

# Engineering of the Myosin- $\text{I}\beta$ Nucleotide-binding Pocket to Create Selective Sensitivity to $N^6$ -modified ADP Analogs\*

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**Distinguishing the cellular functions carried out by enzymes of highly similar structure would be simplified by the availability of isozyme-selective inhibitors. To determine roles played by individual members of the large myosin superfamily, we designed a mutation in myosin's nucleotide-binding pocket that permits binding of adenine nucleotides modified with bulky  $N^6$  substituents. Introduction of this mutation, Y61G in rat myosin- $\text{I}\beta$ , did not alter the enzyme's affinity for ATP or actin and actually increased its ATPase activity and actin-translocation rate. We also synthesized several  $N^6$ -modified ADP analogs that should bind to and inhibit mutant, but not wild-type, myosin molecules. Several of these  $N^6$ -modified ADP analogs were more than 40-fold more potent at inhibiting ATP hydrolysis by Y61G than wild-type myosin- $\text{I}\beta$ ; in doing so, these analogs locked Y61G myosin- $\text{I}\beta$  tightly to actin.  $N^6$ -(2-methylbutyl) ADP abolished actin filament motility mediated by Y61G, but not wild-type, myosin- $\text{I}\beta$ . Furthermore, a small fraction of inhibited Y61G molecules was sufficient to block filament motility mediated by mixtures of wild-type and Y61G myosin- $\text{I}\beta$ . Introduction of Y61G myosin- $\text{I}\beta$  molecules into a cell should permit selective inhibition by  $N^6$ -modified ADP analogs of cellular processes dependent on myosin- $\text{I}\beta$ .**

Myosin molecules carry out mechanical work within cells, hydrolyzing ATP to produce force along actin filaments (1, 2). The myosin superfamily contains at least 15 major classes, eight or more of which are found in vertebrates. Because multiple myosin isozymes are found within each class, the superfamily is large; for example, the murine genome has more than 30 myosin genes (3, 4). Myosin molecules share a common three-domain structure: an actin- and ATP-binding head that carries out chemomechanical transduction, an extended neck or lever domain that binds light chains of the calmodulin family

and amplifies small movements within the head, and diverse tail domains that couple myosin molecules to other cellular structures (2).

Although roles have been identified for conventional myosin isozymes (the myosin-II class), elucidating cellular functions for unconventional myosin isozymes has been notably difficult. Cells may express a dozen or more myosin isozymes simultaneously (5, 6), and many of these isozymes have similar biochemical properties. In a few cell types, inhibition by isozyme-selective antibodies (7), chromophore-assisted laser inactivation (8), or precise localization within specialized structures (9, 10) has led to tentative identification of isozyme function. Roles for other isozymes have been inferred from consequences of null or inhibitory mutations, either those occurring naturally (4, 11–14) or from gene-targeting experiments (15, 16). As with other genes, null mutations can lead to developmental abnormalities or compensatory responses. Accordingly, gene deletion may not be the best approach for understanding myosin function.

An isozyme-selective myosin inhibitor that could be applied acutely would be a powerful tool for unveiling myosin function. Unfortunately, active-site conservation between myosin isozymes assures that nucleotide analogs that bind one isozyme should bind to others. Although inhibitory antibodies can be useful (7), they are often difficult to deliver easily into cells and may not successfully inhibit myosin molecules that are bound to other proteins within cells.

We chose instead to design a mutation that would render a myosin isozyme sensitive to a nucleotide analog that otherwise did not bind native myosin molecules. Once these mutant myosin molecules have been introduced into a cell, processes dependent on that isozyme should become sensitive to the inhibitor. This strategy uses as its inspiration other examples of modification of enzymatic specificity by protein engineering. For instance, the substrate specificity of the GTPase superfamily can be changed from guanine nucleotide- to xanthine nucleotide-dependent by changing a conserved Asp (in the motif NKXD) to Asn (17–24). Dependence on xanthine triphosphates following introduction of mutated GTPases signals the participation of the mutated protein (21). In a second example, nucleotide-binding pockets of protein-tyrosine kinases can be altered to accept certain  $N^6$ -substituted adenosine triphosphates, which otherwise did not serve as substrates for known kinases (25, 26). The mutated kinases were also sensitive to certain pyralazo[3,4-*d*]pyrimidines, membrane-permeant inhibitors that could revert morphological changes associated with kinase-mediated cell transformation (27).

We have focused our attention on myosin- $\text{I}\beta$ , an isozyme hypothesized to mediate adaptation of auditory and vestibular mechanical transduction (28). We have designed a missense mutant of rat myosin- $\text{I}\beta$ , replacing tyrosine-61 with glycine

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(Y61G), which has little effect on ATP hydrolytic activity yet renders the mutant sensitive to  $N^6$ -modified adenosine diphosphates. These analogs inhibit ATP hydrolysis by preventing myosin dissociation from actin, inducing a tightly bound state that arrests actin filament motility in an *in vitro* motility assay. By introducing Y61G myosin-I $\beta$  (equivalent to Y135G in *Dictyostelium discoideum* and chicken muscle myosin-II) into cells, we should be able to selectively inhibit mutant myosin and the cellular processes in which it participates.

#### EXPERIMENTAL PROCEDURES

**Materials**—Life Technologies, Inc. was the source of Grace's medium, lactalbumin hydrolysate, yeastolate, Pluronic F-68, gentamicin, and fetal calf serum. The pBlueBacHis2B transfer vector, linearized DNA from *Autographa californica* nuclear polyhedrosis virus, cationic liposomes, and anti-DLYDDDDK antibodies were purchased from Invitrogen (San Diego, CA). Restriction enzymes and other DNA-modifying enzymes were obtained from New England Biolabs (Beverly, MA). Reagents for unique-site elimination, Superdex 200 columns, phenyl-Sepharose, DEAE-Sephadex, and [ $\gamma$ - $^{32}$ P]ATP were from Amersham Pharmacia Biotech.  $Ni^{2+}$ -charged nitrilotriacetic acid-agarose ( $Ni^{2+}$ -NTA)<sup>1</sup> was obtained from Qiagen (Valencia, CA). Frozen bovine brains and rabbit muscle acetone powder (special order) were purchased from Pel-Freez (Rogers, AR). Microtiter plates were either Immulon from Dynex (West Sussex, UK), or high binding plates from Xenopore (Hawthorne, NJ). All SDS-polyacrylamide gel electrophoresis reagents, dithiothreitol, and Tween 20 were from Bio-Rad. Secondary antibodies were from Southern Biotechnology Associates (Birmingham, AL), whereas bicinchoninic acid protein assay reagents and *p*-nitrophenol phosphate were from Pierce. ATP, ADP, catalase, glucose oxidase, and protein A were from Sigma. Adenosine 5'-*O*-(2-thiodiphosphate) (ADP $\beta$ S) and bovine serum albumin were purchased from Calbiochem, whereas rhodamine-phalloidin was from Molecular Probes (Eugene, OR). Nitrocellulose in isoamyl alcohol was from Ernest Fullam (Latham, NY). Insight II (version 4.0.0), used to prepare the structure in Fig. 1A, was obtained from Molecular Simulations (San Diego, CA).

**Synthesis of ATP Analogs**—Analog 1-6 (see Fig. 5B) were synthesized as described previously (25); 6-chloropurine riboside (Aldrich) was refluxed with aniline, benzylamine, 2-phenylethylamine, 3-methylbenzylamine, 1-methylbutylamine, or 2-methylbutylamine, respectively, in ethanol overnight (29). Triphosphate synthesis was carried out as described previously (25).

$N^6$ -modified nucleoside diphosphates were isolated as a by-product (about 20% of total) of the triphosphate synthesis. Nucleotides were purified on DEAE-Sephadex (A-25) using a linear gradient of 0.1–1.0 M triethylammonium bicarbonate at pH 7.5. These compounds were characterized by  $^1H$  NMR and mass spectral analysis. Nucleotides were also characterized by high pressure liquid chromatography on a strong anion-exchange column (Rainin SAX-83-E03-ETI), using a linear gradient of 5–750 mM ammonium phosphate, pH 3.9, for 10 min, followed by isocratic elution at 750 mM ammonium phosphate for 10 min. Typical retention time difference between diphosphates and triphosphates was about 2 min.

