Increased protein phosphorylation of cytoplasmic dynein results in impaired motor function

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Inhibition of serine/threonine protein phosphatases in rat hepatocytes by okadaic acid and microcystin increased the phosphorylation of several components of the cytoplasmic dynein complex. UV light/vanadate cleavage and Western blot analysis revealed that two of these components with molecular masses of approx. 400 kDa and 74 kDa were dynein heavy- and intermediate-chains respectively. This increased phosphorylation

resulted in inhibition of dynein ATPase activity, and reduced motor-dependent avidity of endosomal/lysosomal membranes for microtubules.

Key words: hepatocytes, microcystin, okadaic acid, Ser/Thr protein phosphatases.

INTRODUCTION

Cytoplasmic dynein is a microtubule (MT)-activated mechanochemical protein that uses energy derived from ATP hydrolysis to drive vesicles along the MT path with the aid of other accessory proteins [1]. This multimeric protein comprises two heavy chains (DHCs; 440–530 kDa), which have ATPase and MT-binding properties, two to three intermediate chains (DICs; 70–74 kDa), and various numbers of light chains (DLCs; 50–55 kDa) [2] or light-intermediate chains (DLICs; 60–70 kDa) [3]. In addition, cytoplasmic dynein-driven vesicle motility requires at least one other component, the dynactin complex [4,5]. Given its ubiquitous function in membrane trafficking, the motor activity of cytoplasmic dynein is expected to be extensively regulated.

Reversible protein phosphorylation plays a critical role in cells. Changes in the phosphorylation status of serine/threonine and tyrosine residues in response to altered activity of appropriate protein kinases (PKs) and protein phosphatases (PPs) cause major changes in protein conformation and function. Major advances in understanding cellular regulatory mechanisms have been made from the ability to perturb the phosphorylation status of cells in a controlled way. Given the organization of the dynein complex [3,6,7], phosphorylation of DICs or dynactin could regulate membrane binding, while phosphorylation of DHCs could modulate MT attachment/ATP hydrolysis.

In hepatocytes, we have previously shown that even a modest degree of PP inhibition by low doses of microcystin (MCY) or okadaic acid (OKA) (specific inhibitors of PP1, PP2A, and also PP4 and PP5 [8]) results in significantly impaired movement of vesicles along MTs [9], events which are correlated with major reductions in receptor-mediated endocytosis of transferrin [10]. At doses where MT-dependent vesicle movements are impaired, there are no major changes in MT integrity; higher doses and/or longer times of exposure to MCY or OKA are needed to evoke the well-described disruption of the cytoskeleton by these inhibitors [11]. Rather, MT-based motor proteins responsible for driving vesicle movements along the MT path appear to be

targets of inhibition of PPs. Here, we investigate the effects of modest PP inhibition in hepatocytes on the activity and function of the motor protein, cytoplasmic dynein, which plays a principal role in receptor-mediated endocytosis [12–14]. We show that OKA and MCY promote increased phosphorylation of several components of the dynein complex, including DHCs and DICs, and that these changes in phosphorylation are associated with reduced dynein function. To our knowledge, this is the first biochemical demonstration of reduced dynein activity associated with increased dynein phosphorylation.

EXPERIMENTAL

Preparation and treatment of hepatocytes

Male Sprague–Dawley rats (Harlan Sprague Dawley, Indianapolis, IN, U.S.A.) fed *ad lib.*, weighing 240–300 g body weight, were used to isolate rat hepatocytes aseptically by the method of Moldeus et al. [15] and as described previously [9,10]. Hepatocytes were cultured 24 h before use. Treatments included MCY-LR (500 nM, 60 min), OKA (250 nM, 60 min), PMA (50 nM, 60 min), forskolin (FOR; 25 μ M, 60 min), staurosporine (STAUR; 5 μ M, 2 h), K252a (7 μ M, 2 h), dibutyl cAMP (2 mM, 60 min) or vehicle (DMSO) controls.

