A Regulatory Light Chain of Ciliary Outer Arm Dynein in Tetrahymena thermophila*

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Ciliary beat frequency is primarily regulated by outer arm dyneins (22 S dynein). Chilcote and Johnson (Chilcote, T. J., and Johnson, K. A. (1990) J. Biol. Chem. 256, 17257–17266) previously studied isolated Tetrahymena 22 S dynein, identifying a protein p34, which showed cAMP-dependent phosphorylation. Here, we characterize the molecular biochemistry of p34 further, demonstrating that it is the functional ortholog of the 22 S dynein regulatory light chain, p29, in Paramecium. p34, thiophosphorylated in isolated axonemes in the presence of cAMP, co-purified with 22 S dynein and not with inner arm dynein (14 S dynein). Isolated 22 S dynein containing phosphorylated p34 showed ~70% increase in *in vitro* microtubule translocation velocity compared with its unphosphorylated counterpart. Extracted p34 rebound to isolated 22 S dynein from either Tetrahymena or Paramecium but not to 14 S dynein from either ciliate. Binding of radiolabeled p34 to 22 S dynein was competitive with p29. Phosphorylated p34 was not present in axonemes isolated from a mutant lacking outer arms. Two-dimensional gel electrophoresis followed by phosphorimaging revealed at least five phosphorylated p34-related spots, consistent with multiple phosphorylation sites in p34 or perhaps multiple isoforms of p34. These new features suggest that a class of outer arm dynein light chains including p34 regulates microtubule sliding velocity and consequently ciliary beat frequency through phosphorylation.

Cilia are ubiquitous cellular nanomachines, found in protists and multicellular eukaryotes, including man, whose repetitive beat depends on a microtubule-based cytoskeleton, powered by molecular motors, the outer and inner rows of dyneins (outer arm dynein, 22 S dynein; and inner arm dyneins, 14 S dynein; respectively). The arrangement of the dynein arms along the axonemes is complex (1). Dynein arm mechanochemistry is thought to regulate beat frequency and beat form by signal transduction mechanisms that change the parameters of microtubule sliding within the axoneme, such that the outer arm dyneins principally regulate beat frequency whereas the inner arm dyneins control beat form (cf. Refs. 2 and 3).

cAMP specifically increases ciliary beat frequency (4), normally measured by an increase of swimming speed, in the protozoan ciliate, Paramecium tetraurelia. The increase occurs in living cells and in cells that have been permeabilized with Triton X-100 and reactivated with Mg²⁺-ATP; it persists in the permeabлизized cells even when cAMP is subsequently removed and it is quenched by simultaneous addition of Ca²⁺ to the medium (5–8). We previously reported on a molecule, p29, whose phosphorylation both *in vivo* and *in vitro* correlated with the cAMP-dependent Ca²⁺-sensitive increase in swimming speed. Further studies revealed that p29 is a component of outer arm dynein (6, 8, 9), which specifically binds to one heavy chain isofrom of the three-headed 22 S outer arm dynein. Phosphorylation of p29 increases *in vitro* microtubule translocation velocity by outer arm dyneins by 40% (6, 9) via activation of dynein mechanochemistry (10). Hence, p29 is regarded as a dynein regulatory light chain of ciliary outer arm dyneins in Paramecium.

Like Paramecium, Tetrahymena thermophila outer arm dynein consists of three different heavy chain isoforms (11) in a three-headed bouquet that sediments at 22 S, but is probably compacted in *situ* (12), together with intermediate chains and light chains. Chilcote and Johnson (13) studied phosphorylation of isolated Tetrahymena 22 S dynein. They were successful in phosphorylating 22 S dynein using the catalytic subunit of cAMP-dependent protein kinase from bovine heart with 500 munit ATP and vanadate as a phosphatase inhibitor. They found that about 0.5 mol of phosphate was added per mol of 22 S dynein, mainly into proteins at molecular masses 78 and 34 kDa (p78 and p34, respectively).

