Mutational analysis of phosphorylation sites in the *Dictyostelium* myosin II tail: disruption of myosin function by a single charge change

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Abstract The dynamic assembly/disassembly of non-muscle myosin II filaments is critical for the regulation of enzymatic activities and localization. Phosphorylation of three threonines, 1823, 1833 and 2029, in the tail of *Dictyostelium discoideum* myosin II has been implicated in control of myosin filament assembly. By systematically replacing the three threonines to aspartates, mimicking a phosphorylated residue, we found that position 1823 is the most critical one for the regulation of myosin filament formation and in vivo function. Surprisingly, a single charge change is able to perturb filament formation and in vivo function of myosin II.

Key words: Myosin; Phosphorylation; Filament formation

1. Introduction

In non-muscle cells, active reorganization of myosin II (referred from this point forth simply as myosin) filaments is essential to a number of processes that take place at distinct locations in the cell. Most dramatically, myosin localizes to the cleavage furrow during cell division [1,2] and has been shown to be essential for cytokinesis in suspension and on a non-adhesive surface [3–5]. Although the cellular function of myosin is completely dependent on its ability to assemble into filaments [6], little is known about the specific features of the myosin tail that allow formation of bipolar filaments.

The myosin tail is comprised almost entirely of heptad repeats common to all coiled-coil forming regions, with the residues at the first and the fourth position packed in the hydrophobic core of the coil [7]. Superimposed on this basic structure, the tail displays alternating regions of positively and negatively charged residues. These regions are thought to be important to intermolecular interactions that form the basis of filament assembly as well as to inter- and intramolecular interactions that favor filament disassembly [8].

The cellular slime mold, *Dictyostelium discoideum*, has proven to be particularly amenable to detailed studies of both the in vivo as well as biochemical properties of myosin function. Analysis of *Dictyostelium* myosin tail fragments has identified two regions at the C-terminal end of the tail that account for the assembly and disassembly properties of the intact molecule. The first region (Fig. 1, hatched) comprises a 34 kDa segment that is both necessary and sufficient for self-assembly in vitro [9]. The second region, located immediately C-terminal to the assembly domain, serves to mediate interactions within the tail and thus the assembly state of myosin. Three threonines within this region have been shown to be targets of two specific myosin heavy chain kinases [10,11]. Phosphorylation of these residues results in the inhibition of filament formation at physiological salt concentrations [12,13].

These observations have been supported by site-specific mutagenesis studies targeting these three threonines, located at amino acid positions 1823, 1833 and 2029 [6]. Substitution of all three of these threonines to alanines prevents phosphorylation at those sites. This results in a myosin that constitutively forms thick filaments and thus is found almost exclusively in a Triton-insoluble cytoskeletal pellet [6]. Substitution of these threonines to aspartates was also studied to simulate a permanently phosphorylated state. These substitutions result in the inhibition of filament assembly similar to that observed with phosphorylated myosin and failure of this mutant myosin to localize to the Triton-insoluble pellet. Moreover, unlike the triple alanine myosin, the triple aspartate myosin is unable to rescue myosin null cells for cytokinesis in suspension and development into fruiting bodies [6].

These studies demonstrate the importance of phosphorylation within this region of the tail in regulating myosin function. However, whether such regulation requires phosphorylation of all three threonines remains unclear. To monitor the influence of phosphorylation at each of these three threonines on the assembly and disassembly properties as well as the in vivo function of myosin, we have systematically mutated these three threonines at amino acid positions 1823, 1833 and 2029 to aspartates in all six possible combinations. These proteins are denoted as DTT, DDT, DTD, TDT, TDD and TTD (see Section 2). Analyses of these mutant myosins both in vitro and in vivo suggest that phosphorylation of threonine 1823 critically determines the regulation of myosin filament formation and that the inability to remove the negative charge at this position completely disrupts in vivo function.

