Implications of Treadmilling for the Stability and Polarity of Actin and Tubulin Polymers In Vivo

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ABSTRACT In this report, we examine how the cell can selectively stabilize anchored filaments and suppress spontaneous filament assembly. Because microtubules and actin filaments have an organized distribution in cells, the cell must have a mechanism for suppressing spontaneous and random polymerization. Though the mechanism for suppressing spontaneous polymerization is unknown, an unusual property of these filaments has been demonstrated recently, i.e., under steady-state conditions, in vitro actin filaments and microtubules can exhibit a flux of subunits through the polymers called "treadmilling." In vivo, however, most, if not all, of these polymers are attached at one end to specific structures and treadmilling should not occur. The function of treadmilling in vivo is, therefore, unclear at present. However, as shown here, the same physicochemical property of coupling assembly to ATP or GTP hydrolysis that leads to treadmilling in vitro can act to selectively stabilize anchored polymers in vivo. I show here that the theory of treadmilling implies that the concentration of subunits necessary for assembly of the nonanchored polymer will in general be higher than the concentration necessary for the assembly of polymers anchored with a specific polarity. This disparity in the monomer concentrations required for assembly can lead to a selective stabilization of anchored polymers and complete suppression of spontaneous polymerization at apparent equilibrium in vivo. It is possible, therefore, that the phenomenon of treadmilling is an in vitro manifestation of a mechanism designed to use ATP or GTP hydrolysis to control the spatial organization of filaments in the cell.

Cells contain organized networks of microtubules and actin filaments. These polymers are not polymerized at random throughout the cell and it is likely that most are anchored at specific sites. Because an organized arrangement of these filaments is important for their function, the cell must have a way of preserving these anchored filaments and suppressing spontaneous filament assembly. This specification could be achieved by making self-assembly kinetically unfeasible, in which case anchored polymers would represent a kinetically stable, but thermodynamically unstable, state. However, as described in this report, it is also possible through the use of ATP or GTP hydrolysis to stabilize selectively anchored filaments and prevent all spontaneous assembly in vivo. The properties of the filaments that lead to selective stabilization will also produce the observed "treadmilling" of subunits in vitro under steady-state conditions. From this we may conclude that the intracellular arrays of anchored microtubules and actin can in fact be thermodynamically stable, a hypothesis that then leads to certain predictions about the polarity of filaments in the cell.

The basis for this analysis is the finding that tubulin and actin monomers bind and hydrolyze GTP and ATP respectively during their polymerization into microtubules and filaments. This hydrolysis does not provide the energy for assembly, as both monomers assemble readily in the presence of nonhydrolyzable nucleoside triphosphate analogs (2, 11, 12, 24, 25, 32). A different function for nucleotide hydrolysis was proposed when Wegner (31) suggested that ATP hydrolysis, coupled with the known polarity of actin filaments, would, under steady-state conditions, allow for a net polymerization at one end and a corresponding depolymerization at the other end of a filament, while the average polymer length does not change. This steady-state flux has been called "head-to-tail polymerization" or treadmilling (31). Wegner showed theoretically that as long as there is a pool of ATP, a continuous flow of subunits through the filament can occur. The observed rapid exchange of free subunits with the actin polymer at steady-state is presumably attributable to such treadmilling. Margolis and Wilson (20), using pulse-chase experiments and selective poisoning of the ends of microtubules, demonstrated in a more direct manner that treadmilling also occurs for microtubules at steady state in vitro.

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Recently, Bergen and Borisy (4) obtained more complete kinetic data for head-to-tail polymerization by measuring the individual on- and off-rates for each end of microtubules assembled from flagellar axonemes. The four rate constants calculated by them are sufficient to determine not only the net flux of subunits but the actual assembly rates at each end. They confirmed that treadmilling could occur for microtubules at steady state, while finding that the flux is small relative to the number of association and dissociation reactions occurring at each end: for every 14 subunits that are added to and come off a microtubule at a steady-state, there is a net gain of one at the plus (fast-growing) end and a net loss of one at the minus (slow-growing) end. Similar support for subunit flux in microtubules has come recently from the work of Karr and Purich (17).

Although treadmilling under steady-state conditions in vitro is an experimentally verified property of microtubules and actin filaments, the exact function of this process in vivo is unclear. It is possible, in principle, for treadmilling to be made to do mechanical work, as suggested by Margolis et al. (21) for mitosis; however, all such schemes must contend with the inefficiency of the process and the lack of obvious molecular mechanisms for utilizing the subunit flux to do useful work.