Phosphorylated p78 and p34 cosedimented with microtubules in an ATP-sensitive manner, as expected for dynein constituents. An endogenous cAMP-dependent protein kinase was present in the preparations. No dephosphorylation was detected, even with the addition of calcineurin (protein phosphatase type II). Exogenous acid or alkaline phosphatase was able to partially dephosphorylate the dynein, which lowered specific activity of dynein ATPase by 30%.

Barkalow et al. (9) showed that p29 from Paramecium binds to Tetrahymena 22 S dynein, which suggested that there was a Tetrahymena ortholog of p29. Accordingly, using a series of fractionation, reconstitution, and analytical technologies, including two-dimensional gel electrophoresis and *in vitro* motility assays, we have investigated whether p34 as identified by Chilcote and Johnson (13) could be this ortholog.

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FIG. 1. Axonemal outer arm dynein assembly is required for cAMP-dependent p34 phosphorylation. Autoradiographic analysis of phosphorylated axonemal proteins after SDS-PAGE in oad-1 at 28 °C (permissive temperature) or 38 °C (non-permissive temperature). Protein phosphorylation was performed in the presence and in the absence of 10 μM cAMP and 1 mM CaCl2. Molecular mass markers (M; from top): 205, 116, 97, 66, 45, 36, 29, 24, and 16 kDa. The graphs summarize relative levels of p34 phosphorylation based on band densities in the autoradiograms (average ± S.E.; n = 4).

EXPERIMENTAL PROCEDURES

Cell Cultures—The following strains of T. thermophila were used in this study: a nuclease-free mutant, SB 255 (14), and oad-1, a temperature-sensitive outer dynein arm-deficient mutant (15, 16). SB 255 cells were grown either in 1-liter cultures in 2-liter capacity flat flasks with shaking at 38 °C or in 8-liter aerated cultures containing Antifoam C (Sigma) at 27 °C. oad-1 cells were grown in 1-liter cultures either at 28 °C (permissive temperature) or at 38 °C (non-permissive temperature). The growth medium was a modified version of a milk medium (5) with a few modifications. Demembranation of cilia and phosphorylation of axonemes were performed according to Hamasaki et al. (6) with a few modifications. Demembranation was accomplished with 0.5% Triton X-100 in axoneme buffer, and axonemes were collected in axoneme buffer supplemented with 1 mM dithiothreitol. Axonemes from SB255 cells were in vitro-thiophosphorylated for 30 min at room temperature in axoneme buffer containing about 300 nM [γ-32P]ATP. The reactions were performed in the presence and in the absence of 10–20 μM cAMP and 10−3–10−4 M CaCl2 (pCa 3–4). All steps were performed in the presence of a mixture of protease inhibitors, Complete™ (Roche Molecular Biochemicals).

Isolation and Phosphorylation of Axonemes—Cilia were collected according to the dibucaine method by Satir et al. (18) with minor modifications. Cells were harvested and resuspended in 2% proteose peptone and 1 mM phenylmethylsulfonyl fluoride, allowing deciliation to occur within 5–10 min. Cell bodies were pelleted and discarded by centrifugation at 1500 × g in axoneme buffer (30 mM HEPES (pH 7.6), 20 mM KCl, 5 mM MgSO4, 0.5 mM EDTA). Demembranation of cilia and phosphorylation of axonemes were performed according to Hamasaki et al. (5) with a few modifications. Demembranation was accomplished with 0.5% Triton X-100 in axoneme buffer, and axonemes were collected in axoneme buffer supplemented with 1 mM dithiothreitol. Axonemes from SB255 cells were in vitro-thiophosphorylated for 30 min at room temperature in axoneme buffer containing about 300 nM [γ-32P]ATP. The reactions were performed in the presence and in the absence of 10–20 μM cAMP and 10−3–10−4 M CaCl2 (pCa 3–4). All steps were performed in the presence of a mixture of protease inhibitors, Complete™ (Roche Molecular Biochemicals).