**Construction of Myosin-I $\beta$  Baculoviruses**—Using the polymerase chain reaction, we modified the pBlueBacHis2B baculovirus transfer vector by adding an *Nco*I site immediately following the enterokinase cleavage site. We also removed an *Nco*I site in the original multiple cloning site by *Kpn*I digestion, removal of 3' overhangs with T4 DNA polymerase and deoxynucleotides, and religation; the modified plasmid was termed pBlueBacHis2B-Nco. The plasmid C myr2 tag pCMV5 (30), containing the cDNA sequence for rat myosin-I $\beta$  with a COOH-terminal myc epitope tag (courtesy of Dr. Martin Bähler), was digested with *Nco*I and *Bam*HI; after purification, myosin-I $\beta$  cDNA was ligated to *Nco*I- and *Bam*HI-digested pBlueBacHis2B-Nco to generate the plasmid pBBHis2B-rmyoI $\beta$ . Sequencing was carried out to ensure the fidelity of the cloning junctions. When expressed, the recombinant myosin-I $\beta$  contained an extra 34 amino acids at its  $NH_2$  terminus, including a hexahistidine tag, an antibody epitope (DLYDDDDK), and an enterokinase protease cleavage site. The COOH terminus contained the 10-amino acid c-myc epitope tag (EQKLISEEDL). Mutagenesis of tyrosine-61 of wild-type myosin-I $\beta$  to glycine was carried out using unique-

site elimination (31), adding a *Kpn*I site, and was confirmed by sequencing. Sf9 cells were cotransfected with pBBHis2B-rmyoI $\beta$  (with or without the Y61G mutation) and linearized *Autographa californica* nuclear polyhedrosis virus genomic DNA using cationic liposomes; recombinant plaques were identified using 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside staining. Virus stocks were manipulated using standard methods (32). *Xenopus laevis* calmodulin, incorporated into baculovirus using the pVL1393 transfer vector, was provided by Dr. James Sellers.

**Protein Purification**—Rat myosin-I $\beta$  was expressed by infecting Sf9 insect cells with recombinant baculoviruses. In a typical purification, 4–8  $\times 10^8$  Sf9 cells, in 400 ml of Grace's medium that contained 3.3 mg/ml lactalbumin hydrolysate, 3.3 mg/ml yeastolate, 20  $\mu$ g/ml gentamicin, 0.1% Pluronic F-68, and 10% fetal calf serum, were infected with myosin-I $\beta$  and calmodulin viruses at multiplicities of infection of 4 and 2, respectively. After shaking at 100 rpm in a 2,500-ml low form culture flask for 48 h at 27 °C, cells were centrifuged at 1,500  $\times g$ , washed with Grace's medium without serum, and recentrifuged. Pelleted cells were usually stored at –80 °C before use. For purification, cells were thawed, resuspended with purification buffer (25 mM Tris, pH 8, 0.5 mM  $MgCl_2$ , 0.5 mM EGTA, 2.5 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 1  $\mu$ M leupeptin, 1  $\mu$ M pepstatin), and lysed by passing twice each through 22- and 25-gauge needles. This and all subsequent purification steps were carried out at 4 °C. The solution was adjusted to 300 mM NaCl and 1 mM ATP and was centrifuged at 400,000  $\times g$  for 30 min. After applying the high-speed supernatant to a 1-ml  $Ni^{2+}$ -NTA column, the column was washed with purification buffer containing 300 mM NaCl. Myosin-I $\beta$  (and contaminating Sf9 cell proteins) was eluted at pH 8.0 with 250 mM imidazole, 25 mM Tris, 200 mM KCl, 2 mM  $MgCl_2$ , 2 mM EGTA, 2.5 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 1  $\mu$ M leupeptin, and 1  $\mu$ M pepstatin.

In some experiments, myosin-I $\beta$  was purified further by cycling on and off actin filaments. 10  $\mu$ M purified actin, stabilized with 10  $\mu$ M unlabeled phalloidin was added to the  $Ni^{2+}$ -NTA eluate; after incubation on ice for 30 min, the solution was centrifuged at 400,000  $\times g$  for 30 min. The supernatant was discarded, and the actin pellet was resuspended with 1 mM ATP, 0.3% Tween 20, 50 mM KCl, 1 mM  $MgCl_2$ , 0.1 mM EGTA, 2.5 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 1  $\mu$ M leupeptin, 1  $\mu$ M pepstatin, and 15 mM HEPES at pH 7.5; the solution was recentrifuged at 400,000  $\times g$  for 30 min. We confirmed that this concentration of Tween 20, included to minimize protein adsorption and aggregation, does not affect myosin ATPase activity (data not shown). Active myosin-I $\beta$  was in the supernatant.

Frog myosin-I $\beta$ , expressed in Sf9 cells with a baculovirus vector<sup>2</sup> was purified to >90% by purification of the  $Ni^{2+}$ -NTA eluate with gel filtration on Superdex 200. Actin was purified from rabbit muscle acetone powder as described (33); its concentration was determined assuming 38.5  $\mu$ M/ $A_{290\text{ nm}}$ . Calmodulin was purified from bovine brain using isoelectric precipitation and phenyl-Sepharose chromatography (34); its concentration was determined assuming 330  $\mu$ M/ $A_{276\text{ nm}}$  (35).

**Measurement of Myosin-I $\beta$  Concentration by Enzyme-linked Immunoassay (ELISA)**—The concentration of myosin-I $\beta$  was measured using an ELISA. Frog myosin-I $\beta$  was used as a standard (typically 0–100 ng/well). The concentration of frog myosin-I $\beta$  was determined assuming 6.8  $\mu$ M/ $A_{290\text{ nm}}$ , assuming two bound calmodulin molecules (36) (calculated from sequences using the ExpASY tool ProParam; see Ref. 37). This value was confirmed by measuring the concentration of frog myosin-I $\beta$  using the Bradford (38) and bicinchoninic acid (39) protein assays, which when averaged together gave a protein concentration similar to that determined from absorbance.

Solutions of partially purified rat myosin-I $\beta$  or purified frog myosin-I $\beta$  were diluted with PBS and applied overnight to wells of a 96-well microtiter plate. After the remaining protein-binding sites were blocked for 1 h with 0.3% Tween 20 and 5 mg/ml bovine serum albumin in PBS (ELISA block), primary antibody was applied in ELISA block for 1–2 h. The primary antibody was anti-DLYDDDDK (1:5,000), which recognized epitope tags on frog and rat myosin-I $\beta$ . After washing with water, bound antibodies were detected with an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (1:1,000) in ELISA block. After incubation with 3 mM *p*-nitrophenol phosphate, alkaline phosphatase activity was measured in a microtiter plate reader at 405 nm.

**ATPase Assay**—The ATPase assay solution contained 1 mM  $MgCl_2$ , 0.1 mM EGTA, and 15 mM HEPES at pH 7.5. Assays also included [ $\gamma$ - $^{32}$ P]ATP (~60,000 cpm/sample) and unlabeled ATP; KCl was added to a final concentration of 50 mM. Myosin ATPase activity was meas-

<sup>1</sup> The abbreviations used are:  $Ni^{2+}$ -NTA, nickel-nitrilotriacetic acid; ADP $\beta$ S, adenosine 5'-*O*-(2-thiodiphosphate); PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

<sup>2</sup> S. Jean and P. G. Gillespie, unpublished data.

ured in 10  $\mu$ l total volume; after mixing components in 1.7-ml siliconized microfuge tubes and initiating the reaction with [ $\gamma$ - $^{32}$ P]ATP or a mixture of actin and myosin, tubes were centrifuged for  $\sim$ 5 s. After incubation at 37  $^{\circ}$ C for 10–40 min, reactions were terminated with silicotungstic acid and sulfuric acid and [ $\gamma$ - $^{32}$ P]P $_i$  was recovered using isobutanol:benzene (1:1) and ammonium molybdate as described (40). To account for the intrinsic ATPase activity of actin and its inhibition by adenine nucleotides, we always included in our assays control samples lacking myosin-I $\beta$  but including actin and appropriate nucleotides.