In this communication, I would like to point out why I believe that treadmilling may provide a clue to the control of the sites of microtubule and actin filament assembly in vivo and show how the cell can use ATP or GTP hydrolysis to specify the spatial organization of filaments within it.

The Critical Concentration for Bidirectional Growth

Consider first the simple case of bidirectional growth from a polar polymer not involving nucleotide hydrolysis. As shown by Asakura (3) and restated by Wegner (31), a polar polymer can have different rates of assembly and disassembly from each end, as shown in Fig. 1. Using the nomenclature of Bergen and Borisy (4), we can write the rates at the plus and minus ends as:

$$\frac{dn^+}{dt} = k_2^+ c - k_1^+ \tag{1}$$

and

$$\frac{dn^-}{dt} = k_2^- c - k_1^-, \tag{2}$$

where dn^+/dt and dn^-/dt are the net rates of elongation in units of number of subunits, n, added per second per polymer at the plus and minus ends respectively; k_2^+ and k_2^- are second-order rate constants for the respective association reactions, k_1^+ and k_1^- are first-order rate constant for the dissociation reactions, and c is the number of monomers per cubic centimeter. The principle of detailed balance (which is closely related to the principle of microscopic reversibility) constrains the equilibrium constant K, to be the same at each end. This principle, based not on thermodynamic laws but on a formulation of statistical mechanics, states that at equilibrium not only must the overall concentration of the individual components (e.g., polymer length and monomer concentration) be constant, but the individual fluxes of each reaction must also go to zero. Therefore, at equilibrium, cyclic reactions are ruled out and at each end the probability of a subunit going on the filament

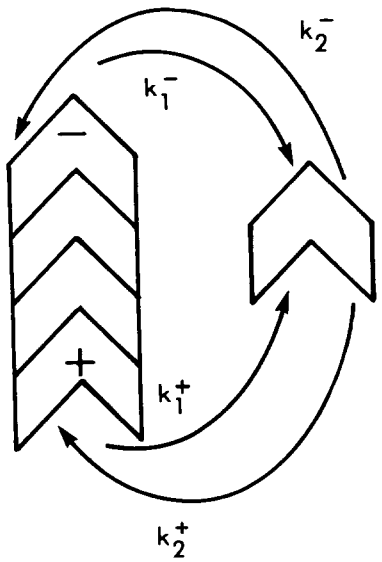

FIGURE 1 Diagram of bidirectional growth from a polar polymer. The fast-growing end is denoted by a plus, the slow by a minus. This figure is meant to be purely diagrammatic. However, the chevron orientation does correspond to the arrowhead arrangement of S1 or heavy meromyosin-decorated actin, where the plus end (fast-growing end) corresponds to the barbed end of the decorated actin filament and the minus end corresponds to the pointed end.

must equal the probability of a subunit coming off the filament. Therefore, there is one equilibrium constant, K, for both ends, and K is given by:

$$K = \frac{k_2^+}{k_1^+} = \frac{k_2^-}{k_1^-} \tag{3}$$

The net assembly rates at each end may then be written as:

$$\frac{dn^+}{dt} = k_2^+(c - K^{-1}) \tag{4}$$

and

$$\frac{dn^-}{dt} = k_2^- (c - K^{-1}) \tag{5}$$

(for plus and minus, respectively), and therefore both ends must have the same critical concentration, C_c, defined as the concentration of monomers at equilibrium where $dn/dt = 0$, (here, $C_c = K^{-1}$). For such a system of bidirectional growth, a plot of the rate vs. concentration for reactions at each end intersects the abscissa at a common point, as shown in Fig. 2.

In this general model, both ends will either grow or shrink together, depending on the monomer concentration. If the filaments are anchored or capped, i.e., one end is blocked from polymerization and depolymerization, the free end will grow when the monomer concentration exceeds the critical concentration. Free fragments of filaments will elongate at a greater rate than capped filaments, because they can add subunits to both ends. Because spontaneous polymerization can take place at any concentration above the critical concentration, spontaneous assembly will occur under the same conditions as filament elongation. At equilibrium, the cell will tend to be filled with an exponential distribution of polymer lengths with many of the filaments not attached to anchorage sites (see Oosawa and Asakura [23] for the ultimate length distribution).

