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Journal

Journal of Cell Biology, 112(6)

ISSN

0021-9525

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Publication Date

1991-03-15

DOI

10.1083/jcb.112.6.1199

Peer reviewed

Nucleotide Specificities of Anterograde and Retrograde Organelle Transport in *Reticulomyxa* Are Indistinguishable

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Abstract. Membrane-bound organelles move bidirectionally along microtubules in the freshwater ameba, *Reticulomyxa*. We have examined the nucleotide requirements for transport in a lysed cell model and compared them with kinesin and dynein-driven motility in other systems. Both anterograde and retrograde transport in *Reticulomyxa* show features characteristic of dynein but not of kinesin-powered movements: organelle transport is reactivated only by ATP and no other nucleoside triphosphates; the K_m and V_{max} of the ATP-driven movements are similar to values obtained for dynein rather than kinesin-driven movement; and of 15 ATP analogues tested for their ability to promote organelle transport, only 4 of them did. This narrow specificity resembles that of dynein-mediated in vitro transport and is dissimilar to the broad specificity of the kinesin motor (Shimizu, T., K. Furusawa, S. Ohashi, Y. Y. Toyoshima, M. Okuno, F. Malik, and R. D. Vale. 1991. *J. Cell Biol.* 112: 1189–1197). Remarkably, anterograde and retrograde organelle transport cannot be distinguished at all with respect to nucleotide specificity, kinetics of movement, and the ability to use the ATP analogues. Since the "kinetic fingerprints" of the motors driving transport in opposite directions are indistinguishable, the same type of motor(s) may be involved in the two directions of movement.

Two types of molecules are currently known that are good candidates for microtubule-dependent organelle motors: kinesin and cytoplasmic dynein. Based on in vitro assays consisting of microtubules and motor molecules adsorbed to either polystyrene beads or glass coverslips, kinesin moves towards the plus-end of microtubules, also known as anterograde movement (Vale et al., 1985; Porter et al., 1987; Cohn et al., 1987; Saxton et al., 1988), while the movement of cytoplasmic dyneins is minus-end directed, or retrograde (Paschal and Vallee, 1987; Gibbons, 1988; Schroer et al., 1989; Schnapp and Reese, 1989). These motors are believed to be associated with cytoplasmic organelles, including vesicular bodies (Pratt, 1986, 1989; Pfister et al., 1989) and ER (Vale and Hotani 1988; Dabora and Sheetz, 1988; Hollenbeck, 1989; also Hering, G. E., and G. G. Borisy, unpublished results), and mediate their movements along microtubules.

Since kinesin and dynein promote movement in only one direction in vitro, it has been assumed widely that in higher eukaryotes, anterograde and retrograde organelle transport are driven by kinesin or dynein, respectively (for review see Vale, 1987). Consistent with this hypothesis, exposure to UV light in the presence of vanadate ions and ATP, which cleaves the heavy chain of dynein and not kinesin (Lee-

Eiford et al., 1986), appears to preferentially block retrograde transport of organelles in reconstituted models of fibroblasts and squid giant axons (Schroer et al., 1989; Schnapp and Reese, 1989). On the other hand, both anterograde and retrograde transport in extruded squid axoplasm are inhibited by a mAb against kinesin (Brady et al., 1990). However, it needs to be emphasized that the evidence for the function of dynein and kinesin as organelle motors in opposite directions along microtubules in cells is still largely indirect (for reviews see Huitorel, 1988; McIntosh and Porter, 1989).

A cell type that currently does not seem to fit the emerging consensus regarding the involvement of cytoplasmic dynein and kinesin in bidirectional organelle transport is the lower eukaryote, *Reticulomyxa*, a giant syncytial freshwater ameba (Koonce et al., 1986). This protozoan extends a peripheral feeding network supported by an extensive array of microtubules. Organelle transport along these microtubules occurs at rates of ~9.5 µm/s in both directions and can be reactivated in vitro (Koonce and Schliwa, 1986). As in higher eukaryotes, bidirectional organelle transport occurs along a predominantly unipolar array of microtubules (Euteneuer et al., 1989a). In contrast to other cell types, however, current evidence suggests the surprising possibility that only one type of motor is involved: biochemical studies have demonstrated the presence of cytoplasmic dynein but have failed to

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reveal kinesin using protocols commonly used to isolate kinesin (Euteneuer et al., 1988), and UV photocleavage inhibits both anterograde and retrograde transport (Euteneuer et al., 1989b).

