

Regulation of 22S Dynein by a 29-kD Light Chain

Kurt Barkalow, Toshikazu Hamasaki, and Peter Satir

Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461

Abstract. Previously, a 29-kD axonemal polypeptide (p29) that copurifies with 22S dynein has been shown to be phosphorylated in a cAMP- and Ca^{2+} -sensitive manner, consistent with a role for this molecule in the signal transduction cascade leading to fast forward swimming in *Paramecium tetraurelia* (Hamasaki, T., K. Barkalow, J. Richmond, and P. Satir. 1991. *Proc. Natl. Acad. Sci. USA.* 88:7912–7922). This study demonstrates the nature of the relationship between p29 and 22S dynein. Chaotropic agents can be used to separate p29 fractions from 22S dynein. When extracted p29 is exchanged into physiological buffers, it regains the ability to recombine with 22S dynein with an apparent dissociation constant of 25 nM; no recombination is seen with 14S dynein or with unrelated control proteins. p29 from *Paramecium* will also recombine with *Tetrahymena* 22 but not 14S dynein.

After chymotryptic digestion of 22S dynein, p29 preferentially binds to a single-headed fragment, homologous to the α H chain of *Tetrahymena* 22S dynein. 22S dynein treated in vitro by *Paramecium* protein kinase A in the presence of cAMP and ATP to phosphorylate p29 translocates bovine brain microtubules significantly ($1.53\times$; $p < 0.001$) faster than before phosphorylation. Similarly, 22S dynein reconstituted in vitro with thiophosphorylated p29 translocates microtubules significantly ($1.31\times$; $p < 0.001$) faster than controls reconstituted with nonthiophosphorylated p29. p29 is the only moiety thiophosphorylated in the reconstituted dynein. We conclude that p29 functions as a 22S dynein regulatory light chain in that it alone is sufficient to control the rate of microtubule translocation by changes in its phosphorylation state.

IN *Paramecium tetraurelia* (Bonini and Nelson, 1988; Satir et al., 1993), as in many ciliated cells (Stommel and Stephens, 1985; Sanderson et al., 1992), ciliary beat and cell swimming are regulated by second messengers. In particular, the membrane-permeable cAMP-analogue, monobutyl cAMP, and the phosphodiesterase-inhibitor, 3'-isobutyl-1-methylxanthine, increase forward swimming speed of living *Paramecium* (Bonini et al., 1986). An increase in cAMP levels occurs in conjunction with a membrane hyperpolarization that leads to fast forward swimming in *Paramecium* (Bonini et al., 1986) and adenylate cyclase activity seems to be tightly coupled to, or part of, a K^+ channel in the cell membrane (Schultz et al., 1992). Further, cAMP has been shown to increase the forward swimming speed of permeabilized demembrated cells (Bonini and Nelson, 1988; Hamasaki et al., 1991).

Likely candidates for the effector molecules in this cAMP cascade probably are parts of, or interact strongly with, the dynein arms. Brokaw and Kamiya (1987) have shown that in *Chlamydomonas reinhardtii* the outer dynein arm seems to be a primary regulator of beat frequency. Changes in beat frequency are measured directly in mussel gill and other cilia as a response to an increase in intracellular cAMP (Mura-

kami, 1987). In the absence of changes in beat form, beat frequency is proportional to the rate at which microtubules slide relative to one another, which is determined by axonemal dynein, presumably the outer arm (Satir et al., 1993).

In *Paramecium*, the isolated outer dynein arm is a three-headed molecule with a sedimentation coefficient of 22S (Larsen et al., 1991; Beckwith and Asai, 1993; Walczak et al., 1993). A 29-kD axonemal polypeptide (p29)¹ that copurifies with 22S dynein (Bonini and Nelson, 1990; Hamasaki et al., 1991) has been shown to be phosphorylated in a cAMP-dependent and Ca^{2+} -sensitive manner in *Paramecium* (Hamasaki et al., 1989). Axonemal polypeptides of similar molecular weight have been identified by virtue of their cAMP-dependent phosphorylation in mussel gill cilia (Stephens and Prior, 1992), in *Tetrahymena* (Chilcote and Johnson, 1990) and in ovine respiratory cilia (Salathe et al., 1993). In *Paramecium*, experimental phosphorylation of p29 is achieved within isolated axonemes via an endogenous kinase. In in vitro microtubule mobility assays, phosphorylated p29 induces 22S dynein to translocate bovine brain microtubules 1.4 times faster than controls (Hamasaki et al., 1991). However, many axonemal proteins are phosphorylated (Table I) and minor quantitative changes in phosphorylation in proteins other than p29 could influence these findings.

Address all correspondence to Dr. Peter Satir, Dept. of Anatomy and Structural Biology, Albert Einstein College of Medicine, 1300 Morris Ave., Bronx, NY 10461.

1. *Abbreviations used in this paper:* H, heavy; p29, 29-kD axonemal polypeptide; PKA, cAMP-dependent protein kinase.

Table I. Phosphorylation of p29 Correlates with Translocation Velocity

Sample type	Experimentally phosphorylated bands										Translocation velocity \pm SD	Versus control
22S dynein preparation	KD										<i>n</i>	
Axonemal treatment*	S	H	79	72	68	54	49	47	29	23		
No cAMP	\pm	-	+	\pm	\pm	+	-	+	-	+	1.81 \pm 0.41 (296)	1.12 \ddagger
+cAMP	+	-	+	+	+	+	-	+	+	+	2.53 \pm 0.55 (340)	1.57 \ddagger
+cAMP + Ca ²⁺	+	-	+	\pm	\pm	+	-	+	-	\pm	1.92 \pm 0.56 (210)	1.12 \ddagger
Dynein treatment												
PKA control	-	-	-	-	-	-	-	-	-	-	1.61 \pm 0.79 (29)	1.00
PKA	-	+	-	-	-	-	+	-	+	-	2.48 \pm 1.08 (44)	1.53 \ddagger
Dynein recombination												
No addition	-	-	-	-	-	-	-	-	-	-	1.05 \pm 0.41 (53)	0.65
+p29 fraction	-	-	-	-	-	-	-	-	-	-	1.60 \pm 0.68 (59)	1.00 \ddagger
+pp29 fraction	-	-	-	-	-	-	-	-	+	-	2.10 \pm 0.78 (47)	1.31 \ddagger

This table compares the phosphorylated polypeptides formed in various preparations of 22S dynein used in microtubule translocation measurements in this study with those demonstrated by Hamasaki et al. (1991). Phosphorylated bands present in kinase only sample are omitted. When p29 is phosphorylated in a cAMP-dependent manner, either before extraction of 22S dynein from the axoneme or in vitro with PKA, translocation velocity compared to a control dynein incubated in cAMP and ATP without PKA in vitro is increased. A comparable increase is seen when unphosphorylated 22S dynein is reconstituted with phosphorylated p29 (pp29). Other phosphorylated polypeptides vary in each preparation. These may have modulatory effects on translocation.

