Mutations in Human Nonmuscle Myosin IIA Found in Patients with May-Hegglin Anomaly and Fechtner Syndrome Result in Impaired Enzymatic Function

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A family of autosomal-dominant diseases including May-Hegglin anomaly, Fechtner syndrome, Sebastian syndrome, Alport syndrome, and Epstein syndrome are commonly characterized by giant platelets and thrombocytopenia. In addition, there may be leukocyte inclusions, deafness, cataracts, and nephritis, depending on the syndrome. Mutations in the human nonmuscle myosin IIA heavy chain gene (MYH9) have been linked to these diseases. Two of the recently described mutations, N93K and R702C, are conserved in smooth and nonmuscle myosins from vertebrates and lie in the head domain of myosin. Interestingly, the two mutations lie within close proximity in the three-dimensional structure of myosin. These two mutations were engineered into a heavy meromyosin-like recombinant fragment of nonmuscle myosin IIA, which was expressed in baculovirus along with the appropriate light chains. The R702C mutant displays 25% of the maximal MgATPase activity of wild type heavy meromyosin and moves actin filaments at half the wild type rate. The effects of the N93K mutation are more dramatic. This heavy meromyosin has only 4% of the maximal MgATPase activity of wild type and does not translocate actin filaments in an in vitro motility assay. Biochemical characterization of the mutant is consistent with this mutant being unable to fully adopt the “on” conformation.

There are at least three isoforms of the conventional (class II) nonmuscle myosins in humans, termed nonmuscle myosin (NM) IIA (NMIA), IIB (NMIB), and IIC (NMIIIC) (1, 2). Like all conventional myosins, these are hexameric proteins composed of two heavy chains and two pairs of light chains. The carboxyl-terminal half of each of the heavy chains dimerize to form a coiled-coil α helix, whereas the amino-terminal half forms a globular head region that binds the light chains, interacts with actin, and hydrolyzes ATP. The heavy chains of these three myosins are products of distinct genes (MYH9 for NMIA and MYH10 for NMIB in humans; the gene name for the human nonmuscle myosin IIA heavy chain gene (9, 10) along with the appropriate light chains were mutated to nucleotides coding for Lys using the QuikChange system. The complete nucleotide sequence of the mutant cDNA was confirmed by DNA sequencing.

The wild type and mutant recombinant heavy meromyosins (HMMs) of human nonmuscle myosin IIA were expressed along with regulatory and essential light chains in the baculovirus/Sf9 system and purified according to Wang et al. (10). The proteins were either used fresh or were frozen and stored in liquid nitrogen until used. The concentration was determined by the Bradford assay using smooth

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‡ The abbreviations used are: NM, nonmuscle myosin; HMM, heavy meromyosin; MOPS, 4-morpholinepropanesulfonic acid; WT, wild type; ELC, essential light chain.

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EXPERIMENTAL PROCEDURES

Construction of Baculovirus Transfer Vectors—A recombinant HMM-like protein of human nonmuscle myosin IIA was expressed in the baculovirus/Sf9 system according to Wang et al. (10). Briefly, the cDNA for wild type human nonmuscle myosin IIA (accession number M81105) was truncated at codon 1337 to create an HMM-like fragment. Nucleotides coding for a FLAG epitope (DYKDDDDK) followed by a stop signal were appended to aid purification. The HMM-like construct was subcloned into the baculovirus transfer vector pVL1392 (Invitrogen). Site-directed mutagenesis was used to generate two mutant constructs. For the N93K mutant HMM, the nucleotides coding for Asn at codon 93 were mutated to nucleotides coding for Lys using the QuikChange system (Stratagene). The primers used were 5′-GCCTCTACAGTTTCCAGAGAGGCTA-3′ and the complementary antisense primer. For the R702C mutant HMM, the nucleotides coding for Arg at codon 702 were mutated to Gys using the primers 5′-GCCTCTACAGTTTCCAGAGAGGCTA-3′ and the complementary antisense primer. The complete nucleotide sequence of the mutant cDNA was confirmed by DNA sequencing.

