Intragenic Suppressors of Dictyostelium Myosin G680 Mutants Demarcate Discrete Structural Elements: Implications for Conformational States of the Motor

Bruce Patterson

Department of Molecular and Cell Biology, University of Arizona, Tucson, Arizona 85721

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ABSTRACT

We are using intragenic suppression to generate inferences about currently inaccessible conformations of the molecular motor myosin. We identified 19 unique suppressors of Dictyostelium G680A and G680V mutants and find that they are structurally and chemically restricted, suggestive of specific, compensatory mechanisms of action. Suppressors cluster in two adjacent elements of the myosin structure, one of which is dominated by substitutions that increase the volume of hydrophobic residues. The suppressors outline a general model for the mechanism of suppression of the G680A and G680V alterations. Secondary phenotypes conferred by suppressors independent of the G680 changes demonstrate that they will be useful substrates for biochemical and genetic characterization.

As the tools of molecular biology have grown ever more powerful, questions about *how* bio-machines such as pumps, switches, and motors perform their jobs have become increasingly interesting and addressable. One technique that has proven powerful in dissecting pathways has yet to be harnessed for the study of mechanics of individual molecular machines: suppressor genetics. We are addressing this deficit by using suppressor genetics to unlock the secrets of the molecular motor myosin. Starting with a conditionally defective allele of myosin of known biochemical defect, we have isolated a family of intragenic suppressors. The locations and properties of these suppressors yield insights into the structural components of the motor and their conformational states during the course of the stroking cycle.

The starting mutations in this study are the G680A and G680V mutations of Dictyostelium myosin II. The G680V allele was isolated as part of a hunt for mutations conferring cold-sensitive myosin phenotypes (Patterson and Spudich 1995, 1996), while G680A was created by site-directed mutagenesis (Patterson *et al.* 1997). The G680V mutation perturbs the myosin cycle biochemically in several ways. The most noteworthy feature of the G680V mutant's ATPase cycle is that, in comparison to wild type, it overoccupies an actin-bound state; this state exhibits a salt sensitivity that is not characteristic of ADP-bound or nucleotide-free (rigor) states. It also increases the affinity of the mutant myosin for two forms of nucleotide, ADP and ATPγS, on the order of 40-fold. We have therefore proposed that this mutation interrupts the motor cycle at a previously uncharacterized point early in actin binding (Patterson *et al.* 1997).

To further understand the defects arising from the alteration of this position in the motor and to discover ways in which the motor could change to compensate for the G680A/V defects, we have isolated intragenic suppressors of each allele. Suppressors potentially provide three important types of information about the functioning of the myosin motor: First, their recovery indicates that despite the apparently pleiotropic defects of the position 680 alterations, the *critical* defect(s) can be reversed or compensated by *single* alterations. Second, their locations within crystal structures can give us a first clue as to what functionalities of the motor require alteration to restore function to motors bearing position 680 deficiencies. Third, by biochemically characterizing the consequences of these new alterations, we will further illuminate the defects for which they are compensating and gain insight into the mechanism of the compensation.

Our suppressor isolation methodology relies on the phenotypic consequences of myosin deficiency in Dictyostelium. Cells lacking wild-type myosin function can be maintained on surfaces such as petri dishes, but they are deficient in cytokinesis in suspension, generation of cortical tension (Pasternak *et al.* 1989), fruiting body construction, and plaque expansion on bacterial lawns (De Lozanne and Spudich 1987; Knecht and Loomis 1987, 1988; Patterson and Spudich 1995). The latter phenotype is the one we have used for suppressor selection: restoration of myosin function gives rise to a clone of cells that distinguish themselves by their rapid invasion and consumption of the bacterial lawn compared to their neighbors possessing unrepaired myosin. The

Address for correspondence: LSS452, Department of Molecular and Cell Biology, University of Arizona, Tucson, AZ 85721.
E-mail: patterso@u.arizona.edu

suppressors identified in this fashion are almost exclusively intragenic.

We combined a PCR-driven marker rescue strategy with in vivo homologous recombination to localize each suppressor mutation to ~400-nt segments of the myosin gene. These regions were sequenced, and the resident suppressor mutation was identified. G680V suppressors have also been expressed in the absence of the position 680 changes, and their consequences were characterized phenotypically.

In our analysis of mutants, we have made several striking findings: (1) the majority of the suppressors reside at positions in the tertiary structure that are distant from residue 680; at most, one of the ~18 suppressors can potentially contact G680V; (2) a significant fraction of the suppressors are deficient for myosin function in the absence of the position 680 change (*i.e.*, the mutations are mutually suppressing); (3) all the mutations affect highly conserved residues; and (4) one "family" of suppressors highlights a central portion of the myosin structure that is suggestive of their mode of action.