To further probe into the question of which and how many motor species are involved in the bidirectional transport of organelles in *Reticulomyxa*, we have used a series of ATP analogues that, based on in vitro microtubule translocation assays, are used by different motors with different efficiencies (Shimizu et al., 1991). The results of this analysis are consistent with the idea that transport is driven by dynein, and not by kinesin motors. Moreover, none of the analogues are able to activate plus end-directed or minus end-directed organelle transport preferentially, suggesting the possibility that one type of motor is involved in both directions of organelle transport.

Materials and Methods

Cells

Stock cultures of *Reticulomyxa* were maintained as described previously (Koonce and Schliwa, 1986). Cells were transferred every 3–4 d into fresh dishes.

Light Microscopy

Small pieces of the cell body were excised, placed onto 18 × 18-mm coverslips in a buffer consisting of 10 mM Hepes and 2 mM MgCl₂, pH 7.0, and were allowed to extend a radial network for ~1 h. The cell body was removed, leaving the undisturbed network. Then the coverslip was inverted onto a slide using coverslip chips as spacers, and the top and bottom sides were sealed with VALAP (equal parts of Vaseline, lanolin, and paraffin). Preparations were viewed with a Zeiss Photomicroscope III equipped with Nomarski differential interference contrast. The light microscopic image was projected into video camera (Series 67; DAGE-MTI Inc., Wabash, MI), whose signal was fed into an image processor (IMAGE I; Interactive Video Systems Inc., Concord, MA). Processed images were displayed on a video monitor at a final magnification of 3,125 and recorded in real time with a video recorder (model NV-8050; Panasonic).

Cell Lysis

Networks were lysed with 50% PHEM buffer (Schliwa and van Blerkom, 1981) consisting of 30 mM Pipes, 12.5 mM Hepes, 4 mM EGTA, 1 mM MgCl₂, pH 6.9, supplemented with 5% hexylene glycol, 1 mM sodium orthovanadate, and 0.2% Brij 58. After lysis for 1 min in this buffer, the cells were rinsed thoroughly with 50% PHEM buffer, followed by experimental solutions.

ATP Analogues and Other Nucleotides

The preparation of ATP analogues was as follows (as described in Shimizu et al., 1991): 3'dATP and FTP were made from the corresponding monophosphate forms. Purine riboside triphosphate, monomethyl ATP, and dimethyl ATP were synthesized from corresponding nucleosides by two-step chemical phosphorylation to the diphosphate forms, followed by enzymatic phosphorylation to the triphosphate forms by pyruvate kinase with phosphoenolpyruvate. The preparation of the phosphorothioate analogues of ATP is described elsewhere (Shimizu et al., 1990). Other nucleotides as well as 2'dATP, 8-bromo ATP, 8-azido ATP, and etheno ATP were purchased from Sigma Chemical Co. (St. Louis, MO), Boehringer Mannheim GmbH (Mannheim, FRG) or Pharmacia Fine Chemicals (Piscataway, NJ). All nucleotides and ATP analogues were purified to remove residual ATP. Their purity was confirmed by HPLC. All nucleotides were substantially free from contaminating ATP except 8-azido ATP which still contained <0.1% ATP. Nucleotides and ATP analogues were used as magnesium salts.

Materials

All materials were obtained from Sigma Chemical Co. unless otherwise indicated.

Results

Lysed *Reticulomyxa* networks reactivated with 1 mM ATP show rapid bidirectional transport of organelles at an average rate of 9.5 μm/s (Koonce and Schliwa, 1986). Transport occurs in association with microtubule bundles of predominantly uniform polarity, with plus ends located away from the cell body (Euteneuer et al., 1989a). ATP also induces splaying and sliding of microtubule bundles (Koonce et al., 1987). We tested the capability of nucleotides other than ATP to reactivate organelle transport and find that reactivation is strictly specific for ATP. No other nucleoside triphosphate (GTP, CTP, UTP, ITP, or TTP) reactivates organelle movements or microtubule sliding at all, even when used at a concentration of 10 mM and reactivation is monitored by time-lapse video recording. This finding is in marked contrast to those obtained in prior studies with kinesin in which all nucleoside triphosphates support microtubule gliding at between 27 and 78% the rate of ATP (Cohn et al., 1989).

ATP supports organelle transport in a saturable manner with Michaelis-Menten kinetics, as demonstrated by linearity in a Lineweaver-Burk plot (Fig. 1). Significantly, the kinetics of retrograde and anterograde transport are virtually identical. Linear regression analysis yields apparent K_m's of 153 and 132 μM and V_{max}'s of 2.15 and 11.6 μm/s for retrograde and anterograde transport, respectively. Although organelle motility is observed at all concentrations of ATP down to 5 μM, there appeared to be a critical concentration of ATP required for activity, as a plot of organelle transport velocity vs. ATP concentration extrapolates to an ATP concentration of ~2 μM for both anterograde and retrograde transport (not shown).