Isolation of Dynein and p34 From Axonemes—Dynein heavy chains 14 S (inner arm dynein) and 22 S (outer arm dynein) were purified according to the procedure of Hamasaki et al. (6) with a few modifications. Axonemes were incubated on ice for 30 min in axoneme buffer with 0.6 M KCl. This separates the dynein molecules from microtubules, the latter being pelleted by centrifugation at 27,000 × g for 15 min. Usually, the dynein-containing supernatant was further incubated on ice for 30 min in axoneme buffer with 1.2 M KCl, allowing dynein-regulatory proteins to be separated from the dynein heavy chains (9). The sample was then loaded onto a Centricon 100 (Millipore, Milford, MA) and centrifuged at 1000 × g at 4 °C until the volume of the retentate, containing dynein heavy chains, was less than 100 μl. The retentate was then carefully layered on top of a 5–30% linear sucrose gradient and centrifuged at 100,000 × g for 15 h, followed by fractionation of the gradient into about 20 fractions each having a volume of about 500 μl. The fractions were assayed for ATPase activity (19) and analyzed by gel electrophoresis to identify fractions containing 14 S and 22 S dyneins. Prior to sucrose gradient separation, fractions containing crude 22 S dynein with thiophosphorylated p34 were incubated with antisera generated against the 22 S dynein-regulatory proteins to be separated from the dynein heavy chains (9). The sample was then loaded onto a Centricon 100 (Millipore, Milford, MA) and centrifuged at 1000 × g at 4 °C until the volume of the retentate, containing dynein heavy chains, was less than 100 μl. The retentate was then carefully layered on top of a 5–30% linear sucrose gradient and centrifuged at 100,000 × g for 15 h, followed by fractionation of the gradient into about 20 fractions each having a volume of about 500 μl. The fractions were assayed for ATPase activity (19) and analyzed by gel electrophoresis to identify fractions containing 14 S and 22 S dyneins. Prior to sucrose gradient separation, fractions containing crude 22 S dynein with thiophosphorylated p34 were incubated with antisera generated against the 22 S dynein heavy chains and then mixed with protein-A-Sepharose 3B beads (Zymed Laboratories Inc., San Francisco, CA), following proce-
Fig. 3. Analysis of dyneins fractionated on a 5–30% linear sucrose gradient. Dyneins were salt-extracted from Tetrahymena SB255 axonemes thiophosphorylated in the presence of cAMP. A, ATPase activity measurements used to identify fractions containing 14 S and 22 S dynein. B, corresponding SDS-PAGE gel, showing the dynein heavy chain (HC) region and autoradiograph (autorad) showing p34.

RESULTS

Effects of cAMP and Ca²⁺ on in Vitro Protein Phosphorylation in oad-1 Axonemes—We initially conducted a series of
phosphorylation experiments with the temperature-sensitive outer dynein arm-deficient mutant of *T. thermophila*, oad-1 (Fig. 1). In axonemes isolated from cultures grown at 28 °C (permissive temperature) where outer arm dyneins are present in this mutant (15, 16) 10 μM cAMP induced the [γ-32P]ATP phosphorylation of several proteins in the p34 range, about 6× over no add controls. In the presence of cAMP, addition of Ca2+ increased the p34 phosphorylation further to about 9× control levels, whereas in the absence of cAMP, Ca2+ did not elicit p34 phosphorylation. Similar changes were seen in a p78 protein. However, at 38 °C (non-permissive temperature) where outer arm dyneins are greatly reduced in the mutant, although similar changes were seen at p78, p34 phosphorylation remained at control levels regardless of whether cAMP and/or Ca2+ were present. These results confirm that p34 is an outer arm dynein-related molecule that is phosphorylated in a cAMP-dependent manner.