³²P-labelling studies

Hepatocytes were labelled in phosphate-free medium containing [³²P]P_i for 2 h (0.3 mCi / 1.5 × 10⁶ cells) before treatments and cell lysis in 50 mM Tris/HCl, pH 8.0/150 mM NaCl/1% (v/v) Nonidet P-40/0.5% (v/v) sodium deoxycholate/0.1% (v/v) SDS containing 0.1 mM PMSF, 100 mM NaF, 1 mM Na₃VO₄ and 0.5 mM EDTA. Dynein was immunoprecipitated from lysates with the 74.1 monoclonal antibody against DICs (Chemicon, Temecula, CA, U.S.A.) and Protein A/G PLUS Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Immunoprecipitates were resolved by SDS/PAGE, transferred to nitrocellulose and ³²P:dynein ratios were determined by

Abbreviations used: MT, microtubule; DHC, cytoplasmic dynein heavy chain; DIC, cytoplasmic dynein intermediate chain; DLC, cytoplasmic dynein light chain; DLIC, cytoplasmic dynein light-intermediate chain; PK, protein kinase; PP, protein phosphatase; OKA, okadaic acid; MCY, microcystin; FOR, forskolin; STAUR, staurosporine; VAN, vanadate; p[NH]ppA, adenosine 5'-[β , γ -imido]triphosphate.

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Western blot analysis and autoradiography. Blots and autoradiograms were scanned with a Biorad GS-670 Imaging Densitometer. Scanned densitometry values fell within the linear range of the enhanced chemiluminescence (ECL) system used for Western blotting. For identification of DHCs, lysates were exposed to UV light in the absence or presence of 100 mM vanadate (VAN) and 2 mM MgATP, before immunoprecipitation of the dynein complex [16].

MT-affinity purification of membranes

Using a cell press (H & Y Enterprise, Redwood City, CA, U.S.A.), hepatocytes were homogenized in complete PMEE' buffer [35 mM Pipes (pH 7.4)/10 mM MgSO₄/1 mM EDTA/0.5 mM EGTA, supplemented with 1 mM dithiothreitol and a protease-inhibitor cocktail] [17]. Homogenates were centrifuged (40 000 *g*) at 4 °C for 15 min. Subsequent MT-affinity purification of membrane vesicles was performed as described previously [13,14]. The supernatant was incubated with exogenous MTs at 37 °C for 15 min (formed from phosphocellulose affinity-purified porcine tubulin) and additional taxol (20 μ M) and MgGTP (1 mM) to polymerize endogenous tubulin. This mixture was depleted of ATP with glucose/hexokinase for 15 min at room temperature, before the addition of adenosine 5'-[β , γ -imido]triphosphate (p[NH]ppA) for 15 min at room temperature, followed by centrifugation (25 min, room temperature, 19 000 *g*). Under these conditions, membranes that were not bound to MTs remained in the supernatant, while membranes associated with MTs remained in the pellet. The pellet was resuspended in complete PMEE' buffer with 10 mM MgATP and 20 μ M taxol, incubated for 15 min at room temperature, and then centrifuged (15 min, room temperature, 19 000 *g*). The pellet from this centrifugation step contained membranes bound to MTs in an ATP-independent manner. Membranes recovered in the supernatant were concentrated by subsequent centrifugation (15 min, room temperature, 100 000 *g*); these membranes exhibited ATP-dependent MT binding, a property showing that their attachment was mediated by active motors. Changes in endosomal/lysosomal markers and motors in MT-binding, ATP-release membranes were our principal focus; the amounts of each marker in this fraction were calculated as a percentage of the recovery of marker across all three membrane pools before comparison across treatments. Membrane-associated dynein and kinesin were quantified by Western blotting and densitometry, whereas β -hexosaminidase and acid phosphatase activities were measured as described previously [18,19].

Motor purification and analysis of ATPase activity

Hepatocytes with or without PP inhibitors were homogenized in complete PMEE' buffer with 1 mM Na₃VO₄ and 2 μ M OKA, and motors were isolated by the MT-affinity, ATP-release procedure described previously [18]. These samples were diluted and concentrated using Microcon 30 micro-concentrators (Amicon, Danvers, MA, U.S.A.) to remove residual ATP remaining in the sample from the ATP released from MTs, before addition of fresh ATP and polymerized MTs (50 μ g/ml or $\approx$ 1 μ M) as described previously [20]. Controls included samples without added MTs and samples incubated in the absence of ATP or motors. ATPase activity was measured as P_i released from ATP in 30 min at 37 °C with the EnzChek Phosphate Assay Kit (Molecular Probes, Eugene, OR, U.S.A.). To examine whether any changes in activity were due to inhibition of the ATPase activity of kinesin, a GTP-release protocol [18] was employed in parallel (without dilution and concentration in Microcon 30 filters) to obtain samples enriched in kinesin only.