Data were plotted as the mean  $\pm$  S.D. of 2–5 (usually 3) samples. Inhibition data in Figs. 4, 6, and 7 were fit with:

$$\text{Velocity (\% of control)} = \frac{[I]}{[I] + IC_{50}} \quad (\text{Eq. 1})$$

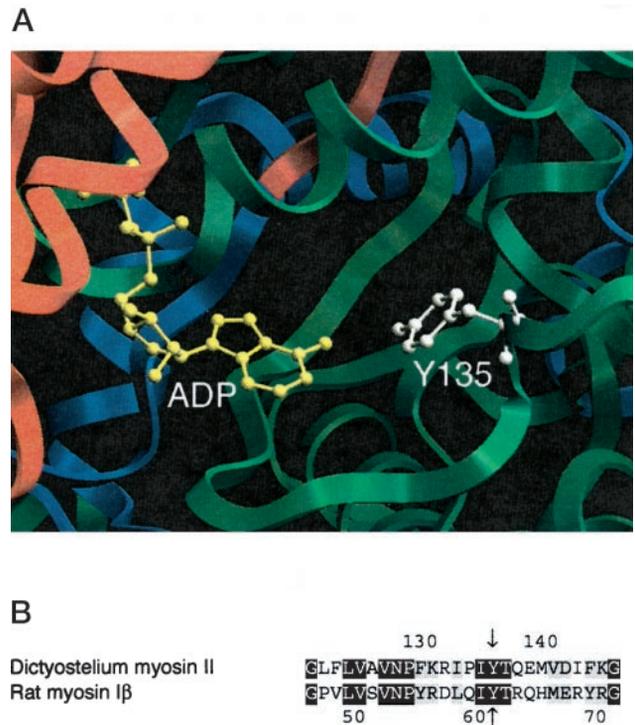
where [I] is the concentration of the nucleotide analog and  $IC_{50}$  is the concentration yielding 50% inhibition.  $IC_{50}$  values were occasionally extrapolated from data sets showing very little inhibition; confidence in these  $IC_{50}$  values was therefore poor (e.g. inhibition of wild-type myosin-I $\beta$  by some analogs). Binding data in Fig. 7 were plotted as described in the legend.

**Actin-Myosin-I $\beta$  Binding Assay**—The standard buffer was ATPase assay solution. Myosin (0.02–0.1  $\mu$ M) and actin (12.5  $\mu$ M) were mixed at room temperature in the presence of various concentrations of adenine nucleotides; the solution was immediately centrifuged at 550,000  $\times g$  for 10 min at 25  $^{\circ}$ C to sediment actin filaments. Supernatants were removed and the concentration of myosin-I $\beta$  was measured by ELISA.

**In Vitro Motility Assay**—We used the sliding filament assay (41) modified to use tail-specific antibodies to immobilize myosin molecules (42, 43). Coverslips coated with nitrocellulose (0.1% in isoamyl alcohol) were assembled into flow chambers; chambers were sequentially incubated with 0.5 mg/ml protein A in PBS for 30 min at room temperature, block solution (1 mg/ml bovine serum albumin in PBS) for 15 min at room temperature, and 0.25 mg/ml anti-myosin-I $\beta$  antibody (R2652)<sup>3</sup> in block solution at 4  $^{\circ}$ C overnight. This antibody recognizes the COOH-terminal 15 kDa of rat myosin-I $\beta$ . Subsequent steps were carried out at room temperature. Chambers were then washed with 1 mg/ml bovine serum albumin in ATPase assay solution and incubated with myosin-I $\beta$  (0.025 mg/ml) for 1 h. Flow chambers were washed, treated for 2 min with 20 nM rhodamine-phalloidin-labeled actin in wash buffer, and finally incubated with motility buffer (ATPase assay buffer containing 5 mM ATP, 5 mM MgCl $_2$ , 10  $\mu$ M calmodulin, 50 mM dithiothreitol, 0.05 mg/ml catalase, 0.25 mg/ml glucose oxidase, and 3 mg/ml glucose). Motility was observed at room temperature (23–25  $^{\circ}$ C) on an Axiovert inverted microscope, equipped with a 63 $\times$  Plan Neofluar objective and 1.6 $\times$  Optivar accessory lens (all from Zeiss, Thornwood, NY). Images were captured with an intensified CCD camera (Photon Technologies International, Monmouth Junction, NJ) using an AG-5 frame-grabber board from Scion (Frederick, MD) in an Apple Macintosh G3. Acquisition was controlled by and filament movement was measured with a modified version of NIH Image, Scion Image 1.62a (Scion). To measure actin filament velocity, we calculated the centroid position (short filaments) or the leading edge (long filaments) of each filament at 5-s intervals and averaged filament velocity over 20 frames. Data were collected from multiple protein preparations each of wild-type and Y61G myosin-I $\beta$ ; velocities of at least 30 filaments in three separate fields were counted.

## RESULTS

**Myosin-I $\beta$  Y61G Mutation**—We sought to design mutations in myosin that would render it sensitive to modified adenosine diphosphates yet maintain normal ATP hydrolysis. In a two-fold approach, we designed nucleotide analogs that were modified on the  $N^6$  amine, the furthest position on the adenine base from the 5'-triphosphate. To these nucleotide analogs, we needed to then create an additional cavity in the nucleotide-binding site of myosin. We identified amino acid positions in the binding pocket with bulky side chains that could be compacted by substitution by examining the crystal structures of *D. discoideum* myosin-II complexed with nucleotides (44–47). In each of these structures, the side chain of tyrosine-135 forms a hydrogen bond with  $N^6$  of the nucleotide (Fig. 1A). We rea-



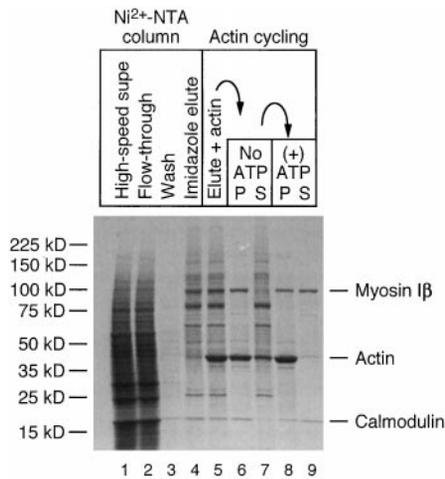
**FIG. 1. Myosin-I $\beta$  Tyr-61 is a suitable residue for substitution.** A, ribbon representation of the *Dictyostelium* myosin-II motor domain complexed to ADP and aluminum fluoride. Note the close contact between adenine ring of ADP, shown in yellow, and Tyr-135 (Y135) (equivalent to tyrosine-61 of rat or mouse myosin-I $\beta$ ), shown in white. The NH $_2$ -terminal domain (25 kDa) is shown in green, the central domain (50 kDa) is shown in red, and the COOH-terminal domain (20 kDa in chicken skeletal muscle myosin) is shown in blue. This image was prepared with Insight II using published data (46). B, alignment of *Dictyostelium* myosin-II (62) with rat myosin-I $\beta$  (30); Tyr-135 of myosin-II and Tyr-61 of myosin-I $\beta$  are indicated by arrows.

soned that this tyrosine might be amenable to substitution for the following reasons. First, in some myosin isozymes, other amino acids occupy the position corresponding to Tyr-135, including leucine in *Acanthamoeba castellanii* myosin-IB, serine in *D. discoideum* myosin-IB, and phenylalanine in *Drosophila melanogaster* myosin-III (*ninaC*) (48). Indeed, the ATPase activity and motility of *A. castellanii* myosin-IB have been studied in detail and resemble closely those of other well characterized myosin isozymes (49, 50). Second, modifications at the C6 position of ATP are tolerated by myosin; even analogs such as 2',3'-*O*-isopropylidene-6-chloropurine ribose (51) or 2-[(4-azido-2-nitrophenyl)amino]ethyl triphosphate and its derivatives (52, 53) are hydrolyzed by myosin-II and support muscle contraction. These data suggest that although a hydrogen bond between Tyr-135 and a nitrogen or oxygen at the C6 position of a nucleotide may be optimal for motor activity (51), it is not essential.