S, sky chain; H, dynein H chain.

* Hamasaki et al., 1991.

\ddagger $p < 0.001$ versus PKA control.

\S $p > 0.05$ versus PKA control, no significant difference.

Analysis of Triton X-100-permeabilized cells, under conditions where p29 is phosphorylated but cAMP is removed, demonstrates that swimming proceeds at nearly twice the speed of control cells.

These previous studies strongly suggest that p29 or its equivalent plays an important role in the regulation of the outer dynein arm. In this study we provide additional evidence to confirm this hypothesis. This has led to a further understanding of the construction of the outer dynein arm of *Paramecium* and the role of p29 as a regulatory light chain of 22S dynein. From a crude mixture of *Paramecium* dyneins we have isolated a fraction containing p29, but lacking the dynein heavy (H) chains. Using this fraction, we document how p29 can specifically reassociate with 22 but not 14S dynein of *Paramecium*, and that this reassociation extends to the outer arm dynein of another ciliate genus, *Tetrahymena*. We are able to localize the p29-binding site for reassociation to a specific H chain of these dyneins, most likely the α H chain.

We further exploit our ability to repopulate 22S dynein with p29 to examine the ability of thiophosphorylated p29 to regulate the activity of reconstituted dynein in vitro, and we also extend the axonemal phosphorylation studies to the in vitro translocation system, using a *Paramecium* cAMP-dependent protein kinase (PKA). In both of these different types of experiments, the number of phosphorylated constituents is more limited than previously, and in the reconstitution experiment only p29 is experimentally thiophosphorylated. Microtubule translocation velocity increases in a manner comparable to that reported by Hamasaki et al. (1991), which implies that p29 phosphorylation is critical in determining changes in translocation velocity in this assay, possibly by altering the mechanochemical properties of outer arm dynein.

Materials and Methods

Axoneme Isolation

Paramecium tetraurelia are cultured axenically and harvested as described

previously (Hamasaki et al., 1989, 1991). The harvested *Paramecium* are deciliated by calcium shock; the cilia are isolated, demembrated, and purified (Hamasaki et al., 1989). *Tetrahymena* are grown axenically (Barkalow et al., 1994) and deciliated by addition of 0.25 mg/ml dibucaine (Ciba-Geigy, Summit, NJ). Complete deciliation occurs within 10–15 min. From this point in the isolation, handling of *Paramecium* and *Tetrahymena* samples is identical. Methods follow Hamasaki et al. (1989, 1991).

Axonemal Phosphorylation and Isolation of Crude Dynein

The axonemes are used directly for dynein extraction when unphosphorylated dynein is required or for thiophosphorylation (used as a stable counterpart of phosphorylation). *Paramecium* axonemes are thiophosphorylated on ice for 30 min in 2 ml of axoneme buffer containing 10 μ M γ -[S]ATP (Boehringer Mannheim, Indianapolis, IN) in the presence or absence of 20 μ M cAMP (Boehringer Mannheim), and/or 1 mCi γ -[³⁵S]ATP (NEN, Boston, MA or Amersham Corp., Arlington Heights, IL) at a total protein concentration of \sim 3 mg/ml. The thiophosphorylated axonemes are repelleted and washed with 5 ml axoneme buffer at 12,000 g at 4°C for 10 min.

In all cases, for dynein extraction the washed pellet is resuspended in axoneme buffer containing 0.6 M KCl and allowed to stand on ice for 30 min. The salt extract is centrifuged at 12,000 g at 4°C for 10 min. The supernatant, containing the extracted dynein, is collected and centrifuged at 100,000 g at 4°C for 30 min to remove large particulates. The supernatant is concentrated in a Centricon 30 or 100 (Amicon, Beverly, MA) by centrifugation at 5,000 or 1,000 g, respectively, at 4°C to a volume \leq 100 μ l, and washed with 0.5 ml axoneme buffer containing 0.6 M KCl. In the case of the Centricon 100, all proteins or protein complexes less than \sim 80 kD flow through a filter. The Centricon retentate is enriched in dynein and will be referred to as crude dynein.

Purification of Unphosphorylated Dyneins and Phosphorylation In Vitro

Paramecium or *Tetrahymena* crude dynein from unphosphorylated axonemes is loaded onto an 11.6 ml, 5–30% linear sucrose gradient in axoneme buffer; no more than 200 μ l is loaded onto a single gradient. The gradients are centrifuged in L5-50 centrifuge (Beckman Instrs., Inc., Fullerton, CA) and an SW41 Ti rotor for 15 h at 35,000 RPM and 4°C, and fractionated into 0.5-ml fractions. Dynein is detected by ATPase activity (Hayashi and Takahashi, 1979; Murphy and Riley, 1962) and SDS-polyacrylamide gel electrophoresis (modified after Laemmli, 1970). Protein concentration is determined by the BCA protein assay using BSA as a standard (Pierce, Rockford, IL). In these gradients two peaks of dynein ATPase are found at 14 and 22S. Fractions containing these dyneins are pooled, frozen, and stored at -80°C for later use in the reconstitution and translocation assays.

