the elution characteristics of rp3 closely paralleled those of a known cytoplasmic dynein component in that both proteins were completely released by a combination of ATP and salt treatment. We previously reported that Tctex1 is also completely eluted from microtubules by sequential treatment with ATP and salt (*16*). Intriguingly, however, in that study (see Figure 4 in ref *16* which employed the same protein samples as those shown here) only ∼50% of Tctex1 was released from microtubules with ATP alone, the remaining 50% being eluted by subsequent salt treatment. Thus, that fraction of brain dynein containing Tctex1 appears less amenable to ATP release than does rp3-containing dynein, raising the possibility that functionally distinct isoforms of cytoplasmic dynein exist.

To further assess the interaction of rp3 with cytoplasmic dynein, proteins eluted from microtubules with ATP were fractionated in a 5−20% sucrose density gradient. Immunological examination of the individual fractions (Figure 4) revealed that rp3 cosedimented with bona fide cytoplasmic dynein proteins at ∼20 S in fractions 6−9. In addition, the sedimentation profile of rp3 was clearly different from that of kinesin (found in fractions 10−13). As reported previously, under our conditions dynactin sediments as a polydisperse structure from the dynein peak to the bottom of the gradient (*15*, *16*). In Figure 4, the p150/135 components of dynactin are clearly present in fractions 1−9. Thus, the rp3 sucrose gradient profile does not coincide with that of dynactin.

To obtain independent confirmation for the association of rp3 with cytoplasmic dynein, the motor enzyme was purified directly from a rat brain homogenate by immunoprecipitation with monoclonal antibody 74-1. Immunoprecipitation of kinesin, dynactin (using antibodies H-2 and 50-1, respectively), and beads alone was used to control for any nonspecific association of rp3 with the bead matrix. Immunological analysis of the resulting precipitates (Figure 5) revealed that rp3 was present exclusively in the dynein sample and was not detectable in either the kinesin or dynactin immunoprecipitates or in the bead control. Thus, the rp3 protein exhibits all the biochemical attributes of a bona fide cytoplasmic dynein component.

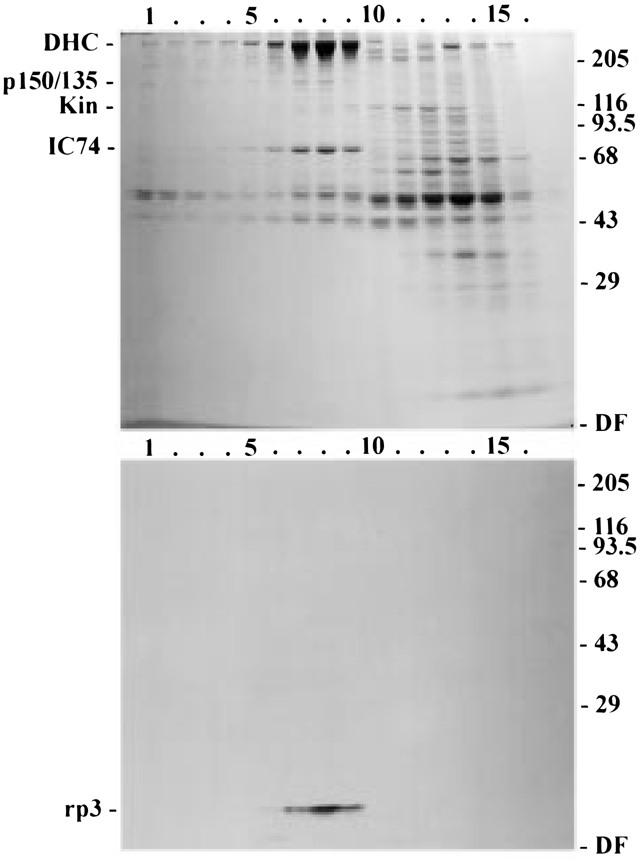

FIGURE 4: The rp3 protein comigrates with cytoplasmic dynein in sucrose gradients. Proteins eluted from microtubules with 5 mM ATP were sedimented through a 5−20% sucrose density gradient. Equal volumes of each fraction were electrophoresed in 5−15% acrylamide gradient gels and either stained with Coomassie blue (upper panel) or blotted to nitrocellulose and probed with the R5270 antibody (lower panel). The bottom of the gradient is at the left. The positions at which the M_r markers and the dynein HC (DHC), IC74 (IC74), kinesin heavy chain (Kin), dynactin (p150/135), and the dye front (DF) migrated are indicated on the upper panel. The locations of rp3 and the M_r markers are marked on the lower panel. The rp3 protein precisely comigrates with the HC and IC74 of cytoplasmic dynein.