Motor contents were determined by Western blot analysis using 74.1 and DK410-4.1 antibodies [18] raised against DICs and kinesin heavy chains respectively. To compare ATPase activity of controls with MCY- or OKA-treated samples, the signal from the quantitative scanning of the blot for each experiment was normalized as a control.

Fluorescence microscopy

Rat hepatocytes seeded on to collagen-coated glass coverslips were exposed to the treatments outlined above, fixed and processed as described previously [9] using a mouse monoclonal anti-(α -tubulin) antibody (YOL1/34) and an FITC-conjugated goat anti-mouse secondary antibody.

Statistics

Raw data obtained from separate preparations were analysed for significance in a Student's *t* test. A *P* value $\leq$ 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Inhibition of PPs causes increased phosphorylation of the dynein complex

Previous work has shown that 500 nM MCY or 250 nM OKA results in comparable reductions in MT-based vesicle movements and receptor-mediated endocytosis [9,10]. As shown in Figure 1, the phosphorylation of several components of the cytoplasmic

Figure 1 Cytoplasmic dynein-associated polypeptides phosphorylated in hepatocytes exposed to OKA or MCY

Hepatocytes were labelled with ³²P[P_i] (0.3 mCi /1.5 $\times$ 10⁶ cells, 2 h) prior to treatment with PP inhibitors (250 nM OKA or 500 nM MCY, 60 min), and lysates were exposed to UV light in the absence (no UV-VAN) or presence (UV-VAN) of 100 mM VAN and 2 mM MgATP. Dynein was immunoprecipitated with the 74.1 monoclonal antibody against dynein and Protein A/G PLUS Agarose. Increased phosphorylation in dynein-associated species at 400 kDa, 150 kDa, 74 kDa, 55 kDa and 45 kDa is evident after both treatments (upper panel). The loss of the high-molecular-mass band in dynein immunoprecipitates from extracts exposed to UV-VAN identifies this species as being DHC. The dynein in each fraction, as determined by Western blotting, is shown in the lower panel. CON, control.

Table 1 Effect of treatment with OKA and MCY on the phosphorylation of components of the dynein complex in hepatocytes

Autoradiograms of immunoprecipitates for Figure 1, lanes 1 (control, NO VAN), 2 (MCY, NO VAN) and 3 (OKA, NO VAN) were scanned by densitometry to quantify the phosphorylation of the dynein complex. Results for MCY (500 nM)- and OKA (250 nM)-treated hepatocytes are given as percentages of control.

Dynein band	MCY	OKA
400 kDa (DHC)	185	168
150 kDa	122	155
74 kDa (DIC)	129	153
55 kDa	142	191
45 kDa	145	257

dynein complex is altered by both agents. In immunoprecipitates from hepatocytes exposed to OKA or MCY, increased phosphorylation of components of the dynein complex at approx. molecular masses of 400 kDa, 150 kDa, 74 kDa, 55 kDa and 45 kDa is seen (Figure 1). UV-VAN photocleavage [16] demonstrated that the 400 kDa species was DHC. The species at $\approx$ 74 kDa was confirmed as DIC by using Western blot analysis to show co-migration of this phosphorylated species with the protein. Although unidentified, the species at 45 and 55 kDa might be DLCs or DLICs, polypeptides exhibiting considerable heterogeneity that are known to be phosphorylated [3]. The Western blot in Figure 1 shows that the treatments did not alter the amounts of dynein recovered by immunoprecipitation.

The increase in phosphorylation of the components of the dynein immunoprecipitate in Figure 1 relative to the same species from untreated cells is shown in Table 1. The increased phosphorylation of DHCs, DICs and the band at 150 kDa were essentially the same for MCY and OKA. DHCs and the 150 kDa band exhibited an approx. 2-fold increase in phosphorylation. Although DIC phosphorylation is pronounced in Figure 1, the magnitude of the increase following OKA and MCY is approx. 1.5-fold, reflecting the large extent of endogenous phosphorylation of this species. The effect of OKA was greater than that of

MCY for the species at 55 and 45 kDa. Similar results (not shown) were obtained in two to three other experiments.