In some experiments, a partially purified cyclic AMP-dependent PKA isolated from *Paramecium* (Hoelstrasser and Nelson, 1989; kindly provided by C. Walczak and D. Nelson, University of Wisconsin, Madison, WI), has been used to phosphorylate 22S dynein *in vitro* (C. Walczak and D. Nelson, personal communication). Unphosphorylated 22S dynein (~0.3 μ g) was incubated with PKA (~10 U), in the presence of 5 μ M cAMP and 0.1 mM ATP (including 30 μ Ci γ -[³²P]ATP) in 10 mM Mg-acetate and 50 mM MOPS, pH 6.5, at room temperature for 10 min. Controls omitted either the dynein or the PKA. The reaction was terminated by the addition of an equal volume of cold 30% trichloroacetic acid and allowed to stand for 30 min on ice. The samples were centrifuged in a microfuge, and resuspended in SDS sample buffer with 1 M Tris, pH 8.0, and incubated for 3 min at 80°C. Samples are analyzed by SDS-PAGE and autoradiography using X-OMAT AR scientific imaging film (Eastman Kodak, Rochester, NY).

p29 Isolation

In general, p29 has been isolated from thiophosphorylated crude dynein. When prepared from axonemes that have been treated either with γ -[³⁵S]ATP or γ -[S]ATP in the absence of cAMP, p29 is unlabeled and not experimentally phosphorylated (Hamasaki et al., 1991); when prepared from axonemes treated with γ -[³⁵S]ATP and cAMP, p29 is thiophosphorylated and labeled; when prepared from axonemes treated with γ -[S]ATP and cAMP, p29 is thiophosphorylated and unlabeled.

To determine the original percent dissociation of p29 from dynein, labeled axonemes are treated with 0.6 M KCl and the resulting crude dynein is allowed to stand on ice for 30 min. The sample is loaded onto a Centricon 100 and centrifuged at 1,000 g at 4°C until the volume of the retentate is <100 μ l. An additional 0.5 ml of axoneme buffer containing 0.6 M KCl is added to wash the retentate. For measurements the Centricon 100 flowthrough is concentrated in a Centricon 10 (Cutoff ~10 kD). p29 does not flow through the Centricon 10. The retentates of the Centricon 100 and 10 are repetitively concentrated to <100 μ l and washed with axoneme buffer. The initial experiments were done in 200 μ l, with a protein concentration of 1.5 mg/ml. In individual retentates and flowthroughs, p29 is detected by SDS-PAGE employing an acrylamide gradient of 5–15%, usually followed by phosphorimaging using a Molecular Dynamics Phosphorimager (Sunnyvale, CA) and/or autoradiography. Percent p29 in the flowthrough is the amount in the Centricon 100 flowthrough, determined quantitatively using the Phosphorimager, divided by amount in Centricon 100 (flowthrough plus retentate) \times 100. Based on competition experiments, we assume the distribution of label is indicative of the distribution of total p29. Additional chaotropic agents tested were 1.2 M KCl with and without 10 mM DTT, and 4 M urea.

The 1.2 M KCl treatment yields a Centricon 10 retentate containing a significant amount of p29 in an enriched fraction lacking the dynein H chains. This fraction, which is used routinely, will be referred to as the p29 fraction. It is routinely obtained starting with crude dynein, 1.5 mg/ml, in a volume of 2 ml in axoneme buffer containing 0.6 M KCl to which is added 2 ml of axoneme buffer containing 1.8 M KCl, processed as above. The Centricon 10 retentate is washed to a final volume of ~500 μ l, a final KCl concentration of ~50 mM, and a final protein concentration of 1 mg/ml. For some experiments, the p29 fraction has been further purified by column chromatography. The p29 fraction is loaded onto a Superdex 75 column (Pharmacia LKB Biotechnology, Piscataway, NJ) pre-equilibrated in axoneme buffer. 0.5-ml fractions are collected and assayed by scintillation counting, SDS-PAGE (proteins detected by silver staining as described by Merrill et al., 1984), and autoradiography. The Superdex 75 column is calibrated using chromatographic standards (M_r 68,000, 45,000, 25,000, and 12,500) (Boehringer Mannheim, Mannheim, Germany).

Tests of Association of p29 to 22S Dynein and Other Proteins

Reconstitution of purified *Paramecium* 22S dynein with p29 was tested by sucrose density gradient purification. Unphosphorylated 22S dynein was incubated with a labeled p29 fraction at room temperature for 2 h, subsequently loaded onto a sucrose gradient and repurified. Gradient fractions have been analyzed by ATPase activity, SDS-PAGE, autoradiography and scintillation counting. As a control, purified *Paramecium* 14S dynein was substituted for 22S dynein.

To test the ability of p29 to associate with 22S dynein or other moieties more generally, a force filtration procedure similar to that described above has been employed. After incubation, the sample is loaded on a Centricon 100 to recover the retentate in a final volume of 40 μ l. The flowthrough is

concentrated in a Centricon 10 to 40 μ l. The samples are analyzed separately by SDS-PAGE and autoradiography.

For the quantitative analysis of reconstitution, one constituent (p29 or 22S dynein) is maintained at a constant concentration in a series of aliquots, while the concentration of the other constituent is systematically increased. Reconstitution is performed at room temperature for a period of 2 h (based on a time course analysis of reconstitution that indicates saturation at >30 min). Controls are for volume, and for protein concentration when appropriate; all samples are treated identically throughout. Samples are collected using the above Centricon procedure. To improve recovery of all fractions, in some experiments the Centricons used are pretreated overnight at room temperature with 1% nonfat instant milk (Carnation, Los Angeles, CA) with 0.02% NaN₃. Before use, the Centricons are extensively washed with distilled water. Retentate and flowthrough are analyzed by SDS-PAGE and quantitatively analyzed using a Phosphorimager. The reassociation of p29, at varying concentrations, with limiting amounts of 22S dynein was also analyzed using a Molecular Dynamics densitometer by scanning a 5–15% acrylamide gel stained with Coomassie brilliant blue R250 and destained using a low (10%) methanol–7.5% acetic acid solution, which preserves p29 staining.

Chymotryptic Digestion of 22S Dynein

Chymotryptic digestion of 22S dynein has been performed essentially as described by Toyoshima (1987a, b). Digestion is achieved at a chymotrypsin/22S dynein ratio of 1:100, by weight, at room temperature for 5 and 30 min. The digestion is stopped with 1 mM PMSF. The resulting fragments are separated by sucrose density gradient centrifugation and analyzed as above.

The structural characteristics of the peak fractions of the dynein fragments are assayed by negative stain electron microscopy, using a carbon-coated mica method described in Barkalow et al. (1994).

Microtubule Translocation Assay

The functional effects of reconstitution are assayed by *in vitro* microtubule translocation as described previously (Vale and Toyoshima, 1988, 1989; Hamasaki et al., 1991). In a field selected for measurement, all microtubules that produce consistent movement of over 30 sec are measured. Qualitatively, percent mobility does not vary with treatment. All 22S dyneins used in these assays are initially unphosphorylated. Reconstitution experiments compare dyneins from retentates obtained using the force filtration recombination of unlabeled 22S dynein and a p29 fraction obtained by 1.2 M extraction of crude dynein from axonemes thiophosphorylated in the presence of γ -[S]ATP with versus without cAMP.