Preparations of Proteins—The wild type and mutant recombinant HMMs of human nonmuscle myosin IIA were expressed along with regulatory and essential light chains in the baculovirus/Sf9 system and purified according to Wang et al. (10). The proteins were either used fresh or were frozen and stored in liquid nitrogen until used. The concentration was determined by the Bradford assay using smooth

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muscle heavy meromyosin as a standard (11). Myosin light chain kinase (12), calmodulin (13), actin (14), and tropomyosin (15) were prepared as previously described. Approximately 10 mg of wild type HMM could be routinely purified from $4 \times 10^8$ Sf9 cells, but the yield of soluble N93K mutant was typically about 0.2–2% of this amount. Western blot analyses of the Sf9 cell pellets prior to extraction of the N93K mutant demonstrated that normal amounts of the protein were being expressed, but subsequent extraction revealed that most of this was not solubilized by the extraction buffer.

Enzymatic Assay—The actin-activated MgATPase activity was measured using the method of Pollard and Korn (16) at 35 °C in 2 mM MgCl$_2$, 1 mM ATP, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM MOPS (pH 7.0). The actin concentration was typically varied from 0.5 to 30 μM. The kinetic constants, $V_{\text{max}}$ and $K_{\text{ATPase}}$ (the actin concentration required for half maximal activation of the MgATPase activity), were determined by fitting the data to the Michaelis-Menten equation using SigmaPlot (Jandel Scientific). The K$^+$-EDTA ATPase activity was measured in 0.5 M KCl, 2 mM EDTA, 10 mM MOPS (pH 7.2), and 1 mM ATP at 35 °C.

The in vitro motility of the HMM fragments were measured as described (10, 17). The conditions were 80 mM KCl, 5 mM MgCl$_2$, 1 mM ATP, 0.1 mM EGTA, 1 mM dithiothreitol, 20 mM MOPS (pH 7.4), 200 nM tropomyosin, 0.7% methylcellulose, 2.5 mg/ml glucose, 0.1 mg/ml glucose oxidase, and 2 mg/ml catalase at 30 °C.

RESULTS

The enzymatic properties of a nonmuscle myosin II HMM-like fragment expressed in the baculovirus/Sf9 system have been previously characterized and shown to be an excellent model for the tissue-purified protein (10). In this report, two separate point mutations in the myosin heavy chain (N93K and R702C) corresponding to mutations recently reported in some families with May-Hegglin anomaly and Fechtner syndrome were separately engineered into this background (4). The unmutated HMM will be referred to as WT-HMM, and the HMMs bearing either one of the two mutations will be referred to as R702C-HMM and N93K-HMM. All three proteins were expressed in the baculovirus/Sf9 system (Fig. 1). Both mutants bound regulatory and essential light chains in the same ratios with the heavy chain as was found in WT-HMM. The amounts of N93K-HMM obtained were variable due to the fact that most of it tended to be in an unextractable form in the Sf9 cells, and the protein that was purified aggregated during and after purification.