2. Materials and methods

2.1. Plasmid construction

All DNA manipulations were carried out using standard methods [14]. Expression plasmids encoding the myosin tail variants T1823D, (pLittleDTT); T1823D, T1833D (pLittleDDT); T1823D, T2029D (pLittleDTD); T1833D (pLittleTDT); T1833D, T2029D (pLittleTDD); T2029D (pLittleTTD) and T1823A, T1833D, T2019D (pLittleADD) were based on the vector pLittleMyo which contains the wild-type (wt) *Dictyostelium* mhcA gene inserted into pLittle, an extrachromosomally replicating expression vector [15]. In brief, the vector pBIGAsp [16] was digested with *Bgl*II and *Sma*I. The obtained fragment, containing most of the mhcA gene, was ligated with pBluescript II SK(+) (Stratagene, La Jolla, CA, USA) linearized with *Sma*I and *Bam*HI. This plasmid pBlueMyo was used to construct the tail

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variants described above by using polymerase chain reaction (PCR)-based mutagenesis (Quickchange™, Stratagene, La Jolla, CA). The PCR products were sequenced to confirm the presence of the desired point mutations. pBlueMyo containing the mutated gene fragments was digested with *Kpn*I and the fragment containing the myosin coding sequence cloned in pLittleΔKK (pLittleMyo digested with *Kpn*I and religated without insert). The right orientation of the insert was checked by digesting the plasmids with *Hin*dIII.

2.2. Cell culture

HS1, a myosin null cell line [17], was transformed with wt-myosin (pBIGMyo) and DDD-myosin (pBIGAsp), and the plasmids encoding the different myosin tail variants using standard electroporation conditions [18]. Individual clones were grown at 21°C in HL5 media [19], supplemented with Pen-Strep (60 U/ml of penicillin, 60 μg/ml streptomycin) and 10 mg/ml G418 (Geneticin; Life Technologies, Gaithersburg, MD, USA) on plastic dishes or in suspension. To observe development, cells were spotted onto *Klebsiella aerogenes* lawns on SM agar plates [19].

2.3. Protein purification

Myosin and its variants were purified from stably transformed *Dictyostelium* cells grown in suspension (variants TDD-, TTD-, TDT-, ADD- and wt-myosin) or on plastic Petri dishes (variants DDD-, DDT-, DTT-, DTD-myosin). Proteins were typically purified from 30 g of cells. Purification was performed essentially as described by [17] with the following modifications. The washed cell pellet was resuspended in 9 vol./g cells of cold lysis buffer (25 mM HEPES pH 7.4, 50 mM NaCl, 2 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM TPCK, 0.2 mM TLCK and 0.5% Triton X-100). The cell suspension was gently stirred on ice for 10 min and the actomyosin precipitate was collected by centrifugation at 20 000×*g* for 30 min. The pellet was resuspended in 3 vol./g cells of 10 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM DTT and the suspension was than diluted with 6 vol./g cells of 10 mM HEPES pH 7.4, 2 mM EDTA, 1 mM DTT. The suspension was incubated on ice for 10 min. The actomyosin precipitate was collected by another centrifugation step. Extraction of the myosin was achieved by resuspending the actomyosin pellet in 4 vol./g cells of buffer containing 10 mM HEPES pH 7.4, 300 mM NaCl, 3 mM $MgCl_2$, 2 mM ATP and 1 mM DTT. The solution was centrifuged at 18 000×*g* and the supernatant was collected. RNase A (50 μg/ml) was added and the solution was concentrated by dialyzing against Aquacode III (Calbiochem, La Jolla, CA, USA) solution in 10 mM HEPES pH 7.4, 150 mM NaCl, 4 mM $MgCl_2$, 1 mM DTT. The concentrated dialysate was adjusted to 2 mM ATP and immediately centrifuged at 50 000×*g* for 20 min. The supernatant was diluted with 2 vol. of 10 mM HEPES pH 7.4, 200 mM NaCl, 40 mM $NaPP_i$, 1.2 M KI and immediately loaded on a gel filtration column (Bio-Gel A-15m, 100–200 mesh, Bio-Rad, Richmond, CA, USA) previously equilibrated with 10 mM HEPES pH 7.4, 200 mM NaCl, 20 mM $NaPP_i$, 0.6 M KI. The concentration of protein was determined using the Bradford assay [20], with rabbit skeletal myosin as the standard.

2.4. Electrophoresis and immunoblots

Equal amounts of *Dictyostelium* whole cell lysates were loaded onto two sodium dodecyl sulfate/7.5% polyacrylamide gels. These gels were either stained with Coomassie brilliant blue or transferred onto nitrocellulose paper. The blot was probed with My6, an anti-*Dictyostelium* myosin II monoclonal antibody, followed by an appropriate secondary antibody conjugated to horseradish peroxidase (Bio-Rad, Richmond, CA, USA). An enhanced chemiluminescence system (Amersham, La Jolla, CA, USA) was used to visualize the signals.

