We have investigated the folding of the myosin motor domain using a chimera of an embryonic striated muscle myosin II motor domain fused on its COOH terminus to a thermal stable, fast folding variant of green fluorescent protein (GFP). In in vitro expression assays, the GFP domain of the chimeric protein, S1795GFP, folds rapidly enabling us to monitor the folding of the motor domain using fluorescence. The myosin motor domain folds very slowly and transits through multiple intermediates that are detectable by gel filtration chromatography. The distribution of the nascent protein among these intermediates is strongly dependent upon temperature. At 25 °C and above the predominant product is an aggregate of S1795GFP or a complex with other lysate proteins. At 0 °C, the motor domain folds slowly via an energy independent pathway. The unusual temperature dependence and slow rate suggests that folding of the myosin motor is highly susceptible to off-pathway interactions and aggregation. Expression of the S1795GFP in the C2C12 muscle cell line yields a folded and functionally active protein that exhibits Mg$^{2+}$-ATP-sensitive actin-binding and myosin motor activity. In contrast, expression of S1795GFP in kidney epithelial cell lines (human 293 and COS 7 cells) results in an inactive and aggregated protein. The results of the in vitro folding assay suggest that the myosin motor domain does not fold spontaneously under physiological conditions and probably requires cytosolic chaperones. The expression studies support this conclusion and demonstrate that these factors are optimized in muscle cells.

Muscle myosin, the prototype of the myosin II family, is a large asymmetric, multidomain protein composed of six polypeptides: two heavy chains of molecular weight 220,000 and two pairs of nonidentical light chains of molecular weights 17,000–23,000. The myosin catalytic or motor domain is a compact structure formed by the amino-terminal ~780 residues of the heavy chain (1). The core folding motif is a T-strand, mostly parallel, β-sheet that is flanked by three α-helices on each side and forms the ATP-binding pocket. This structural motif is shared with the kinesin family of microtubule-based motors and with G-proteins involved in signal transduction (2, 3). Myosin contains large insertions in the connecting loops of the core motif. These insertions form an extended actin-binding surface and distinguish the myosin structure from kinesin and the G-proteins. The core motif that is the heart of the myosin motor domain is discontinuous and includes segments of primary structure from the entire length of the 780-residue catalytic domain. Thus, it is not surprising that the myosin motor domain has not been refolded once denatured in vitro (4) or successfully expressed in bacterial expression systems as, for example, the much more compact and structurally related kinesin motor domain (5).

The myosin light chain-binding domain corresponds to an 85-A long α-helix that emerges from the myosin motor domain and is stabilized by the binding of two nonidentical light chains. This domain is believed to act as a lever arm to amplify and transmit structural changes in the catalytic domain into relative motion of actin and myosin (6). The light chain binding helix terminates in a proline that starts the long α-helical coiled-coil rod domain.

In our previous analysis (7) of the myosin folding pathway using heavy meromyosin (HMM),1 we demonstrated that light chain binding and folding of the S2 domain of the rod precede motor domain folding. We inferred in that study that folding of the myosin motor domain was rate-limiting and was mediated by cycles of chaperonin binding and release. To confirm this hypothesis, we were interested in investigating the folding of the motor domain in the absence of the influence of the myosin light chains or dimerization of the heavy chain.

Here we present an analysis of the folding of a skeletal muscle myosin motor domain::GFP chimeric protein. The chimeric protein was designed to eliminate the light chain-binding region and replace it with a fast folding variant of green fluorescent protein (GFP) (8). When synthesized in a rabbit reticulocyte lysate, we observe slow folding kinetics for the motor domain in the absence of the influence of the myosin light chains or dimerization of the heavy chain.

The skeletal muscle myosin heavy chain is encoded by the epitope-tagged embryonic chicken myosin cDNA previously described (7, 9). The 3' end of the myosin motor domain was amplified by PCR and a PsI site was introduced with the 5' primer, CCTGGACATCGCTGGTTTTG, and the 3' primer, TGCTTGGACACTGTTGCGAGTGAGTGC, from the

1 The abbreviations used are: HMM, heavy meromyosin; CCT, chaperonin containing TCP-1; GFP, green fluorescent protein; Hsp, heat shock protein; mAb, monoclonal antibody; S1795GFP, motor domain::GFP chimeric protein; S1, myosin subfragment 1; S2, myosin subfragment 2; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid.