**p34 Is a 22 S Outer Arm Dynein Light Chain**—Treatment of axonemes with 0.6 M KCl extracts most of the dynein, yielding so-called crude dynein. Further incubation of the dynein fraction in 1.2 M KCl dissociates some of the 22 S dynein into its constituent proteins (9). After high salt treatment, the dynein-containing fraction, centrifuged through a Centricon 100, produces a retentate containing the dynein heavy chains and a flowthrough containing axonemal proteins between 10 and 100 kDa, including dynein light chains.

Fig. 2A shows SDS-PAGE and autoradiographic analysis of proteins in *T. thermophila* SB 255 in the salt-extracted thio-phosphorylated axonemes, in the retentate heavy chain-containing fraction, and in the 10–100 kDa light chain-containing fraction. p34 is the major protein of the light chain fraction that becomes phosphorylated upon stimulation with cAMP. Phosphorimaging measurements again show a 5× increase on p34 labeling upon cAMP addition, which is enhanced by Ca2+, whereas in Ca2+ alone, p34 phosphorylation remains at control levels (Fig. 2B). Fig. 3 shows ATPase activity measurements of fractions collected from the crude dynein within a 5–30% linear sucrose gradient. The ATPase peak in fractions 7–11 corresponds to 14 S inner arm dyneins, and the peak in fractions 14–18 correspond to 22 S outer arm dynein (Fig. 3A), as confirmed by SDS-PAGE showing the dynein heavy chains (Fig. 3B). The corresponding autoradiogram shows that p34 remaining with the dynein heavy chains comigrates with 22 S dynein and not with 14 S dynein (Fig. 3B). Some p34 (seen in fractions 1–5) dissociates from the dynein heavy chains during this procedure.

To test further whether p34 is a dynein light chain, we immunoprecipitated 22 S dynein heavy chains using 22 S dynein antisera, protein-A-Sepharose, and p34 immunoprecipitated with the dynein heavy chains (Fig. 4A). The antisera did not immunoprecipitate thio-phosphorylated p34 when fractions containing thio-phosphorylated p34 but no heavy chains (10–100-kDa light chain-containing fraction) were used for the experiments. As expected, no immunoprecipitate was produced in the absence of antisera. We also examined the phosphorylation pattern of 22 S dynein under native gel electrophoresis conditions. If p34 is a light chain, it should remain attached to 22 S dynein under native conditions, and upon denaturation in SDS-PAGE the amount of phosphorylation in p34 plus the residual radioactivity in the dissociated single-headed dynein heavy chains and their constituent components should quantitatively match the amount of phosphorylation in the native 22 S complex. After denaturation, the σ and γ dynein heavy chains are seen to be phosphorylated at a constitutively low level independently of cAMP addition. A number of additional bands are also phosphorylated. Although the level of p34 phosphorylation varies, the level of phosphorylation in the bands other than p34 in fractions 14–17 of the gel is virtually constant. The phosphorylated bands seen in the autoradiogram after SDS-PAGE (Fig. 4B) disappear under native conditions, and fraction by fraction the amount of phosphorylation under denaturation conditions is switched to the native 22 S complex (Fig. 4C). The variation in p34 phosphorylation is reproduced in the complex,
which supports the conclusion that p34 is a light chain of native 22 S dynein.

Reconstitution and Competition Experiments—To show whether p29 and p34 are functional orthologs, we performed a series of experiments on the ability of radiolabeled p34 to reconstitute with Tetrahymena and Paramecium dyneins. Fig. 5 shows reconstitution of radiolabeled p34 with Tetrahymena and Paramecium dyneins. Fig. 5 shows reconstitution of radiolabeled p34 with Tetrahymena and Paramecium dyneins in our standard assay. In all cases bovine serum albumin was added to the reactions both as a control for nonspecific binding of p34 to protein bovine serum albumin was added to the reactions both as a hand, p34 reconstituted with both labeled p34 was found only in the flowthrough. On the other hand, p34 reconstituted with both Paramecium and Tetrahy-