MATERIALS AND METHODS

Strains and media: Initial experiments were performed in HS10, a cell line in which the myosin heavy chain sequence has been deleted completely (Ruppel *et al.* 1994). The nonaggregating SPERA cell line was derived as described in results and was used for isolation of all G680V suppressors excepting the first two isolates of N483S.

Cell culture conditions were as described by Sussman (1987). Bacterial lawns were made by flowing 1 ml of an overnight culture of *Klebsiella aerogenes* over the entire surface of an SM/16 plate [as described in Sussman (1987) for SM plates, except that glucose, peptone, and yeast extract were used at 1/16th of the recommended levels]. Excess liquid was aspirated off, and the plates were incubated overnight at room temperature. All suppressor isolation experiments were performed at 22°.

Plasmids and transformation: All plasmids introduced into Dictyostelium were based on the pLittle or pTIKL chassis. Myosin genes were introduced as *Bam*HI-*Sac*I fragments that included either the wild-type Dictyostelium promoter (Patterson and Spudich 1996) or the actin-15 promoter (Egelhoff *et al.* 1990). Plasmids were introduced via electroporation using the following protocol: cells were harvested from confluent, 100-mm petri dishes after thorough aspiration of all media. Cells were harvested using gentle trituration with 1.5 ml ice-cold E-pore buffer (50 mm sucrose, 10 mm $NaPO_4$) and spun gently in an Eppendorf tube for 30 sec. Cells were resuspended in 300 μl E-pore buffer. A total of 45-μl aliquots were added to 1–2 ml of 0.2–2 mg/ml DNA (in 2 mm Tris pH 8.0, 0.2 mm EDTA) and transferred to an ice-cold 0.1-cm electroporation cuvette. Electroporation conditions were 800 V, 3 μF, 1000 Ω resistance. After electroporation, 350 μl of cold HL-5 supplemented with heat-killed *K. aerogenes* was added to the cuvettes. The mixture was then added to chilled six-well petri dishes containing 1.5 ml HL-5. Cultures were incubated at 22° for 24 hr, and G418 was added to bring cultures to 10 μg/ml G418. After an additional 48 hr, the media was replaced with HL-5 containing 8 μg/ml G418. Resulting transformants were combined for all further experiments. All sequencing was performed on pBluescript or pBC plasmids (Stratagene, La Jolla, CA) bearing PCR-derived fragments of the myosin gene cloned using convenient restriction sites.

Mutagenesis and suppressor isolation: To isolate suppressors, HS10 or SPERA cells transformed with plasmid bearing a mutant myosin were grown to confluency in a six-well plate. Cells were harvested by trituration and spun gently. Cells were then resuspended in 100 μl HL-5. 10-μl aliquots were dripped onto bacterial lawns (usually 8 spots per 100-mm plate or 18 spots per 150-mm plate). After 24 hr, plaques had cleared and the plates were irradiated with UV light at 800 $\mu J/cm^2$ in a Stratalinker (Stratagene). Revertant "blisters" were usually visible within 5–7 days. Revertants were harvested by scraping the leading edge of a plaque with a toothpick and transferring to a six-well plate. DNA was prepared from these cells using a DNA prep kit (Qiagen, Chatsworth, CA).

Polymerase chain reaction: PCR was performed using Pfu polymerase (Stratagene). The oligos used were as follows: for the 5′ segment of the myosin-coding sequence, PIHDRT, 5′-AGCGGATCCAATTCATGATAGAACTTCA-3′, and HLN, 5′-GTCTGTCGACGATGAGGATTTTTCAACGTTGA-3′, for the internal segment, VLEQQIL, 5′-TGTTCTAGAACAACAAA TTCTCCAAGCC-3′ and ELDQ, 5′-TCGGGATCCTGTTGTA ATGGATCTTTGTTC-3′, and for the 3′ segment, GLDSQT, 5′-TGGTCTAGATTCACAAGCCACTATC-3′ and ARIE, 5′-TTGTTCTCGAGCTTCTTCAATACGAGC-3′. Subcloning was performed into an engineered myosin vector containing one or more of the following engineered or endogenous restriction sites: *Bsp*HI at nucleotide 12 (the A in ATG encoding the start methionine being 1), *Xba*I at 630, *Sal*I at 937, *Eco*NI at 1164, *Xho*I at 1231, *Xba*I at 1521, *Bgl* II at 1782, and *Xho*I at 2270. In all cases, successful introduction of the mutation was recognized by amplifying the mutation on a PCR fragment with an endpoint at one of these restriction sites that encoded a silent nucleotide change (*e.g.*, changing the *Bgl* II site at 1782 to a *Bam*HI site such that a hybrid site was created upon insertion into the wild-type sequence). Sequencing subclones for unmapped G680V suppressors were generated by preparing two PCR products, using oligos PIK 5′-GACTGGTAC CAATCAAATTCGATGGTGTC-3′ and GAS 5′-pGAAGCAC CACTAATGAAACCA-3′ as one pair and QERK 5′-TGA CGAGCTCTGTCAAGAGAGAAAAGCT-3′ and QA-TID 5′-pA AATCGATAGTGGCTTGTGAA-3′ as the second. The first product was cut with *Kpn*I, the second with *Sac*I, and the two pieces were cloned end to end into pBluescript (Stratagene) cut with *Kpn*I and *Sac*I and sequenced.