For the *in vitro* phosphorylation experiments, unphosphorylated 22S dynein is perfused into the translocation chamber and motility is recorded. The translocation chamber is then washed with gliding buffer followed by phosphorylation buffer. Then phosphorylation buffer containing 1 mM ATP, with or without 10 μ M cAMP or PKA (~10 U), is perfused into the chamber and incubated at room temperature for 10 min. This buffer is then replaced with gliding buffer. Motility of the treated dynein is analyzed within 10 min of phosphorylation.

Results

When thiophosphorylated crude dynein is isolated from axonemes in 0.6 M KCl, 28% of p29 as dissociated from dynein H chains, based on quantitative analysis of the radiolabeled p29 as discussed in the Materials and Methods. When this mixture is further subjected to high salt (1.2 M KCl), an extract is obtained in which a total of 60% of p29 is dissociated. Addition of a reducing agent (10 mM DTT) to the mixture does not increase the dissociation. Dissociation is slightly enhanced (71%) when 4 M urea is used in lieu of the 1.2 M KCl. The final high salt extract (using the 1.2 M KCl treatment) is subjected to Centricon force filtration steps to separate the dissociated p29 from the H chains and to obtain proteins of M_r > 10 < 100 kD in a filtrate. p29 retains its label throughout and is a principal labeled band of the filtrate (Fig. 1, p29 fraction).

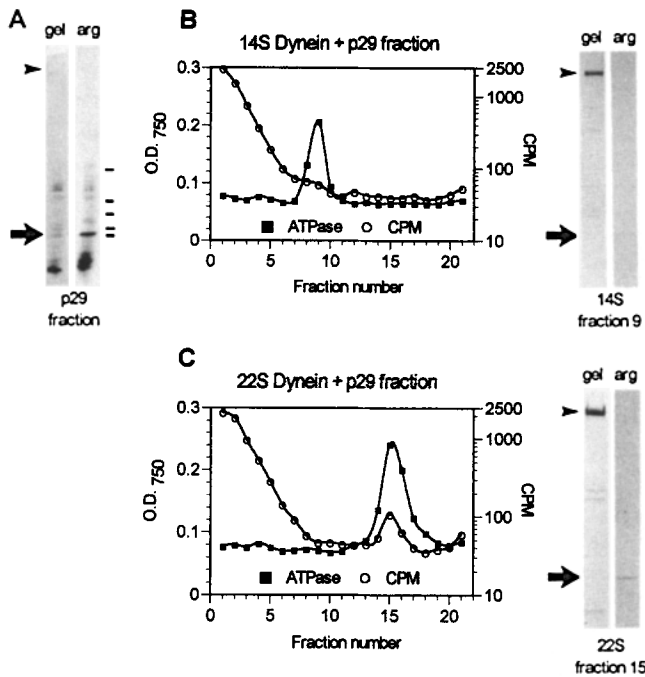


Figure 1. Recombination of p29 fractions with *Paramaecium* dyneins. (A) p29 fraction from thiophosphorylated labeled axonemes. Coomassie blue-stained lane from a 5–15% linear gradient SDS-PAGE (*gel*) and corresponding autoradiogram (*arg*) are shown. (B) Unphosphorylated 14S dynein recombined with the labeled p29 fraction. Sucrose density gradient fractions (right, *gel* and *arg* of fraction 9). (C) Unphosphorylated 22S dynein recombined with the labeled p29 (*right, gel* and *arg* of fraction 15). A peak of radioactivity corresponding to labeled p29 cosediments with 22S dynein. 60 μ g of 22 and 14S dynein and 50 μ g of p29 fraction are used. Samples were not concentrated prior to SDS-PAGE. Indicators for all Figs. p29, arrow; dynein H chain region, arrowhead; lines, M_r standards (from top) 66, 45, 36, 32, and 24 kD (some gels have a sixth standard at 20.1 kD).

Reconstitution of Dyneins with p29

When placed in physiological buffers, the dissociated p29 will reassociate with 22S dynein. To demonstrate reassociation, we purified unlabeled crude dynein by sucrose density centrifugation. Based on ATPase activity, fractions containing 14S and 22S dynein were obtained and incubated for 2 h with the labeled p29 fraction before a second sucrose gradient centrifugation of each dynein was performed. Fig. 1 shows that although most of the label is found unassociated with either dynein, a significant peak of p29 radioactivity reassociates and cosediments with 22S dynein. p29 is the only radiolabeled band to reassociate in this way. In contrast, very little, if any, p29 radioactivity cosediments with 14S dynein.

In further experiments, this reconstitution assay has been simplified. The dynein to be tested for reassociation is incubated with the labeled p29 fraction and subjected to force filtration in a Centricon 100, through which the dynein H chains and bound proteins do not pass. The amount of label that remains with the dynein, after extensive washing, is taken as a measure of reconstitution. The results of this assay with 22 and 14S dynein, BSA and no added proteins (buffer

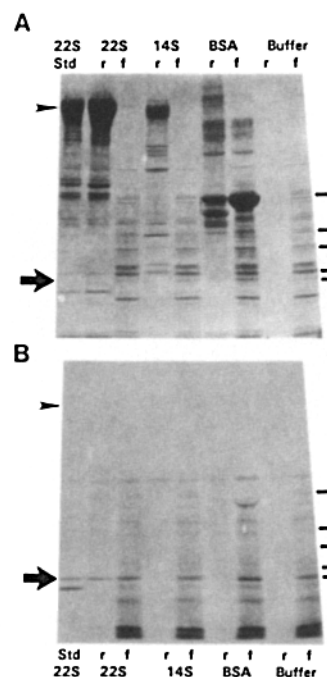


Figure 2. Force filtration assay for recombination. (A) Coomassie blue-stained gel of a 5–15% linear gradient SDS-PAGE. (B) Corresponding autoradiogram. Labeled p29 fraction is incubated with various unlabeled substrates (22S dynein, 14S dynein, BSA, and buffer only) and subjected to force filtration with a Centricon 100 filter as described (see Materials and Methods). Retentate (*r*) and flowthrough (*f*) are shown for each sample, in this and other figures. p29 recombines only with 22S dynein to remain in the retentate. In the BSA sample, the high molecular mass bands probably represent artifacts of BSA oligomerization. (*Left lane*) Standard of thiophosphorylated 22S dynein before KCl extraction. All recombinations were done with 50 μ g p29 fraction and 60 μ g 22S, 14S, or BSA in 375 μ l, volumes matched by addition of appropriate amount of axoneme buffer.