The enzymatic activity was initially accessed by measuring the MgATPase activities of the three proteins in the absence of actin (Table I). For all three proteins, this activity was very low ($<0.007$ s$^{-1}$). Because the enzymatic activity of NMIIA is known to be regulated by light chain phosphorylation, the samples were treated with myosin light chain kinase. Glycerol gel electrophoresis of the kinase-treated samples revealed that the regulatory light chains of each of the myosins incorporated a single mole of phosphate per mole (data not shown). The MgATPase activity of phosphorylated WT-HMM, as expected, was greatly activated by actin (Fig. 2). That of R702C-HMM was activated to a lesser extent, whereas the activity of N93K-HMM was barely activated by actin (Fig. 2). The data for each HMM was fitted to the Michael-Menten equation to determine the kinetic constants, $V_{\text{max}}$ and $K_{\text{ATPase}}$ (Table I). The $V_{\text{max}}$ of R702C-HMM was about 25% of that of WT-HMM, and its $K_{\text{ATPase}}$ was slightly lower than that of WT-HMM. In contrast, the $V_{\text{max}}$ of N93K HMM was less than 5% of that of wild type. The low activity was not a result of the protein becoming dephosphorylated during the course of the assay, as it retained its original level of phosphorylation during the ATPase time course (data not shown). To determine whether the cause of the low activity of N93K was due to gross denaturation of the protein, the nonphysiological K$^+$-ATPase activity was measured. The data in Table I show that the N93K-HMM had an even higher K$^+$-EDTA ATPase activity than WT-HMM or R702C-HMM, indicating that the protein was able to bind and hydrolyze nucleotides under these conditions and was, therefore, not denatured. The low activity of the N93K HMM could possibly be due to the inability to be activated by light chain phosphorylation. The MgATPase activities of all three HMM molecules in the unphosphorylated state were measured in the presence of 30 μM actin. The MgATPase activities of unphosphorylated WT-HMM (0.039 s$^{-1}$) and R702C-HMM (0.016 s$^{-1}$) were much lower than that of their phosphorylated forms, indicating that the enzymatic activity of these proteins was well regulated. The activity of unphosphorylated N93K-HMM (0.012 s$^{-1}$) was similarly low but was only increased 2–3-fold by phosphorylation.

An important property of myosin is its ability to translocate actin. This can be measured in an in vitro motility assay using rhodamine-phalloidin-labeled actin filaments. WT-HMM translocated actin filaments at a rate of 0.28 μm/s when phosphorylated (Fig. 3). In the absence of phosphorylation, WT-HMM did not move actin filaments. Phosphorylated R702C-HMM translocated actin filaments at about one-half the velocity of WT-HMM (Fig. 3). In contrast, no movement of actin filaments was observed for phosphorylated N93K-HMM, but actin filaments did remain bound to the surface (Fig. 3).

An experiment in which phosphorylated N93K-HMM and rabbit skeletal muscle HMM are mixed in the motility assay affords a means to better examine the nature of the low activity of N93K-HMM. There are several alternative explanations for its low activity. First, it is possible that most (96%) of the myosin is totally inactivated with respect to its enzymatic activity and that 4% of the myosin retains the activity of wild type myosin. Chemical modification of smooth and skeletal muscle myosins can generate such a condition. Typically these
inactivated myosins still retain the ability to bind tightly to actin and have a diminished $K_{\text{ATPase}}$ measured under nonphysiological conditions (18). When such inactivated myosins are mixed with actively cycling myosins in the in vitro motility assay, even small amounts of the inactivated myosins are sufficient to arrest the movement of the actively cycling myosins (18). Dramatic shearing of the actin filaments also occurs in this case. Neither of these phenomena occurred when phosphorylated N93K-HMM was mixed with rabbit skeletal muscle HMM (Fig. 4, triangles) or with phosphorylated WT-HMM (data not shown). Coupled with the observation that N93K-HMM had a $K_{\text{ATPase}}$ similar to that of WT-HMM further argues against the possibility that this mutation merely results in a dead, rigor-like myosin that would account for the low enzymatic activity (Table I). Second, the mutation could render the HMM incapable of interacting with actin. This can be ruled out on the basis that when phosphorylated N93K-HMM alone is applied to the coverslip surface, actin filaments remain bound (though not moving) even in the presence of ATP. Third, the mutation may have simply slowed the rate-limiting step for the kinetic cycle of the mutant such that it is only 4% that of WT-HMM, but the mutant myosin is otherwise proceeding through its cross-bridge cycle as a myosin should, and is possibly even moving actin filaments at such a slow rate so as to be undetectable by our methods. The rate that would be predicted by this expla-

| Human nonmuscle myosin IIA HMM | Basal MgATP$_{\text{max}}$ (s$^{-1}$) | pHMM Actin-Activated MgATP$_{\text{max}}$ (s$^{-1}$) | $V_{\text{max}}$ (s$^{-1}$) | $K_{\text{ATPase}}$ (M) | $K_{\text{EDTA}}$ ATPase (s$^{-1}$) |
|-------------------------------|---------------------------------|---------------------------------|----------------|----------------|----------------|
| Wild type                     | <0.007                          | 0.92 ± 0.15                     | 0.20 ± 0.05   | 3.0 ± 0.14     | 0.54 ± 0.14     |
| R702C                         | <0.007                          | 0.20 ± 0.05                     | 0.20 ± 0.20   | 0.47 ± 0.13     |
| N93K                          | <0.007                          | 0.03 ± 0.002                    | 0.13           |