Suppressor mapping: Plasmids recovered from revertant cells were initially purified and reintroduced into Dictyostelium to check whether or not the suppressor was plasmid linked. Because 100% of recovered plasmids passed this test, however, it was eventually dropped. Suppressors were localized by preparing three PCR fragments using the DNA recovered from each revertant. The three fragments were generated using oligos PIHDRT-HLN, VLEQQIL-ELDQ, and GLDSQT-ARIE. Each fragment was then cotransformed with either plasmid bearing either the G680A or G680V myosin, as appropriate. Fragments were cut with *Eco*NI (for the PIHDRT-HLN fragment), *Bst*XI (for the VLEQQIL-ELDQ fragment), or *Bst*XI and *Bgl* II (for the GLDSQT-ARIE fragment). Transformants were assayed for plaque expansion rate, and those showing 150% or greater rates of expansion compared to the parent plasmid were judged to result from introduction of the suppressor. The location of the suppressor was deduced from examining which PCR fragments conferred suppressor activity. The appropriate region of DNA was then recovered by PCR and sequenced. Once we had established that the majority of G680V suppressors could be found among amino acids 90–250 or 400–500, we designed a sequencing strategy

to recover these regions. We then sequenced them without engaging in mapping, and we mapped only those suppressors that were not identified by sequencing this region.

Growth rate measurements: Plaque expansion rates were measured on lawns of *K. aerogenes* that were prepared as described above. Each Dictyostelium strain to be measured was grown to confluence in a six-well petri dish. Cells were harvested by trituration and concentrated by low-speed centrifugation followed by resuspension in 10 μl HL-5. Eight microliters of the suspension was dripped onto a bacterial lawn. Plaque size measurements were made on successive days by comparing the plaque to a set of standard circles. Measurements were continued as long as most plaques demonstrated roughly linear growth rates. Final growth rates were calculated by figuring the total growth over the linear phase divided by the number of days grown. All rates were compared to those of wild type spotted on the same plate to control for variability in plates, lawns, and other environmental variables. The rate compared to that of wild type was generally observed to be reproducible ±10 percentage points.

RESULTS

Isolation of suppressors: The primary methodology we used in suppressor isolation took advantage of one of the striking phenotypes of myosin deficiency in Dictyostelium: poor colony expansion rates on bacterial lawns. We had previously demonstrated that this phenotype was sufficiently powerful for marker rescue experiments (Patterson and Spudich 1995). We reasoned that it represented an ideal tool for the identification of intragenic suppressors. In the isolation of G680A suppressors, we took advantage of a second deficiency of Dictyostelium cells expressing functionally impaired myosin: failure to erect normal fruiting bodies. Thus, we sought mutant derivatives of G680A myosin-expressing cell lines that simultaneously acquired the ability to expand rapidly on bacterial lawns and restored the ability of the cell line to produce recognizable fruiting bodies. Mutagenesis was achieved by exposing plaques of cell lines expressing only G680A myosin to UV light, followed by outgrowth and screening for fast-growing "blisters" of mutant cells that gave rise to fruiting bodies. Of 15 revertant cell lines isolated in this manner, 14 contained second-site mutations in the G680A mutant myosin II gene, while the 15th was a true revertant (it also bore a second nearby change that we have not characterized).

Suppressors of G680V were difficult to acquire by the method described above; indeed, only two suppressor events were identified through several rounds of screening dozens of mutagenized plaques. To increase our sensitivity of detection, we took advantage of our observation that myosin-null cell lines occasionally give rise to faster-expanding plaques after mutagenesis (B. Patterson, unpublished data). Reasoning that these changes might confer still more rapid expansion in the presence of functional myosin, we performed two rounds of selection for more rapidly expanding myosin-null cell lines. The resulting cell line was designated the SPERA line. While SPERA cells were only mildly enhanced in plaque expansion compared to their predecessors (1–1.2 *vs.* 0.6–0.8 mm/day), addition of the wild-type myosin gene resulted in marked improvement in plaque expansion rates over parental cells transformed with wild-type myosin genes (6–7 *vs.* 2.5–3.5 mm/day). One important side effect of the mutation(s) resulting in rapid plaque expansion is that cells no longer aggregate or form fruiting bodies. We have not determined whether blocking aggregation is sufficient to convert wild-type Dictyostelium cells to a rapid aggregation phenotype, although preventing aggregation using 2 mm caffeine does not enhance the plaque expansion rate (B. Patterson, unpublished data).

