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13. Phage from 40 plaques were transferred by toothpick to tubes containing all the components necessary for PCR (*16*) including these two oligonucleotides:
(i) TCGAAAGCAAGCTGATAAACCG
(ii) ACAGACAGCCCTCATAGTTAGCG
After 40 cycles, the PCR products from phage with and without an insert (296 bp compared to 227 bp) could be easily distinguished on a 2 percent agarose gel.
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Polarity and Velocity of Sliding Filaments: Control of Direction by Actin and of Speed by Myosin

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Myosin filaments, which are responsible for a large repertoire of motile activities in muscle and nonmuscle cells, can translocate actin filaments both toward and away from their central bare zone. This bidirectional movement suggests that there is enough flexibility in the head portion of the tightly packed myosin molecules in the native myosin filaments to move actin filaments not only in the expected direction, but also in the direction opposite to that predicted by the regular structure of muscle—away from the center of the myosin filament.

MYOSIN FILAMENTS EXHIBIT A tight packing of the rod portion of the myosin molecules with the heads forming projections of opposite polarity on either side of a narrow central bare zone (*1*). It is commonly accepted that the cyclical interaction of myosin heads with polar actin filaments generates force that pulls the actin filaments toward the center of the bipolar myosin filament resulting in shortening of the muscle (*1*). Recently, an in vitro motility assay has been developed that allows for the visualization of the movement of fluorescently labeled actin filaments over a surface randomly coated with myosin (*2, 3*). In this assay, the myosin molecules are not directly imaged and therefore their precise orientation is not known.

We have isolated large native thick filaments from clam adductor muscles with the use of a gentle technique (*4*). These myosin filaments can be directly visualized by video-enhanced differential interference contrast (DIC) microscopy (*5*) and appear as spindle-shaped filaments of lengths up to 20 μm (Fig. 1) (*6*). When bound to a glass surface, these myosin filaments translocated fluorescently labeled actin filaments in the in vitro motility assay (Fig. 2A). Direct comparison between the DIC and fluorescence images revealed that fluorescently labeled actin filaments could bind at any position on the myosin filament and commence directed movement (Fig. 2). Actin filaments moved both toward and away from the center of the myosin filament. Both long (2 to 8 μm) and short (<1 μm) actin filaments exhibited this behavior. The movement of actin filaments away from the center of the myosin filament is opposite that which normally occurs in muscle contraction. Analysis of the movement demonstrated that those actin filaments traveling toward the center of the myosin filament moved at a fast rate of 8.8 ± 1.4 μm/s, whereas those that were traveling away from the center moved at the much slower rate of 1.0 ± 0.3 μm/s (Fig. 2B). Some actin filaments traveled the entire length of the thick filament. In these cases the actin filament would bind to a myosin filament and commence moving at the fast rate until it crossed the bare zone, where it would slow abruptly upon encountering myosin heads of the opposite polarity. A single actin filament could reverse direction of travel by detaching and rapidly reattaching after undergoing a 180° or 360° end-to-end flip (Fig. 3A). This occurred most frequently with short actin filaments, which undergo rapid Brownian movements upon detachment. The direction of travel after the flip was correspondingly recovered or reversed, consistent with the intrinsic polarity of the actin filament. On some occasions a longer actin filament sliding off the end of the myosin filament reattached through its initial leading end and proceeded to move back down the same myosin filament in the opposite direction. Two actin filaments traveling in opposite directions on a single myosin filament could pass one another unimpeded, which is not surprising given the relatively large diameters of the myosin filaments (Fig. 3B). When this occurred one filament would be traveling at the fast speed and the other at the slower speed.