**FIG. 2.** Effect of actin on the MgATPase activity of phosphorylated wild type and mutant HMM molecules. The MgATPase activity of WT-HMM (closed circles), R702C-HMM (open circles), and N93K-HMM (closed inverted triangles) was measured at different actin concentrations. The MgATPase activity of myosin in the absence of actin was subtracted from each data point. Data sets were fitted to the Michaelis-Menten equation (solid lines) to determine the kinetic constants, $V_{\text{max}}$ and $K_{\text{ATPase}}$. The data shown are representative of a single preparation of each HMM. In total, three such preparations contributed to the data presented in Table I.

**FIG. 3.** In vitro motility of phosphorylated wild type and mutant HMM molecules. The average rate of actin filament sliding was determined from three preparations each of WT-HMM, R702C-HMM, and N93K-HMM. The plot shows the mean velocity with the standard deviation. No detectable movement of actin filaments was seen with the N93K-HMM preparation. All proteins were phosphorylated on the coverslip surface by incubation in a buffer containing 50 mM KCl, 20 mM MOPS (pH 7.0), 4 mM MgCl$_2$, 0.1 mM EGTA, 0.2 mM CaCl$_2$, 1 mM ATP, 1 mM calmodulin, and 1 µg/ml myosin light chain kinase.
nation, 10 nm/s, should be detectable in our assay and was not observed. Cuda et al. (19) and Harris et al. (18) demonstrated that when two actively cycling myosins with different inherent rates of motility are mixed in the motility assay, the more slowly cycling myosin dominates the velocity.

Phosphorylated N93K-HMM did not dominantly retard the movement of skeletal muscle HMM in the experiment shown in Fig. 4, further suggesting that the mutation has not merely rendered the HMM a very slow but otherwise normal motor. As a control, we show that phosphorylated WT-HMM, as expected, does dominantly retard the movement of rabbit skeletal muscle HMM (Fig. 4, circles). Fourth, it is possible the N93K mutation does not allow the HMM to fully adopt the active state upon phosphorylation, and thus the molecule mostly remains in an inhibited state that is able to interact weakly with actin filaments but cannot support their movement. The behavior of noncyclcizing unphosphorylated smooth and nonmuscle myosins when mixed with an actively cycling myosin has also been studied and modeled (18, 19). The unphosphorylated noncyclclying unphosphorylated smooth and nonmuscle myosins do so in a non-dominant manner. As a control, we show in Fig. 4 that unphosphorylated WT-HMM has such an effect on rabbit skeletal muscle HMM (squares). This is also the behavior observed when phosphorylated N93K-HMM was mixed with skeletal muscle HMM, which supports the latter case that this mutation is not able to adopt the "on" conformation (Fig. 4, triangles).

**DISCUSSION**

Platelets are unusual in that the only myosin II isoform they contain is the nonmuscle myosin IIA isoform, whereas most cells express at least nonmuscle myosin IIB and sometimes nonmuscle myosin IIC, in addition to nonmuscle myosin IIA (20). In platelets, myosin and actin form a dense cortical meshwork (21). The linkage of mutations in MYH9 (the heavy chain gene for nonmuscle myosin IIA) to several related human disorders, such as May-Hegglin anomaly, Sebastian syndrome, Fechtner syndrome, Alport syndrome, and Epstein syndrome, involving abnormalities in platelets and leukocytes is therefore not surprising (4, 5, 8, 22). Fechtner, Alport, and Epstein syndrome patients also display sensorineural deafness and nephritis. Interestingly, another MYH9 mutation (R702H) not examined here was linked to DFNA17, a nonsyndromic hereditary deafness with no apparent involvement of platelets (7). Thus, mutations in MYH9 account for these phenotypically distinct MYHIIA syndrome disorders and are important in the pathogenesis of macrothrombocytopenia, sensorineural deafness, cataracts, and nephritis.