When the G680V myosin gene was introduced into the SPERA cell line, plaque expansion rates were comparable to cells lacking myosin. After UV mutagenesis, however, we were able to recover rapid plaque expansion mutants. While only ~10% of these proved to express the G418 resistance anticipated for cells containing the G680V-expressing plasmid, all of these contained myosins bearing intragenic suppressors to the G680V mutation.

Localization and sequencing of suppressors: To map the suppressing mutations, we developed a strategy related to gap repair used in *Saccharomyces cerevisiae* gene studies. We induced homologous recombination between PCR fragments derived from the suppressor plasmids and from plasmids containing only the G680A or G680V mutations by using restriction enzymes to introduce "gaps" in the target plasmids. A series of PCR fragments were generated such that in sum, they overlapped to include DNA encoding the entire myosin head domain (2500 nt) but were individually 800–1000 nt in length. Plasmids bearing the starting mutation, but not the suppressor, were cut at sites spanned by the PCR fragment being tested (in imitation of procedures used to enhance homologous recombination in Saccharomyces). Cut plasmid and PCR fragment were coelectroporated into the SPERA cell line, and transformants were selected for G418 resistance arising from a marker borne on the target plasmid. Populations of transformants arising from a single transformation were mixed and tested for plaque expansion rate; those exhibiting rates indicative of suppression of the mutation were concluded to have arisen from PCR fragments bearing the suppressor.

Once the region bearing the intragenic suppressor mutation had been narrowed down to ~500 nt or less, a fragment spanning the region was cloned into a vector for sequencing. The nucleotide sequence was then determined, and in virtually all cases, it contained a suppressor mutation. In those cases where no suppressor was detected, the adjacent region was mapped, and in all cases, a suppressor was found; we conclude that this inaccuracy in the mapping arises from "run-on" PCR products that we failed to destroy in the nuclease step

(see materials and methods). The DNA sequence changes and predicted amino acid alterations are shown in Table 1.

Expression of suppressors: For a suppressor to be fully useful, it must have altered properties beyond merely countering the defect caused by the original mutation. For this reason, we separated several of the suppressors from the generative position 680 mutation and tested their properties *in vivo*. Mutants were separated by using PCR of a region containing only the suppressor followed by replacement of a fragment from an otherwise wild-type myosin gene using convenient restriction sites. Mutants generated in this manner were then tested for myosin function using the plaque expansion rate assay described above. While many of the suppressors of the G680V mutation behaved essentially as nulls in the plaque expansion assay (Table 2), those that suppressed *only* G680A were indistinguishable from wild type (B. Patterson, unpublished data). However, several G680A suppressors were also capable of suppressing G680V and conferring growth defects in isolation of G680 changes; conversely, several G680V suppressors (such as G182C and E476Q) confer little, if any, plaque expansion rate phenotype on their own.

DISCUSSION

We have pioneered a suppressor-driven approach to myosin structure/function. By isolating intragenic revertants of G680V, a Dictyostelium myosin II cold-sensitive mutant, we have (1) demonstrated that the initial G680V defect can be repaired (at the level of biological phenotype) by single amino acid alterations, (2) demonstrated a compensatory relationship between changes in amino acid volume at (at least) two noncontiguous positions in the myosin structure, (3) generated tools for further understanding both the initial defect and its remediation, and (4) heightened our focus on the enigmatic 466–496 α-helix.

Position 680 mutations can be suppressed by altering highly conserved residues within myosin: The first question of this approach was whether the G680A and G680V mutations could be suppressed at all. These mutations change an absolutely conserved glycine residue and give rise to mutants with a variety of apparent defects, including altered nucleotide affinity and extended actin-binding times. While the fact that the mutants are only cold sensitive rather than completely inactive suggested that they were not irreparably altered "monsters," the search for compensatory changes within myosin was unprecedented.

The isolation of 19 different intragenic suppressors clearly demonstrates that the mutations at position 680 can be compensated by several alterations elsewhere in the motor domain. Indeed, by visual inspection of the three-dimensional locations of the suppressors, it is difficult to neatly assign all the suppressors to a single functional element of the motor, such as P_i release. This suggests the possibility that there may be a variety of ways to "tweak" the motor back to function. Preliminary biochemical analysis of the suppressors suggests that this is indeed the case (Y. Wu and B. Patterson, unpublished results).

Even in the absence of biochemical characterization, the locations of the mutations indicate that they are altering critical residues. All of the suppressors affect highly conserved residues, some absolutely so (see Table 1). For example, P128Q, G182C, N235D, L453F/Y, G240N, and E476Q all change residues absolutely conserved among all myosins, while M91I, N483S, and L676F change residues absolutely conserved in myosin II.