2.5. 4,6-Diamidino-2-phenylindole (DAPI) staining

Cells expressing the different myosin variants were fixed and stained with DAPI, as described previously [19].

2.6. Salt-dependence filament assay

Myosin assembly was analyzed using a filament sedimentation assay [22] modified by [23]. Aliquots (100 μl) of purified myosin (0.2 mg/ml) were mixed with an equal volume of two times concentrated KCl solutions in TL 100.1 polycarbonate tubes. After a 15 min incubation on ice, assembled myosin was sedimented at 55 000 rpm in a TLA 100.1 rotor for 10 min at 4°C. The supernatant fractions containing monomeric myosin were analyzed with the Bradford assay [20]. The fraction soluble was calculated by dividing the amount of protein in the supernatant by the total protein.

2.7. Cell imaging

Cells were imaged with a Zeiss Axiovert 100 microscope and a 1.6× Optovar lens (Zeiss, Thornwood, NY, USA). Images were collected on a cooled charge-coupled device camera as described [2].

3. Results

3.1. Expression of mutant MHC genes in Dictyostelium

To examine the relative importance of the three threonine residues, 1823, 1833 and 2029, of the *Dictyostelium* myosin tail in regulation of myosin thick filament formation and myosin function in vivo, we constructed plasmids that express all six possible combinations of aspartates and threonines at the positions 1823, 1833 and 2029. Furthermore, we constructed a plasmid encoding a myosin heavy chain in which threonine 1823 was replaced by an alanine and threonines 1833 and 2029 were changed to aspartates. Transformation of the myosin null cell line HS1 with the plasmids pLittleDTT, pLittleDDT, pLittleDTD, pLittleTDD, pLittleTTD, pLittleTDT, pLittleADD as well as pLittleMyo and pBigAsp resulted in cells that express DTT-myosin, DDT-myosin, DTD-myosin, TDD-myosin, TTD-myosin, TDT-myosin, ADD-myosin, wt-myosin and DDD-myosin, respectively. The expression level

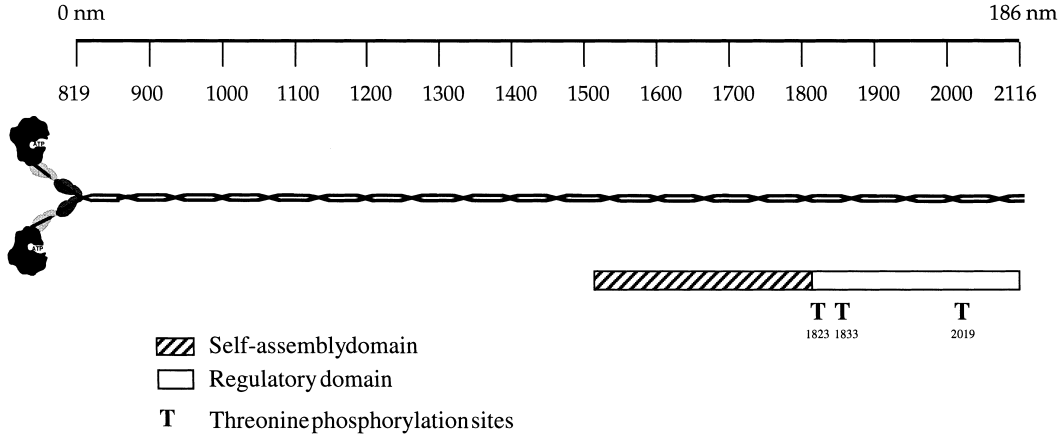

Fig. 1. Schematic representation of functional domains within the tail of *Dictyostelium* myosin.

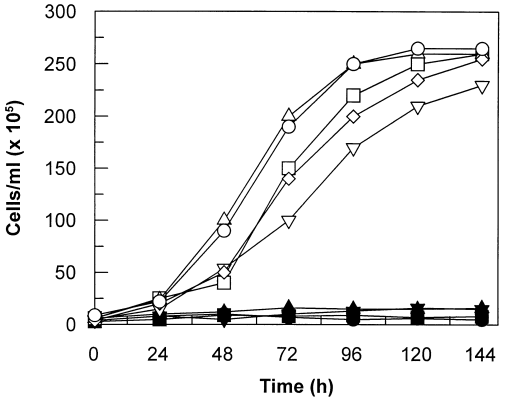

Fig. 2. Growth curves of *Dictyostelium* cells expressing different myosin heavy chain variants. At time point 0 h, 200 μl aliquots of cells grown on plates were placed into 20 ml suspension culture. Cells transformed with pLittleDTD (▲), pLittleDDT (■), pLittleDTT (▼), pLittleTDD (□), pLittleTTD (▽), pLittleTDT (△), pLittleADD (◇), pLittleMyo (○) and pBigAsp (●).

of all the myosin variants was comparable to wt-myosin in wt cells as seen by Western blot analysis (data not shown).