Message for the rp3 protein is highly abundant in brain, retina, and fibroblasts (see ref *30*) but is present only at relatively low levels in many other tissues (specifically esophagus, heart, liver, lung, muscle, spleen, and testis). Therefore, to assess whether this dynein component is present in large amounts only in certain tissues as is Tctex1 (*16*), cytoplasmic dynein was purified from rat brain, kidney, liver, spleen, and testis homogenates by immunoprecipitation with antibody 74-1. For electrophoretic analysis, the dynein loadings were normalized on the basis of quantitative densitometry of the Coomassie blue-stained IC74 band (note that, in brain, the IC74 band is not so tightly focused apparently due to the presence of multiple isoforms that are distinguished following one-dimensional electrophoresis). When probed with the R5270 and R5205 antibodies, Tctex1

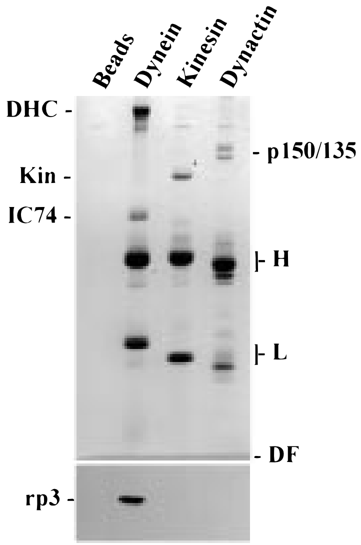

FIGURE 5: The rp3 protein is immunoprecipitated by the 74-1 antibody. Cytoplasmic dynein, kinesin, and dynactin were purified directly from a rat brain homogenate by immunoprecipitation with monoclonal antibodies 74-1, H-2 and 50-1, respectively. These samples and a bead control were electrophoresed in a 5−15% acrylamide gradient gel and either stained with Coomassie blue (upper panel) or blotted to nitrocellulose and probed with the R5270 antibody (lower panel). The positions at which various components of the immunoprecipitates [including antibody heavy (H) and light (L) chains] migrated are indicated. The rp3 protein was detected exclusively in the cytoplasmic dynein sample. DF, dye front.

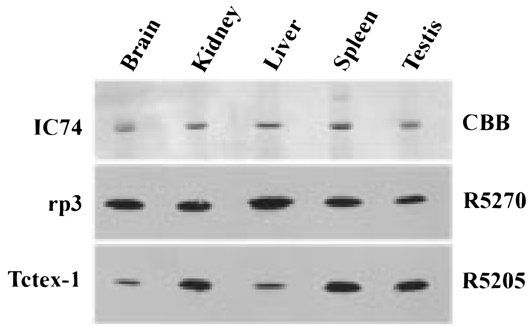

FIGURE 6: Tissue-specific differences in the distributions of the rp3 and Tctex1 LCs. Cytoplasmic dynein was purified from rat brain, kidney, liver, spleen, and testis by immunoprecipitation with monoclonal antibody 74-1. Sample loadings were normalized on the basis of densitometry of the Coomassie blue-stained IC74 band (upper panel). Identical samples were blotted to nitrocellulose and probed with R5270 and R5205 to detect rp3 and Tctex1, respectively (lower panels). The rp3 protein is present in all tissues and is most abundant in liver dynein. In contrast, the amount of Tctex1 is significantly reduced both in that tissue and also in brain.

was found at low levels in brain and liver dynein as described previously (*16*). In contrast, the rp3 protein was relatively abundant in all samples, with clearly the most present in liver dynein and the least associated with the testis enzyme (Figure 6).

To further analyze M_r 14 000 LC heterogeneity, cytoplasmic dynein was purified by immunoprecipitation using the 74-1 antibody both from a whole rat brain homogenate and also from cultured PC-12 cells that had been metabolically labeled with $[^{35}S]$methionine. Samples subsequently were separated by two-dimensional gel electrophoresis to assess whether additional isoforms were present. Sequential immunostaining of the whole brain dynein sample using the R5270 and R5205 antisera revealed that only single immunoreactive species for both rp3 and Tctex1 were present (Figure 7a). Although somewhat spread out possibly due to charge heterogeneity, no obvious additional spots were observed in these samples. Autoradiography of the analogous region from a two-dimensional gel loaded with metabolically labeled PC-12 cell dynein also revealed only two discrete spots (Figure 7b). This suggests that there are single isoforms for both proteins. Thus, M_r 14 000 LC heterogeneity in cytoplasmic dynein is achieved at the level of the individual proteins and does not appear, at least in brain or adrenal-derived PC-12 cells, to be further complicated by the presence of posttranslational modifications as is found for other components of this complex.