We reasoned that we could substitute glycine for the equivalent residue in rat myosin-I $\beta$ , tyrosine-61 (Fig. 1B), and engineer a myosin mutant (Y61G) that would both hydrolyze ATP and be inhibited by  $N^6$ -modified adenosine diphosphates. We therefore constructed *A. californica* nuclear polyhedrosis virus recombinant baculovirus stocks capable of directing expression of wild type or Y61G rat myosin-I $\beta$ , each with an NH $_2$ -terminal hexahistidine tag for purification and NH $_2$ - and COOH-terminal epitope tags for antibody detection.

**Hydrolysis of ATP by Wild Type and Y61G Myosin-I $\beta$** —To produce myosin-I $\beta$ , we coinfectd Sf9 insect cells with myosin-I $\beta$  and *Xenopus* calmodulin baculoviruses, harvesting cells after 48 h of expression. We purified myosin-I $\beta$  using Ni $^{2+}$ -NTA chromatography and, in some cases, actin-affinity purifi-

<sup>3</sup> Y.-D. Zhao and P. G. Gillespie, unpublished data.

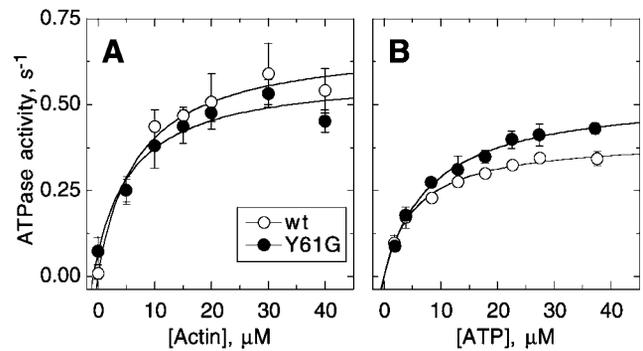


**FIG. 2. Purification of rat myosin-I $\beta$ .** Coomassie Blue-stained, 4–15% acrylamide SDS-polyacrylamide gel electrophoresis gel of the rat myosin-I $\beta$  preparation. Starting from a high speed supernatant from infected Sf9 cells (lane 1), myosin-I $\beta$  was purified using Ni<sup>2+</sup>-NTA chromatography. Myosin-I $\beta$  was often further purified using actin cycling. Actin was added to the Ni<sup>2+</sup>-NTA eluate (lane 5); centrifugation was used to separate actin-bound proteins (in the pellet, P; lane 6) from other proteins (in the supernatant, S; lane 7). ATP was used to release myosin from actin; purified active myosin was in the ATP supernatant (lane 9). ATPase and actin-myosin binding assays generally used the Ni<sup>2+</sup>-NTA eluate (lane 4); *in vitro* motility assays used the ATP supernatant of the actin cycling step (lane 9). A band of ~70 kDa in the ATP eluate, presumably corresponding to the contaminating band stained by Coomassie, was recognized by an antibody directed against the head of myosin-I $\beta$  (data not shown). Although a wild-type myosin-I $\beta$  preparation is shown here, results with Y61G myosin-I $\beta$  were indistinguishable.

cation (Fig. 2). Typical preparations from 400-ml cultures (~6 × 10<sup>8</sup> cells) yielded ~100  $\mu$ g of myosin-I $\beta$  without actin cycling and ~50  $\mu$ g with cycling; preparations could be completed in several hours. Because actin-activated ATPase activity in the Ni<sup>2+</sup>-NTA eluate was almost exclusively from myosin-I $\beta$  (data not shown), we often used this fraction as the source of myosin-I $\beta$ , although it was only partially pure (Fig. 2). In contrast to brush-border myosin-I (54), ADP's affinity was sufficiently low that hydrolysis of 10  $\mu$ M ATP proceeded linearly with time until substrate depletion became apparent (data not shown).

Y61G myosin-I $\beta$  hydrolyzed ATP with most properties very similar to those of wild type (Fig. 3; Table I). Within experimental error, concentrations of ATP and actin yielding half-maximal velocity were identical. The maximal ATP hydrolytic rate ( $V_{\max}$ ) of wild-type myosin-I $\beta$  stored at 4 °C was stable for several weeks. By contrast, the  $V_{\max}$  of Y61G myosin-I $\beta$  was less stable, decaying with a  $t_{1/2}$  of less than a week (data not shown). When ATPase assays were carried out within several hours of protein isolation, the maximum velocity of ATP hydrolysis by Y61G myosin was about 1.4-fold greater than that of wild-type myosin.

**Inhibition of ATP Hydrolysis by N<sup>6</sup>-substituted ATP Analogs**—To identify nucleotide analogs that bind to Y61G but not wild-type myosin-I $\beta$ , we screened nucleotides for inhibition of [ $\gamma$ -<sup>32</sup>P]ATPase activity. In a specific activity dilution experiment, unlabeled ATP inhibited [ $\gamma$ -<sup>32</sup>P]ATPase activity with  $K_i$  values for Y61G and wild-type myosin-I $\beta$  of 15  $\mu$ M, similar to the directly measured  $K_m$  values. Although ADP $\beta$ S inhibited mutant and wild-type myosin-I $\beta$  equally, ADP was a substantially less potent inhibitor of Y61G myosin-I $\beta$  than wild type (Table II). These data are consistent with the higher ATPase activity seen with freshly isolated Y61G myosin-I $\beta$ ; more rapid ADP dissociation, consistent with the reduced affinity, could



**FIG. 3. ATPase activity of wild-type and Y61G rat myosin-I $\beta$ .** A, dependence of ATPase activity on actin concentration. Myosin ATPase was measured with 250  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP. In this experiment, the  $K_{0.5}$  for actin ( $K_{\text{ATPase}}$ ) was 7  $\mu$ M for wild type and 7  $\mu$ M for Y61G. B, dependence of ATPase activity on ATP concentration. Myosin ATPase was measured with 12.5  $\mu$ M actin. In this experiment, the  $K_m$  for ATP was 6  $\mu$ M for wild type and 8  $\mu$ M for Y61G.

**TABLE I**  
*Hydrolysis of ATP by wild type and Y61G myosin-I $\beta$*

ATPase activities were measured from Ni<sup>2+</sup>-NTA eluates in the presence of 50 mM KCl. Myosin-I $\beta$  concentration was determined by ELISA. Means  $\pm$  S.D. are reported;  $n$  refers to the number of independent preparations assayed.

Parameter	Wild type	Y61G
ATP hydrolysis rate (no actin) <sup>a</sup>	0.1 $\pm$ 0.1 s <sup>-1</sup> ( $n$ = 11)	0.1 $\pm$ 0.1 s <sup>-1</sup> ( $n$ = 4)
ATP hydrolysis rate (25 $\mu$ M actin) <sup>a</sup>	0.5 $\pm$ 0.3 s <sup>-1</sup> ( $n$ = 11)	0.7 $\pm$ 0.3 s <sup>-1</sup> ( $n$ = 4)
$K_m$ (ATP)	9 $\pm$ 6 $\mu$ M ( $n$ = 4)	10 $\pm$ 2 $\mu$ M ( $n$ = 4)
$K_{0.5}$ (actin) <sup>b</sup>	10 $\pm$ 6 $\mu$ M ( $n$ = 5)	9 $\pm$ 5 $\mu$ M ( $n$ = 5)

<sup>a</sup> Assayed with 250  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP. Wild-type myosin-I $\beta$  preparations were assayed within 2 days of protein purification; Y61G myosin-I $\beta$  activities were measured within 4 h of purification. Four wild-type myosin-I $\beta$  preparations measured within 4 h of purification had activities of 0.1  $\pm$  0.1 s<sup>-1</sup> without actin and 0.5  $\pm$  0.2 s<sup>-1</sup> with actin.

<sup>b</sup> Actin concentration yielding half-maximal activity ( $K_{\text{ATPase}}$ ).

modestly accelerate ATPase activity.

