INTRODUCTION

Molecular motors carry out a myriad of functions in the cell, ranging from vesicle (Hirokawa, 1998) and RNA transport (Nasmyth and Jansen, 1997) to spindle assembly and chromosome motility in dividing cells (Endow, 1999c). The finding of a large number of motors that fall into one of the three families of motors known to exist in eukaryotes, the actin-based myosins (Sellers and Goodson, 1995) or microtubule-based kinesins (Bloom and Endow, 1995) and dyneins (Hirokawa, 1998), has been revolutionary in permitting analysis of diverse biological processes as the roles of the proteins in the cell are discovered. The importance of molecular motors in the cell is exemplified by the process of cell division, in which many of the kinesin microtubule motors, together with cytoplasmic dynein, produce force for spindle assembly and chromosome attachment to the spindle (Endow, 1999c). Cytoplasmic myosin then functions during cytokinesis to complete the process of cell division by accumulating in the cleavage furrow and providing motile force for the contractile ring (Goldberg et al., 1998). Vesicle trafficking similarly involves motors from all three families (Hirokawa, 1998; Brown, 1999), while specific cellular roles are played by myosin in muscle contraction (Cooke, 1986), dyneins in flagellar motility (Gibbons, 1996) and kinesins in regulation of microtubule dynamics (Inoué and Salmon, 1995; Waters and Salmon, 1996).

The cellular roles of the motors and interactions between the motors are only beginning to be understood. The fact that the cellular functions of many of the motors are as yet unknown means that the effects of mutants that cause loss of function have not always been straightforward to predict, and some of these effects have been unexpected. For example, mutants of the unconventional myosins, myosin VI and VII, cause deafness in mice and humans, and are the basis of previously described syndromes, Snell’s Walzer and shaker, that are associated with behavioral abnormalities in mice (Avraham et al., 1995; Gibson et al., 1995; Weil et al., 1995). These myosins are present in hair cells of the inner ear, where their roles in hearing are currently being investigated.

Another example of an unexpected effect of a mutant comes from work on the kinesin-related KIF3B microtubule motor. In the KIF3B-knockout mouse, loss of KIF3B is associated with loss of bilateral asymmetry (Nonaka et al., 1998), which is needed to form organs such as the heart in mammals. Hirokawa and co-workers propose that the basis of the loss of asymmetry is defective transport of substances required for formation of cilia in the early embryo (Nonaka et al., 1998). They hypothesize that the cilia cause a leftward flow of extraembryonic fluid in the area of the node, which is a transient structure present during gastrulation, and therefore play a key role in the formation of left-right morphogen gradients. These unforeseen mutant effects reflect the inability to predict the cellular roles of the newly discovered molecular motors.
myosin, kinesin and dynein motors, and underscore the necessity for cellular studies. But to fully understand the roles of molecular motors in cells, it is necessary to determine how the motors work.

THE MOTOR MECHANISM

Molecular motors are unique in their ability to convert the chemical energy from ATP hydrolysis into work, which permits them to bind to and move along actin filaments or microtubules. The process of energy conversion by motor proteins is not understood, and considerable effort is now being devoted to unraveling the possible mechanisms. The discovery of large families of related motors whose members differ from one another in directionality of movement on their filament, ATPase activity and motor velocity offers an unprecedented opportunity to perform studies to uncover the mechanism by which these proteins function. This information will be valuable, first, because it represents basic information about energy conversion in living organisms and may lead to the discovery of new principles and, second, because it will help us to understand how the motors work in the cell. Practical uses of the anticipated information will be to design mutants that disrupt specific cellular functions, or to create motors for transport of drugs or other substances to specific cells or regions of cells.