Besides changing only conserved residues, suppressors show striking clustering in the primary structure. By displaying the positions of unique changes (changes at the same position were only counted once unless the changes produced different amino acids), we detect distinct groupings (see Figure 1). Four-fifths of the mutants affect residues between positions 100 and 200 or 450 and 500. This interval comprises approximately one-fifth of the motor domain-encoding sequences. Viewing the tertiary structure (Figure 2A), we detect no changes at either end of the molecule, and there was a clustering of alterations in a belt or disk centered around the ATP-binding site. This localization clearly excludes all the actin-binding sites as well as the light chain-binding domain.

Analysis of G680A and G680V suppressors: The chemical changes represented in the collection are extremely nonrandom. While at the DNA level we detect all six distinguishable transitions and transversions, the predicted amino acid changes are dramatically skewed: more than one-third of the changes are L → F alterations (counting each change at a given position once regardless of duplication), whereas of the remaining changes, only two (G → C and I → M) are represented twice, and the rest are represented once. While it is difficult to calculate the effect of UV light mutagenesis on different codons (*e.g.*, there are 6 leucine codons, and we have only recovered those of the form TTPurine changed to phenylalanine), it would nonetheless appear that alteration of leucine to phenylalanine is overrepresented in the collection. These changes are distributed evenly in the primary structure, with one in each of the intervals shown in Figure 1, except for only the 50–100 interval.

Even more striking is the fact that of the 19 amino acid changes observed, only one, N483S, substitutes a markedly smaller amino acid for a larger one (Table 1). This position is also distinctive in that it is the only one which could directly interact with position G680. This alteration is thus the only possible instance of a "lock and key" type of suppression in which an alteration in a directly interacting amino acid compensates for the primary lesion by restoring the wild-type structure. It

TABLE 1

Suppressors of G680A and G680V myosin mutants

Mutant[a]	Codon change[b]	No. Suppressors[c] G680A	No. Suppressors[c] G680V	Volume change[d]	Percent Conserv.[e]	Chick no.[f]
M91I	ATG → ATC	|	—	−2	II = M; LMF	91
G120C	GGT → TGT	|	—	+41	II = G, GA	120
L123F	TTG → TTT	|	—	+35	LMF	123
P128Q	CCA → CAA		|	+32	100	128
L175F	CTC → TTC	|		+35	CLIMV	175
L176F	TTA → TTT		|	+35	II = L; LIMV	176
I177M	ATC → ATG		|	+2	CIV	177
G182C	GGT → TGT	|	||	+41	100	182
T189I*	ACC → ATT		|	+45	TSA	189
I193M	ATT → ATG	|	—	+2	IQLM	193
L216F	CTC → TTC	|	|||||	+35	ILV	223
N235D	AAT → GAT		|	−11	100	242
G240N*	GGT → AAT		|	+69	100	247
L453F	TTG → TTC,T	|	|||||	+35	100	462
L453Y*	TTG → TAC	|		+35	100	462
E476Q	GCA → CAA	|	||	+6	100	485
N483S	AAT → AGT		|||	−36	II = N; INT	492
L638F	TTA → TTT	|	|	+35	AVLQM	657
L676F	TTA → TTT	|		+35	II = L; ILV	695
V192F	GTC → TTC		(+)	+61	II = V; AILV	192
G240C	GGT → TGT		(+)	+41	100	247
G240V	GGT → GTT		(+)	+75	100	247
G680A	GGT → GCT			+25	100	699
G680V	GGT → GTT			+75	100%	699

[a] Deduced amino acid changes and their positions are indicated.

[b] The nucleotide sequence alteration(s), that gives rise to the supressors; those marked with an asterisk contain two nucleotide changes.

[c] The number of times a given suppressor was independently identified as a suppressor of G680A or G680V. A minus sign in the G680V column indicates that the G680A suppressor indicated failed to show suppression of G680V when tested by *in vivo* recombination. A plus sign in the G680V column indicates that the suppressor was not isolated in the mutant hunt and was generated by recombinant DNA techniques.

[d] The predicted difference in size in cubic angstroms of the mutant amino acid residue compared to that of the wild type (Chothia 1975).

[e] The conservation of the wild-type residue at this position in an alignment of 80 myosins (Sellers and Goodson 1995); 100 indicates the residue at this position is invariant, and the common residues are shown otherwise. Wherever a residue is highly conserved in myosin II is indicated as "II = X", with amino acids found in other myosins listed afterward.

[f] The corresponding residue number for the chicken crystal structure.

should also be noted that this residue, along with E476, is on the opposite side of the 466–496 α-helix that we believe to be a primary focus of G680V suppressors (see below).

The fact that the G680V mutation, itself a substitution of a large hydrophobic residue for a smaller one, is suppressed by *increasing* the size of other, primarily hydrophobic residues illuminates the mechanism of suppression. It is highly unlikely that these changes reverse or directly compensate the defect introduced by the G680V alteration [presumably derived from increased volume of the side chain; the G680A change is biologically and biochemically milder (Patterson *et al.* 1997)]. One plausible interpretation of this finding is that the G680V mutation "damages" or destabilizes one of the *states* (conformations) of the myosin motor (or inhibits a transition out of a state), and that the suppressor mutations restore the *balance* between conformations (or ease a transition between states) by similarly destabilizing or inconveniencing a competing state.