The robust hydrolysis of ATP by Y61G myosin-I $\beta$  encouraged us to screen a large collection of N<sup>6</sup>-modified adenine nucleotides (25, 26). By assaying with [ $\gamma$ -<sup>32</sup>P]ATP, we could carry out our initial screen with the more readily available nonradioactive N<sup>6</sup>-modified adenosine triphosphates, even if the analogs were hydrolyzed by myosin. Using ATP near its  $K_m$ , we measured the inhibition of ATPase activity by a 10-fold greater concentration of a variety of analogs. Several analogs inhibited Y61G myosin-I $\beta$  to a substantially greater degree than wild type, including those modified with a phenyl group and several analogs modified with aliphatic groups (Fig. 4, A and B).

Using a wider range of analog concentrations, we measured  $K_i$  values for inhibition of ATP hydrolysis by several N<sup>6</sup>-modified adenosine triphosphates (Fig. 5, Table II). Our goal was not necessarily to find the most potent analogs, but rather those which are highly selective, where selectivity is defined as the ratio of wild-type to Y61G  $K_i$  values. ATP derivatives modified on the N<sup>6</sup> position with an aromatic group were selective for Y61G over wild type, with increasing potency and selectivity as the aromatic group was moved away from the N<sup>6</sup> position. Both N<sup>6</sup>(benzyl) ATP and N<sup>6</sup>(2-phenethyl) ATP were >80-fold more selective for Y61G over wild type (Fig. 5). We tried to improve selectivity by adding a substituent to various positions of N<sup>6</sup>(benzyl) ATP; although  $\alpha$ -L-methyl, 2-methyl, and 4-methyl additions reduced selectivity (Fig. 4), N<sup>6</sup>(3-methyl benzyl) ATP was somewhat more effective than N<sup>6</sup>(benzyl) ATP (Fig. 5). We also found that N<sup>6</sup> derivatives with aliphatic side chains were potent inhibitors of Y61G myosin-I $\beta$  (Figs. 4 and

TABLE II

Inhibition of wild type and Y61G myosin-I $\beta$  by ATP and ADP analogs

Y61G or wild-type myosin-I $\beta$  was assayed using 10  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP in the presence of 25  $\mu$ M actin and 50 mM KCl.  $K_i$  values were calculated from

$$K_i = \frac{IC_{50}}{1 + ([S]/K_m)}$$

where  $IC_{50}$  is the half-blocking concentration at [S], or 10  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP, and  $K_m$  values are taken from Table I. If not reported,  $n = 1$ . Selectivity was  $K_i$  (wild type)/ $K_i$  (Y61G).

Analog	$K_i$		Selectivity
	Wild type	Y61G	
	$\mu$ M		<i>fold</i>
<b>ATP analogs</b>			
ATP	15	15	1
$N^6$ (phenyl) ATP	560	82	7
$N^6$ (benzyl) ATP	920	11	84
$N^6$ (2-phenethyl) ATP	200	2.3	87
$N^6$ (3-methyl benzyl) ATP	230	2.4	96
$N^6$ (1-methyl butyl) ATP	78	2.9	27
$N^6$ (2-methyl butyl) ATP	560	6.3	89
<b>ADP analogs</b>			
ADP	20 $\pm$ 2 ( $n = 3$ )	74 $\pm$ 19 ( $n = 3$ )	0.27
ADP $\beta$ S	470	620	0.75
$N^6$ (benzyl) ADP	13.3 $\pm$ 0.1 ( $n = 2$ )		
$N^6$ (2-phenethyl) ADP	170 $\pm$ 40 ( $n = 5$ )	4 $\pm$ 3 ( $n = 5$ )	43
$N^6$ (3-methyl benzyl) ADP	400 $\pm$ 130 ( $n = 3$ )	8 $\pm$ 3 ( $n = 3$ )	50
$N^6$ (2-methyl butyl) ADP	195 $\pm$ 4 ( $n = 3$ )	3.3 $\pm$ 0.4 ( $n = 3$ )	59

5); the most selective of these was  $N^6$ (2-methyl butyl) ATP. Because of poor inhibition of wild-type myosin-I $\beta$ , selectivity values were relatively poorly constrained for some of these analogs, particularly those modified with phenyl, benzyl, 3-methyl benzyl, or 2-methyl butyl side groups.

Several of the  $N^6$ -substituted ATP analogs were substrates for myosin-I $\beta$ . Using a colorimetric phosphate release assay, preliminary results indicated that 100  $\mu$ M  $N^6$ (benzyl) ATP,  $N^6$ (2-phenethyl) ATP, and  $N^6$ (2-methyl butyl) ATP were hydrolyzed by wild-type and Y61G myosin-I $\beta$  at velocities similar to those observed with ATP (data not shown).

Because adenosine diphosphates, rather than triphosphates, should be effective inhibitors of myosin-I $\beta$  motor function, we synthesized several  $N^6$ -modified adenosine diphosphates and tested their effects on ATP hydrolysis by myosin-I $\beta$ .  $N^6$ (benzyl) ADP, although a potent inhibitor of Y61G myosin-I $\beta$ , affected wild-type myosin-I $\beta$  with complex inhibitory properties and was therefore not investigated further (Fig. 6). Somewhat less selective than the triphosphates,  $N^6$ (2-phenethyl) ADP,  $N^6$ (3-methyl benzyl) ADP, and  $N^6$ (2-methyl butyl) ADP nevertheless all inhibited Y61G myosin-I $\beta$  at much lower concentrations than they did wild type (Fig. 6, Table II).

**Actin-Myosin-I $\beta$  Binding**—Because our goal was to find an inhibitor of Y61G myosin-I $\beta$  that tightly arrested myosin upon actin filaments, we complemented the ATPase-inhibition studies by investigating the effects of ADP analogs on the myosin-actin-ATP binding equilibrium. Following incubation of actin, myosin, and nucleotides, we sedimented actin-bound myosin-I $\beta$  by centrifugation and assayed unbound myosin-I $\beta$  in the supernatant with an ELISA assay. Useful inhibitors should promote association of Y61G myosin-I $\beta$  with actin, even in the presence of ATP.

In the absence of ATP, wild-type myosin-I $\beta$  bound to actin with a  $K_d$  of <10 nM with or without 5 mM ADP (data not shown). ATP dissociated the actomyosin complex with  $K_{0.5}$  values of  $\sim$ 10  $\mu$ M for both wild type and Y61G (data not shown); excess ADP could reverse the dissociation elicited by ATP and drive all of the myosin into an actomyosin-ADP complex (Fig.

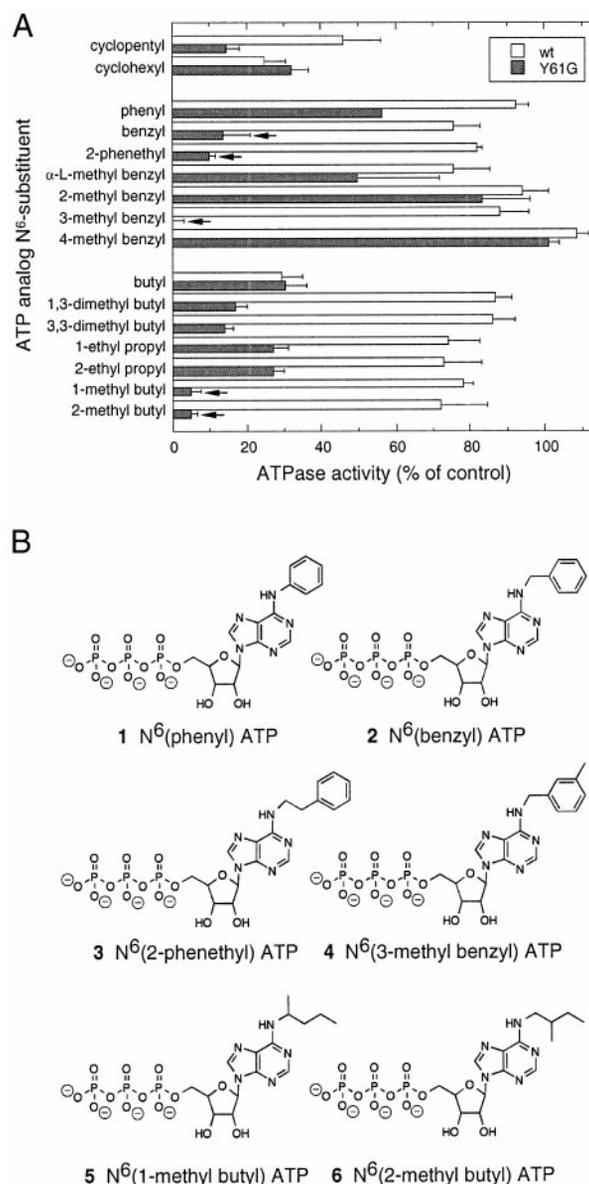


FIG. 4. **Initial screen of  $N^6$ -modified adenosine triphosphates.** A, myosin ATPase was measured with 10  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP and 25  $\mu$ M actin; analogs were included at 100  $\mu$ M. The  $N^6$  substituent is indicated on the left. Arrows indicate inhibition of Y61G myosin-I $\beta$  by highly selective analogs that we chose to characterize further. B, structures of analogs selected for thorough characterization based on results of Fig. 4A.