A milestone towards the goal of understanding the changes that take place in the motors during the conversion of chemical to mechanical energy has been the solution of crystal structures of myosin in three different nucleotide states: a ‘rigor’ state without nucleotide (Rayment et al., 1993), a putative transition state with bound ADP-AlF$_4^-$ (Fisher et al., 1995; Dominguez et al., 1998), and a third distinct conformational state that may correspond to a prehydrolysis state (Houdusse et al., 1999). A comparable advance for the kinesins has been the solution of crystal structures of monomeric and dimeric forms of the motors in the Mg-ADP state (Sack et al., 1999). The crystal structures of the kinesins provide information about the dimeric forms of the motors, currently unavailable for the myosins, but are uninformative about the changes that take place in the motors during nucleotide hydrolysis because they all correspond to the same state, presumably a post-hydrolysis state. Nonetheless, the structures have been valuable in revealing similarities between the kinesin motors and myosin (Kull et al., 1996), and between the kinesins and G proteins (Sabin et al., 1996; Fig. 1). These structural similarities provide a basis for comparing the mechanisms of protein function. The kinesin and myosin motor proteins share with the G proteins the ability to hydrolyze nucleotide at a greatly enhanced rate in the presence of effector proteins. Specific effector proteins exist for different G proteins, but the effector proteins for the myosins and kinesins are actin filaments and microtubules, the cytoskeletal filaments along which the motors move. This means that the very act of binding to their filament greatly increases the ability of the motors to convert energy from nucleotide hydrolysis into the mechanical energy that enables them to move along the filament.

ANALYSIS OF MOTOR FUNCTION

The realization that molecular motors might be fundamentally different from other nucleotide-hydrolyzing proteins in possessing the ability to convert chemical energy into work has led to the question of how to obtain information about the motor mechanism. Strategies that have been used successfully for other proteins, such as the G proteins, are to characterize the proteins functionally and structurally using available biochemical and structural methods (Bourne et al., 1991), and then to mutate specific residues of the proteins and determine the effects of the mutations on protein structure and function (Raw et al., 1997). Findings from such structure/function studies have resulted in working models of G protein function (Sprang, 1997). Further tests by state-of-the-art methods, such as time-resolved or millisecond-resolution X-ray crystallography (Scott et al., 1996; Stoddard et al., 1998), could confirm the conformational changes in protein structure proposed to occur during the hydrolysis cycle, which are based on the ‘still’ images from static crystal structures.

This general approach is one that is being used to study the mechanism of myosin function and has led to several noteworthy achievements in recent years. The solution of the first myosin motor domain structure was a breakthrough (Rayment et al., 1993) that has been rapidly followed by new myosin structures (Fisher et al., 1995; Dominguez et al., 1998; Houdusse et al., 1999), as noted above. Mutant analysis coupled with expression of functional myosin proteins in baculovirus is underway (Ruppen and Spudich, 1995) and should provide information about the myosin mechanism of function. But despite the recent surge in progress on myosin, the kinesins have continued to attract attention. The kinesins offer several advantages over myosin, including the smaller size of the motors compared with myosin, which is a distinct advantage for structural analysis, and the ease of expressing the kinesin proteins in bacteria compared with the technicalities of baculovirus expression. On the other hand, a major barrier to rapid progress on the kinesins has been the inability to crystallize the motors in different conformations that correspond to different nucleotide states of the motor. Although seven crystal structures of kinesin proteins have been reported to date (Sack et al., 1999; Kozielksi et al., 1999), all of the structures are in the same nucleotide state, with bound Mg-ADP. Efforts to identify different structures of the kinesins bound to other nucleotides, including attempts to use nucleotide analogues to grow crystals of motors in different states, have not been successful – the crystals have been in the Mg-ADP conformation despite the bound nucleotide (Muller et al., 1999). This means that the crystal structures cannot be used to determine the conformational changes that occur during nucleotide hydrolysis, or to identify regions of the kinesin motor domain that move or change conformation during hydrolysis.