It is notable that several of the suppressors alter residues in regions of assigned function or that have been biochemically characterized in other studies. Perhaps the most notable of these is the G182C change that alters an absolutely conserved residue in the "P loop" (Smith and Rayment 1996a). This domain is conserved not only among myosins, but can be identified in diverse proteins such as kinesin, ras, and F_1-ATPase. In all cases, the P-loop backbone hydrogen bonds with the β- and γ-phosphates of ATP. In F_1-ATPase, the residue corre-

TABLE 2

Growth phenotypes associated with suppressors of G680V

Mutant	Single	Double with G680V
G680V	+	—
P128Q	++	+++
L176F	++++	++++
I177M	++	+++
G182C	++++	+++
T189I	+	++++
V192F	+	++++
L216F	+	++++
G240V	++	+++
L453F	+	+++
E476Q	++++	++++
N483S	+	++++
L638F	++	++++

Growth phenotypes of suppressors with and without the G680V mutation. Plaque expansion rates of Dictyostelium SPERA cells transformed with single- or double-mutant myosins are shown. Data are generated by comparing the plaque expansion rate to that of SPERA cells transformed with wild-type myosin. To accurately represent the precision of the data, it is shown in 20% increments, where + represents 20–40% wild-type rate (untransformed SPERA cells fall into this category), ++ is 41–60%, +++ is 61–80%, and ++++ is 80–100%.

sponding to G182 is also a glycine, and its alteration to cysteine has been genetically characterized and found to give rise to cold sensitivity (Shen *et al.* 1994). P128Q affects a residue that directly contacts the adenine moiety.

Several of the mutations alter residues that are close to highly conserved elements of P-loop proteins or that feature prominently in models of myosin function. L453 is adjacent to residues 455 and 457, about which critical rotations and angle changes take place in the conformational alterations that have been seen in myosin to date (Fisher *et al.* 1995; Smith and Rayment 1996b). N235 and G240 flank residue R238, one of the residues proposed to control the egress of P_i (Yount *et al.* 1995). Shimada *et al.* (1997) recently created alanine mutations at positions 235 and 240. Their characterization indicates that N235A and G240A show decreased ATPase activities, and G240A is greatly impeded in *in vitro* motility of actin filaments. We also tested G240V and G240C, which we had recovered in our initial cold-sensitive mutant hunt (Patterson and Spudich 1995; identified in strains HS85 and HS87; B. Patterson, unpublished data), and found that they also suppressed the G680V mutation.

The tightest clustering observed among the suppressors is the group L175F, L176F, I177M, T189I, I193M, and L453F. This group defines a discrete cluster of residues in the myosin structure (Figure 2, B and C). Visual inspection of this region of myosin suggested that changes at residue V192 might have similar effects on the structure. Because we had recovered V192F in our original screen for cold-sensitive mutants (in strains HS94 and HS109; Patterson and Spudich 1995; B. Patterson, unpublished results), we assayed its ability to suppress the G680V mutation. Indeed, the V192F/G680V double mutant grows much better than the G680V parent mutation. Of all the substitutions in this region that have been shown to suppress the G680V mutation, virtually all mutant residues occupy larger volumes than those they replace (Chothia 1975). The two termini of the grouping, L453 and L176, are closely juxtaposed with highly conserved helix 466–496 in the vanadate structure of Fisher *et al.* (1995), whereas only L453F approaches closely in the beryllium fluoride structure of Smith and Rayment (1996b). We are currently characterizing these mutants biochemically to see if their properties are similar and to determine how they perturb the motor cycle.

Three observations lead us to consider the possibility that the Cluster suppressors (L175F, L176F, I177M, T189I, V192F, I193M, and L453F) were acting via steric constraint of helix 466–496: (1) the close apposition of the "Cluster" mutants, particularly residues L453 and L176, to the 466–496 helix, (2) our previous observation

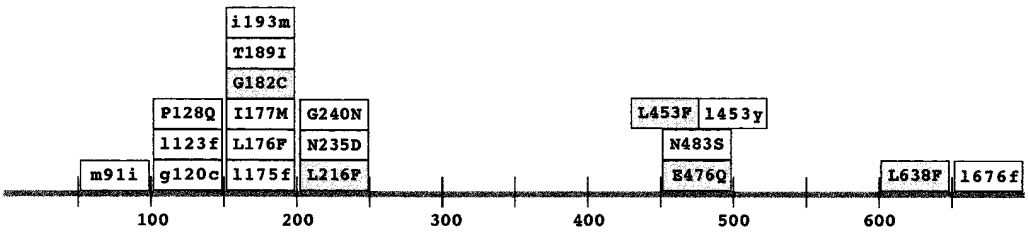

Figure 1.—Distribution of G680 suppressor mutations in the motor domain. Unique mutants identified in each of the 50 amino acid segments of the first 700 amino acids of Dictyostelium myosin II are indicated. Suppressors isolated against G680A appear in lowercase, while suppressors isolated against G680V appear in capitals. Those G680V suppressors also isolated against G680A are shown with a gray background.