We also observed that the actin filament was very flexible and behaved more as a rope than as a rigid rod when moving in this assay. When a long actin filament traveling at the fast rate crossed into the region of opposite polarity of the myosin filament, its leading end slowed abruptly while the tail end, which was still in contact with myosin heads of the correct polarity, continued to move fast. Because of the difference in the sliding speeds of each end of the actin filament, it formed a flexible loop in the middle portion that was over the bare zone and was free of attachment (Fig. 3C). Actin filaments could also interact simultaneously with more than one adjacent myosin filament, producing independent sliding actions and often undergoing large changes in angles (Fig. 3D). The implications of this flexibility may be more commonly manifest in nonmuscle systems where the actin filaments are typically much longer than the myosin filaments and where a single actin filament may possibly interact with more than one myosin filament (7). This flexible nature of the long actin filaments indicates that the actions of well-separated myosin heads are not necessarily integrated along the actin polymer chain. Actin filaments often exist in bundles within cells. Such bundles may allow for a structure with more

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stiffness that would allow for better integration.

These experiments demonstrated that actin filaments could move in a direction opposite to that which occurs in intact skeletal muscle, where it is thought that actin filaments always move toward the center of the myosin filament during active contraction (*1*). Any segment of the bipolar myosin filament could move actin in both directions, and the direction of travel is determined only by the polarity of the actin filament. Several reports have shown that the heads of isolated myosin molecules are capable of great rotational movements that probably occur at the subfragment 1–subfragment 2 (S1-S2) junction (*8*), and similar observations have been made on myosin heads projecting from myosin filaments (*9*). Reedy *et al.* recently demonstrated that in mutated *Drosophila* flight muscle, in which peripheral thick and thin filaments are misregistered, the heads of myosin can bind with opposite rigor cross bridge angles to flanking actin filaments that are of the opposite polarity (*10*). These results suggest that the heads of myosin are able to swivel 180° relative to each other. Two recent reports have addressed the question of whether actin can interact in a productive manner with myosin heads of the opposite polarity. Stephenson *et al.* (*11*) showed that the myofibrillar Mg^{2+} adenosine triphosphatase (ATPase) activity does not decrease at short sarcomere length, suggesting that actin can activate the Mg^{2+} ATPase activity of myosin heads from parts of the filament of opposite polarity. Toyoshima *et al.* (*12*) described bidirectional movement of actin filaments along tracks of myosin heads created by binding actin filaments decorated with skeletal muscle heavy meromyosin (HMM) to a nitrocellulose surface. However, the artificial constraints imposed on the HMM molecules by binding to nitrocellulose cannot be compared to the constraints imposed upon myosin molecules in the specifically packed native myosin filaments. We now provide definitive data that the specific packing of myosin in the native filament organization can also generate force to translocate actin both toward and away from the central bare zone.

It is clear from the present results that the power stroke cannot be derived exclusively from vectorial changes in the S2 region. Movement of actin filaments by myosin heads of the reversed polarity would not be accounted for by models of muscle contraction involving vectorial processes occurring in the S2 region such as shortening induced by a helix-coil transition (*13*) or a peeling of the S2 region off of the surface of the myosin filament (*14*) since these effects would work against the observed reversed direction of actin travel. In fact, previous work by Toyoshima *et al.* (*2*) has already shown that "tail-less" myosin heads, subfragment 1, are sufficient to move actin. Our

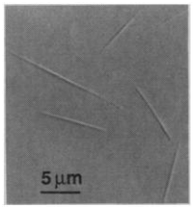

Fig. 1. Video-enhanced light micrograph of isolated native myosin filaments from clam muscle. The DIC image was obtained with a Zeiss-Axiomat microscope in the critical illumination mode (*5*) equipped with a ×100, 1.4 NA objective and a Newvicon video camera (Dage-MTI). The mean myosin filament length was 9.5 ± 3.8 μm.