To obtain additional information about the signalling pathways converging on dynein in the presence of PP inhibitors, we examined the changes in phosphorylation that occurred upon pretreatment with two different Ser/Thr kinase inhibitors, STAUR and K252a. As shown in Figure 2(A), pretreatment of hepatocytes with either inhibitor substantially reduced the basal and OKA-induced phosphorylation of DICs. Another experiment showed that two activators that enhance Ser/Thr phosphorylation, PMA (PKC) and FOR (cAMP-dependent PK via adenylate cyclase), were able to mimic the phosphorylation of the 55 kDa dynein polypeptide seen with PP inhibitors (Figure 2B). These studies suggested that the phosphorylation of these two dynein polypeptides might be Ser/Thr-directed. The phosphorylation of the other dynein polypeptides indicated in Figure 1 was unchanged by these interventions, suggesting the involvement of additional, as-yet-unidentified PKs.

Dynein phosphorylation is associated with reduced ATPase activity

In intact hepatocytes exposed to PP inhibitors, we have shown that MT-dependent vesicle movements are significantly impeded [9]. These changes are not associated with any common changes in the MT array (Figure 3) [9]. Another explanation for reduced vesicle movements might be that PP-inhibitor-induced changes in dynein phosphorylation could lead to reduced dynein ATPase activity, thus hindering vesicle movements. We found that MT-activated ATPase activity of MT-binding, ATP-release motors from hepatocytes exposed to either of the PP inhibitors was markedly reduced relative to motors from untreated hepatocytes. MCY reduced ATPase activity to 26.8 ± 3.2 , and OKA to 70.7 ± 26.8 % of controls (Table 2). To exclude the possibility that the changes in MT-activated ATPase activity in the ATP release fractions were due to reduced activity of kinesin, the other MT-based motor protein, we measured the MT-activated ATPase activity of motors in MT-affinity, GTP-release fractions. GTP releases only kinesin from MTs [18]. In our assays, isolation of motors by GTP release yielded samples containing kinesin

Figure 2 Serine/threonine PK inhibitors and activators modulate the phosphorylation of two dynein polypeptides

Dynein was immunoprecipitated from extracts from ^{32}P -labelled hepatocytes, as described in the legend to Figure 1. (A) Phosphorylation of DIC (upper panel) after exposure to the following (shown in lanes from left to right): CON (control); STAUR ($5 \mu\text{M}$, 2 h); K252a ($7 \mu\text{M}$, 2 h); OKA (250 nM, 60 min); STAUR/OKA (STAUR at $5 \mu\text{M}$ for 60 min, and additional OKA at 250 nM for 60 min); and K252a/OKA (K252a at $7 \mu\text{M}$ for 60 min, and additional OKA at 250 nM for 60 min). (B) Phosphorylation of the 55 kDa band after exposure to the following for 60 min: CON; MCY (500 nM); OKA (250 nM); PMA (50 nM); and FOR (25 μM). Dynein in each fraction, as determined by Western blotting, is shown in the lower panels.

Figure 3 MT distribution in hepatocytes

MT array in control hepatocytes (A); hepatocytes exposed to 500 nM MCY for 60 min (B); or 250 nM OKA for 60 min (C). MTs were labelled using an anti- α -tubulin primary antibody and an FITC-conjugated secondary antibody. Bar $\approx 10 \mu\text{m}$.

only (dynein in these samples was $< 10\%$ of that in the ATP-release samples), whereas extracts obtained by ATP-release contain both kinesin and dynein. As shown in Table 2, MT-affinity, GTP-release motors from hepatocytes exposed to PP inhibitors showed no changes in MT-activated ATPase activity relative to controls: ATPase activity for MCY- and OKA-

treated cells was 114.6 ± 43.6 and 133.8 ± 52.9 respectively. Neither was there a significant change in the kinesin levels in ATP versus GTP fractions (results not shown). The reduced MT-dependent ATPase activity measured in motors isolated from hepatocytes exposed to PP inhibitors is therefore not due to kinesin, and is attributable to dynein. These effects are directly correlated with increased phosphorylation of DHC and other polypeptides (Figure 1 and Table 1).

Motor-dependent avidity of endosomal/lysosomal membranes for MTs is significantly reduced by PP inhibitors

To understand further how changes in ATPase activity or phosphorylation of other elements of the dynein complex might contribute to altered dynein function, we used an MT-affinity protocol for isolation of membranes from control and PP-inhibitor-treated hepatocytes. Membranes with active motors exhibit MT binding in the presence of the non-hydrolysable ATP analogue, p[NH]ppA, but are released in the presence of ATP [13,14]. Since liver dynein participates in receptor-mediated endocytosis, specifically in trafficking from endosomes to lysosomes [12–14], we therefore analysed changes in motor-mediated endosomal/lysosomal membrane recovery with MTs, which may indicate further functional changes in dynein.