Further similarities between organelle transport in *Reticulomyxa* and dynein-driven motility are revealed in experiments with a series of ATP analogues that include three deoxy derivatives, seven analogues modified on adenine, and five phosphorothioate analogues (Shimizu et al., 1991). As summarized in Table I, organelle transport exhibits a remarkably narrow substrate specificity. Only two of the deoxy derivatives are capable of reactivating transport with >10% the efficiency of ATP. Two analogues, dideoxy ATP and methyl ATP, promote very slow movements at a rate of 0.3–0.4 μm/s. There is no significant difference in the rates for anterograde and retrograde transport with any of the ATP analogues. Furthermore, the number of organelles moving either towards or away from the cell body is approximately equal (between 45 and 55% in either direction) with all analogues that elicit motility.

Discussion

Nucleotide Specificity Suggests That Organelle Transport Is Driven by a Dynein-like Motor

Organelle transport in lysed *Reticulomyxa* networks is reactivated only by ATP. This strict preference is similar to that of bovine brain and *Caenorhabditis elegans* cytoplasmic dynein (Paschal and Vallee, 1987; Lye et al., 1987) as well as *Tetrahymena* axonemal dynein (Vale and Toyoshima, 1988), which all produce microtubule gliding in the presence of ATP only. In contrast, GTP, UTP, ITP, TTP, and CTP support kinesin-driven microtubule gliding at ~25%–75% the rate of ATP (Cohn et al., 1989). Our observations
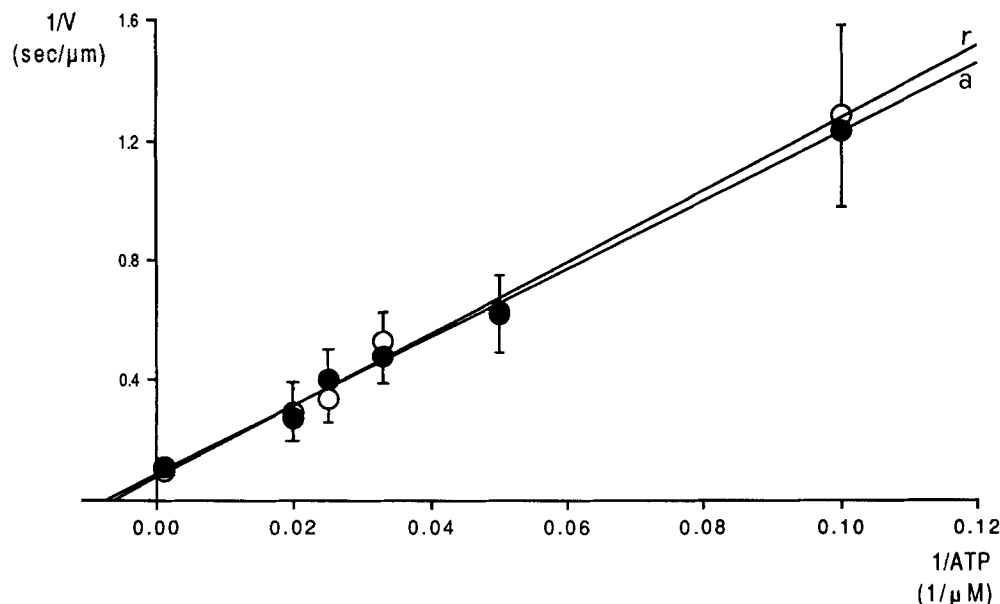
Figure 1. Double-reciprocal plot of the effect of MgATP on anterograde (a; ●) and retrograde (r; ○) organelle transport in lysed *Reticulomyxa* networks. Each point represents the mean velocity (±SD) of at least 30 particles from at least four experiments. The lines were fit to the data by linear regression ($r = 0.99$ for both), yielding V_{max}'s of 11.6 μm/s for anterograde and 12.5 μm/s for retrograde transport. The corresponding apparent K_m's are 132 and 153 μM, respectively.

also differ from those of Leopold et al. (1990) on extruded squid axoplasm. These authors find that both anterograde and retrograde transport can be sustained by nucleoside triphosphates other than ATP at up to 57% the rate of ATP. Other nucleotides also reactivate movements in lysed models of fish melanophores (Rozdzial and Haimo, 1986), but the rates are very slow (<10% those of ATP). However, in contrast to the *Reticulomyxa* cell model where the transport machinery is completely exposed and separated from other cell constituents, extruded axoplasm and fish melanophores are substantially more complex.