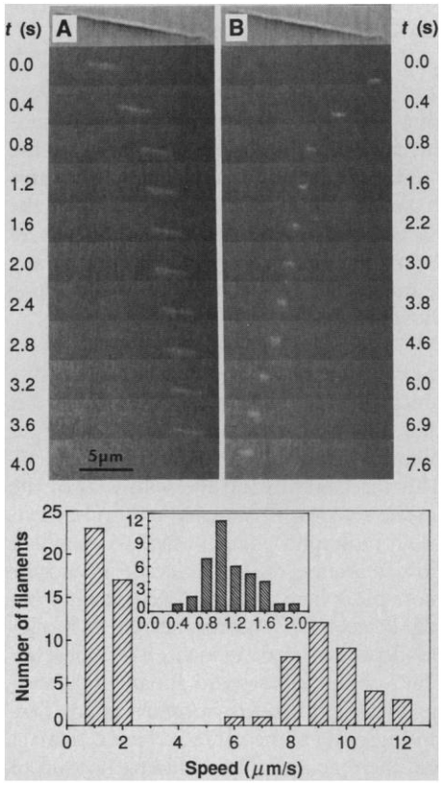

Fig. 2. Video analysis of the bidirectional sliding rates of fluorescently labeled actin filaments along single myosin filaments. (**A**) Sequential fluorescence images of actin filaments sliding along a single myosin filament (upper panel). Note that in each case (both left and right sequences) when an actin filament was moving toward the center of the myosin filament the speed was fast and when it was moving away from the center the speed was slow. (**B**) Distribution of speeds. The mean and standard deviation of the slow rates was 1.0 ± 0.3 μm/s; and that of the fast rates was 8.8 ± 1.4 μm/s. The inset shows an expansion of the data from 0 to 2.0 μm/s. All filaments moving 2.0 μm/s or less were used for the computation of the standard deviation of the speed of the slow rates, whereas all filaments moving at 6.0 μm/s or greater were used for the fast rates. The conditions of the motility assay are described in (*17*).

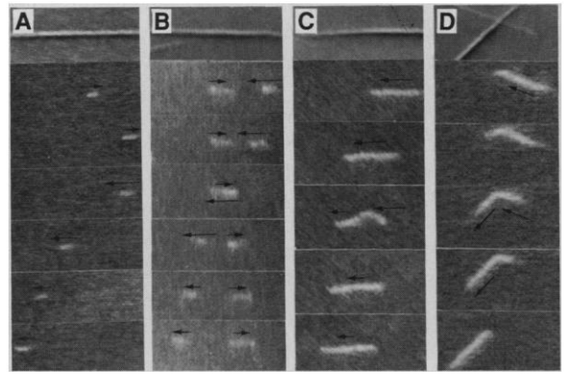

Fig. 3. Sequential video images showing different trajectories of fluorescently labeled actin filaments translocating along individual myosin filaments. The DIC image of the myosin filament is shown at the beginning of each sequence of fluorescence images. (**A**) An actin filament sliding away from the center of the myosin filament momentarily detached and then reattached to the same myosin filament and started sliding in the reverse direction. Arrows show the direction of movement, and the length of the arrow indicates whether the filament was moving at the slow (short arrow) or the fast speed (long arrow). (**B**) Two filaments passed each other unimpeded and changed speeds on a single thick filament. (**C**) A long actin filament whose leading end had just entered into the region of opposite polarity is shown. When this occurred the forward movement of the actin filaments slowed dramatically, but the movement of the tail end of the actin filament, which was still in contact with myosin heads of the correct polarity, continued to move fast and form a loop over the bare zone. (**D**) A single actin filament transferred from one myosin filament to an adjacent one after undergoing a large change in the angle of movement.

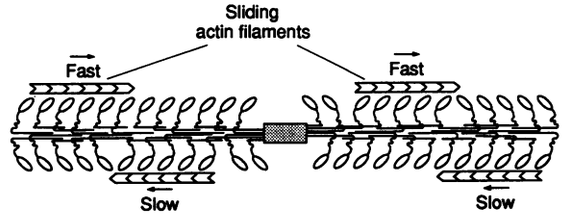

Fig. 4. Schematic diagram showing the allowed sliding interactions of the polar actin filaments with the bipolar myosin filament. The arrows indicate the direction of movement. The myosin heads are schematically shown at the ends of their power strokes. The "reverse chevrons" concept for the myosin heads contacting actin moving away from the center of the myosin filament is taken from Reedy *et al.* (*10*). The crosshatched area represents the bare zone.

results also suggest that kinetic or structural constraints are imposed on the rotated myosin heads, since the speed of actin filaments sliding away from the center of the myosin filament is about nine times less than that of actin filaments sliding toward the center of the myosin filament. The rotated myosin heads may be constrained so that the detachment of the rotated myosin heads from actin would occur at a much slower rate than that which normally occurs. Alternatively, the distance of the power stroke of the rotated head may be less than if the head is not rotated.