7A). Consistent with the reduced effectiveness of ADP for inhibition of Y61G ATPase activity, the ADP concentration required to reverse the dissociating effects of 100  $\mu$ M ATP was substantially greater for Y61G than for wild-type myosin-I $\beta$  (Fig. 7A).

The  $N^6$ -substituted ADP analogs locked Y61G myosin-I $\beta$  tightly to actin;  $N^6$ (2-phenethyl) ADP,  $N^6$ (3-methyl benzyl) ADP, and  $N^6$ (2-methyl butyl) ADP all were significantly more potent against Y61G than wild type in reversing ATP-elicited actomyosin dissociation (Fig. 7, B–D). The selectivity of  $N^6$ (3-methyl benzyl) ADP appeared to be reduced in this assay compared with the selectivity seen in the ATPase assay; by contrast, selectivity exhibited by  $N^6$ (2-methyl butyl) ADP and  $N^6$ (2-phenethyl) ADP appeared to be adequate for promoting tight binding of Y61G myosin-I $\beta$ , but not wild type, to actin filaments.

**In Vitro Motility**—We observed actin filament sliding on

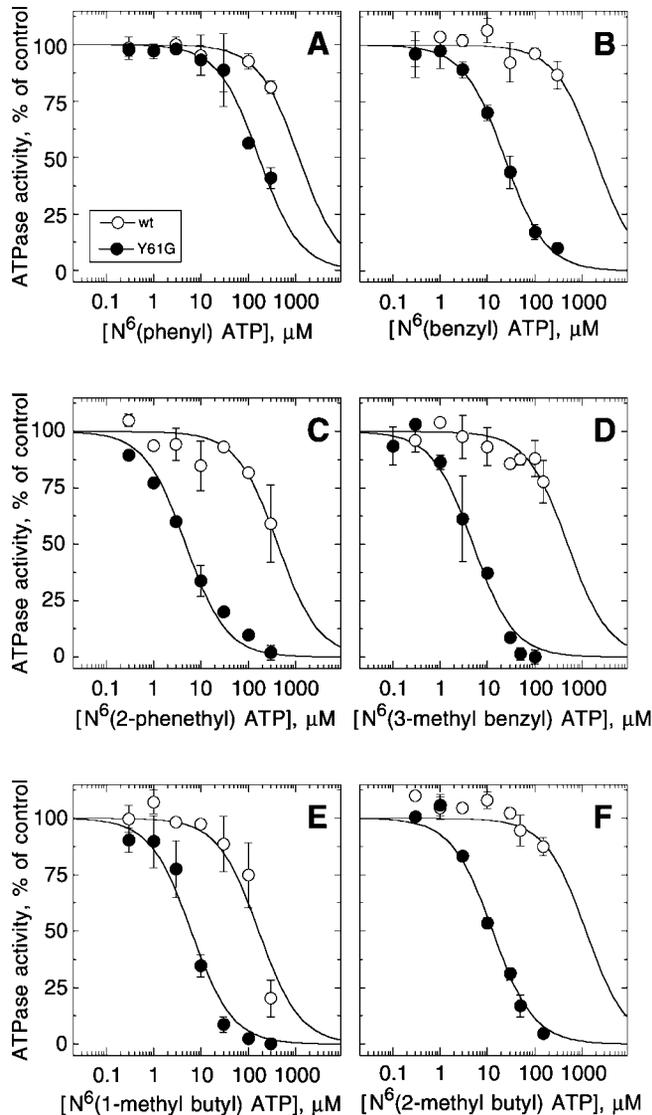


FIG. 5. Inhibition of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis by  $\text{N}^6$ -modified adenosine triphosphates. ATPase activity was measured as in Fig. 4 legend. Analogs were: A,  $\text{N}^6$ (phenyl) ATP; B,  $\text{N}^6$ (benzyl) ATP; C,  $\text{N}^6$ (2-phenethyl) ATP; D,  $\text{N}^6$ (3-methyl benzyl) ATP; E,  $\text{N}^6$ (1-methyl butyl) ATP; F,  $\text{N}^6$ (2-methyl butyl) ATP.

surfaces coated with rat myosin-I $\beta$  only when we both purified myosin by actin-affinity cycling and oriented myosin molecules on the surface using a tail-specific polyclonal antibody (Fig. 8). The actin velocity observed for wild-type rat myosin-I $\beta$  in the presence of 5 mM ATP ( $0.033 \pm 0.007 \mu\text{m s}^{-1}$ ;  $n = 90$ ) was considerably slower than that reported for bovine myosin-I $\beta$  ( $\sim 0.4 \mu\text{m s}^{-1}$ ; Ref. 55). Under the same conditions, actin filament velocity on Y61G myosin-I $\beta$  ( $0.117 \pm 0.027 \mu\text{m s}^{-1}$ ;  $n = 74$ ) was almost 4-fold faster than on wild type. Myosin in the  $\text{Ni}^{2+}$ -NTA eluate did not exhibit *in vitro* motility, presumably because of a large number of ATP-insensitive, actin-binding myosin molecules (see Fig. 2, lane 8).

To examine the effects of  $\text{N}^6$ -modified analogs, we employed a lower concentration of ATP (0.5 mM) to ensure inhibition at relatively low analog concentrations. As with other myosin isozymes, even though this concentration of ATP is saturating for ATP hydrolysis (Fig. 3, Table I), motility was slowed by about 30% for wild type and 60% for Y61G myosin-I $\beta$  as compared with the rate when 5 mM ATP was used. The apparent  $K_m$  values for ATP in the *in vitro* motility assay were 0.2 mM for wild-type and 0.8 mM for Y61G myosin-I $\beta$  (data not shown).

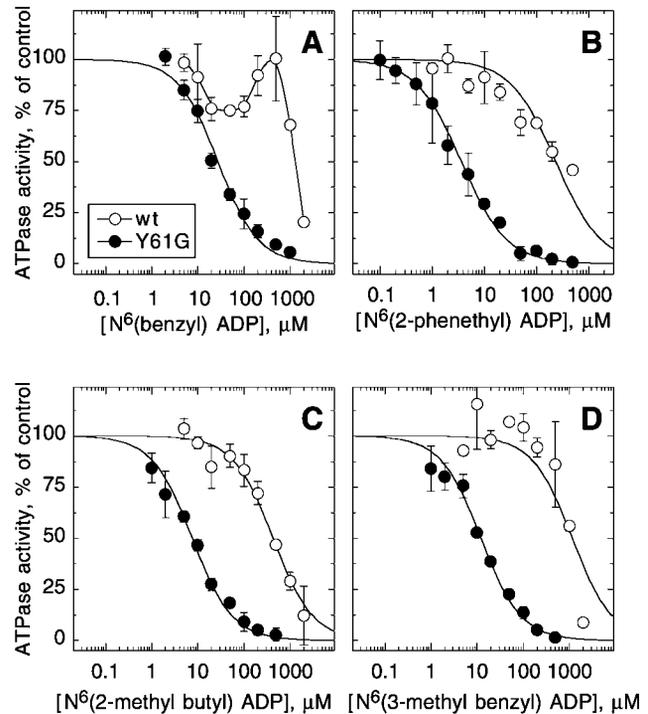


FIG. 6. Inhibition of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  hydrolysis by  $\text{N}^6$ -modified adenosine diphosphates. ATPase activity was measured as in Fig. 4 legend. Analogs were: A,  $\text{N}^6$ (benzyl) ADP; B,  $\text{N}^6$ (2-phenethyl) ADP; C,  $\text{N}^6$ (2-methyl butyl) ADP; D,  $\text{N}^6$ (3-methyl benzyl) ADP.