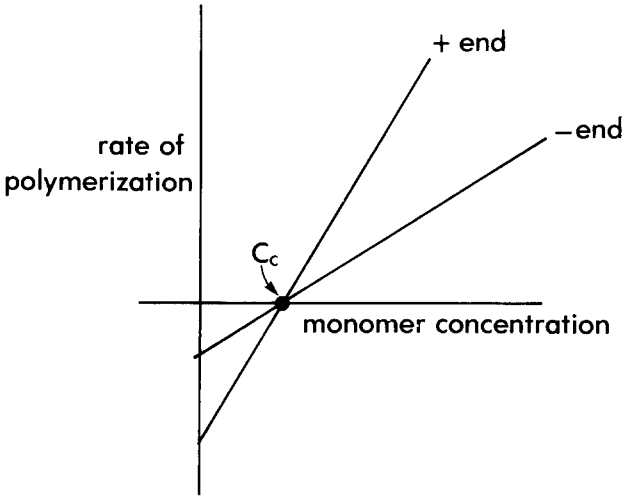

FIGURE 2 A plot of the rate of polymerization vs. monomer concentration for elongation of a polar polymer showing bidirectional growth. In this diagram, C_c denotes the critical concentration, which is the equilibrium monomer concentration. Below C_c, each end of the filament shortens, as indicated by the negative rates of polymerization.

The Critical Concentration for Treadmilling

In the treadmilling scheme of Wegner (31), assembly is coupled to nucleoside triphosphate hydrolysis to allow pathways of monomer addition and removal in which certain steps can be made to proceed at a negligible rate. A scheme appropriate to both actin and tubulin is shown in Fig. 3, where the kinetically significant steps are shown as bold face arrows.[1] The net rates at each end are given by:

$$\frac{dn^+}{dt} = k_2^+ c - k_1^+ \tag{6}$$

and

$$\frac{dn^-}{dt} = k_2^- c - k_1^-. \tag{7}$$

Although the equations are the same form as those given before for bidirectional growth, Eqs. 1 and 2, the pairs of rate constants k_2^+ and k_1^+ and k_2^- and k_1^- are no longer the forward and reverse rates of the same reactions. Therefore k_2^+/k_1^+ need not in general be equal to k_2^-/k_1^-. The overall growth rate is the sum of Eqs. 6 and 7, and it defines a critical concentration where the overall rate is zero, but where, in general, the two separate processes at each end are proceeding in opposite directions. This critical concentration C_c^s is given by Wegner (31):

$$C_c^s = \frac{k_1^+ + k_1^-}{k_2^+ + k_2^-}. \tag{8}$$

Note that this scheme allows the critical concentration to be different for each end. The critical concentrations for the plus and minus ends are given by:

$$C_c^+ = \frac{k_1^+}{k_2^+} \tag{9}$$

and

$$C_c^- = \frac{k_1^-}{k_2^-}. \tag{10}$$

There is no real restriction on the relative magnitudes of the individual rate constants, as discussed by Bergen and Borisy (4). The plus end can be defined merely as the end with the lower critical concentration. In general, compared to the minus end, it may have a slower on rate with a slower off rate, a faster on rate with a faster off rate, as well as a faster on rate and a slower off rate. Bergen and Borisy (4) and Karr and Purich (17) actually found that the plus end of microtubules had a greater off rate than the minus end, though it had a greater on rate as well. An example of a plot of the rates vs. concentration for a filament showing this treadmilling type of assembly is shown in Fig. 4. Unlike the situation in Fig. 2, the intercept at zero rate for the two ends is not the same. (This is only one of a number of such plots, which have as a common feature that $C_c^+ \neq C_c^-$.)

Implications of Treadmilling for Anchored or Capped Polymers

Most, if not all, microtubules and actin filaments in the cell are probably attached to anchorage sites and, in general, one end of each filament will be blocked and one end will be free. As a consequence, no treadmilling could actually take place. If