This lack of success has caused workers to consider different approaches to uncover the conformational states of the kinesin motors. One idea is to use mutants to ‘trap’ the motor in different conformations that reflect different nucleotide states of the motor. Mutants could potentially block the motor at different steps of the nucleotide-hydrolysis cycle – even the steps that normally proceed very rapidly, at millisecond rates – and thus reveal details of the hydrolysis mechanism that would be difficult or impossible to observe using currently available biochemical methods. The task, then, is to find mutants that will be informative. This task is one that confronts both kinesin and myosin motors workers and one for which general strategies have been elusive.
Fig. 1. Structural similarities between a kinesin, a myosin and a G protein. The nucleotide-binding or P loop (light blue), and switch I (SwI, red) and switch II (SwII, dark green) regions of the G proteins are conserved in the kinesin and myosin motors. The crystal structures are of p21\textsuperscript{ras}, the cellular Harvey-Ras p21 protein (PDB 821P; Franken et al., 1993), the kinesin Kar3p motor domain (PDB 3KAR; Gulick et al., 1998), and chicken skeletal muscle myosin S1 subfragment (MyoII, PDB 2MYS; Rayment et al., 1993). The residues mutated in the kinesin (N650K) and myosin (S474V, S465V in Dictyostelium) uncoupling mutants are depicted as ball-and-stick models in purple. The mutated residues both lie in the switch II loop/helix that follows the switch II region in the Kar3p or myosin motor domain. The microtubule- or actin-binding region of the motor is shown in dark pink. The residue numbers and sequences of the P loop, and switch I and switch II regions are shown at the bottom. PDB, Protein Data Bank.
**MOTOR MUTANTS**

The problem that has perplexed many workers is how to identify residues that are needed for motor function and mutational changes that will define essential functions without having to carry out exhaustive genetic or molecular mutagenesis screens. Even given the available crystal structures and the identification of residues that are involved in nucleotide hydrolysis or binding to microtubules or actin filaments, it is not clear which residues to target or which residue to change the targeted residues to. There are also other problems inherent in targeted mutagenesis that complicate the analysis of mutants. For example, point mutants could have effects that are specific to a given protein rather than affecting an activity that is required for basic motor function, or they could inactivate the motor by destroying the motor ATPase or the ability of the motor to bind to its filament, making it impossible to detect activity using standard ATPase or filament-binding assays. This means that it is important to consider both the type of mutant and the available assays that can be used to determine mutant effects when designing or selecting mutants for analysis. Fortunately for projected structural studies, the effects of single residue changes on overall structure are usually not a major concern, since considerable evidence indicates that proteins exist in stably folded conformations that are not easy to change by single missense mutations. Proteins maintain their folded conformations by extensive, relatively weak electrostatic forces, including hydrogen bonds and van der Waals forces, together with hydrophobic interactions and stabilizing effects of disulfide bonds (Branden and Tooze, 1991). Mutation of a single residue can disrupt some of these interactions, but generally affects local structure rather than overall structure. Thus, mutants resulting from single residue changes are not expected to produce global alterations in protein structure and, in contrast, have the potential to provide valuable information regarding possible conformations in which the protein can exist.

**ATPase mutants**

Several types of molecular motor mutants are likely to be informative about the motor mechanism of function. They include mutants that interfere with the nucleotide-hydrolysis cycle, which are expected to affect both the ability of the motor to bind and hydrolyze nucleotide and motor interactions with microtubules or actin filaments. These mutants will be informative not only about the mechanism of motor function but also about the cellular phenotypes that are caused by motor mutations. The mutants could show a complete inability to hydrolyze nucleotide, or they could affect only the filament-stimulated ATPase of the motor. Both types of mutant are expected to alter the binding of the motor to its filament – the effect would depend on the step of hydrolysis that is defective. For example, mutants that cannot bind nucleotide and therefore are unable to hydrolyze ATP would be ‘rigor’ mutants that bind very tightly to their filament, whereas defective filament-activated ATPase activity could result in either weaker or stronger binding to microtubules or actin filaments, depending on the nucleotide state in which the mutants are blocked. The steps that are blocked can be determined by careful biochemical analysis of nucleotide-hydrolyzing ability and correlated with the ability of the mutant motor to bind to its filament. Functionally interesting conformations of the motor – for example, the force-producing steps – are likely to be only transiently stable. By stabilizing or increasing the lifetime of these steps, the ATPase mutants have the potential to reveal many or all of the steps in the nucleotide-hydrolysis cycle. Crystallization of the mutant motors could produce different crystal forms that reveal conformational changes that occur in the motors during hydrolysis. Analysis of the ATPase mutants is also expected to reveal the coupling between the nucleotide-hydrolysis cycle and interactions of the motor with its filament. At least some of the ATPase mutants should fall into the class of ‘uncoupling’ mutants.