3.2. Cytokinesis

To assay competence for myosin-dependent cytokinesis in suspension, we placed transformants of all myosin variants in suspension culture and monitored their growth. Cells expressing wt-myosin grew in suspension with a doubling time of approximately 10–15 h, but sometimes slower (Fig. 2). Unlike cells growing on plates, where all the cell lines were able to grow by myosin-independent cytokinesis [4,21], cells expressing myosin variants in which threonine 1823 was replaced by an aspartate could not survive in suspension. However, replacement of aspartate 1823 with alanine in the ADD-myosin variant recovered the ability of the cells to grow in suspension. From these studies, we suggest that threonine at position 1823 is a critical phosphorylation site for regulation of myosin assembly/disassembly and function in vivo.

We then investigated the shape and size of the different transformants in suspension by video microscopy. All the cell lines transformed with a plasmid encoding a myosin variant with a wt-threonine or an alanine at position 1823 behaved like wt cells, in that they appeared generally round and actively extended and retracted projections (pseudopodia and filopodia). All transformants expressing myosin variants with position 1823 replaced by an aspartate were increased in size like myosin null cells [3]. They were capable of extending and retracting filopodia and lamellipodia and of forming streams that led to aggregates. These cells also ingested bacteria by phagocytosis, but at a lower rate than the wt cells.

Mutant cells that had been grown in suspension were plated onto coverslips, fixed and stained with the DNA stain, DAPI. Immunomicroscopy of stained cells showed that all constructs with an aspartate at position 1823 of the myosin heavy chain lead to the formation of large multinucleated cells in suspension, which is consistent with abnormalities in the cell division process (Fig. 3). In contrast, all cells containing a threonine or an alanine at position 1823 were mostly single nucleated like wt cells. The presence or absence of a negative charge at positions 1833 and 2029 seemed to have only a slight influence on the formation of multinucleated cells (Fig. 3) or on growth in suspension (Fig. 2). These results are consistent with threonine 1823 being the most critical site for regulation of thick