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Construction of a Motor Domain::GFP Fusion Protein—The skeletal muscle myosin heavy chain is encoded by the epitope-tagged embryonic chicken myosin cDNA previously described (7, 9). The 3' end of the myosin motor domain was amplified by PCR and a PsI site was introduced with the 5' primer, CCTGGACATCGCTGGTTTTG, and the 3' primer, TGCTTGGACACTGTTGCGAGTGAGTGC, from the
p4HMMa plasmid (7). The 1-kb PCR product was digested with BglII and PstI and inserted between the BglII and PstI cloning sites of p4HMMa, effectively truncating the motor domain coding region at amino acid residue 795. The GFP coding sequence was truncated at the 5' end and was introduced as PCR with the 5' primer, GTACTGCAGAAGAACTTTTCACTGGAG, and the 3' primer sequence, GGTTGACACTATA. The 825-bp GFP insert was digested with PstI cloned into the newly created PstI site of the truncated myosin expression vector. The 5' end of the S1795GFP coding sequence is adjacent to the plasmid Sp6 promoter in the final vector facilitating expression in vitro in a coupled transcription-translation assay. The complete coding region of the vector, p4-S1795GFP, was confirmed by DNA sequencing.

Coiled Transcribed Coupled Translation/Translation Folding Assay—The plasmid p4-S1795GFP was transcribed with Sp6 RNA polymerase and translated in a reticulocyte lysate using the TNT coupled transcription-translation system (Promega Corp., Madison, WI). The reactions were performed as per the manufacturers recommendations in the presence of 50 μCi/100-μl reaction of [35S]methionine (1175 Ci/mmol, PerkinElmer Life Sciences) at 30°C for 2 h. Translation reactions (200 μl) containing newly synthesized S1795GFP were depleted of Mg2+ ATP and exchanged into column buffer by rapid gel filtration on a Sephadex G-50 spin column (Amersham Biosciences) equilibrated with 20 mM MES, pH 6.9, 100 mM KCl, 1 mM MgCl2, 0.5 mM EGTA, 1 mM DTT (7). The effluent from the Sephadex column was divided into aliquots and either stored on ice or incubated with 5 mM Mg2+ ATP for an additional 60 min at 30°C.

The aliquots were analyzed on a Superose 6 HR 10/30 gel filtration column (Amersham Biosciences), equilibrated in the same buffer at 25°C. The elution characteristics of the 10-mm inner diameter × 30-cm long Superose 6 column (flow rate of 0.25 ml/min) were calibrated using myosin, HMM, myosin S1, and myosin light chains, hemoglobin, serum albumin, and reticulocyte CCT (purified as described in Ref. 10) and recorded with both a UV monitor and an in-line Gilson model 121 fluorometer with GFP filter set (Gilson, Madison, WI). Column fractions (0.5 ml) were analyzed by scintillation counting and SDS-polyacrylamide gel electrophoresis (7). The radioactive S1795GFP in the column fractions was quantitated by autoradiography of the dried SDS gels and densitometry (Amersham Biosciences).

Construction of Replication Defective Adenovirus for Expression of S1795GFP—The AdEasy shuttle vector, pShuttleCMV (American Type Culture Collection, Rockville, MD) is a kidney cell line transformed with the early region of human adenovirus 5. These cells contain the genes necessary to package pAdEasy DNA and amplify the replication competent adenovirus (11). The 293 cells were maintained in growth medium containing Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 5% penicillin-streptomycin (10, 0, and 40 μg/ml), 0.2 mM hypoxanthine, 0.6 mM aminopterin, 0.04 mM thymidine (HAT), 5% CO2, and 5% FCS. The adenovirus was harvested once virus plaques were evident and about 50% of the cells were rounded and detached from the surface. The infected 293 cells were collected and infected with the S1795GFP virus at a multiplicity of infection (MOI) of 3–5. When most of the infected 293 cells round-up and begin to detach, the virus was harvested from the cells by cycles of freeze-thaw. Viruses titers of 103–104 plaque-forming units/ml are routinely achieved by this method.