**Uncoupling mutants**

Uncoupling mutants were proposed to exist because of the unusual coupling of nucleotide hydrolysis to force generation (discussed above) that is a basic property of the molecular motors. The realization that uncoupling mutants might exist and would be informative to study led to the search for and recovery of such mutants in Dictyostelium myosin (Ruppel and Spudich, 1996) and to the recent identification of a kinesin uncoupling mutant (Song and Endow, 1998). The uncoupling mutants separate essential motor functions so that one function is increased or decreased, whereas another remains unchanged or is changed in the opposite direction. Uncoupling mutants can be either partial loss-of-function mutants or dominant mutants. One example is the Dictyostelium S465V (S474 of chicken) myosin mutant (Table 1), which translocates actin filaments much more slowly than wild type, at one-tenth the velocity, but has an elevated basal ATPase activity that is nearly equal to the actin-activated ATPase activity of wild type (Ruppel and Spudich, 1996). The S465V mutation thus uncouples ATPase activity and motility. We recently reported a kinesin uncoupling mutant (Table 1) in which microtubule-stimulated ATP hydrolysis is completely blocked even though the basal ATPase activity of the mutant is unchanged from that of wild type (Song and Endow, 1998). The basis of this defect is the uncoupling of nucleotide and microtubule binding: the mutated motor binds tightly both to ADP and microtubules, unlike wild-type kinesin motors, which bind weakly to microtubules in the presence of ADP. The inability of the motor to release nucleotide at an enhanced rate when bound to microtubules is correlated with an inability to move on microtubules. The mutated residue is present in the microtubule-binding region of the motor (Fig. 1), some

<table>
<thead>
<tr>
<th>Table 1. Uncoupling mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Myosin S465V mutant</td>
</tr>
<tr>
<td>Kinesin uncoupling mutant</td>
</tr>
<tr>
<td>Loss-of-function mutant</td>
</tr>
<tr>
<td>Null mutant</td>
</tr>
<tr>
<td>Wild type</td>
</tr>
</tbody>
</table>

The myosin S465V mutant of Dictyostelium (Ruppel and Spudich, 1996) and the kinesin uncoupling mutant (Song and Endow, 1998) cause an increase in one motor activity but a decrease in another, uncoupling or separating motor functions. The effects of loss-of-function and null mutants are shown for comparison. Wild-type motors show all three activities.
distance from the nucleotide-binding cleft of the motor, thus the effect of the mutation on nucleotide binding was unexpected. It is consistent, however, with the proposal that the structural element affected by the mutation is involved in communication between the nucleotide- and microtubule-binding regions of the motor (Sablin et al., 1996).

Surprisingly, despite the difference in their biochemical effects, the kinesin and myosin uncoupling mutations map to analogous structural elements in the two motors: a loop/helix that is thought to correspond to the ‘switch II’ loop/helix of the G proteins (Fig. 1), which changes in conformation on nucleotide exchange. The myosin mutation lies in the cleft that divides the 50-kDa domain of the myosin motor into the upper and lower regions, which, like the structural element affected by the kinesin uncoupling mutation, has been proposed to participate in communication between the ATP- and filament-binding sites (Ruppel and Spudich, 1996). The difference in biochemical effects between the myosin and kinesin uncoupling mutants indicates that the mechanisms of the myosin and kinesin motors could differ considerably despite the structural homology of the motors. The uncoupling mutants thus have the potential to provide insights into motor function that cannot be obtained from loss-of-function or null mutants. Other uncoupling mutants are likely to be discovered, since there are probably several different ways of uncoupling nucleotide hydrolysis from force generation by the motors. The effects of these mutants are difficult to predict because the mechanism of coupling between nucleotide hydrolysis and motility is not well understood. The uncoupling mutants could therefore provide further surprises as they reveal unexpected aspects of motor function.

MUTAGENESIS SCREENS AND MUTANT SELECTION

Molecular screens

How can informative ATPase or uncoupling mutants be identified? Several strategies can be used to obtain mutants for analysis (Table 2). These strategies include extensive molecular mutagenesis coupled with biochemical or phenotypic analysis – for example, random mutagenesis or alanine-scanning mutagenesis has been used to mutagenize specific regions of myosins (Porter and Montell, 1993; Ruppel and Spudich, 1996; Sasaki and Sutoh, 1998) or surface residues of specific regions. Molecular mutagenesis does offer the advantage that mutational changes can be introduced into targeted regions of a protein, e.g. the motor domain, but this advantage does not outweigh the demands on labor and time. This approach is therefore one that is not a method of choice for most laboratories, given limitations on manpower and financial resources.