of the conspicuousness of the 466–496 helix as a target for cold-sensitive mutants [residues 467 and 494 are helix elements that can mutate to cold sensitivity, as can nearby residues 501, 680, and 691 (Patterson and Spudich 1996)], and (3) the fact that the G680V mutation introduces a bulkier side chain on an opposing face of the 466–496 helix. These relationships can be seen in Figure 2, B and C. The L638F mutation can be viewed similarly; while it is not contiguous with residues in the Cluster, it is positioned diametrically opposite the G680V change that it suppresses, and it contacts the 466–496 helix near residues 473–477.

The E476Q and N483S mutations reside in the highly conserved α-helix (residues 466–496 in Dictyostelium myosin) mentioned above. It is interesting to note that these residues, separated by two turns of the helix, both face the outside of the molecule away from the 175, 176, 177, 189, 192, 193, 453 Cluster discussed above. The E476Q alteration is identical to one introduced and characterized by Ruppel and Spudich (1996). This mutant dramatically perturbs the properties of the motor; while its basal ATPase is indistinguishable from that of wild type, and its ATPase upon actin activation is 1/3 that of wild type, its ability to translocate actin filaments in vitro is only ~1/20th that of wild type. These results indicate that ATP hydrolysis has been largely uncoupled from actin filament translocation. It is not immediately obvious why these properties compensate for the defects in G680A and G680V, but the alteration of the link between actin binding and (presumably) P_i release is intriguing. Two features of the N483S mutation are notable: it represents substitution of a smaller residue on the opposite side of the helix from the Cluster alterations (which introduce larger residues), and its position in three dimensions is close to that of the G680V mutation.

A final critical point is that the majority of the G680V suppressors confer significant biological defects when isolated from the generative mutation. As with intergenic suppression (Adams and Botstein 1989), we interpret this phenomenon as an indicator of specific suppression [as opposed to global enhancement of stability (Shortle and Lin 1985), for example]. The existence of strong biological phenotypes caused by the isolated suppressors bodes well for both biochemical analysis and isolation of "chains of revertants" (Jarvik and Botstein 1975). Both types of experiments are currently underway.

Cold-sensitive myosin mutations suppress one another: One unanticipated result of this work was the finding that three of our original cold-sensitive mutants could suppress the cold-sensitive G680V mutation. These were V192F, G240C, and G240V. The suppressor screen identified changes at position T189 and I193, prompting us to test the V192F alteration, while recovery of G240N prompted us to test the G240C and G240V mutants. In all cases, we observed suppression of the