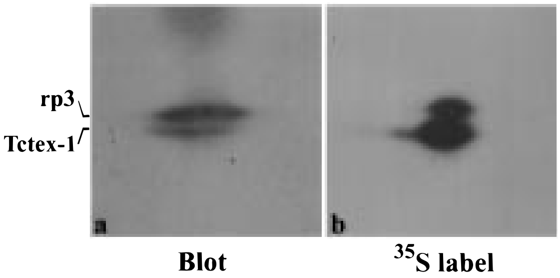

FIGURE 7: Two-dimensional electrophoretic analysis of the M_r 14 000 LCs. (a) Cytoplasmic dynein immunoprecipitated from whole rat brain homogenate using monoclonal antibody 74-1 was separated by two-dimensional gel electrophoresis and blotted to nitrocellulose. The blot was probed sequentially with the R5205 and R5270 antibodies to detect Tctex1 and rp3, respectively. Only single spots are evident for the two LC proteins. (b) Immunoprecipitated dynein obtained from PC-12 cells that had been metabolically labeled with $[^{35}S]$methionine was subject to two-dimensional gel electrophoresis. The M_r 14 000 LC region of the autoradiograph is shown. Both Tctex1 and rp3 appear as single electrophoretic species.

To determine the associations of the LCs with other components of the dynein complex, purified cytoplasmic dynein was dissociated by treatment with 0.6 M KI (*13*), and the various subparticles were separated by centrifugation in KI-containing sucrose gradients. Electrophoretic and immunological analysis of the resulting fractions revealed that KI caused cytoplasmic dynein to sediment as two distinct particles as described previously (*33*). The HCs were found at $\sim$12 S, indicating that they had dissociated from each other (*41, 42*). Following KI treatment, the HC particles have previously been shown also to contain the LICs (*13*). IC74 was found nearer the top of the gradient as was the comigrating Tctex1 LC. This suggests that IC74 and Tctex1 interact directly, as the ICs and LCs of other dyneins do not otherwise cofractionate in sucrose gradients (see ref *43*). Examination of these same fractions with the R5270 antibody did not reveal any detectable rp3 immunoreactivity. In subsequent tests using whole brain immunoprecipitates (not shown), KI treatment was found to result in a very significant decrease in signal intensity for both antibodies R5270 (vs rp3) and R4058 (vs M_r 8000 LC). In the latter case, however, a low signal was detected in the KI sucrose gradient fractions following very prolonged exposure and revealed that the M_r 8000 LC also cofractionated with IC74 (Figure 8, lower panel) as predicted previously (*15*).

The M_r 8000 and 14 000 LCs are present in cytoplasmic dynein at a ratio of one per IC74 (*16*). Quantitative densitometry of sucrose gradient-purified cytoplasmic dynein in Coomassie blue-stained gels indicates that there is one

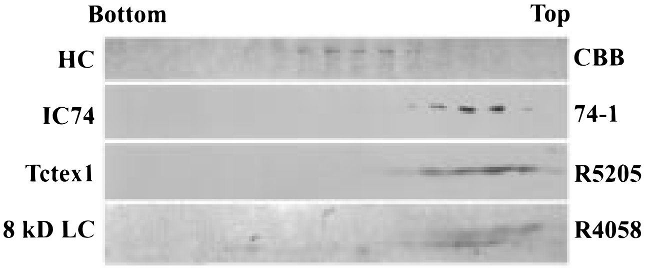

FIGURE 8: KI-induced dissociation of dynein. Purified cytoplasmic dynein was treated with 0.6 M KI to dissociate the complex into discrete subparticles and sedimented through a 5−20% KI-containing sucrose gradient. Fractions were electrophoresed in 5−15% acrylamide gels and either stained with Coomassie blue or blotted to nitrocellulose. The panels show the Coomassie blue-stained HC and immunoblots of identical fractions probed to reveal IC74 (antibody 74-1), Tctex1 (R5205), and the M_r 8000 LC (R4058). The HC sedimented at ~12 S, whereas IC74 and both LC species were present in the upper portion of the gradient. Note that KI treatment seriously affected the immunoreactivity of both the M_r 8000 LC and rp3; in the latter case our antibody could detect no signal even following very prolonged exposure.

Table 1: Stoichiometry of IC74 within Cytoplasmic Dynein[a]

component	relative stoichiometry[b]	copies per dynein particle
HC	1.00	2
IC74	1.13, 1.14, 1.17	2

[a] Determined by quantitative densitometry of sucrose gradient-purified cytoplasmic dynein in Coomassie blue-stained gels. [b] Values are quoted relative to the HCs.

copy of IC74 per HC (Table 1). Therefore, individual dynein particles contain two copies of the HC, IC74, and both LCs.

To assess whether members of the Tctex1 LC family interact with similar or distinct cargoes within the cell and to further address whether these proteins are indeed present in a cell type-specific as well as a tissue type-specific fashion, the R5205 and R5270 antibodies were used to investigate the location of Tctex1 and rp3 in three rat cell types: normal kidney fibroblasts (NRK), brain oligodendrocytes, and the B104 neuronal cell line. For both brain-derived cell lines, no Tctex1 staining over background was observed, consistent with the low amount of this protein observed in whole brain following immunoprecipitation (Figure 6). In contrast, rp3 was present in many punctate perinuclear particles (Figure 9). These particles appeared to coincide with a subset of those stained to reveal IC74 of cytoplasmic dynein. The rp3-positive puncta were not stained by either wheat germ agglutinin or the dye LysoSensor. Thus, they do not appear to derive from the Golgi or to represent acidic membrane-bounded organelles.