Fig. 5. Reconstitution of thiophosphorylated p34 with Tetra-
hymena and Paramecium 22 S but not 22 S dynein. Control (C), no dyneins were present in the reconstitution reaction. All reactions were carried out in the presence of 20 μg of bovine serum albumin (BSA). The abbreviations are as follows: r, retentate from samples collected with Microcon 100 (≥100-kDa proteins); fl, flowthrough from Microcon 100, concentrated in Microcon 10 (10–100-kDa proteins). With 22 S dynein, p34 is found in the retentate, but this did not affect the retention presence of dyneins, a small amount of bovine serum albumin

in the retentate from the Centricon membranes. In the absence of any of the dyneins, radiolabeled p34 identified by autoradiogram was found exclusively in the Centricon 100 flowthrough, as was bovine serum albumin. In the presence of dyneins, a small amount of bovine serum albumin was found in the retentate, but this did not affect the retention of p34. 14 S dynein did not rebinding p34 in either ciliate, and labeled p34 was found only in the flowthrough. On the other hand, p34 reconstituted with both Paramecium and Tetrahy-

dynein 22 S dynein, where much of the labeled p34 remained with the retentate.

To test whether p34 competes with p29 for binding to 22 S dynein, we added increasing concentrations of cold-labeled p34 or p29 to a constant amount of 35S-labeled p34 in our assay (Fig. 6). Cold-labeled p34 competed with the binding of radiolabeled p34 to Tetrahymena 22 S dynein (Fig. 6B) and cold-labeled p29 competed effectively with the binding of radiolabeled p34 to 22 S dynein (Fig. 6A), qualitatively displacing p34 radiolabel from the retentate to the flowthrough.

p34 Resolved by Two-dimensional Gel Electrophoresis—Fig. 7 shows a two-dimensional gel analysis of the 10–100-kDa light chain fraction thiophosphorylated in the absence and in the presence of cAMP. The silver stain pattern of proteins is reproducible in the two gels, as are virtually all the radiolabeled spots (Fig. 7A). After cAMP treatment, a ladder of at least five unique phosphorylated spots (Fig. 7B), focusing in the pH range from 5 to 6, corresponding to faint spots in the silver-stained gels, is found in the p34 region. These spots increase slightly in molecular mass, whereas at the same time they decrease in pI. The p34 spots are the only significant thiophosphorylated molecules in the 10–100-kDa fractions that were consistently radiolabeled in the presence of CAMP, and not present in the autoradiogram when thiophosphorylation occurred in the absence of CAMP.

cAMP Treatment of 22 S Outer Arm Dynein Increases the Microtubule Translocation Velocity—The *in vitro* translocation velocity of microtubules on 22 S dynein isolated from axonemes that had been thiophosphorylated in the presence and in the absence of cAMP was determined. The length of the microtubules that moved on the dynein varied, although the distribution of the lengths was essentially similar in all experiments,
which allowed us to compare the velocity of translocation from one experiment to another (6). 22 S dynein from axonemes thiophosphorylated in the absence of cAMP produced an average in vitro velocity of microtubules of $0.83 \pm 0.08 \text{ m s}^{-1}$ (mean $\pm$ S.E.) ($n = 37$). This contrasted to microtubule velocity produced by 22 S dynein from axonemes phosphorylated in the presence of cAMP, where the average velocity increased to $1.42 \pm 0.09 \text{ m s}^{-1}$ ($n = 40$) ($t$ test, $p$, 0.0001).