Infection of Cells with AdsS1795GFP and Fluorescent Microscopy—COS 7 cells at 70–80% confluence were infected with the AdsS1795GFP virus at a multiplicity of infection of ~100. The infected cells were incubated in 5% CO2 at 37°C and monitored for green fluorescence and growth arrested at 48–96 h post-infection. C2C12 myoblasts and myotubes were infected at 10-fold higher multiplicity of infection (~1000) because these cells appear less sensitive to this human virus. Myoblasts were infected while subconfluent and maintained in growth medium for ~72 h prior to harvest. Myotubes were infected 2–4 days postfusion once they were well differentiated. Infected myotubes were maintained for 4–5 days in fusion medium prior to harvest of the recombinant protein.

Preparation of S1795GFP from Muscle and Non-muscle Cells—The recombinant protein was isolated from 10–20 100-mm dishes of infected COS 7 cells, C2C12 myoblasts, or myotubes. The cell layer was rinsed twice with 5–10 ml of cold phosphate-buffered saline and then harvested in sucrose/Triton extraction buffer (STE: 100 mM NaCl, 300 mM sucrose, 10 mM imidazole, pH 7.0, 1 mM EGTA, 0.5% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM Mg2+ ATP, 1 mM DTT). The combined, scraped cell layer was transferred to a Dowex Homerogenizer and disrupted with 10–15 strokes on ice. The extract was separated from cell debris by centrifugation for 10 min at 10,000 × g at 4°C. The majority of the fluorescence (>90%) is in this Triton-soluble fraction. The STEB extract was fractionated with ammonium sulfate using steps of 0.5%, 1.0%, 2.5%, and 4.0% saturation. The samples were incubated 30 min at 0°C between steps and the precipitate was pelleted for 20 min at 10,000 × g at 4°C. The redissolved pellets were dialyzed against a low salt buffer: either 20 mM MES, pH 6.9, 100 mM KCl, 1 mM MgCl2, 0.5 mM EGTA, and 1 mM DTT for gel filtration or 25 mM imidazole, pH 7.0, 1 mM DTT for anion exchange chromatography. Samples were fractionated by anion exchange chromatography on a HiTrap Q column (Amersham Biosciences) in 25 mM imidazole buffer, pH 7.0, 1 mM DTT. Bound protein was eluted with a linear gradient of 0–1 M NaCl over 22 min at a flow rate of 1 ml/min. The distribution of the S1795GFP in each fraction was quantitated by fluorescence spectroscopy. Column fractions were collected and analyzed by SDS-PAGE and Western blotting. Blots were probed with the anti-myosin mAb 4H7.6, and an anti-GFP rabbit polyclonal IgG antibody (Molecular Probes, Inc., Eugene, OR) and developed with a chemiluminescence detection kit (Pierce).

The exchange column fractions containing the S1795GFP were extracted from muscle cells were pooled and F-actin was added to a final concentration of 0.2 mg/ml. The sample was dialyzed against 25 mM imidazole, pH 7.6, 25 mM KCl, 4 mM MgCl2, 0.2 mM CaCl2, 1 mM DTT for 4 h to remove the Mg2+ ATP and then sedimented at 300,000 × g for 20 min. The F-actin pellet was resuspended in the same buffer with 5 mM Mg2+ ATP added and sedimented again at 300,000 × g for 20 min to release the bound S1795GFP.