The apparent K_m and V_{max} of organelle transport in *Reticulomyxa* are also more similar to those described for dyneins than kinesins. Double-reciprocal plots of motility rates vs. ATP concentration show a linear relationship that obeys Michaelis-Menten kinetics. The values for K_m and V_{max} (~140 and ~12 μm/s, respectively) calculated from

these plots are in the same range as those reported for other dynein-mediated motile processes, but different from kinesin-mediated transport. As the comparison in Table II demonstrates, this is true irrespective of the assay system used. The apparent K_m's of kinesin-mediated motility range from 10 to 60 μM, while those of dynein processes are in the range of 100-210 μM. V_{max}'s are 0.5-0.9 μm/s for kinesin and 8-19 μm/s for dynein.

Reticulomyxa organelle transport is similar to dynein-mediated movements also with respect to the ATP analogues that support movement. Microtubule gliding assays demonstrate significant differences in the ability of kinesin and dynein to use these analogues for microtubule motility (Shimizu et al., 1991). Only two of these analogues support organelle transport at a rate of >2 μm/s (Table I). When the "analogue profile" presented in Table I is compared with that of dynein or kinesin-driven microtubule gliding in vitro (Ta-

Table I. Effectiveness of ATP Analogues in Reactivating Organelle Transport

Compound	Velocity		Microtubule sliding	n
	Anterograde	Retrograde		
	μm/s	*μm/s*		
ATP	9.2 ± 4.1	9.3 ± 2.7	yes	8
2' Deoxy ATP	5.0 ± 2.5	5.7 ± 2.4	yes	3
3' Deoxy ATP	2.0 ± 0.9	2.5 ± 1.1	yes	5
2'3' Dideoxy ATP	0.3 ± 0.1	0.3 ± 0.1	yes	4
N-Methyl ATP	0.4 ± 0.2	0.3 ± 0.1	yes	5
N,N-Dimethyl ATP	0	0	no	5
Formycin triphosphate	0	0	no	4
8-Bromo ATP	0	0	no	4
8-Azido ATP	0	0	no	4
Etheno ATP	0	0	no	4
Purine riboside triphosphate	0	0	no	3
ATPαS(Sp)	0	0	no	4
ATPαS(Rp)	0	0	no	2
ATPβS(Sp)	0	0	no	2
ATPβS(Rp)	0	0	no	2
ATPγS	0	0	no	3

Velocities are given as the mean ± SD from at least six different particles for each direction in each independent experiment. n, number of experiments.

Table II. K_m and V_{max} of Several Microtubule-based Transport Systems

Transport system	Assay	K_m	V_{max}	Reference
		μM	$\mu m/s$	
Drosophila kinesin	MT gliding	44	0.9	Saxton et al., 1988
Sea urchin kinesin	MT gliding	10–20	0.5	Porter et al., 1987
Sea urchin kinesin	MT gliding	60	0.6	Cohn et al., 1989
Bovine kinesin	MT gliding	30	0.6	Howard et al., 1989
Sea urchin axonemes	Reactivated sliding	140	14	Yano and Miki-Noumura, 1980
Sea urchin flagella	Reactivated sliding	210	19	Oiwara and Takahashi, 1988
Tetrahymena 22S dynein	MT gliding	100	8	Vale and Toyoshima, 1988
Reticulomyxa	Organelle transport			this paper
anterograde		132	11.6	
retrograde		153	12.5	

MT, microtubule.

ble III), strong similarities to dynein and significant differences to kinesin are revealed. 22S dynein from *Tetrahymena* cilia exhibits a narrow substrate specificity, with only four of the derivatives supporting microtubule translocation at >10% ATP efficiency. Significantly, the analogues that reactivate *Reticulomyxa* organelle transport are the same that also *Tetrahymena* dynein can use effectively. The only major difference is the response to one of the phosphorothioate analogues, ATPαS(SP), which can be used mechanochemically by dynein (at 18% the rate of ATP), but is ineffective in reactivating organelle transport. On the other hand, bovine brain kinesin is rather promiscuous and exhibits a broad spectrum of substrate specificity, with 8 of the 15 compounds promoting microtubule gliding at >10% the rate of ATP, and 3 more causing motility at lower rates. Thus the "profile" of ATP analogues that support organelle transport in *Reticulomyxa* is remarkably similar to that of dynein-mediated microtubule gliding.