We found that the recovery of endosomal (acid phosphatase) and lysosomal (β -hexosaminidase) compartment markers in membranes exhibiting MT-binding ATP release was significantly reduced in hepatocytes treated with PP inhibitors (Table 3). As shown in Table 3, exposure of hepatocytes to PP inhibitors did not significantly alter the recovery of dynein or kinesin with membranes exhibiting MT-binding ATP release. These findings suggest that the specific activity of dynein associated with endosomal/lysosomal membranes is decreased, since comparable membrane-associated dynein is associated with reduced avidity of endosomal/lysosomal membranes for MTs. In fact, endosomal/lysosomal membranes might even recruit additional dynein in an attempt to recover normal MT-binding capabilities, thus explaining how equivalent amounts of dynein are associated with recovery of significantly fewer endosomal/lysosomal membranes. Since dynein normally mediates the association of endosomal/lysosomal membranes with MTs [13,14], we suggest that changes in dynein phosphorylation reduce its ability to tether endosomal/lysosomal membranes to MTs.

PP inhibition alters dynein phosphorylation and function

Our previous work has shown that increases in the phosphoryl-

Table 2 Effect of treatment with OKA or MCY on ATPase activity of hepatocyte motors released from MTs by ATP or GTP

MT-dependent ATPase activity of kinesin and dynein (ATP-release of motors), and of kinesin alone (GTP-release of motors) was measured after incubation of hepatocytes for 60 min with MCY (500 nM) or OKA (250 nM). ATPase activity was measured as the increase in absorbance from the hydrolysis of the substrate ATP to P_i over a period of 30 min at 37°C . ATPase activity is given per $10 \mu\text{l}$ of extract; ATP-release and GTP-release samples are not directly comparable (see the Experimental section). The relative dynein concentration of ATP-release samples, and of kinesin for GTP-release samples, was determined by Western blotting of extracts followed by quantification of the intensity of the band (in arbitrary units). The intensity value for the controls of each preparation was normalized to allow comparison of ATPase activity/motor content between experiments. Results are given as means $\pm$ S.E.M. ($n = 3$); $*P = 0.05$.

Treatment	ATPase activity...	ATP-release of motors		GTP-release of motors	
		/10 μl extract	/Dynein content normalized	/10 μl extract	/Kinesin content normalized
Control		0.157 ± 0.030	0.157 ± 0.030	0.204 ± 0.082	0.204 ± 0.082
MCYST		0.112 ± 0.034	$0.042 \pm 0.005^*$	0.232 ± 0.084	0.234 ± 0.089
OKA		0.106 ± 0.030	0.111 ± 0.042	0.189 ± 0.060	0.273 ± 0.108

Table 3 Effect of treatment with OKA or MCY on the affinity of endosomal and lysosomal membranes for MTs in hepatocytes

Membranes were prepared by MT-affinity purification from untreated hepatocytes or hepatocytes treated for 60 min with OKA (250 nM) or MCY (500 nM). Activities (units/mg protein) for acid phosphatase (endosomal marker) and β -hexosaminidase (lysosomal marker) of MT-affinity, ATP-release membranes were calculated under each condition specified. Dynein and kinesin content across cellular membranes were determined by Western blot analysis and densitometry. All results are expressed as percentages of the recoveries from the MT-affinity, ATP-release membranes of control hepatocytes. Results (means $\pm$ S.E.M.) represent data from four to five preparations for OKA and MCY treatment. * $P \leq 0.05$.

Treatment	Activity of lysosomal and endosomal membrane markers		Motor recovery	
	β -Hexosaminidase	Acid phosphatase	Dynein	Kinesin
MCY	61.70 $\pm$ 12.60*	51.31 $\pm$ 7.53*	140 $\pm$ 26	120 $\pm$ 22
OKA	55.78 $\pm$ 9.50*	61.30 $\pm$ 9.24*	155 $\pm$ 8	100 $\pm$ 8