Genetic screens

A second strategy, although one that has not yet been widely adopted by motors workers, is to select mutants for analysis from existing collections recovered from genetic screens. Several such screens have been carried out for myosins (Bejsovec and Anderson, 1988; Patterson and Spudich, 1995) and kinesins (Saxton et al., 1991; Hoyt et al., 1993). A potential disadvantage of using mutants recovered in genetic screens is that the mutants could map to any region of the protein and therefore probably include those that are specific for the myosin or kinesin that was the target of the screen, as well as those that affect basic motor functions. This means that a selection step, which could itself be as time-consuming as screening mutants obtained by molecular mutagenesis, must be performed to identify informative mutants for analysis.

Unexpectedly, two previously reported genetic screens yielded mutants all of which mapped to the motor domain of a myosin or kinesin protein. Bejsovec and Anderson (1988) screened for dominant muscle defects in C. elegans that caused paralysis, targeting the unc-54 muscle myosin heavy chain gene. They found that the most strongly dominant alleles altered highly conserved residues in the nucleotide-binding region of the motor domain, whereas other mutant alleles affected the actin-binding region (Bejsovec and Anderson, 1990). They hypothesized that the basis of the dominance and localization of the mutations to the motor domain was the effects of the mutants in disrupting assembly of thick filaments. The sensitivity of thick filament assembly to perturbations in the ability of myosin to bind to actin and hydrolyze nucleotide thus provided an effective in vivo selection for mutants affecting motor function. The second screen that produced mutants with defective motor function was for yeast suppressors of the temperature-induced lethality of cin8-ts kip1-Δ cells (Hoyt et al., 1993), which are null for two kinesin proteins, Cin8p and Kip1p, that overlap in function (Saunders and Hoyt, 1992). All seven extragenic suppressors recovered in the screen mapped within or just adjacent to the conserved motor domain of Kar3p (Hoyt et al., 1993), a third kinesin protein, which is thought to produce force in the mitotic spindle that opposes the force produced by Cin8p and Kip1p (Saunders and Hoyt, 1992). The recovery of kar3 mutants as suppressors of cin8-ts kip1-Δ lethality is consistent with the idea that the motors produce antagonistic forces: loss of Kar3p motor function should suppress the unopposed force produced by Kar3p in cin8 kip1 null cells.

These and other available mutant collections provide ample material for analysis, but the informative mutants must still be identified from among the other mutants. Several criteria can be used to select mutants that are likely to be informative.

Table 2. Motor mutants for analysis

<table>
<thead>
<tr>
<th>Mutant source</th>
<th>Disadvantages</th>
<th>Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular</td>
<td>Labor-intensive</td>
<td>Target motor</td>
</tr>
<tr>
<td>Genetic</td>
<td>Target entire protein</td>
<td>Global effects</td>
</tr>
<tr>
<td>Rational</td>
<td>Limited by criteria</td>
<td>Site-specific</td>
</tr>
<tr>
<td>Natural</td>
<td>Limited availability</td>
<td>Global effects</td>
</tr>
</tbody>
</table>

Molecular motors – a paradigm for mutant analysis 1315
These include the degree of conservation of the mutated residue. The extensive sequence alignments now available for the myosins (Hodge and Cope, 1996) and kinesins (Greene et al., 1996) can be used to determine how highly conserved the mutated residue is. The mutants can also be mapped onto the available crystal structures to identify the structural element that is affected. Mutants that alter highly conserved or invariant residues that are present in structural elements thought to be involved in nucleotide hydrolysis or binding of the motor to microtubules or actin filaments are good candidates for analysis. The same mutation can be expressed in different myosins or kinesins to determine whether the effect of the mutation is specific to a given motor protein or general to motors of its family or class. The mutations that are general to motor function should cause similar effects in different motors, rather than being specific to a given motor.