The rp3 protein was also present in elongated structures. The latter were observed in the layer of cytoplasm above the nucleus and in some cases appeared to align along the microtubules present in this region for at least part of their length. These elongated structures were observed in both formaldehyde- and methanol/acetone-fixed cells. It is possible that these structures derive from microtubule minus end-directed membranous organelles such as recycling endosomes. Another possibility is that they represent vesicles involved in endoplasmic reticulum to Golgi trafficking.

In the kidney-derived NRK cell line, both Tctex1 and rp3 were found in multiple puncta throughout the cytoplasm (Figure 10). No organelles reminiscent of the rp3-positive elongated structures (see Figure 9) were observed in NRK cells.

DISCUSSION

In this report, we demonstrate that the protein rp3 originally identified by Roux et al. (*30*) is a LC of cytoplasmic dynein. This polypeptide is ~55% identical with Tctex1, which we have shown previously to be a dynein component (*16*). Biochemical and immunological analyses of these two proteins have revealed several intriguing features. First, these polypeptides are differentially expressed in both a tissue-specific and a cell type-specific manner. Second, these LCs are associated with IC74 and the M_r 8000 LC and thus define within the cytoplasmic isozyme an IC/LC complex similar to that found in flagellar outer arm dynein. Third, immunofluoresence analysis suggests that rp3-containing dynein specifically marks an elongated structure in both glial (oligodendrocytes) and neuronal (B104) cells but not in kidney cells. Finally, the dynein particles with which these two LCs associate appear to elute under different conditions from microtubules, suggesting that they may define functionally distinct subsets of cytoplasmic dynein.

Considerable heterogeneity in cytoplasmic dynein polypeptides has been observed for the HCs, ICs, and LICs (*10, 13, 14, 17−21*). By identifying rp3 as a cytoplasmic dynein LC, we now extend that concept to include at least one of the three LC classes so far identified within this dynein motor complex. However, in this case no alternatively spliced or phosphorylation variants were observed following two-dimensional electrophoresis. This suggests that M_r 14 000 LC activity is modulated only by the incorporation of different polypeptides into discrete subsets of cytoplasmic dynein rather than by posttranslational modification.

Recently, we found that a *Chlamydomonas* flagellar outer arm dynein LC (and its murine homologue Tctex2) were distantly related to Tctex1/rp3 (*29*). Moreover, at least in *Chlamydomonas*, Tctex1 is associated with both cytoplasmic dynein and one of the flagellar inner arms (*22*). The current databases contain several additional sequences, from mammals (W64276 and N79692),[4] *Caenorhabditis elegans* (D1009-5 and T05C12-5), and *Trypanosoma brucei* (TBU86346), that share significant similarity with, but are clearly distinct from, various members of this family. Together, these observations suggest the existence of an extensive group of related LCs associated with both flagellar and cytoplasmic dyneins. It will be of great interest to determine whether these other proteins also are cytoplasmic dynein components perhaps expressed only in specific cells or tissues and/or if some represent unrecognized components of flagellar dyneins.

Previously, Roux et al. (*30*) demonstrated that the amount of rp3 message varied considerably between tissue types. Specifically, they found large amounts of message in brain and retina but lesser amounts in many other tissues. At the protein level, we observed the most rp3 (on a per dynein basis) in liver and brain and the least in the testis enzyme.

---

[4] Neither the W64276 nor the N79692 sequence tags encode full-length proteins.