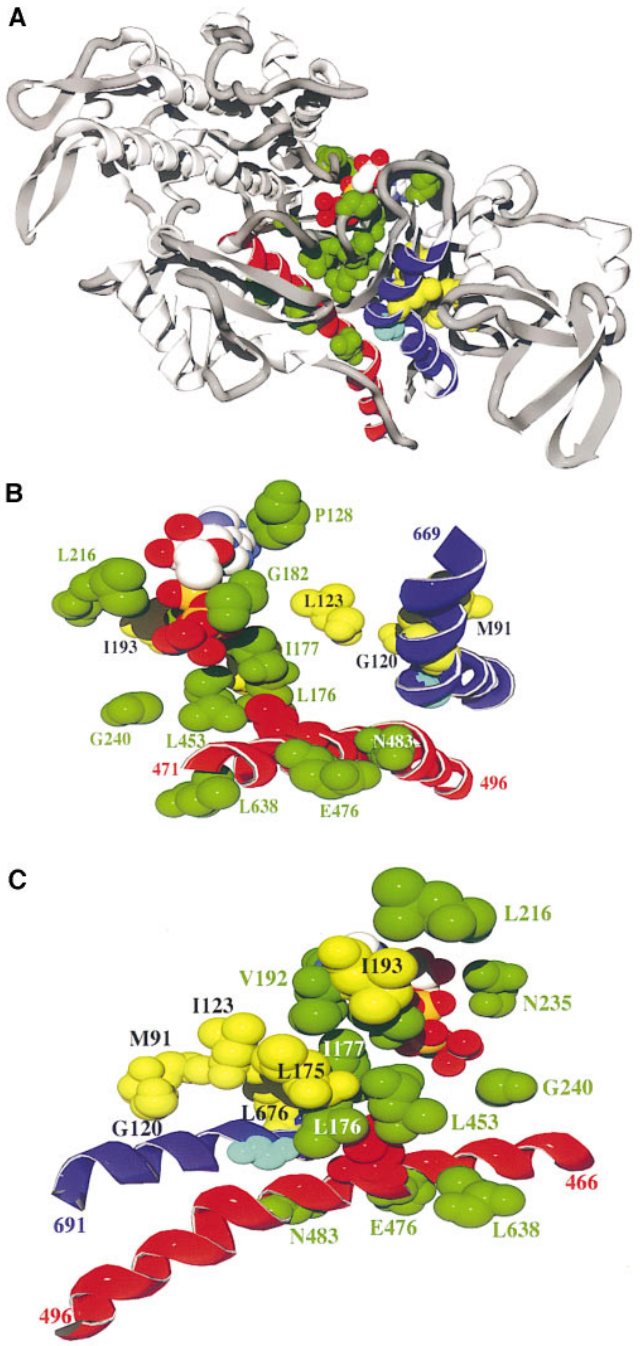

Figure 2.—Location of suppressors in the ADP · vanadate crystal structure of the Dictyostelium myosin II motor domain (Smith and Rayment 1996b). (A) Ribbon representation of the entire structure. (B) Isolation of the region containing position G680, the suppressors, and ADP. (C) A 180° rotation of B. In all figures, helix 466–496 is shown in red, helix 669–691 is in blue, and position G680 is space filled and colored cyan. Position L478 is space filled in red. Positions capable of giving rise to G680V suppressors are space filled and colored dark green, while those giving rise to suppressors of G680A are space filled and colored lime. ATP is space filled using conventional atomic coloring, with the vanadate atom in gray. G680V suppressors are labeled in dark green or white; G680A suppressors are labeled in black. Helix termini are numbers without residue letters and are colored as the helix they label.

G680V mutation. The discovery that 3 of our original ~20 cold-sensitive mutants can suppress a fourth suggests the possibility that there are only a small number of ways in which cold-sensitive myosin function can be induced, and that the states or changes induced are in some way complementary. We have also observed this phenomenon in our characterization of actin-binding site, cold-sensitive mutants, where at least two more of the original cold-sensitive mutants have been found in suppressor screens (E. Misner and B. Patterson, unpublished results; Patterson and Spudich 1996).

A mechanical model for G680V and its suppressors: The striking feature of the locations of the suppressors vis-à-vis the G680V mutation is their relationship to the 466–496 helix. The addition of a valine side chain at position 680 would impinge upon this helix and constrain potential movements of the helix. It is therefore noteworthy that the Cluster group of suppressors (positions 175–177, 189, 192, and 453) increase the volume of residues on another face of the helix. Yet another suppressor, L638F, increases the volume of another side chain that is oriented toward the helix, opposite position G680. Finally, the change at position N483, on the opposite face of the helix from the Cluster, suppresses when changed to a *smaller* residue (the E476Q change is difficult to interpret in this light). These observations suggest that the effect of the suppressor changes is to "rebalance" constraints on the movement or positioning of the helix induced by the G680V change. One prediction of this model is that the defects introduced by the Cluster suppressors in the absence of the G680V change arise by "unbalanced" shifting of the helix opposite that induced by G680V. In this regard, it is interesting to note that helix 466–496 undergoes significant rotation and bending between the Dictyostelium myosin vanadate or AlF structures (Fisher *et al.* 1995; Smith and Rayment 1996b) as compared to the Dictyostelium BeF and chicken myosin crystal structures (Rayment *et al.* 1993; Fisher *et al.* 1995). Biochemical analysis of the G680V alteration suggests that its primary defect lies in reluctant P_i release; our preliminary results are consistent with the possibility that the Cluster mutants induce hyperactive P_i release. Intriguingly, residue R238, which may comprise one of the "guardians" of the back door (Yount *et al.* 1995), is intimately associated with the 466–496 helix, and is flanked in the primary structure by residues 235 and 240, which also appear as G680V suppressors.

Future directions: The mutants already in hand demonstrate the feasibility of our approach and offer several hints as to pathways of communication and conformational change operating within the myosin motor. We are currently engaging in biochemical characterization of these new mutants, primarily in the absence of the G680V mutation, to more precisely define the perturbations they introduce. Preliminary data indicate that the Cluster mutants share common biochemical defects consistent with compensation of the G680V-induced perturbations (Y. Wu, M. Nejad and B. Patterson, unpublished results). By comparing these changes to each other and to those we have characterized for G680V, we intend to map out a more complete picture of the mechanics of the myosin motor. We are also investigating the possibility that the 466–496 helix represents a key effector of myosin conformations because a large number of cold-sensitive mutants and suppressors (including G680V, the Cluster, E476Q, N483S, and L638F) lie in and around it. Details of ongoing work can be found at http://research.biology.arizona.edu/myosin/.

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