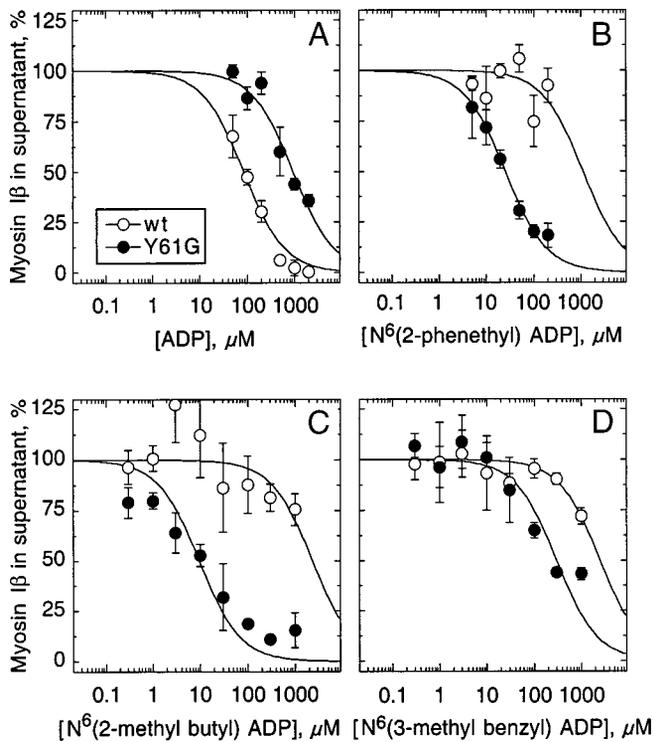
When 30 or 100  $\mu\text{M}$   $\text{N}^6$ (2-methyl butyl) ADP was included along with 0.5 mM ATP in the motility solution, actin filament velocity on surfaces coated with wild-type myosin-I $\beta$  was unaffected; 300  $\mu\text{M}$   $\text{N}^6$ (2-methyl butyl) ADP reduced the velocity modestly (Fig. 8, C and G). By contrast, actin velocity on surfaces coated with Y61G myosin-I $\beta$  was completely abolished by the  $\text{N}^6$ (2-methyl butyl) ADP, even at 30  $\mu\text{M}$  (Fig. 8, F and G). Actin filaments remained unfragmented, further indicating the lack of myosin force production in the presence of the analog.

The inhibitors were effective against Y61G myosin-I $\beta$  at higher ATP concentrations as well. Even at 2 mM ATP, 30  $\mu\text{M}$   $\text{N}^6$ (2-methyl butyl) ADP completely arrested filament movement on Y61G myosin-I $\beta$  (data not shown).

In the absence of  $\text{N}^6$ (2-methyl butyl) ADP, small fractions of wild-type myosin-I $\beta$  significantly slowed motility on mixtures of myosin-I $\beta$  (Fig. 8H). When inhibited by  $\text{N}^6$ (2-methyl butyl) ADP, however, Y61G myosin molecules acted in a dominant manner to block actin movement on myosin mixtures. In the presence of 2 mM ATP, actin filament movement was slowed to near zero by 100  $\mu\text{M}$   $\text{N}^6$ (2-methyl butyl) ADP, even when Y61G myosin-I $\beta$  was present at less than 50% of the total myosin (Fig. 8H). These data show that inhibition by  $\text{N}^6$ -modified ADP analogs should be apparent even if the mutant myosin-I $\beta$  molecules make up a small fraction of the total.

## DISCUSSION

*Designing and Testing the Myosin-I $\beta$  Y61G Mutation*—To determine which roles myosin-I $\beta$  plays in specific cellular processes, we intend to replace or supplement wild-type myosin-I $\beta$  in cells with a mutant myosin-I $\beta$  that we can inhibit selectively. This goal demands several features of a mutant myosin. First, in the presence of ATP alone, activities of the mutant myosin, including hydrolysis rate, unloaded velocity along actin filaments, and force production, should be as close as possible to those of wild type. Second, the mutant must be inhibited by a pharmacological agent at concentrations where the

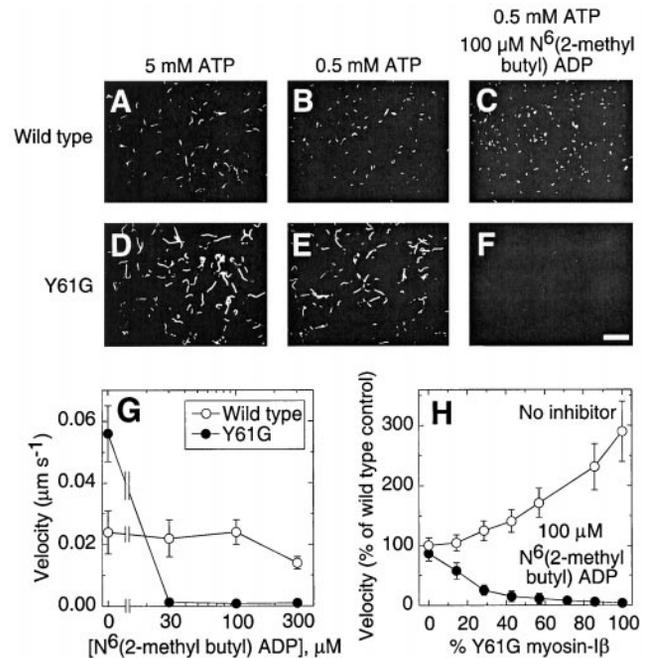


**FIG. 7. Effect of  $N^6$ -modified ADP analogs on ATP-actin-myosin binding.** Binding of myosin-I $\beta$  in the  $Ni^{2+}$ -NTA eluate was measured at 25 °C in the presence of 100  $\mu$ M ATP and 12.5  $\mu$ M actin. Results are plotted with 100% corresponding to the amount of myosin remaining in the supernatant with actin and ATP only and 0% corresponding to the amount of myosin remaining in the supernatant with actin and no nucleotides. *A*, for ADP, the  $K_{0.5}$  (half-maximal concentration for promoting myosin association with actin) was 85  $\mu$ M for wild type and 940  $\mu$ M for Y61G. *B*, for  $N^6$ (2-phenethyl) ADP, the  $K_{0.5}$  was >1,000  $\mu$ M for wild type and 25  $\mu$ M for Y61G. *C*, for  $N^6$ (2-methyl butyl) ADP, the  $K_{0.5}$  was >2,000  $\mu$ M for wild type and 10  $\mu$ M for Y61G. *D*, for  $N^6$ (2-methyl)benzyl ADP, the  $K_{0.5}$  was >2,000  $\mu$ M for wild type and 300  $\mu$ M for Y61G.

agent has few or no effects on the wild-type myosin. Finally, when inhibited, the mutant myosin should remain tightly bound to actin, interfering with the activity of other functionally coupled myosin molecules.

The Y61G mutation of myosin-I $\beta$  provides all three features. First, hydrolysis of ATP and chemomechanical transduction by Y61G and wild-type myosin-I $\beta$  were similar. For example, the half-maximally activating concentrations of actin and ATP concentration were nearly identical for Y61G and wild-type myosin-I $\beta$  and both translocated actin filaments. Second, several  $N^6$ -substituted adenine nucleotides were effective inhibitors of Y61G but not wild-type myosin-I $\beta$ . We were able to achieve this selectivity by engineering a cavity in the nucleotide-binding site of myosin-I $\beta$ , accommodating the bulky  $N^6$  substituents of the nucleotide analogs we used. The unfavorable van der Waals contacts between the  $N^6$  substituent and Tyr-61 in wild-type myosin-I $\beta$  presumably prevented these analogs from binding at high affinity. Finally, even when Y61G made up only a fraction of total myosin-I $\beta$ , actin filament translocation could be fully inhibited by  $N^6$ -modified ADP analogs.