Table III. Comparison of the Relative Rates of Reactivated Organelle Transport with Dynein and Kinesin-driven Microtubule Gliding

Compound	Organelle transport		Dynein	Kinesin
	Anterograde	Retrograde		
ATP	100	100	100	100
2' Deoxy ATP	54	61	28	91
3' Deoxy ATP	21	27	44	72
2'3' Dideoxy ATP	3	3	21	69
N-Methyl ATP	4	3	6	47
N,N-Dimethyl ATP	0	0	0	20
Formycin triphosphate	0	0	0	10
8-Bromo ATP	0	0	0	3
8-Azido ATP	0	0	0	0
Etheno ATP	0	0	0	13
Purine riboside triphosphate	0	0	0	2
ATPαS(SP)	0	0	18	18
ATPαS(RP)	0	0	0	0
ATPβS(SP)	0	0	0	0
ATPβS(RP)	0	0	0	0
ATPγS	0	0	5	2

All compounds were used at a concentration of 1 mM. Motility rates are given in percent of the rate in ATP. The data in columns two and three are from Shimizu et al. (1991) using 22S ciliary dynein from *Tetrahymena* and bovine brain kinesin.

It is difficult to determine how close the similarities in the nucleotide fingerprints ought to be to identify the motor involved in powering a motility-related event. This question might be easier to answer once several dyneins and kinesins from different sources have been compared using these nucleotides. In the present case, however, the resemblances in the enzymatic signatures of *Tetrahymena* dynein and the *Reticulomyxa* organelle motor are undeniable.

The conclusion from the nucleotide requirement studies that organelle transport in *Reticulomyxa* is dynein based is consistent with prior biochemical studies. The characteristics of the microtubule-dependent motor isolated from *Reticulomyxa* suggest that it is a dynein-like molecule with heavy chains of ∼440 kD (Euteneuer et al., 1988). These heavy chains are cleaved into two lower molecular weight components by UV photolysis, and UV cleavage inhibits reactivated organelle transport in both anterograde and retrograde directions (Euteneuer et al., 1988, 1989b). So far no evidence for kinesin has been found.

One Motor May Drive Anterograde and Retrograde Transport

Organelles are transported in both directions along microtubules in many eukaryotic cells (Schliwa, 1984). A large body of indirect evidence suggests that the two candidates for these motors are dynein (minus-end directed) and kinesin (plus-end directed). These motors thus far have shown distinct differences in their nucleotide sensitivities and kinetic properties. In fact, even different isoforms of dynein can be distinguished by their nucleotide-utilizing properties. For example, two different dynein species (14S and 22S) that are both found in *Tetrahymena* cilia have clearly distinct V_{max}'s (Vale and Toyoshima, 1988) and K_m's (Toyoshima, Y. Y., and R. D. Vale, unpublished observations) and also show different sensitivities to the ATP analogues (Shimizu et al., 1991). For example, 14S dynein can use 8-azido ATP and FTP, whereas 22S dynein can not. Thus, one should be able to establish whether similar or different motors are involved in a cellular transport process on the basis of its nucleotide "fingerprint."

In light of this potential for crisp distinctions between motors with even minor differences in their nucleotide specificities, the most significant finding of the present study is that the two directions of organelle transport in *Reticulomyxa* are not distinguished by any criterion: they share the exclusive use of ATP; their K_m's and V_{max}'s are very similar; and

the usage of the ATP analogues is identical. If an ATP analogue produced movement at a lower velocity, it did so to the same extent in both anterograde and retrograde directions. Since we find that the nucleotide fingerprints of anterograde and retrograde directions are indistinguishable, whereas all other motors tested can clearly be distinguished by these criteria, we suggest that the same or similar motor(s) mediate(s) transport in the two directions of movement. The current finding does not exclude the possibility, however, that two different motors with distinct biochemical but identical enzymatic properties are involved.

The *Reticulomyxa* organelle motor, if truly endowed with the ability to move bidirectionally along microtubules, poses interesting questions regarding the mechanism of force transduction. The most widely accepted hypothesis of force generation is the crossbridge model which states that the motor undergoes a large conformational change that alters its angle relative to the attached filament and thereby produces relative movement between the motor and the filament (Huxley, 1969). It is difficult to envisage how such a mechanism could operate in reverse. Alternatively, it is possible that the *Reticulomyxa* motor possesses two distinct binding sites for tubulin that cause it to attach in opposite orientations on the microtubule. In such a case, the same conformational change in the motor could be used to elicit bidirectional transport. It is also unclear how the cell governs the choice of direction, although a posttranslational modification of the motor is a likely possibility. Equally intriguing is the problem of how individual organelles are able to selectively bind or activate the anterograde or retrograde forms of the motor so that they are transported in only one direction. Individual organelles do change the direction of movement rapidly and frequently, though. Insight into all of these questions will require detailed analysis of the purified motor.

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