Mutants can also be selected on the basis of their genetic effects. Although the effects of ATPase or uncoupling mutants in live cells cannot be predicted with certainty, mutants that have dominant effects in vivo are likely to cause interesting changes in motor function. Dominant mutants are mutants that show defects in combination with a wild-type gene – the cell is still mutant even though a wild-type gene is present. Although several different properties of proteins or protein-protein interactions can result in dominance, one interaction that has been widely used to obtain information about protein function in vivo is interference of the mutant protein with the function of the wild-type protein. Such antagonistic protein interactions have been termed ‘antimorphic’ (Muller, 1932) or ‘dominant negative’ (Herskowitz, 1987). An example of a mutant recovered in a genetic screen and selected for analysis on the basis of its dominant mutant effects in vivo is the kinesin uncoupling mutant discussed above. This mutant was originally recovered as a kar3 suppressor mutant in the genetic screen for suppressors of cin8 kip1 lethality (Hoyt et al., 1993). The kar3 mutant showed a dominant phenotype in genetic tests, which can now be explained by the strong binding of the mutant motor to microtubules even in the presence of ADP.

The mutant also affects a residue that is invariant among the known kinesin proteins, which indicates that it is likely to affect basic motor function rather than an activity specific to Kar3p. When we tested the same mutation in another kinesin protein, Ncd, we observed the same effects on ADP and microtubule binding (Song and Endow, 1998), thus the mutational change has a general effect on the kinesin motors and affects an essential motor property: the ability to hydrolyze ATP at an enhanced rate when the motor is bound to microtubules. The myosin uncoupling mutants that were recovered previously in Dictyostelium (which is haploid) showed ‘intermediate’ effects and were not reported to have been tested for dominance over wild type. It is not certain that all uncoupling mutants will be dominant, although some are likely to have dominant negative effects because of their unusual biochemical properties. The mec-54 myosin mutants, all of which showed dominant effects in vivo, are likely to be a rich source of interesting mutants that will yield valuable information about myosin function.

The mutants found in genetic screens will be important to study because they should affect all of the basic motor functions needed in live cells, rather than a subset of the functions required for motility in vitro. These mutants can not only identify the regions of the motor that are essential for function, but also reveal the key residues that are required and tell us how the motor can be altered to produce unexpected effects, such as those of the myosin and kinesin uncoupling mutants. Without the use of these mutants, it is not obvious how to obtain information about essential motor functions – for example, how ATP hydrolysis is stimulated by binding of the motor to its filament (Song and Endow, 1998). These mutants can reveal the coupling between nucleotide hydrolysis and motor-filament interactions by uncoupling these basic motor functions in different ways. The uncoupling mutants analyzed to date validate the strategy of studying mutants recovered in genetic screens by showing that the mutants can provide unexpected and important information about essential motor regions and their function that could not be predicted from the crystal structures alone.

Rational design
A third source of mutants for analysis is rational design – identifying and mutating functionally important motor regions on the basis of rational considerations. These regions can often be identified as those containing highly conserved or invariant residues, but are not necessarily limited to these regions. Further useful targets are conserved structural elements that might not consist of conserved residues (Branden and Tooze, 1991). Information from related proteins on which work is more advanced, such as the G proteins, can be used to identify functionally or structurally important regions in the motor proteins by analogy. Based on the structural analogies between the G proteins, the myosins and the kinesins, the switch I and switch II regions (Fig. 1) that are proposed to undergo changes during nucleotide hydrolysis are likely to be informative. Mutating conserved residues in these regions of the motors could trap the motors in a prehydrolysis or transition conformation, or change the kinetics of hydrolysis to reveal intermediate states. Determining the ability of the motor to bind to its filament in the prehydrolysis and transition states will be critically important to understanding how the motor interacts with its filament. Although the kinesin motors are believed to bind tightly to microtubules in the presence of ATP (Brady, 1985) and weakly in the presence of ADP (Ma and Taylor, 1995; Crevel et al., 1996; Pechatnikova and Taylor, 1997), and myosin is thought to bind tightly to actin filaments in the presence of ADP and weakly in the presence of ATP (Sellers and Goodson, 1995), it is not certain what happens during transition states for either the kinesins or myosins. Transition states in nucleotide hydrolysis are likely to correspond to the force-generation steps of the motor (Howard, 1996), thus interactions between motors and microtubules or actin filaments during these states will be extremely important in understanding how molecular motors generate force and move along their cytoskeletal tracks. It is also likely that important principles will be learned by targeting the motor regions analogous to those that are thought to change in conformation in G proteins. The current thinking is that although conserved switch regions exist in the kinesins, myosins and G proteins, fundamental differences exist in the mechanism by which these proteins function. For example, the G proteins use switch II residues to catalyze nucleotide hydrolysis (Frech et al., 1994), whereas myosin uses a switch I residue (Shimada et al., 1997). What residues do the kinesins use? This question can be addressed by
careful design of mutants, based on structural homologies and sequence considerations.