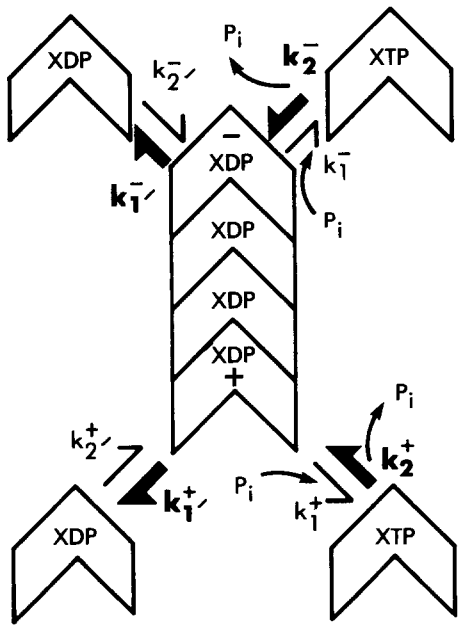

FIGURE 3 Treadmilling of a polar polymer. A kinetic scheme taken from Wegner (31) shows all the kinetic steps for assembly of a monomer in the nucleoside triphosphate (or *XTP* form) or the nucleoside diphosphate (or *XDP* form) to either end of a polar polymer. The major pathways are shown in bold face. In excess XTP, it is assumed that k_2^+ and k_2^- do not occur, because of the low concentration of the XDP monomer. Similarly, k_1^+ and k_1^- are thought to be improbable steps. Again, the chevrons indicate the measured polarity of the decorated actin filament. For microtubules GTP and GDP and for actin ATP and ADP should be substituted XTP and XDP.

[1] To obtain treadmilling it is not necessary to assume that XTP hydrolysis accompanies monomer addition at both ends. In other words, one end can be in equilibrium with the XTP form of the monomer while the other end binds the XTP form in the forward reaction and dissociates the XDP form in the reverse reaction.

the filaments are anchored with their plus ends free then, as shown in Fig. 4, the critical concentration of the nucleated polymer will be C_c^+. If the monomer concentration rises above C_c^+ but is below C_c^s, the nucleated polymer will grow until the free monomer concentration again reaches C_c^+. Under these conditions (at concentrations below C_c^s) no spontaneous polymerization can occur because disassembly at the minus end will exceed assembly at the plus end. In this concentration range, fragments of filaments, having two free ends, will be unstable as well. In other words, within the concentration range C_c^+ to C_c^s, shown shaded in Fig. 4, the cell can elongate anchored or capped polymers without risking either spontaneous assembly or elongation of broken fragments of filaments.

Suppression of spontaneous polymerization could, of course, also be achieved kinetically, i.e., if the rate of spontaneous nucleation were very slow compared to the rate of elongation of anchored filaments, as has been shown for actin. In that case, only anchored polymers would exist in any appreciable numbers. It is very difficult to estimate what the exact rates of nonnucleated assembly would be in vivo and it is therefore difficult to know over what time-scale kinetic suppression of nonnucleated assembly could be operable. However, a depressed rate of spontaneous assembly would not prevent possible rapid polymerization from broken fragments of filaments in vivo.

Finally, the theory of treadmilling leads to certain predictions about filament polarity under steady-state conditions. If there are any filaments anchored at the minus end, the steady-state concentration of monomers should ultimately tend to C_c^+. Any filaments anchored at their plus ends and filaments with both ends free will disappear as the monomer concentration drops below C_c^- and C_c^s respectively, as a result of the elongation of filaments anchored at their minus ends. A prediction of this analysis is that, at steady-state, all anchored filaments should have the same polarity: anchored at the minus and free at the plus end.

All of the polarity determinations for microtubules support this claim. Flagellar microtubules in vivo grow from what is the plus end of basal bodies as determined in vitro (1, 6, 7, 19, 33). In vitro studies of the growth of microtubules from the kinetochore of metaphase chromosomes indicate that they have the expected polarity (5, 8, 28). Similarly, growth from isolated mitotic centers has the same polarity: the plus end pointing away from nucleating sites (5). Thus, for microtubules, which have been shown by many experiments to be in dynamic equilibrium with their subunits, the expected polarity from organizing centers is found.

For actin the situation is more complex. Actin nucleation sites have not been well described and there is less information available as to how dynamic the monomer-polymer equilibrium is in vivo. In vitro polymerization experiments on cellular nucleating centers equivalent to those on microtubules have not been widely performed. In the one case where this has been done, Tilney and Kallenbach (29) found that actin polymerized just above the critical concentration from the actomere of echinoderm sperm grows with the barbed end free and the pointed end attached to the actomere, as determined by decoration with the S1 fragment of myosin. In vitro studies (14, 18, 34) demonstrated that the barbed end is the fast-growing, or plus, end and thus the actin polarity of the actomere is in agreement with the above theory. However, all other determinations of actin polarity by S1 decoration *in situ* indicate that actin has the opposite polarity relative to putative membrane