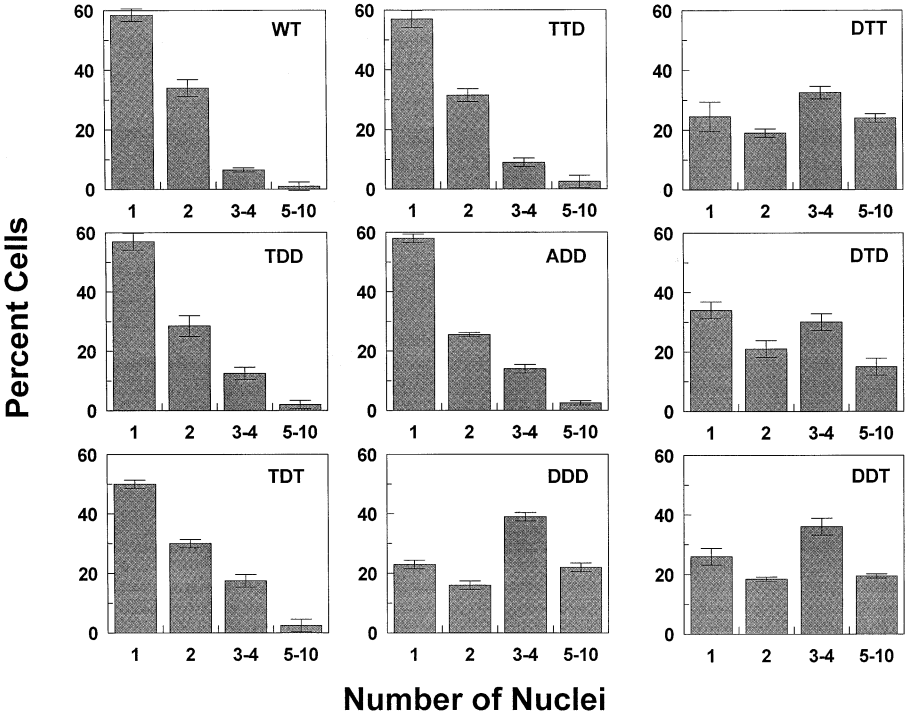

Fig. 3. Quantitative analysis of the number of nuclei in wt-myosin, TDD-myosin, TDT-myosin, TTD-myosin, ADD-myosin, DDD-myosin, DTT-myosin, DTD-myosin and DDT-myosin expressing cells. Cells grown for 10 generations in shaking culture were plated on glass coverslips for 15 min before methanol fixation and nuclei staining with DAPI. From each transformed cell line, 200 cells were counted.

filament formation and myosin function in vivo. The majority of the D1823 containing myosin heavy chain variants was between three and four nuclei. Furthermore, since between 10 and 20% of these cells contain more than five nuclei, it would appear that although mutant cells proceed to an advanced stage in mitosis, they do not complete cytokinesis. This phenomenon was already described for the DDD-myosin variant [6]. The defect in cytokinesis was apparently corrected for when large, multinucleated cells grown in suspension were transferred to a surface, after which their average size and number of nuclei decreased in a short period of time by the myosin-independent cytokinesis B [5,33].

3.3. Developmental properties

Myosin null cells are unable to complete development [3,4,21]. Transformation of plasmids that express myosin wt heavy chain complement this defect. In contrast, the DDD-myosin variant is not able to rescue development [6]. On bacterial lawns, DDD-myosin expressing cells were unable to form sorocarps and arrested at the mound stage. To test the ability of our myosin heavy chain variants to complement this developmental defect, we transformed myosin null cells with the corresponding plasmids and plated them on bacterial lawns. Cells complemented with wt-myosin, TTD-myosin, TDT-myosin, TDD-myosin and ADD-myosin aggregated and formed sorocarps on *Klebsiella* lawns within 4–5 days (Fig. 4). Transformation with pLittleDTT or pLittleDTD allowed the cells to aggregate and form short fingers mostly, and further development occurred only rarely (e.g. in Fig. 4, DTD has passed the sporulation stage). The myosin heavy chain variants DDD-myosin and DDT-myosin were arrested at the mound stage, like myosin null cells (Fig. 4). All of the above in vivo tests suggest that threonine 1823 is the most critical site for regulation of myosin thick filament formation.

3.4. Biochemical characterization of purified myosin heavy chain variants

Filament formation of *Dictyostelium* myosin in vitro is highly dependent upon ionic strength [24]. wt-Myosin has its greatest solubility in buffers without salt and at salt concentrations above 200 mM. The highest degree of assembly is between 50 and 100 mM KCl. In contrast, the DDD-myosin

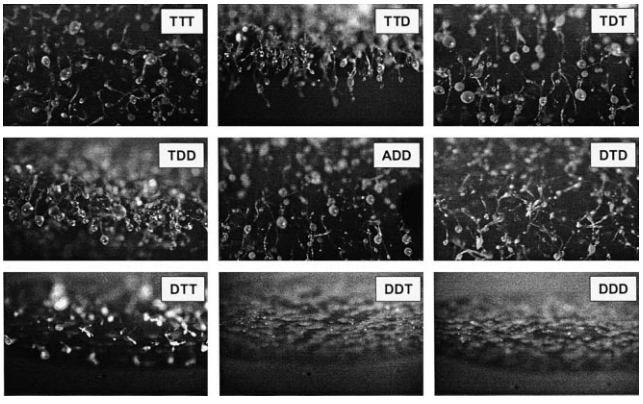

Fig. 4. Developmental phenotypes of *Dictyostelium* cells expressing different myosin heavy chain variants. Myosin null cells were transformed with pLittleDTT, pLittleDDT, pLittleDTD, pLittleTDD, pLittleTTD, pLittleTDT, pLittleADD as well as pLittleMyo and pBigAsp and plated on *Klebsiella* lawns. The different myosin variants are indicated.