In the in vitro motility assay the rate of translocation of the actin filament represents an unloaded velocity and does not directly give a measure of force production of the myosin (*15*). We do not know whether actin filaments traveling away from the center of the myosin filament can produce as much force as that which can be produced when the actin filaments are traveling toward the center. We note that vertebrate striated muscles that have undergone extensive contractions have reduced unloaded shortening velocities and force (*16*). During normal active contraction in vertebrate skeletal muscle the precise arrangement of the myosin and the actin filaments and the Z-line limit the extent to which the actin filament can cross the bare zone (*1*). In the cytoplasm of nonmuscle and some smooth muscle cells, actin filaments display a broad range of lengths and maintain very complex relation with myosin filaments (*7*). Our results suggest that any given orientation of the polar actin filaments can interact with any portion of the myosin filament and still contribute to force generation and movement.

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Autophosphorylation of Protein Kinase C at Three Separated Regions of Its Primary Sequence

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The major autophosphorylation sites of the rat βII isozyme of protein kinase C were identified. The modified threonine and serine residues were found in the amino-terminal peptide, the carboxyl-terminal tail, and the hinge region between the regulatory lipid-binding domain and the catalytic kinase domain. Because this autophosphorylation follows an intrapeptide mechanism, extraordinary flexibility of the protein is necessary to phosphorylate the three regions. Comparison of the sequences surrounding the modified residues showed no obvious recognition motif nor any similarity to substrate phosphorylation sites, suggesting that proximity to the active site may be the primary criterion for their phosphorylation.

THE CALCIUM- AND PHOSPHOLIPID-dependent protein kinase C is a central mediator of signal transduction in the nervous system and other cell types (*1*). It phosphorylates substrates on serine or threonine residues in response to the lipophilic diacylglycerol (DAG) produced by phospholipase C (*2*). In addition to phosphorylating substrates, protein kinase C also phosphorylates itself (*3–6*). Autophosphorylation has been shown to alter the affinity of the protein for the tumor-promoting phorbol esters (*4*), to increase its sensitivity to calcium (*4*), and to increase its rate of H1 histone phosphorylation (*5*). Autophosphorylation has also been proposed to alter the association of protein kinase C with membranes (*7*) and to increase the enzyme's sensitivity to proteolytic down-regulation (*8*).

Autophosphorylation of protein kinase C follows an intrapeptide mechanism in which a single polypeptide chain phosphorylates itself (*9*). The evidence for this surprising finding is obtained with mixed micelles (*10*) that harbor a single protein molecule per micelle. The rate of autophosphorylation is independent of protein concentration and is the same as the rate measured with lipid bilayers, a more physiological condition (*9*). This mechanism contrasts with that of the insulin receptor (*11*), the type II cyclic adenosine monophosphate (cAMP)–dependent protein kinase (*12*), the cyclic guanosine monophosphate (cGMP)–dependent protein kinase (*13*), and the calcium calmodulin–dependent protein kinase (*14*); these are multisubunit kinases and apparently undergo interpeptide autophosphorylation. Surprisingly, both the catalytic kinase domain and the regulatory lipid-binding domain of protein kinase C are phosphorylated in this reaction (*4, 5, 9*). These observations raise important questions about the understanding of protein structure and the regulation of this critical enzyme, and therefore, we have identified the modified residues in the primary sequence.

The gene encoding a rat β isozyme of protein kinase C (PKC βII) was cloned into a baculovirus expression vector and the protein was purified from Sf9 insect cells infected with the recombinant baculovirus (Fig. 1). The purified PKC βII was identical to the enzyme purified from rat brains by all criteria examined. The recombinant protein comigrated on an SDS-polyacrylamide gel

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