only) are shown in Fig. 2. The leftmost lane is 22S dynein isolated from axonemes thiophosphorylated in the presence of cAMP and γ -[35 S]ATP (Hamasaki et al., 1991), which serves as a reference standard for radiolabeled p29 (arrow). In the remaining four lane pairs, the radiolabeled proteins seen by autoradiography (Fig. 2, B) derive solely from the p29 fraction. When no reconstitution occurs, the p29 radiolabeled band is found entirely in the flowthrough (*f*). Where a portion of p29 is selectively found in the retentate (*r*), there is a specific reconstitution. This occurs only between p29 and 22S dynein. Reconstitution plateaus after 30 min of incubation and remains unchanged up to 4 h. The amount of p29 in the retentate is reduced slightly (to 76% of control) by the addition of 1 mM ATP but enhanced somewhat (to 119% of control) by 0.1% Triton X-100, conditions that are used in *in vitro* translocation experiments. Triton X-100 increases overall recovery of p29 at least twofold.

In reconstitution assays where a constant, limiting amount of 22S dynein and large amounts of the p29 fraction are used (Fig. 3), p29 appears as a Coomassie blue-stainable band. Densitometric analysis demonstrates that under these conditions the p29 band increases in the 22S dynein retentate in proportion to the increase in the amount of the p29 fraction used. No other band shows this proportional increase.

The p29 fraction has been analyzed by fast performance column chromatography. Based on elution of M_r calibration standards, the relative molecular mass of p29 is 35 kD. In the eluate fractions, p29 is enriched and appears as a single minor band that is \sim 2% of total protein. It is the sole radiolabeled band in these fractions. When these fractions are incubated appropriately, specificity of reconstitution is maintained and p29 only reconstitutes with 22S dynein, not with 14S dynein.

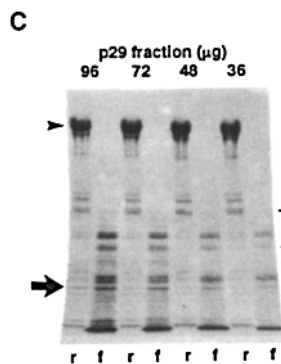
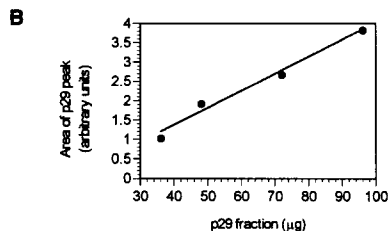
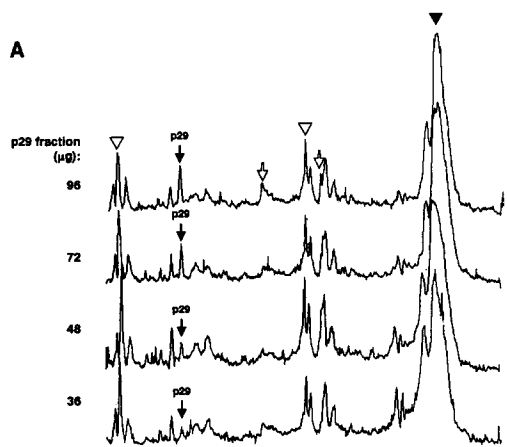


Figure 3. Densitometric analysis of p29 recombination with *Paramecium* 22S dynein. (A) Retentate lanes from Coomassie blue-stained gel are scanned; M_r increases from left to right. Black arrowhead indicates dynein H chains. 30 μg of 22S dynein is used throughout, while the p29 fraction added is systematically increased. The p29 peak is indicated relative to reference proteins (open arrowheads); other changes in top scan (open arrows)

probably do not represent recombination. (B) p29 recombination increases linearly with respect to the amount of p29 fraction added. (C) Coomassie blue-stained gel used in scanning of retentate lanes shown in A. Retentate (r) and flowthrough (f) are shown from recombinations starting with 96, 72, 48, or 38 μg p29 fraction and 30 μg 22S dynein. All recombinations were done in 300 μl adjusted by addition of axoneme buffer.

Quantitative Analysis of p29 Binding to Purified 22S Dynein

The binding of p29 to 22S dynein is saturable (Fig. 4). With increasing dynein concentration, a maximum of $\sim 70\%$ of p29 rebinds to 22S dynein. Saturation occurs at a 22S dynein concentration of $\sim 1.2 \times 10^{-7}$ M, giving an apparent dissociation constant of 2.5×10^{-8} M. Based on Coomassie blue staining of the p29 band in the p29 fraction, the concentration of p29 in Fig. 4 is 4.6×10^{-9} M. Correcting for recovery and assuming that one p29 binds per 22S dynein, the concentration of 22S dynein with rebound p29 has been determined. On this basis, a double reciprocal plot (Fig. 4, inset) also yields an apparent dissociation constant of 2.5×10^{-8} M. By direct measurement, where 22S dynein is limiting, a maximum of $\sim 17\%$ of 22S dynein has rebound p29.