**DISCUSSION**

Previous results by Chilcote and Johnson (13) showed that 22 S outer arm dynein in *Tetrahymena* is associated with a 34-kDa protein (p34) that is phosphorylated in vitro by cAMP-dependent protein kinase, resulting in increased phosphate incorporation into the protein. Accordingly, in our studies, thiophosphorylation indicated by $^{35}$S incorporation probably represents new phosphate addition, rather than turnover of existing phosphorylated p34. Two-dimensional gel electrophoresis shows that p34 is largely unphosphorylated prior to cAMP addition. At least five phosphorylated protein spots appear in a ladder in the p34 region after cAMP treatment. Sequentially the spots increase slightly in molecular mass, while at the same time they decrease in pI, which probably indicates that p34 can be phosphorylated at multiple sites or alternatively that multiple isoforms of p34 of slightly different mass and pIs are present in the axoneme. If multiple phosphorylations of p34 occur, this might mean that sequential phosphorylation of a single dynein regulatory light chain leads to increased outer arm dynein activity. Attempts are now being made to sequence p34 from these gels.

Our results support the conclusion that p34 is a functional ortholog of an outer arm dynein regulatory light chain that probably regulates ciliary beat frequency, found in several cilia, of which p29 in *Paramecium* axonemes is the prototype (6, 9). We earlier demonstrated that p29 rebinds to *Tetrahymena*, as well as to *Paramecium* outer arm dynein (9). Here we show that p34 also rebinds to outer arm dynein, but not inner arm dynein, in both ciliates, with some differences when binding is to dyneins of the same versus the contrasting organisms, and that cold-labeled p29 competes with the binding of radiolabeled p34. p34 is a constitutive dynein regulatory light chain, because it coimmunoprecipitates with 22 S dynein heavy chains (20), and it migrates together with heavy chains of outer arm dynein both in a gradient separation of dyneins and in native gels. We calculate that $\sim 2\%$ of the molecular weight of outer arm dynein is p34, assuming that there is one p34 for each $\alpha$-heavy chain in the molecule, an assumption consistent with the measurements of Barkalow et al. (9) on p29. The temperature-sensitive mutant, oad-1, when deficient in outer arms (15, 16) shows no p34 phosphorylation. Further, the in vitro translocation velocity of microtubules by *Tetrahymena* 22 S outer arm dynein increases by about 70% after thiophosphorylation in the presence of cAMP, an increase that is attributable to cAMP-dependent phosphorylation of an outer arm dynein regulatory light chain leading to faster ciliary beat. An increase in p34 phosphorylation should be directly proportional to a corresponding increase in ciliary beat frequency (10) over a necessary wide range of frequencies.

The presence of high Ca$^{2+}$ ($pCa$ 4) increases cAMP-induced
phosphorylation of p34 in *Tetrahymena* axonemes by about 60%, although Ca\(^{2+}\) alone has no effect. Similarly, Chilcote and Johnson (13) found that Ca\(^{2+}\) and calmodulin alone did not elicit new p34 phosphorylation. Therefore, Ca\(^{2+}\) in the absence of cAMP must be insufficient to activate the relevant endogenous kinases, but, speculatively, it could cause conformational changes to increase the accessibility of p34 to phosphorylation.

In mammalian respiratory cilia (23–25), Ca\(^{2+}\) also increases ciliary beat frequency synergistically with cAMP, possibly via a similar action on dynein regulatory light chain phosphorylation. This is different from *Paramecium*, where unusually the effect of Ca\(^{2+}\) on cAMP-dependent phosphorylation of p29 is inhibitory, because Ca\(^{2+}\) inhibits the relevant *Paramecium* cAMP-dependent protein kinase (5).

*Tetrahymena* cilia can be readily isolated in larger quantities than either *Paramecium* or mammalian cilia, which may prove significant for the study of certain minor components of the axoneme, such as p34 and its orthologs. Similar approaches have proven useful in identifying dynein light chains using *Chlamydomonas* (26). Gene transformation and knockout procedures have been developed for *Tetrahymena* (27, 28). The biochemical characterization of p34 presented here provides the basis for a unique opportunity to study function of the dynein regulatory light chain in further detail through biochemical and molecular genetic approaches that will yield fundamental information regarding the molecular mechanisms of beat frequency control in the ciliary axoneme.

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