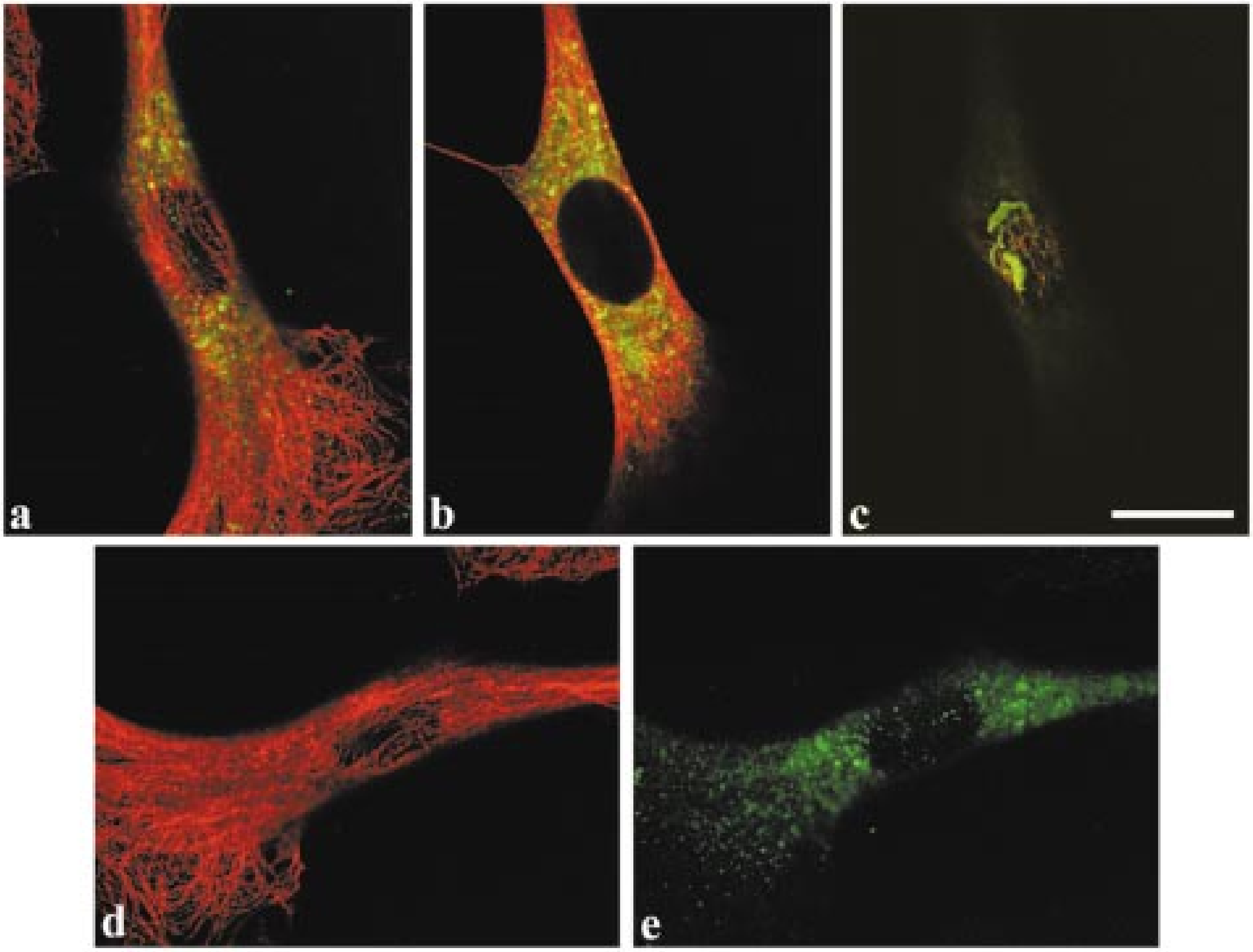

FIGURE 9: Localization of the rp3 LC in neuronal cells. The upper row (a−c) shows three frames from a confocal z-series through a formaldehyde-fixed B104 neuronal cell double labeled to reveal rp3 (green) and microtubules (red). The optical sections derive from near the substratum (a), the central portion of the cell (b), and the region of cytoplasm on top of the nucleus (c). Below (d, e) are the individual channels for the section shown in (a) to more clearly reveal the punctate perinuclear distribution of rp3. The origin of the rp3-positive elongated structure located above the nucleus in (c) is unclear at present. The scale bar = 20 μm.

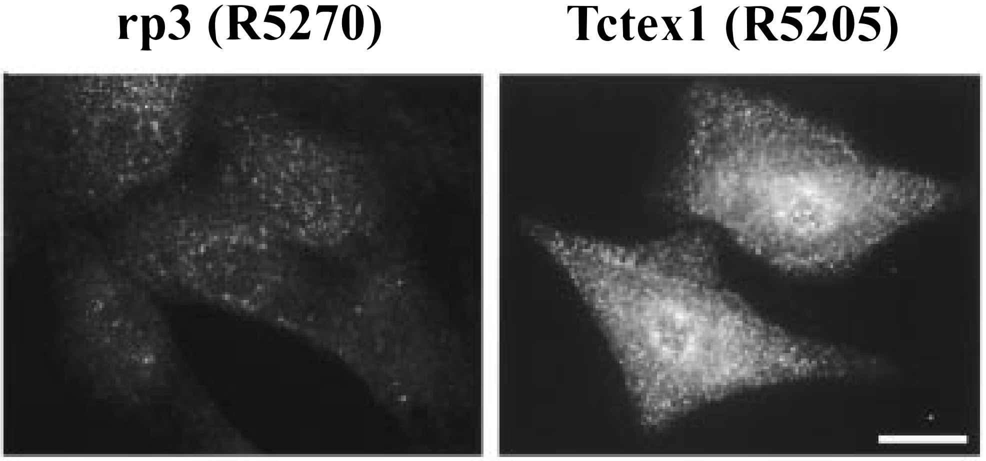

FIGURE 10: Localization of rp3 and Tctex1 in NRK cells. Immunofluoresence microscopy of kidney-derived NRK cells stained with antibodies R5270 and R5205 to identify the rp3 and Tctex1 LCs, respectively. Both LCs were found in numerous punctate structures located throughout the cytoplasm. The contrast on these images has been adjusted to maximize signal intensity. However, Tctex1 staining was considerably more intense than that observed for rp3. The scale bar = 20 μm.