ation status of hepatocytes via inhibition of PPs by OKA or MCY led to decreases in several parameters of MT-based vesicle movements, including frequency, velocity and run length [9]. Since the effects on vesicle movements were similar with both inhibitors, we attributed the modulation of vesicle movements to the common inhibition of PP2A that occurred. However, the possible involvement of PP4 or PP5 cannot be ruled out, since their activities are also inhibited by MCY and OKA [8]. Because MT-dependent vesicle transport is an important component of receptor-mediated endocytosis, we also investigated whether PP inhibitors affected receptor-mediated endocytosis. We found that, under the same experimental conditions that caused inhibition of MT-dependent vesicle transport, PP inhibitors reduced both the uptake and steady-state accumulation of transferrin, a ligand internalized by receptor-mediated endocytosis [10]. Impaired MT-based vesicle transport was not associated with an altered organization of MTs, as shown in Figure 3 and [9]. To understand the mechanism underlying the effects of PP inhibitors on MT-dependent membrane trafficking in endocytosis in hepatocytes, we have focused here on exploring changes in the phosphorylation/function of cytoplasmic dynein.

We find that exposure of cultured rat hepatocytes to MCY or OKA is associated with increased phosphorylation of several components of the cytoplasmic dynein complex. These changes in phosphorylation are corroborated by two different effects on dynein activity. First, dynein ATPase activity is markedly reduced (Table 2). An essential function of dynein as a motor is its ability to generate force from the hydrolysis of ATP to transport membrane cargo along MTs. We propose that the observed effects on ATPase activity are derived largely from phosphorylation of DHCs, since ATP binding and hydrolysis are mediated by DHCs. The reduced frequency and velocity of MT-dependent vesicle movements in PP-inhibitor-treated hepatocytes [9] could easily reflect inhibition of the ATPase activity of DHCs. Although MCY and OKA both reduce dynein ATPase activity, the effects of MCY are more pronounced. PP1 and PP2A differ in their specificity of dephosphorylation. Inhibition of PP1 by MCY might result in increased phosphorylation of different sites that are not PP2A-sensitive, but are nevertheless important in the regulation of dynein ATPase activity.

Increased dynein phosphorylation is also associated with significantly reduced motor-dependent recovery of endosomal/lysosomal membranes with MTs (Table 3). Since OKA and MCY similarly influence the recovery of endosomal/lysosomal membranes with MTs, these regulatory changes are likely to be mediated via the common inhibition of PP2A by both toxins, although again we cannot rule out the possible involvement of PP4 or PP5. We propose that the reduced recovery of endosomal/

lysosomal membranes is attributable to effects on dynein associated with endosomal/lysosomal membranes. It is unclear whether these proposed effects on membrane-associated dynein are related to changes in DHC ATPase activity, or whether phosphorylation of DICs and/or other components of the dynein complex play a role. However, this reduced ability of dynein to tether endosomal/lysosomal membranes to MTs is likely to contribute to the inhibition of MT-dependent vesicle movements and endocytosis seen in hepatocytes exposed to PP inhibitors [9,10].

Different PKs (both serine/threonine and tyrosine PKs) can associate with the dynein complex [21,22]. At least some of the phosphorylation events triggered by PP inhibitors on DICs and the 55 kDa dynein-associated protein appear to involve Ser/Thr kinases (Figure 2). Furthermore, functional changes in ATP-dependent tethering of endosomal/lysosomal membranes to MTs, comparable with those elicited by PP inhibitors, were observed in hepatocytes treated with dibutyryl cAMP, a treatment that increases Ser/Thr protein phosphorylation via activation of cAMP-dependent PK. Incubation of hepatocytes with 2 mM dibutyryl cAMP for 60 min resulted in reduced recovery of endosomal/lysosomal markers in MT-binding, ATP-release membranes; the recovery of acid phosphatase and β -hexosaminidase was 53 % and 73 % of the control respectively ($n = 2$), with no apparent change in dynein content of membranes. Taken together, these findings support a role for reversible Ser/Thr phosphorylation in the regulation of dynein; nevertheless, we have not excluded the possibility that tyrosine phosphorylation plays a role in the control of dynein activity.

To our knowledge, this is the first biochemical demonstration that reduced dynein activity occurs concomitantly with an increased phosphorylation of dynein. Only limited information is available in any system on dynein phosphorylation/activity relationships. DHCs [23], DICs [21,24,25] and DLCs or DLICs [3,26] may be phosphorylated under different conditions. Changes in dynein phosphorylation induced by OKA [23] or cell-cycle changes [26] are linked to a reduced association of dynein with cellular membranes. Moreover, increased DHC phosphorylation in some specialized systems (neurons, *Xenopus* oocytes) has been correlated with some parameters of increased motor activity, although ATPase activity was not measured [24,27]. Clearly, phosphorylation of different dynein polypeptides or phosphorylation at distinct sites within the same polypeptide might lead to different effects on dynein activity. Since the phosphorylation of cytoplasmic dynein in hepatocytes exposed to PP inhibitors is altered on at least five different dynein-associated polypeptides, these effects might contribute towards changes in dynein activity in addition to those noted here on ATPase and membrane-MT binding.