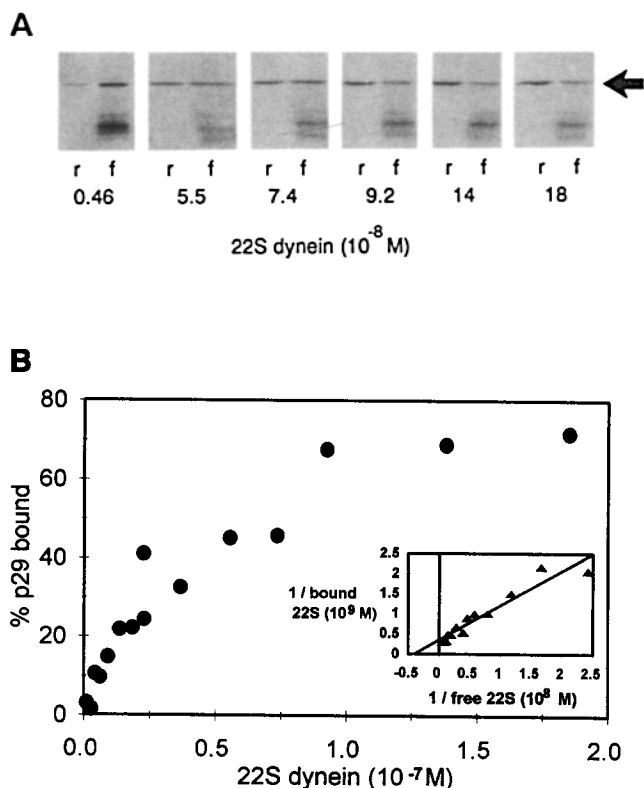


Figure 4. Saturation analysis of p29 recombination with *Paramecium* 22S dynein. (A) Arg of 29-kD region showing a recombination series where the p29 concentration (4.6×10^{-9} M) is maintained and 22S dynein concentration is varied as shown. (B) Saturation curve showing percent p29 in the retentate versus dynein concentration. (Inset) A double reciprocal plot. Bound 22S is the molar concentration of 22S dynein that has rebound p29, based on densitometric measurements. p29 concentration was determined from gels comparable to Fig. 3 using Sigma SDS-7 as protein concentration standards. All recombinations were done in a final volume of 600 μl adjusted by addition of axoneme buffer.

Competition Experiments

We have tested the ability of nonradiolabeled p29 fractions to compete with radiolabeled p29 for reconstitution with 22S dynein. Two different nonradiolabeled p29 fractions have been used: one is isolated from axonemes treated with γ -[S]ATP only (control, C); the other is isolated from axonemes treated with γ -[S]ATP and cAMP to achieve a nonradiolabeled, experimentally phosphorylated p29 (phosphorylated, P). The results are shown in Fig. 5. When a noncompetitive protein, such as BSA, is added to the incubation mixture such that the total protein added to the 22S dynein is tripled, there is no effect on the amount of p29 that is retained by the 22S dynein. When a competitive nonradiolabeled fraction is used, radiolabeled p29 is diluted out of the retentate fractions, in proportion to the amount of competitor added. This is the case when either the control or the phosphorylated nonradiolabeled extracts are used for competition.

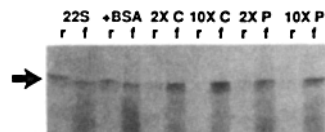


Figure 5. Competition experiments. Aliquots of labeled p29 fraction (12 μg each) are recombined with unphosphorylated 22S dynein (38 μg) in the presence of a competitor

for recombination volumes were adjusted with axoneme buffer to 400 μl . The arg of the 29-kD region is shown. Competitors are: buffer alone (22S); 24 μg BSA (+BSA); 24 or 120 μg of unlabeled, unphosphorylated p29 fraction (2 \times or 10 \times C); 24 or 120 μg unlabeled, thiophosphorylated p29 fraction, (2 or 10 \times P). Both unphosphorylated and phosphorylated unlabeled p29 fractions compete for the labeled p29 equally well.

Recombination of *Paramecium* p29 with *Tetrahymena* Dynein

Recombination of *Paramecium* p29 has been tested with *Tetrahymena* dyneins (Fig. 6). p29 reassociates specifically with *Tetrahymena* 22S dynein to about the same extent as with *Paramecium* 22S dynein. *Tetrahymena* 14S dynein does not bind p29. In preliminary experiments, neither *Chlamydomonas* 12 nor 18S outer arm dynein bind p29.

Recombination of p29 with *Tetrahymena* 22S Dynein Fragments

When *Tetrahymena* 22S dynein is treated with chymotrypsin according to the procedure of Toyoshima (1987a,b), one-headed (12–14S), and two-headed (18–20S) fragments are

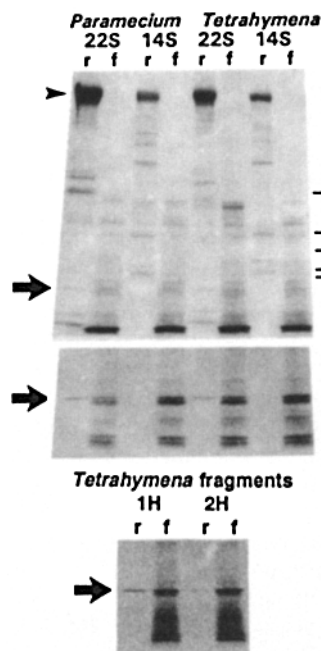


Figure 6. Recombination of *Tetrahymena* dyneins and dynein fragments with *Paramecium* p29. (Top) *Tetrahymena* 22S dynein is equally competent as *Paramecium* 22S dynein for recombination with *Paramecium* p29. In each reconstitution the same amount of labeled p29 fraction is used. *Tetrahymena* 14S dynein does not recombine with p29. Coomassie blue-stained gel, 5–15% linear gradient SDS-PAGE, with corresponding arg of 29-kD region. (Bottom) Recombination of p29 with chymotryptic fragments of *Tetrahymena* 22S dynein. The *Tetrahymena* one-headed fragment (1H) preferentially recombines with *Paramecium* p29, as compared to the two-headed fragment (2H). Arg of 29-kD region. Quantitative

structural analysis of one- and two-headed fractions demonstrates that one-headed fraction is nearly homogeneous. One-headed fraction: 89% one-headed, 4% two-headed, 0% three-headed, 7% structure is obscured ($n = 28$). Two-headed fraction: 17% one-headed, 71% two-headed, 9% three-headed, 3% structure is obscured ($n = 105$). All recombinations done in 300 μl with 30 μg 22 and 14S dyneins and 20 μg p29 fraction. For chymotrypsin digest 100 μg 22S is digested and run on sucrose gradient. Peak one- and two-headed fractions were used (400 μl) and recombined with 25 μg p29 fraction.

obtained. We tested the ability of unlabeled proteolytically generated fragments of *Tetrahymena* dynein to reassociate with labeled p29. p29 reassociates preferentially with the 12–14S single-headed dynein (Fig. 6).