The large amount of rp3 message in brain presumably is a reflection of the considerable quantity of dynein motor present in this organ. More importantly, Roux et al. observed a very significant difference in the amount of rp3 message present in adult vs fetal brain, raising the exciting possibility that the rp3 protein is expressed in a developmentally regulated fashion as are the various isoforms of IC74 (*21*). Intriguingly, message for Tctex1 in brain is regulated in the opposite fashion to rp3, being highly expressed only during fetal development and for a few days post partum (*44*).

Previous electron microscopic studies of dyneins containing truncated HCs have led to a generic model of the dynein particle where the C-terminal portion of the HCs comprises the globular heads with the N-terminal region corresponding to the stems of the complex (*45*; reviewed in ref *46*). For cytoplasmic dynein, the LICs have been found to interact directly with the HCs (*13*) and have been predicted to bind toward the base of the particle (*47*) although there is as yet no direct evidence for this localization. However, recent immunoelectron microscopic studies have placed IC74 at the base of the soluble dynein complex (*33*) as was found previously for the related IC69 within the *Chlamydomonas* outer arm (*48*). Structural and subfractionation studies of flagellar outer arm dyneins from several organisms have defined a basal component comprising two ICs of ∼70−80 kDa and several chains of less than ∼22 kDa, including multiple copies of the M_r 8000 LC (*41*−*43*, *49*, *50*; reviewed in ref *51*). Here we demonstrate that, in mammalian cytoplasmic dynein, both the M_r 8000 LC dimer and at least one member of the Tctex1 LC family (Tctex1) cofractionate with IC74 following dissociation of the enzyme by the chaotrope KI. The cytoplasmic dynein particle has previously been reported to contain either two or three copies of IC74 (*52*−*55*). The densitometry reported here strongly supports the lower value. This analysis indicates the presence of an IC/LC complex within cytoplasmic dynein containing two copies each of IC74 and the M_r 8000 and 14 000 LCs (the location of the M_r 22 000 LC is unknown at present) and further underlines the structural similarities between the cytoplasmic isozyme and the flagellar outer arm.

We were unable to determine directly the intradynein association of rp3, as the epitope recognized by our antibody was severely compromised by KI treatment. Thus, we predict that this component is in the IC/LC complex simply by analogy with the demonstrated association of Tctex1. A similar, but slightly less drastic, decrease in immunoreactivity was also observed with the antibody against the M_r 8000 LC. The most likely explanation for this observation is that prolonged KI treatment results in the iodination of the hydroxyl moiety of Tyr residues. We note that the M_r 8000 LC contains five Tyr residues (5.6% of the total) and that rp3 contains a Tyr immediately preceding the 20-residue sequence used for antibody production; in contrast to the situation with rp3, the Tctex1 antibody was raised against the entire recombinant molecule. Thus in both cases, iodination might directly affect epitopes recognized by our antibodies. An alternative explanation is that KI treatment specifically caused these dynein components to become denatured, leading to complete dissociation and the possibility of loss due to aggregation or nonspecific interactions.

Our analysis suggests that both Tctex1 and rp3 form part of the IC/LC complex at the base of the dynein. However, the data do not yet address the more difficult question of whether individual dynein particles always contain either two Tctex1 or two rp3 LCs. Clearly, this situation must occur in those tissues expressing mostly one or the other protein (e.g., rp3 in brain or Tctex1 in testis). It is though quite possible that heteromeric dynein complexes containing both Tctex1 and rp3 exist in those tissues which express significant amounts of both proteins.

Immunofluorescence analysis of several cell types revealed a perinuclear punctate staining pattern for both LCs. This is highly reminiscent of the signals observed with other cytoplasmic dynein-specific antibodies which demonstrate that this motor associates with lysosomes, endocytic vesicles, and possibly also mRNA-containing granules (e.g., *15*, *56*, and *57*). Importantly, the staining observed for each LC was cell type-dependent: only rp3 was found in brain-derived cells whereas both LCs were present in the kidney NRK line. These observations strongly support the differential expression of these proteins deduced from immunoprecipitation experiments. Intriguingly, in both B104 cells and oligodendrocytes the rp3 antibody strongly stained an elongated structure situated along microtubules in the cytoplasm above the nucleus. This structure was not observed with Tctex1 nor was it found in the NRK cell line. The origin of this structure remains unclear at present. However, its presence in two distinct neural cell types and its identification following both formaldehyde and methanol/acetone fixation would argue against an artifactual origin. This structure is not stained by wheat germ agglutinin or by LysoSensor and thus does not appear to represent the Golgi or lysosomes; it is possible that it originates through the coalescence of minus end-directed organelles.