Two of the reported MYH9 mutations, N93K and R702C, were engineered into the heavy chain of human nonmuscle myosin IIA. The heavy chain cDNA was truncated at codon 1377 and tagged with the FLAG epitope at the carboxyl terminus to express a soluble HMM-like fragment that could be easily affinity purified (10). The expression of the HMM fragment was chosen as it is typically soluble at low ionic strengths, which affords ease in kinetic experiments, retains the regulatory behavior of the whole myosin, and has been shown to express well in the baculovirus/Sf9 system (10). In the case of both mutations, homodimers with the same mutation on each heavy chain were generated. Both of these mutations are autosomal-dominant in humans (8, 22). This raises the possibility that an affected individual may have both homodimeric as well as heterodimeric molecules (with respect to the mutation). Assuming random association of the heavy chains in the cell, ~50% of the myosin molecules should be heterodimeric and have one mutant and one wild type heavy chain. The rest should be homodimers with two mutant heavy chains (25%) or with wild type heavy chains (25%). If, however, the dimerization of the two heavy chains occurs at the time of translation on the ribosome, then homodimers of either the mutant myosin heavy chain or of the wild type heavy chain could be preferentially produced. It is also likely, *in vivo*, that mutant molecules co-polymerized into filaments with wild type myosin molecules could also impair the behavior of the otherwise normal wild type myosin. In our study only homodimeric molecules (with respect to their mutation status) are produced. It would thus be expected that our study should give the extreme example of functional impairment of function. We did not address how an HMM with one normal heavy chain and one mutant heavy chain would behave in this study.

Both mutations impaired the activity of the myosin, but to different degrees. The R702C mutation had the more modest effects. When phosphorylated, R702C-HMM exhibited about 25% of the maximal actin-activated MgATPase activity and moved actin filaments at about one-half the velocity of WT-HMM. In contrast, the impairment found with the N93K mutation was severe. This fragment had only about 4% of the maximal actin-activated MgATPase activity as WT-HMM and did not move actin filaments in the *in vitro* motility assay. Its MgATPase activity was barely activated by phosphorylation,
Fig. 5. Localization of the mutant amino acids. The crystal structure of a recombinant smooth muscle myosin motor domain plus ELC fragment with bound ADP-AlF₄ (PDB accession number 1BR1) was used to map the positions of the two mutations. The heavy chain segment is shown in gray, yellow, and green, and the ELC is in magenta.

The yellow segment of the heavy chain corresponds to the SH1 helix; the green segment corresponds to the converter domain. The position of Asn-93 (Asn-96 in the smooth muscle sequence) is depicted as a cpk model colored red. The position of Arg-702 (Arg-715 in smooth muscle myosin) is depicted as a cpk model colored orange. The light blue atoms represent the ADP and AlF₄ atoms.

and the mechanism for its impairment may be that it is unable to assume the active conformation. This mechanism is supported by the fact that phosphorylated N93K-HMM slows the movement of fast skeletal muscle HMM in a non-dominant manner, implying that it is able to interact with actin in the weakly bound state. This could occur if the mutation prevented the acceleration of release of products, particularly the release of Pi, as has been shown to occur in Dictyostelium myosin bearing an E476K mutation, for example (23).

The coordinates of the three-dimensional structure of the highly homologous smooth muscle myosin (24) can be used as a model to locate the likely positions of the two May-Hegglin NMIIA mutants. Despite their distant positions in the primary sequence of the myosin heavy chain, N93 and R702 are located in close proximity in the myosin three-dimensional structure (Fig. 5). The sequence in Fig. 5 depicted in yellow is that of the so-called SH1 helix, a critical helical segment that unwinds at some stage during the myosin ATP hydrolysis cycle (25, 26).