The Y61G mutation has properties that may limit its utility *in vivo*, however. The maximal velocity of ATP hydrolysis is higher for Y61G than wild-type myosin-I $\beta$ ; this feature and the diminished affinity of ADP for Y61G myosin-I $\beta$  may signal an accelerated ADP release rate. Because this rate can control the rate of myosin motility (1), more rapid ADP dissociation could account for faster actin filament translocation exhibited by Y61G myosin-I $\beta$ . By decreasing the fraction of the ATPase



**FIG. 8. Inhibition of *in vitro* motility of Y61G myosin by  $N^6$ (2-methyl butyl) ADP.** Myosin-I $\beta$  was captured on glass coverslips with the R2652 anti-myosin-I $\beta$  antibody; actin filaments labeled with rhodamine-phalloidin were introduced to assess myosin motility. All assays were done in the presence of 10  $\mu$ M calmodulin, which was required for efficient motility, and 50 mM dithiothreitol, 0.05 mg/ml catalase, 0.25 mg/ml glucose oxidase, and 3 mg/ml glucose, which were required to minimize photobleaching. Because we subtracted the initial frame of a sequential collection of images from a frame 104.5 s later, the resulting panels (A–F) show movement of labeled actin filaments (63). For filaments that have not moved farther than their length (the majority), the track length is proportional to their velocity. *A*, surface coated with wild-type myosin-I $\beta$ , in the presence of 5 mM ATP; *B*, surface coated with wild-type myosin-I $\beta$ , in the presence of 0.5 mM ATP; *C*, surface coated with wild-type myosin-I $\beta$ , in the presence of 0.5 mM ATP and  $N^6$ (2-methyl butyl) ADP. Filament movement was unaffected. *D*, surface coated with Y61G myosin-I $\beta$ , in the presence of 5 mM ATP. Note the longer tracks, corresponding to more rapid filament velocity. *E*, surface coated with Y61G myosin-I $\beta$ , in the presence of 0.5 mM ATP. *F*, surface coated with Y61G myosin-I $\beta$ , in the presence of 0.5 mM ATP and  $N^6$ (2-methyl butyl) ADP. Filament movement was completely eliminated. Scale bar of 10  $\mu$ m (also applies to A–E). *G*, filament velocity on wild-type or Y61G myosin-I $\beta$  in the presence of 0.5 mM ATP and varying concentrations of  $N^6$ (2-methyl butyl) ADP. Results plotted are mean  $\pm$  S.D. of filament velocities from three to five independent myosin-I $\beta$  preparations (for each point,  $n \geq 30$ ). *H*, filament velocity on mixtures of wild-type and Y61G myosin-I $\beta$ . Motility carried out with 2 mM ATP; 100  $\mu$ M  $N^6$ (2-methyl butyl) ADP was also added to some samples. Results are mean  $\pm$  S.D. of filament velocities from one to two independent myosin-I $\beta$  preparations ( $n \geq 30$ ); because of some day-to-day variability, all preparations were referenced to results from each preparation with 100% wild-type myosin-I $\beta$  and no inhibitor.

cycle spent tightly bound (the duty ratio; see Ref. 56), accelerated ADP dissociation would also reduce the average force production of a motor. Increased unloaded velocity and decreased force production of Y61G myosin-I $\beta$  molecules might lead to unintended consequences for cellular processes requiring myosin-I $\beta$ . Differences in activity between wild-type and Y61G myosin-I $\beta$  should nevertheless be useful for predicting the behavior of mixtures of myosin molecules.

**Y61G Acts as a Dominant Mutation**—Because myosin-I molecules spend most of their ATPase cycle time detached from actin, multiple molecules must work together to generate work along actin filaments (50). Preventing dissociation from actin of a few mutant myosin-I $\beta$  molecules should therefore halt processes dependent on ensembles of myosin-I $\beta$  molecules. Forces of  $\sim$ 10 pN are required to dissociate single, tightly bound myosin-II molecules from actin (57); because the average force

production of an active myosin molecule is  $\sim 2$  pN (58), a small fraction of tight myosin-actin interactions should slow or stop an ensemble.

Our data show directly that the inhibited Y61G molecules act in dominant manner to slow motility on mixtures of wild-type and mutant myosin-I $\beta$  (Fig. 8H). Even when Y61G molecules make up  $<30\%$  of the total, motility in the presence of 2 mM ATP and 100  $\mu$ M  $N^6$ (2-methyl butyl) ADP is slowed by  $\sim 80\%$  compared with the control. These data show that this mutant-inhibitor pair effectively blocks myosin-I $\beta$ -based motility and should do so even in the presence of an excess of wild-type myosin-I $\beta$  molecules.

*Y61G Myosin-I $\beta$  in Cells*—Introduction of a modest fraction of Y61G molecules into a myosin-I $\beta$  ensemble should permit dramatic inhibition by  $N^6$ -substituted ADP analogs of cellular activities dependent on the ensemble. This inhibition strategy can only be applied in certain narrowly defined circumstances, however. Because these analogs may bind to and interfere with other nucleotide-binding proteins, inhibition of these proteins must not interfere with assays for myosin-I $\beta$ . Furthermore, delivery of the membrane-impermeable  $N^6$ -substituted ADP analogs into cells may be difficult. Although microinjection could initially generate an elevated concentration of analog in a cell, enzymes such as creatine kinase, adenylate kinase, or nucleoside diphosphate kinase might metabolize these analogs, preventing the sustained analog concentrations that may be required for some assays. By contrast, assays for myosin-I $\beta$  that function with permeabilized cells should be suitable for this strategy; so too would introduction via tight-seal whole-cell recording electrodes.

Complicating interpretation of an inhibition experiment using  $N^6$ -substituted ADP analogs, an inhibited myosin-I $\beta$  ensemble might block travel of other myosin isozymes along actin filaments. Such a block arises, for example, upon the addition of substantial amounts of  $N$ -ethylmaleimide-modified myosin fragments to cells (59). Nevertheless, success in a reciprocal activation experiment could circumvent this concern. In this experiment, ADP or ADP $\beta$ S (60) would be used to block motility of all myosin molecules, presumably inhibiting an assayed cellular activity. If Y61G myosin-I $\beta$  molecules are present, however, addition of  $N^6$ -substituted adenosine triphosphates should overcome the block and selectively rescue myosin-I $\beta$  ensembles, allowing only myosin-I $\beta$  motor function. Recovery of the assayed cellular activity would strongly implicate myosin-I $\beta$  as the responsible isozyme.

Determining whether myosin-I $\beta$  is the molecular motor that mediates adaptation in hair cells, the sensory cells of the inner ear, is particularly suitable for application of this combined strategy. Myosin-I $\beta$  is tightly localized within a specific, critical subcellular compartment of the hair cell (9), myosin molecules mediating adaptation are thought to be clustered (28), and adaptation is easily assayed using whole cell electrodes (60, 61). If Y61G myosin-I $\beta$  molecules are introduced into hair cells, we expect that whole cell dialysis with a combination of ATP and a  $N^6$ -substituted ADP analog will block adaptation. Conversely,  $N^6$ -substituted ATP analogs should reverse the block of adaptation exerted by ADP or its analogs (60).

*Y61G Mutation in Other Myosin-Isozymes*—This strategy should easily translate to other myosin isozymes that possess a bulky residue at the position equivalent to myosin-I $\beta$  Tyr-61. Nearly all myosin isozymes so far identified have a residue with a large side chain, usually tyrosine, at this position; most myosin-dependent cellular processes therefore should not be affected by the presence of  $N^6$ -substituted ADP analogs. Replacement or supplementation of myosin isozymes with the

appropriate mutant molecules may prove to be a useful strategy for revealing myosin function.

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*Addendum*—Kapoor and Mitchison (64) have recently reported mutagenesis of a member of the kinesin superfamily that renders it sensitive to  $N^6$ -modified nucleotide analogs.

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