**Natural variants**

A final source of informative mutants that should not be overlooked is natural variants, proteins within a family that differ from most of the other family members and arise by spontaneous changes followed by natural selection. Naturally occurring variants are an important source of structural changes coupled to changes in function, and could perform novel functions in the cells in which they are found. Kinesin motors such as *Drosophila* Ncd and other members of the C-terminal motor kinesin subfamily (Endow, 1999a) can be considered to be natural variants of the kinesins. These motors differ in domain organization from other kinesin proteins in that the conserved motor domain is C-terminal to the coiled-coil dimerization domain, instead of N-terminal, as in the remainder of the kinesin family. The C-terminal motor kinesins differ strikingly in motility from other kinesins, moving to the minus end of microtubules instead of the plus end. This difference in polarity of movement has allowed workers to examine the molecular basis of motor directionality (Endow, 1999b). Further work is expected to provide basic insights into motor function, since directionality is a fundamental property of molecular motors.

Another example of a naturally occurring variant is myosin VI, which differs from other myosins in that it has two small insertions and a third large insertion in the conserved motor domain (Wells et al., 1999). The larger, 53 amino acid insertion is present in the myosin ‘converter’ region, a region at the domain (Wells et al., 1999). The larger, 53 amino acid insertion and a third large insertion in the conserved motor VI, which differs from other myosins in that it has two small insertions and a third large insertion in the conserved motor domain (Wells et al., 1999). The helical rod, or putative lever arm, of myosin VI appears to point in the opposite direction to that of myosin II when the motors are bound to actin filaments. This led Wells et al. (1999) to propose that the converter region acts as a ‘pivot point’ for the lever arm. These findings demonstrate that small structural changes can cause profound changes in protein function and underscore the importance of careful sequence analysis to identify functionally important regions of the motors.

**PERSPECTIVES**

Together with the mutants from molecular and genetic screens, the mutants obtained by rational design or discovered as natural variants have the potential to provide basic information about the motor mechanism. These mutants should allow workers to probe the mechanism of motor function in detail and extend the information provided by the crystal structures and biochemistry. The analysis of these mutants should take into account the interactions of the motors both with microtubules or actin filaments and with nucleotides that change during the hydrolysis cycle. To solve a problem as complex and important as energy conversion by molecular motors, it will be necessary to use ingenuity in mutant design and interpretation, together with an arsenal of available methods of analysis. This will include structural methods, such as X-ray crystallography and NMR (nuclear magnetic resonance), and sensitive fluorescence methods – e.g. fluorescence anisotropy (Rosenfeld et al., 1996; Sweeney et al., 1998) and FRET (fluorescence resonance energy transfer; Suzuki et al., 1998) – together with high-resolution microscopy methods to visualize single motors and fluorescent nucleotides in motility assays (Funatsu et al., 1995; Ishijima et al., 1998).

In vivo analysis and tests of mutant function should not be overlooked: these studies will be extremely valuable in testing interpretations from in vitro studies. Ideally, the in vivo tests of mutant function should include live analysis using recently developed methods to visualize proteins in the cell (Chalfie et al., 1994) and permit study of the biophysics of the motors in the cell. Finally, the approaches and strategies for mutant design and selection described here are not limited to molecular motors, but are applicable to other proteins under study whose mechanisms of function have yet to be determined.

Special thanks to T. Salmon for thought-provoking and insightful comments about the ideas presented here, R. Cross for reading the manuscript, and H.-W. Park for notes on protein folding. Work in my laboratory is supported by grants from the NIH and HFSP.

**REFERENCES**