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REFERENCES

- 1 Hirokawa, N. (1998) *Science* **279**, 519–526
- 2 Holzbaur, E. L. and Vallee, R. B. (1994) *Annu. Rev. Cell Biol.* **10**, 339–372
- 3 Gill, S. R., Cleveland, D. W. and Schroer, T. A. (1994) *Mol. Biol. Cell* **5**, 645–654
- 4 Holleran, E. A., Karki, S. and Holzbaur, E. L. (1998) *Int. Rev. Cytol.* **182**, 69–109
- 5 Schroer, T. A. (1994) *Curr. Op. Cell Biol.* **6**, 69–73
- 6 Karki, S. and Holzbaur, E. L. F. (1995) *J. Biol. Chem.* **270**, 28806–28811
- 7 Vaughan, K. T. and Vallee, R. B. (1995) *J. Cell Biol.* **141**, 1507–1516
- 8 Huang, X. and Honkanen, R. E. (1998) *J. Biol. Chem.* **273**, 1462–1468
- 9 Hamm-Alvarez, S. F., Wei, X., Berndt, N. and Runnegar, M. (1996) *Am. J. Physiol.* **271**, C929–C943
- 10 Runnegar, M., Wei, X., Berndt, N. and Hamm-Alvarez, S. F. (1997) *Hepatology* (St. Louis) **26**, 176–185
- 11 Eriksson, J. E. and Goldman, R. D. (1993) *Adv. Protein Phosphatases* **7**, 335–357
- 12 Hamm-Alvarez, S. F. and Sheetz, M. P. (1998) *Physiol. Rev.* **78**, 1109–1129
- 13 Goltz, J. S., Wolkoff, A. W., Novikoff, P. M., Stockert, R. J. and Satir, P. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7026–7030
- 14 Oda, H., Stockert, R. J., Collins, C., Wang, H., Novikoff, P. M., Satir, P. and Wolkoff, A. W. (1995) *J. Biol. Chem.* **270**, 15242–15249
- 15 Moldeus, P., Hogberg, J. and Orrenius, S. (1978) *Methods Enzymol.* **51**, 60–70
- 16 Paschal, B. M. and Vallee, R. B. (1993) *Methods Cell Biol.* **39**, 65–74
- 17 Vilalta, P., Zhang, L. and Hamm-Alvarez, S. F. (1998) *J. Cell Sci.* **111**, 1841–1852
- 18 Hamm-Alvarez, S. F., da Costa, S., Yang, T., Wei, X., Gierow, P. and Mircheff, A. K. (1997) *Exp. Eye Res.* **64**, 141–156
- 19 Sonee, M., Barron, E., Yarber, F. A. and Hamm-Alvarez, S. F. (1998) *Am. J. Physiol.* **274**, C1630–C1639
- 20 Matthies, H. J. G., Miller, R. J. and Palfrey, H. C. (1993) *J. Biol. Chem.* **268**, 11176–11187
- 21 Karki, S., Tokito, M. K. and Holzbaur, E. L. (1997) *J. Biol. Chem.* **272**, 5887–5891
- 22 Campbell, K. S., Cooper, S., Dessing, M., Yates, S. and Buder, A. (1998) *J. Immunol.* **161**, 1728–1737
- 23 Lin, S. X., Ferro, K. L. and Collins, C. A. (1994) *J. Cell Biol.* **127**, 1009–1019
- 24 Dillman, J. F. and Pfister, K. K. (1994) *J. Cell Biol.* **127**, 1671–1681
- 25 Pfister, K. K., Salata, M. W., Dillman, J. F., Vaughan, K. T., Vallee, R. B., Torre, E. and Lye, R. J. (1996) *J. Biol. Chem.* **271**, 1687–1694
- 26 Niclas, J., Allan, V. J. and Vale, R. D. (1996) *J. Cell Biol.* **133**, 585–593
- 27 Allan, V. (1995) *J. Cell Biol.* **128**, 879–891

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