Control of In Vitro Microtubule Sliding Velocity by p29 Phosphorylation

An in vitro microtubule translocation assay has been used to test for the functional capacity of the reassociated p29 to modulate the rate at which 22S dynein translocates brain microtubules. As a control, unphosphorylated *Paramecium* 22S dynein has been isolated and, without further treatment, in vitro translocation has been measured as in Hamasaki et al. (1991). The velocity of translocation ranges from about 0.5–3.3 μms^{-1} , with an average of $1.61 \pm 0.79 \mu\text{ms}^{-1}$ (Table I, Fig. 7). Microtubules have been washed out and the bed of dynein has then been treated with cAMP and ATP, with or without PKA. Microtubules are reintroduced and translocation rate remeasured. In the absence of PKA, velocity does not increase; in fact the average velocity is slightly slowed ($1.3 \pm 0.59 \mu\text{ms}^{-1}$, $n = 32$) (p versus control: $0.1 > p > 0.05$). After PKA perfusion, the translocation velocity increases by 1.53 \times to $2.48 \pm 1.08 \mu\text{ms}^{-1}$ ($p < 0.001$), with 19% of the microtubules having velocities $>3.3 \mu\text{ms}^{-1}$. Comparison between the untreated and PKA-treated 22S dynein reveals that several dynein components have been phosphorylated by the PKA treatment, including the dynein H chains, a ~ 50 kD protein and p29 (Fig. 7).

22S dynein has also been tested for its ability to translocate microtubules after reconstitution with various p29 fractions. Without reconstitution, this dynein, which has been extensively washed in the Centricon, produced microtubule translocation velocities that were more sluggish than usual, but addition of a control p29 fraction where the p29 was not thiophosphorylated increased translocation velocity to control levels (Table I). Compared to reconstitution with control p29 fractions, reconstitution with thiophosphorylated p29 increases translocation rate by 1.31 \times to $2.1 \pm 0.78 \mu\text{ms}^{-1}$ ($p < 0.001$) (Table I) with 10% of the microtubules being translocated at velocities $>3.3 \mu\text{ms}^{-1}$ (Fig. 7). These preparations have no differences in H chain phosphorylation. Moving on the dynein substratum reconstituted with the thiophosphorylated p29, a few microtubules translocate at velocities up to 10 μms^{-1} for short intervals (not shown).

Discussion

The results presented here clarify and further our understanding of the relationship and interaction of p29 and 22S dynein. In isolated 22S dynein, p29 has been found to be substoichiometric in the past (Travis and Nelson, 1988; Hamasaki et al., 1991), presumably because of the loss of $\sim 28\%$ of p29 during isolation of crude dynein with 0.6 M KCl. We assume that this corresponds to the unpopulated sites of isolated 22S dynein, i.e., the 17% or so of the dynein molecules that can rebind p29. The molar ratio of p29/22S dynein would then be ~ 0.6 , suggesting that, in the intact axoneme, p29 is a component of a large proportion, perhaps all, of the outer arms, a conclusion consistent with that of Walczak and Nelson (1994). p29 is not associated with 22S dynein via a covalent linkage, since it is extracted by chaotropic agents

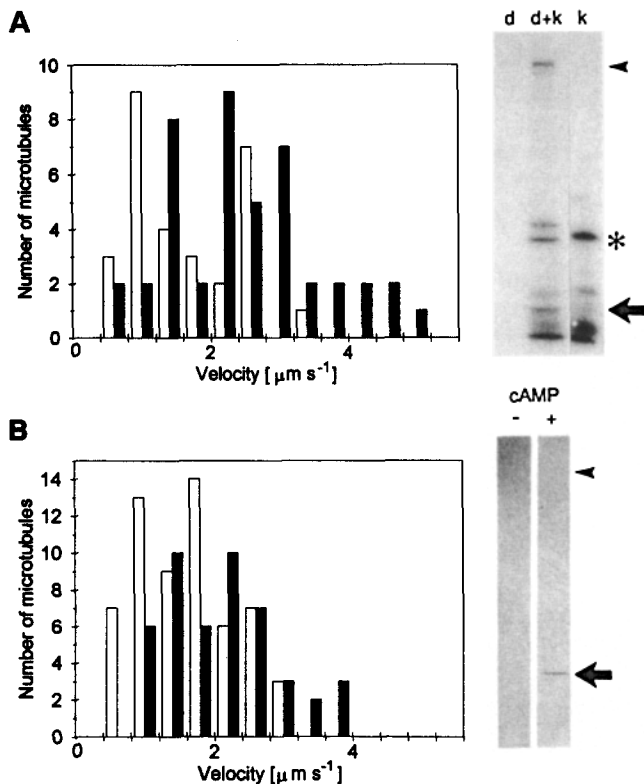


Figure 7. Effect of p29 phosphorylation on microtubule translocation in vitro. (A, left) Histogram shows distribution of microtubule translocation velocities by *Paramaecium* 22S dynein before treatment with PKA (open bars) and after treatment (black bars). (Right) After PKA treatment, p29, dynein H chain(s), and a ~ 50 -kD protein in the 22S dynein substratum are phosphorylated. Arg after SDS-PAGE using a 5–15% gel. (d) 22S dynein incubated with cAMP and γ -[35 S]ATP; no radiolabeled bands are found; (d+k) 22S dynein incubated with cAMP, γ -[35 S]ATP and PKA; (k) kinase only incubated with cAMP and γ -[35 S]ATP (autophosphorylated PKA, *). (B, left) Histogram showing distribution of microtubule velocities by *Paramaecium* 22S dynein recombined with unlabeled p29 (open bars) versus thiophosphorylated p29 (black bars). (Right) after recombination, p29 is the only thiophosphorylated protein present in the 22S dynein substratum. Arg of retentate fractions of 22S dynein recombined with labeled p29 fractions thiophosphorylated in the absence (–, unlabeled p29) or presence (+, thiophosphorylated p29) of cAMP. Recombinations were done in 130 μ l with 30 μ g 22S dynein and 30 μ g p29 fraction.

alone, and in the presence of a reducing agent no effect on the interaction with 22S dynein is seen. The association is fairly strong, with an apparent dissociation constant of 25 nM. Even after treatment with 4 M urea a small population of p29 remains associated with the H chains.