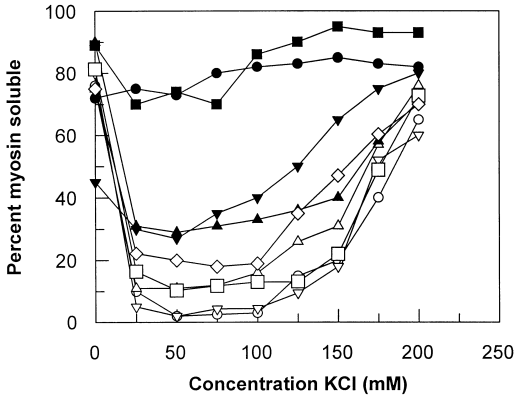

Fig. 5. Salt-dependence of filament formation of wt-myosin and its variants. Purified proteins were dialyzed into buffer without salt. Filament formation was induced by adding KCl in various concentrations and the mixture was incubated for 15 min on ice. Filaments were sedimented by centrifugation. The amount of soluble myosin was determined in a Bradford assay. DTD-myosin (▲), DDT-myosin (■), DTT-myosin (▼), TDD-myosin (□), TTD-myosin (▽), TDT-myosin (△), ADD-myosin (◇), wt-myosin (○) and DDD-myosin (●).

variant has little filament assembly capacity at any salt concentration between 0 and 200 mM [6]. To characterize the in vitro assembly properties of the different myosin heavy chain variants, in each case, the isolated protein was diluted with buffers to obtain different salt concentrations. The samples were centrifuged to separate the soluble and precipitated fractions and the amount of soluble protein was determined in a Bradford assay. Fig. 5 shows solubility curves of the myosin heavy chain variants. The highest degree of assembly for wt-myosin was observed in the 50–100 mM ranges. This as well as the inability of DDD-myosin to form filaments is consistent with previously published solubility data for both proteins [6,24]. The TTD-myosin variant had the same filament assembly properties as wt-myosin (Fig. 5). All myosin variants containing an aspartate at position 1833 and either a threonine or an alanine at position 1823 (TDT-myosin, TDD-myosin and ADD-myosin) were also able to form filaments. The highest degree of filament formation these variants could obtain was between 80 and 90% at physiological salt conditions (50 mM for *Dictyostelium* [25]). Substitution of threonine 1823 to an aspartate had a negative impact on filament formation properties of the myosin. At 50 mM KCl, approximately 30% of the DTT-myosin stayed soluble. The same solubility behavior was seen for the DTD-myosin variant. These observations suggest that a negative charge in position 2029 has no significant effect on the filament formation properties of the protein. In contrast, changing threonine residues 1823 and 1833 in the myosin heavy chain to aspartate increased the solubility of the myosin at all salt concentrations to more than 80% as seen for the DDT-myosin and DDD-myosin in Fig. 5. These data strongly suggest a dominant role of position 1823 on the filament formation of myosin. If this residue cannot get dephosphorylated, myosin stays in monomeric form to a large extent. In order to rule out phosphorylation of the unmutagenized threonine residues in the myosin variants after purification of the protein, we treated samples with alkaline phosphatase. Heavy chain phosphate, but not light chain phosphate, is removed by treatment with bacterial alkaline phosphatase [24]. The salt curves of the alkaline phospha-

tase-treated proteins were identical to the untreated samples (data not shown).

4. Discussion

The in vivo results reported here lead to the somewhat surprising conclusion that the nature of the amino acid at positions 1833 and 2029 in *Dictyostelium* myosin has little influence on the ability of cells to divide in suspension. The residue at position 1823 critically determines the regulation of myosin thick filament formation and in vivo function. The comparison of cells expressing different myosin variants in respect to development provides a similar picture.

From the in vivo results, we conclude that the amino acid at position 1823 has to be able to get dephosphorylated during the life cycle of the cell. However, it is not necessary that this residue allows phosphorylation to sustain growth in suspension or development as seen in the ADD-myosin variant. These data are in accordance with previously published results on a AAA variant [6]. Cells expressing AAA-myosin were able to grow in suspension and formed fruiting bodies on bacterial lawns. The in vivo data suggest that positions 1833 and 2029 do not have strong influence on cell growth and sporulation.