Most myosin crystal structures (including the one shown in Fig. 5) show this region to be helical, but in a recent report (25) on a scallop S1-MgADP complex this helix was shown to be unwound. Its disruption is linked to a state in which the subdomains of myosin are not tightly coupled and is thought to represent myosin in a detached state that would be unable to bind strongly to actin (25). Arg-702 lies directly within the SH1 helix (orange atoms), and Asn-93 (magenta atoms) lies in close proximity on an adjacent helix (Fig. 5). Arg-702 is highly conserved in most classes of myosin and is invariant in the conventional myosin II class (27). In smooth muscle, it forms a side chain hydrogen bond with a conserved glutamine residue located four amino acids down stream (Asn-706 in nonmuscle IIa), which would probably not occur in the mutant form. This would probably result in destabilization of the SH1 helix. Asn-93 is well conserved among vertebrate nonmuscle and smooth muscle myosins (although not universally conserved within the myosin II subclass) (27). It lies on helix C within the amino-terminal segment of the heavy chain that abuts the SH1 helical segment (28). Helix C makes several important stabilizing contacts with SH1 helix (25). Asn-93 hydrogen bonds with Ser-96, and mutation to the bulkier lysine would likely destabilize helix C, which, in turn, could weaken its interactions with and destabilize the SH1 helix. If the SH1 helix were unwound, the subdomains of myosin would be loosely coupled and hence incapable of generating a power stroke. The severity of the mutation on enzymatic function serves to highlight the importance of this critical region of myosin. Interestingly, two other MYHIIA syndrome mutations have been identified that are also located near the SH1 helix region, R706H (7) and A95T (29).

Although there are many mutations that have been engineered into either Dictyostelium or smooth muscle myosins that dramatically slow the actin-activated MgATPase activity, none of these are located in a comparable position to Asn-93 in nonmuscle myosin II (27). There are disease-causing mutations in human β-cardiac myosin that lie in proximity to the SH1 helix, but these are not found in the amino terminus (27).

The primary effects of these mutations are seen in platelets where there is no compensating nonmuscle myosin IIB present. The process of platelet formation from megakaryocytes is still poorly understood. Recent results suggest that they originate from intermediate proplatelet structures on the megakaryocytes (30). The megakaryocytes of these patients have not yet been studied to determine whether their proplatelet-like processes are abnormally large. The giant platelet phenotype found in association with the mutation suggests a role for NMIIB in the process of platelet formation. Most of the MYHIIA syndrome disease phenotypes include the presence of inclusion bodies in leukocytes. Recently, these inclusion bodies were found to contain aggregated nonmuscle myosin II by immunocytochemical staining (22). This is consistent with our observation that N93K-HMM tends to aggregate in solution. The R702C mutation has been found in several different families. In some of these families no leukocyte inclusions are found, which is consistent with our observation that this mutation is more soluble. The aggregation observed in vitro with N93K-HMM was not reversible by increasing the ionic strength, and this suggests that the myosin aggregation seen in the leukocytes of the patients are probably also irreversible and cannot be depolymerized and repolymerized into filaments at different locations in the cell.

It is not clear why one mutation (R702C) is associated with the added complications of Fechtner syndrome, which includes microophthalmia, sensorineural deafness, and cataracts, whereas the other mutation (N93K) is only associated with the platelet and leukocyte disorders. Possibly, the tendency of N93K mutation to induce aggregation may actually serve to effectively remove this myosin from the cellular compartments where its lowered enzymatic activity would otherwise inhibit function. In this way the more severe mutation in vitro may not exert as great of an effect in the cell as the less severe R702C mutation, which is not always associated with cellular aggregations. Myosin II may be performing different tasks in these diverse cell types.

The fact that a wide variety of other organs exhibit no apparent adverse effects of these mutations probably reflects a functional redundancy with NMIIB, NMIIC, and possibly other myosins. It is also likely that the genetic background plays a role in this distinction.

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