22S dynein treated with urea or 1.2 M KCl retains less p29 than the standard 22S dynein. High resolution negative stain techniques demonstrate that the 22S dynein extracted using 1.2 M KCl can be recognized as a three headed structure, although its appearance is altered. The three-headed molecule, in general, appears more unfolded, perhaps due to an unraveling. In fields scored for one-, two-, and three-headed structures this 1.2 M KCl extracted dynein has significantly more (2.5 times) single headed molecules. These observations suggest that the 22S dynein bouquet structure is perturbed by the 1.2 M KCl treatment in comparison to standard

dynein preparations. In vitro microtubule translocation assays demonstrate that this 1.2 M KCl extracted 22S dynein translocates microtubules significantly slower ($0.72 \pm 0.23 \mu\text{m s}^{-1}$; $p < 0.001$) than matched controls using our standard 22S dynein. These results raised concerns about the structural and functional integrity of the 1.2 M KCl extracted 22S dynein and our ability to compare it directly to the standard 22S dynein phosphorylated in situ before extraction. Therefore, we selected the standard (0.6 M KCl extracted) dynein instead in reconstitution assays.

The specificity of the 22S dynein–p29 interaction is demonstrated by a competition experiment. The amount of competing p29 directly affects the amount of radiolabeled p29 that binds to the 22S dynein. The addition of a non-dynein protein such as BSA has no effect on the binding. Densitometric analysis of retentates where p29 can be visualized by Coomassie staining demonstrates that only the p29 band varies proportionately in the retentate, when increasing amounts of p29 fraction are recombined with a constant amount of 22S dynein. When p29 is further enriched on a sizing column, it elutes with an M_r of 35 kD, suggesting that it is not complexed with a protein greater than ~ 10 kD, if any, although specificity of reassociation is maintained. These results strongly suggest that p29 is binding to 22S dynein as a single entity, rather than associating via an accessory factor in the p29 fraction.

The ability of the *Paramecium* p29 to associate specifically with *Tetrahymena* 22 but not 14S dynein demonstrates the similarity of the axonemal dyneins of these two genera and suggests that p29 or its homologues may be more widely used as a cAMP-dependent regulator of outer arm dynein. After chymotryptic digestion, p29 preferentially binds to a 12–14S single-headed dynein fragment. This single-headed fragment from 22S dynein is different from the usual 14S dynein, a single-headed dynein to which p29 does not bind. If 14S dynein is in part a γ H chain breakdown product of 22S dynein, as suggested by Walczak et al. (1993) and Beckwith and Asai (1993), this H chain is unlikely to be the site of p29 association.

Proteolysis of *Tetrahymena* 22S dynein, as described by Toyoshima (1987a,b), has shown that the single-headed fragment contains polypeptides uniquely originating from the intact α H chain. The small amount of binding to the 18–20S-fraction could be the result of contamination by one- and/or three-headed structures or it might indicate a weaker association of p29 with the two-headed molecule. It seems likely that p29 could preferentially be associated with the homologue of the α H chain in *Paramecium* 22S dynein. Apparently the *Paramecium* p29 does not associate with *Chlamydomonas* 12 or 18S outer arm dynein, but cAMP-dependent effects are different in the two organisms.

With the idea in mind that p29 should be considered a regulatory light chain, perhaps specific to the α H chain, of certain outer arm dyneins, we have attempted to clarify the functional role of p29. Hamasaki et al. (1991) reported that cAMP-dependent phosphorylation of p29 prior to 22S dynein isolation resulted in a 1.4 times increase in microtubule translocation rate measured in an in vitro assay. In this study, we have shown that 22S dynein reconstituted with thiophosphorylated p29 translocates microtubules significantly (1.31 times, $p < 0.001$) faster than controls reconstituted with fractions where p29 is not experimentally phosphorylated. Fur-

ther, when the field of unphosphorylated 22S dynein used for translocation is phosphorylated by a *Paramecium* PKA in the presence of cAMP and ATP, the identical dynein molecules translocate microtubules 1.53 times faster than in cAMP and ATP alone. As is also true for *Tetrahymena* (Chilcote and Johnson, 1990) both the dynein H chains and p29 become phosphorylated in such in vitro incubations (C. Walczak and D. Nelson, personal communication; Fig. 7). However, in the reconstituted dynein, p29 is the only moiety phosphorylated in a cAMP-dependent manner (Table I). Therefore, the ability of phosphorylated p29 to modulate 22S dynein activity is functional after reconstitution. The common denominator in the PKA experiments, the reconstitution experiments and previous experiments with axonemally phosphorylated dyneins is that cAMP-dependent phosphorylation of p29 results in a significant increase in microtubule translocation rate by 22S dynein. This confirms the hypothesis that p29 alone is sufficient to control the rate of microtubule translocation by changes in its phosphorylation state. In vivo, H chain or other phosphorylation events could, of course, contribute to changes in microtubule sliding rates and some of the variability seen in vitro could be due to changes in dynein constituents other than p29. Nevertheless, our results make it probable that p29 is the molecule in the ciliary axoneme that is directly in the cAMP-dependent signal transduction cascade.

The relatively small percentage increase in translocation rate seen after phosphorylation of p29 in these assays may reflect the relatively small percentage of 22S dynein molecules that are reconstituted with a phosphorylated p29. In the course of several seconds' translocation at the density of 22S dynein used in our chamber, a 10- μ m-long microtubule would encounter a few, perhaps only one or two appropriately phosphorylated, reconstituted dyneins. Hamasaki et al. (1991) suggest that these dyneins could act as pacemakers for the increase in translocation rate. This suggestion gains some plausibility because it has been demonstrated that a very few special motors (in this case, kinesin) can override the force generation of a considerable number of dyneins (Vale et al., 1992), but perhaps other, more complicated, explanations are necessary. For example, upon reconstitution, the phosphorylated p29 could act catalytically (C. Walczak and D. Nelson, personal communication) to increase phosphorylation in some other dynein constituent.

It remains unclear how p29, a dynein regulatory light chain, in its phosphorylated state modulates the 22S dynein mechanochemical cycle. Two principal mechanisms can be envisioned. The mechanochemistry of dynein could be changed such that each step, i.e., the distance a microtubule is moved in each ATPase hydrolysis cycle, could be increased. This would require substantial changes in the dynein conformation. It also would likely require a high degree of cooperativity between adjacent arms so as to avoid steric difficulties. Alternatively, p29 in its phosphorylated state could decrease cycle time, i.e., the duration of a single mechanochemical cycle, thus increasing the number of steps per unit time. There is some precedence for this latter mechanism. Phosphorylation of myosin light chain is thought to decrease the cycle time of smooth muscle and nonmuscle myosins by accelerating the rate limiting step, which is product release (Butler et al., 1989, 1990). This could potentially be tested for dynein reconstituted with thiophosphorylated p29.

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