On the other hand, the in vitro filament assembly measurement unveiled finer effects by position 1833 that were not obvious from the in vivo assays. From this assay, the purified myosin variants were further characterized to determine the influence of each single threonine-aspartate substitution, which mimics a phosphorylation event, on the ability to form filaments. As described earlier [6], the introduction of three aspartate residues completely blocks filament formation. The same result was obtained by introducing two negative charges at positions 1823 and 1833 (DDT). However, two negative charges are not sufficient to disrupt filament formation if introduced in positions 1833 and 2029 (TDD) as seen in the TDD-myosin and ADD-myosin variants, both of which show filament assembly curves similar to wt-myosin. However, myosin with a threonine at position 1833 recovers the ability to form thick filament even when position 1823 contains an aspartate residue. As seen in Fig. 5, DTT and DTD form thick filaments better than DDT.

The mechanism for *Dictyostelium* myosin filament assembly is thought to proceed in two stages. The initial slower step occurs by sequential association of myosin II monomers into parallel dimers and antiparallel tetramers. The next step is rapid lateral addition of myosin dimers to bipolar nuclei to form thick filaments [27]. Previous to the initial step, myosin monomers are thought to equilibrate between the folded and straight conformations. This is based on a study showing that a folded conformation of the myosin molecule is promoted by phosphorylation of the heavy chain by *Dictyostelium* myosin heavy chain kinase, and the folded monomers are excluded from filaments formed upon addition of salt [26]. As already seen for the in vivo data shown above, position 1823 is most critical for the ability of myosin to form filaments. By changing threonine at position 1823 to aspartate, myosin filament formation is dramatically affected via two possible ways. The first way is by shifting the equilibrium between the folded and the straight conformations previous to the initial step of filament assembly. A negative charge at position 1823 probably favors the folded monomers and therefore reduces the available straight monomers to form dimers. Dimers are the prerequisite to tetramers and the following thick filament assembly. A genetic screen to search for suppressors of the 3×Asp cells also leads to a complete recovery of wt function by changing the aspartate at position 1823 to a tyrosine [28]. This result further supports the proposal that missing the negative charge at this position results in a preference of the straight conformation and therefore favors filament assembly.

The second way that position 1823 could contribute is at the initial slower step where myosin monomers assemble into parallel dimers and antiparallel tetramers. The region of the myosin tail that interacts in the parallel dimer has been mapped previously using rotary shadowing electron microscopy [26]. Although D1823 lies outside of the region of contact common to all parallel dimers, it is the closest of the three phosphorylation sites to the assembly domain. Furthermore, all three residues are within the final 42 nm of the tail, which is the region that overlaps in the antiparallel tetramers [26]. A study using monoclonal antibodies is consistent with the importance of the end of the tail in antiparallel interactions. Only small parallel aggregates form in the presence of a monoclonal antibody against *Dictyostelium* myosin that binds at the carboxy-terminus of the tail [29]. Therefore, it is reasonable that the T1823D change affects both the parallel dimerization and the following antiparallel tetramerization. The interaction between two monomers in a myosin filament appears to be along more than half of the myosin tail [26]. It is surprising therefore that the introduction of a single charge has such a dramatic effect on this interaction. Two analogous cases are described in which a single charge change disrupts filament assembly. One is paramyosin in *Caenorhabditis elegans* [30] and another is the R1880P mutation in *Dictyostelium* myosin [23]. Recent work provided evidence that, besides charge-charge interactions, hydrophobic interactions are likely to play a major role in myosin filament assembly [31]. The disruption of dimer formation due to introduction of a negative charge in a hydrophobic interaction core is a common phenomenon. An example for the disruption of dimer formation by changing one hydrophobic amino acid for a negatively charged amino acid was described for the elongation factor Ts from *Thermus thermophilus* [32].

What is the structural basis for the inhibition of filament formation based on phosphorylation? The tail of the myosin heavy chain contains a 7 residue repeat, characteristic of α-helical coiled-coil proteins in which the first and fourth amino acids are highly hydrophobic [8]. This pattern allows the formation of an internal hydrophobic core of the coil. Whereas T1833 and T2029 lie adjacent to but not within the hydrophobic core [10], T1823 is located at a core position [11]. Moreover, T1823 lies within a stretch of amino acids where 11 out of 23 residues are positively charged. Two negatively charged regions surround this positively charged region. A proposed mechanism of filament formation would be the charge-charge interaction of the positive stretch of amino acids to the negative stretch along another myosin molecule [11]. Phosphorylation of T1823 introduces a negative charge into the hydrophobic core of the coiled-coil. The result could disturb the α-helical coiled-coil structure, leading to a conformational change of the myosin rod. Our results show that the phosphorylation of T1823 is the key for the regulation of myosin thick filament assembly in vitro and in vivo.

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