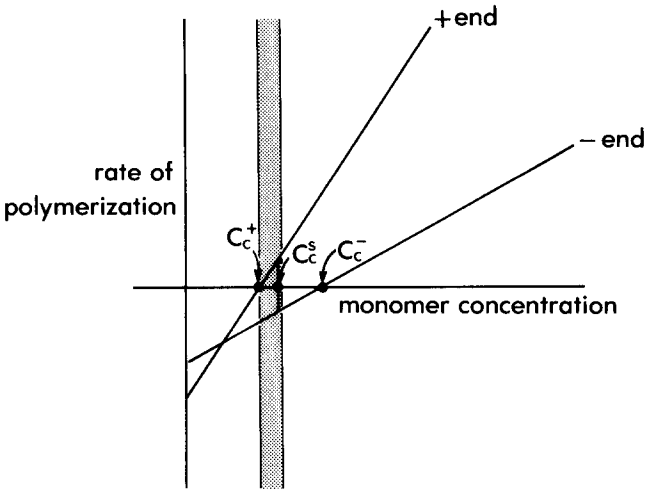

FIGURE 4 A plot of the rate of polymerization vs. monomer concentration for a polymer showing treadmilling. In this figure, C_c^+ denotes the critical concentration for the plus end and C_c^-, the critical concentration for the minus end. The point C_c^s corresponds to the steady-state monomer concentration, where the overall rate, which is the sum of the rates off the plus end and minus end, is zero. At this concentration, treadmilling takes place, because there is a net flux of subunits off the minus end and a corresponding net flux onto the plus end. The shaded area denotes the concentration range in which elongation can proceed from the plus end, but where free filaments are unstable.

attachment sites. In skeletal muscle, the intestinal brush border, microvilli in coelomocytes or sea urchin eggs, and fibroblast and neuroblastoma cells, the plus end of the actin filaments seems to be inserted into the region of the membrane (10, 13, 16, 22, 26, 27). As discussed above, filaments that are truly attached at the plus end and free at the minus end would be expected to be thermodynamically unstable. There are several possible explanations for this apparent conflict with theory. First, it is not at all clear that these membrane sites are nucleation and not insertion sites, because unlike the situation in the microtubule experiments and the actomere experiment, the state of the other end of the filaments is unknown. Second, in some cases it may be true that the polymers are not in equilibrium with monomers, either because both ends of the polymer are capped or because the in vivo conditions do not allow for rapid equilibration. Finally, the relationship between the structural polarity and the rates of elongation could be in error if the rate measurements were made far from the critical concentration and if the shapes of the rate curves are like one shown by Bergen and Borisy (Fig. 2*h* in reference 4). An understanding of the actin data will first depend on identification of the actual actin nucleation structures in cells and, consequently, on unambiguous determination of the actin polarity relative to these centers. This analysis could then lead to an understanding of the in vivo characteristics of the actin monomer-polymer equilibrium.

In summary, microtubules and possibly actin may use ATP and GTP to produce a disparity in the critical concentration for assembly at the two ends of the polymer, thereby suppressing nonanchored growth. Under in vitro conditions, this would produce treadmilling, but under in vivo conditions where generally one end of the filament is blocked no flux is possible. Under these conditions, however, the critical monomer concentration for assembly of the anchored array will be below that

for free microtubules and actin filaments. As long as there is a pool of ATP and GTP the anchored filaments will remain stable, and spontaneous polymerization will be suppressed. Considered in this perspective, the long microtubules emanating from the perinuclear region in fibroblast cells (9, 30) could represent a metastable state that must be preserved by continuous GTP hydrolysis. I would predict that individual microtubules in this cellular milieu would be unstable. If one could inject individual microtubules into such cells, for example, I would expect them to gradually dissolve. Similarly, if one injected nucleation sites accessible at their minus end, but blocked at their plus end, they would not support polymerization. However, if one injected into these cells nucleation sites with plus ends free, they would support assembly. I would predict further that treadmilling would not in general occur in vivo and that in the cell all anchored filaments in dynamic equilibrium with their subunits would have the same polarity, attached at their minus and free at their plus ends.

In some ways, the use of ATP or GTP hydrolysis to suppress an unwanted assembly reaction that is normally thermodynamically favored is reminiscent of the proposed use of ATP or GTP hydrolysis to suppress mistakes in codon-anticodon recognition that would normally be expected from by the relative binding constants of the tRNA species (15, 35). The use of ATP as other than a direct source of energy may then be a widely occurring phenomenon and it is, therefore, not unlikely that other assembly systems in dynamic equilibrium with their subunits would also use nucleoside triphosphate hydrolysis to regulate the sites of their polymerization reactions.

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