In conclusion, we demonstrate here that Tctex1 and rp3 define a family of differentially expressed cytoplasmic dynein LCs. Further detailed study of dyneins containing either or both of these proteins will allow their role in cytoplasmic dynein activity to be determined.

ACKNOWLEDGMENT

We thank Louis Brill for biochemical dissection of dynein, Vladislav Shick for technical assistance, Dr. R. John Lye for helping K. T. Do with immunocytochemistry of NRK cells, and Dr. Juris Ozols for discussion of the potential consequences of KI treatment for the immunoreactivity of proteins.

REFERENCES

1. Cottingham, F. R., and Hoyt, M. A. (1997) *J. Cell Biol. 138*, 1041−1053.
2. Pfarr, C. M., Coue, M., Grissom, P. M., Hays, T. S., Porter, M. E., and McIntosh, J. R. (1990) *Nature 345*, 263−265.
3. Saunders, W. S., Koshland, D., Eshel, D., Gibbons, I. R., and Hoyt, M. A. (1995) *J. Cell Biol. 126*, 617−624.
4. Steuer, E. R., Wordeman, L., Schroer, T. A., and Sheetz, M. P. (1990) *Nature 345*, 266−268.
5. Cortésy-Theulaz, I., Pualoin, A., and Pfeffer, S. R. (1992) *J. Cell Biol. 118*, 1333−1345.
6. Li, Y.-Y., Yeh, E., Hays, T., and Bloom, K. S. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10096−10100.
7. Paschal, B. M., and Vallee, R. B. (1987) *Nature 330*, 181−183.
8. Schroer, T. A., Steuer, E. R., and Sheetz, M. P. (1989) *Cell 56*, 937−946.
9. Holzbaur, E. L. F., Mikami, A., Paschal, B. M., and Vallee, R. B. (1994) in *Microtubules* (Hyams, J. S., and Lloyd, C. W., Eds.) pp 251−267, Wiley-Liss, Inc., New York.
10. Paschal, B. M., Mikami, A., Pfister, K. K., and Vallee, R. B. (1992) *J. Cell Biol. 118*, 1133−1143.
11. Wilkerson, C. G., King, S. M., Koutoulis, A., Pazour, G., and Witman, G. B. (1995) *J. Cell Biol. 129*, 169−178.
12. Sondek, J., Bohm, A., Lambright, D. G., Hamm, H. E., and Sigler, P. B. (1996) *Nature 379*, 369−374.
13. Gill, S. R., Cleveland, D. W., and Schroer, T. A. (1994) *Mol. Biol. Cell 5*, 645−654.
14. Hughes, S. M., Vaughan, K. T., Herskovits, J. S., and Vallee, R. B. (1995) *J. Cell Sci. 108*, 17−24.

15. King, S. M., Barbarese, E., Dillman, J. F., III, Patel-King, R. S., Carson, J. E., and Pfister, K. K. (1996) *J. Biol. Chem. 271*, 19358−19366.
16. King, S. M., Dillman, J. F., III, Benashski, S. E., Lye, R. J., Patel-King, R. S., and Pfister, K. K. (1996) *J. Biol. Chem. 271*, 32281−32287.
17. Vaisberg, E. A., Grissom, P. A., and McIntosh, J. R. (1996) *J. Cell Biol. 133*, 831−842.
18. Vaughan, K. T., and Vallee, R. B. (1995) *J. Cell Biol. 131*, 1507−1516.
19. Dillman, J. F., III, and Pfister, K. K. (1994) *J. Cell Biol. 127*, 1671−1681.
20. Pfister, K. K., Salata, M. W., Dillman, J. F., III, Vaughan, K. T., Vallee, R. B., Torre, E., and Lye, R. J. (1996) *J. Biol. Chem. 271*, 1687−1694.
21. Pfister, K. K., Salata, M. W., Dillman, J. F., III, Torre, E., and Lye, R. J. (1996) *Mol. Biol. Cell 7*, 331−343.
22. Harrison, A., Olds-Clarke, P., and King, S. M. (1998) *J. Cell Biol. 140*, 1137−1147.
23. King, S. M., and Patel-King, R. S. (1995) *J. Biol. Chem. 270*, 11445−11452.
24. Benashski, S. E., Harrison, A., Patel-King, R. S., and King, S. M. (1997) *J. Biol. Chem. 272*, 20929−20935.
25. Espindola, F. S., Cheney, R. E., King, S. M., Suter, D. M., and Mooseker, M. S. (1996) *Mol. Biol. Cell 7*, 372a (abstract).
26. Jaffrey, S. R., and Snyder, S. H. (1996) *Science 274*, 774−777.
27. Silver, L. M. (1993) *Trends Genet. 9*, 250−254.
28. Olds-Clarke, P. (1997) *Rev. Reprod. 2*, 157−164.
29. Patel-King, R. S., Benashski, S. E., Harrison, A., and King, S. M. (1997) *J. Cell Biol. 137*, 1081−1090.
30. Roux, A.-F., Rommens, J., McDowell, C., Anson-Cartwright, L., Bell, S., Scgappert, K., Fishman, G. A., and Musarella, M. (1994) *Hum. Mol. Genet. 3*, 257−263.
31. Meindl, A., Dry, K., Herrmann, K., Manson, F., Ciccodicola, A., Edgar, A., Carvalho, M. R. S., Achatz, H., Hellebrand, H., Lennon, A., Migliaccio, C., Porter, K., Zrenner, E., Bird, A., Jay, M., Wittmer, B., D'Urso, M., Meitinger, T., and Wright, A. (1996) *Nat. Genet. 13*, 35−42.
32. Paschal, B. M., Shpetner, H. S., and Vallee, R. B. (1991) *Methods Enzymol. 196*, 181−191.
33. Steffen, W., Hodgkinson, J. L., and Wiche, G. (1996) *J. Struct. Biol. 117*, 227−235.
34. Pfister, K. K., Wagner, M. C., Stenoien, D. L., Brady, S. T., and Bloom, G. S. (1989) *J. Cell Biol. 108*, 1453−1463.
35. Paschal, B. M., Holzbaur, E. L. F., Pfister, K. K., Clark, S., Meyer, D. I., and Vallee, R. B. (1993) *J. Biol. Chem. 268*, 15318−15323.
36. King, S. M., Otter, T., and Witman, G. B. (1986) *Methods Enzymol. 134*, 291−306.
37. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol. 215*, 403−410.
38. Devereux, J., Haeberli, P., and Smithies, O. (1984) *Nucleic Acids Res. 12*, 387−395.
39. Ainger, K., Avossa, D., Morgan, F., Hill, S. J., Barry, C., Barbarese, E., and Carson, J. H. (1993) *J. Cell Biol. 123*, 431−441.
40. Barbarese, E., Koppel, D. E., Deutscher, M. P., Smith, C. L., Ainger, K., Morgan, F., and Carson, J. H. (1995) *J. Cell Sci. 108*, 2781−2790.
41. Pfister, K. K., and Witman, G. B. (1984) *J. Biol. Chem. 259*, 12072−12080.
42. Tang, W.-J. Y., Bell, C. W., Sale, W. S., and Gibbons, I. R. (1982) *J. Biol. Chem. 257*, 508−515.
43. King, S. M., Gatti, J.-L., Moss, A. G., and Witman, G. B. (1990) *Cell Motil. Cytoskeleton 16*, 266−278.
44. Kai, N., Mishina, M., and Yagi, T. (1997) *J. Neurosci. Res. 48*, 407−424.
45. Sakakibara, H., Takada, S., King, S. M., Witman, G. B., and Kamiya, R. (1993) *J. Cell Biol. 122*, 653−661.
46. Witman, G. B., Wilkerson, C. G., and King, S. M. (1994) in *Microtubules* (Hyams, J. S., and Lloyd, C. W., Eds.) pp 229−249, Wiley-Liss, Inc., New York.
47. Vallee, R. B., Shpetner, H. S., and Paschal, B. M. (1989) *Trends Neurosci. 12*, 66−70.
48. King, S. M., and Witman, G. B. (1990) *J. Biol. Chem. 265*, 19807−19811.
49. King, S. M., Wilkerson, C. G., and Witman, G. B. (1991) *J. Biol. Chem. 266*, 8401−8407.
50. Mitchell, D. R., and Rosenbaum, J. L. (1986) *Cell Motil. 6*, 510−520.
51. Witman, G. B., King, S. M., Moss, A. G., and Wilkerson, C. G. (1991) In *Comparative Spermatology, 20 Years After* (Baccetti, B., Ed.) pp 439−443, Raven Press, New York.
52. Collins, C. A., and Vallee, R. B. (1989) *Cell Motil. Cytoskeleton 14*, 491−500.
53. Neely, M. D., Erickson, H. P., and Boekelheide, K. (1989) *J. Biol. Chem. 265*, 8691−8698.
54. Paschal, B. M., Shpetner, H. S., and Vallee, R. B. (1987) *J. Cell Biol. 105*, 1273−1282.
55. Vallee, R. B., Wall, J. S., Paschal, B. M., and Shpetner, H. S. (1988) *Nature 332*, 561−563.
56. Lin, S. X. H., and Collins, C. A. (1992) *J. Cell Sci. 101*, 125−137.
57. Lin, S. X. H., Pfister, K. K., and Collins, C. A. (1996) *Cell Motil. Cytoskeleton 34*, 299−312.
58. Hillier, L., et al. (1995) *The WashU-Merck EST Project*.

BI9810813