Actin Binding and Motility Assays—The samples were dialyzed against 25 mM imidazole, 25 mM KCl, 4 mM MgCl2, 0.2 mM CaCl2, and 1 mM DTT. F-actin was added to a final concentration of 0.25 mg/ml in 2 mM Mg2+ ATP before centrifugation in an Airfuge at 300,000 × g for 20 min. The supernatants were recovered and diluted for quantitative florescence. Skeletal muscle actin was prepared from an acetone powder of chicken pectoralis muscle (12). The in vitro motility assay was done essentially as described previously (9, 12). An anti-GFP monoclonal antibody, 3E6 (Molecular Probes, Inc., Eugene, OR) and 36800
To analyze the folding of the motor domain of a skeletal muscle myosin, we designed a chimeric protein composed of the first 795 residues of an embryonic chicken striated muscle myosin II gene fused to GFP. The myosin motor domain::GFP chimeric protein is designated S1\textsubscript{795}GFP. The truncated myosin motor domain lacks the long α-helix that forms the essential and regulatory myosin light chain-binding sites. This light chain-binding domain acts as a semi-rigid lever arm (14). It is replaced by a fast folding variant of GFP that contains the S65T mutation to enhance fluorescence sensitivity and two additional COOH-terminal mutations found to improve thermal stability and accelerate folding (8).

The design of the fusion junction was done with the aid of the molecular models of both myosin S1 and GFP using the interactive molecular graphics package molmol (15). The goal was to minimize the introduction of the extraneous intervening sequence between the two protein domains of the chimera. The myosin α-helix that projects from the converter domain and forms the light chain-binding domain was truncated within the IQ motif that forms the essential light chain-binding site (Fig. 1). The myosin α-helix was joined to a short α-helix on the amino terminus of the GFP structure (16). To optimize the position of the GFP with respect to the motor domain, the first 4 residues of GFP were deleted to make the fusion. These residues form the first turn of an NH\textsubscript{2}-terminal GFP α-helix and were replaced by the last turn of a myosin α-helix. Once the design was completed and the expression vector constructed, a molecular model was prepared by merging the two sets of atomic coordinates. The resulting model of the 116-kDa protein is shown in Fig. 1 together with details of the junction between the two domains.

**In Vitro Expression of S1\textsubscript{795}GFP—**A complete cDNA for this chimeric protein was prepared using PCR and standard cloning techniques and a series of expression vectors were constructed and sequenced. The S1\textsubscript{795}GFP cDNA was cloned into a plasmid designated S1\textsubscript{795}GFP. The truncated myosin motor domain lacks the long α-helix that forms the essential and regulatory myosin light chain-binding sites. This light chain-binding domain acts as a semi-rigid lever arm (14). It is replaced by a fast folding variant of GFP that contains the S65T mutation to enhance fluorescence sensitivity and two additional COOH-terminal mutations found to improve thermal stability and accelerate folding (8).

The expression of the single chain S1\textsubscript{795}GFP was quite efficient as measured by incorporation of \(^{35}\)S]methionine into the...
nascent protein (data not shown). We were interested in determining if folding intermediates could be detected, as were found for co-expression of nascent heavy meromyosin with myosin light chains (7). The biosynthesis of S1795GFP was stopped and unincorporated [35S]methionine and Mg2+-ATP were depleted from the translation assay mixture by rapid buffer exchange on a Sephadex G-50 spin column. Aliquots of the assay mixture were then analyzed by gel filtration chromatography (Fig. 2). The newly synthesized S1795GFP was detected using an in-line fluorescent detector set to the GFP fluorescence excitation and emission wavelengths and by measuring the incorporation of [35S]methionine. The column fractions were also analyzed by SDS-PAGE, and the elution profile of the radioactive 116-kDa S1795GFP was quantitated by densitometry of the autoradiograph. The fluorescent protein was found in a broad peak eluting from the Superose 6 column between 10 and 15 ml (Fig. 2, A and B). The fluorescence signal derived from the GFP domain of the chimeric protein correlates with the elution profile of the radioactive 116-kDa protein detected by SDS-PAGE, confirming that both signals mark the elution of the newly synthesized S1795GFP protein.

The GFP domain of the chimeric protein must fold and autoxidize rapidly in the in vitro reaction to produce the fluorescence signal detected in the column effluent. However, the broad distribution of fluorescence and radioactivity in the column effluent suggests that the chimeric protein is a heterogeneous mixture of conformers and is not monodisperse. The molecular weight of S1795GFP (116,000) and the predicted shape of the chimera (Fig. 1) are very similar to that of the myosin proteolytic subfragment, S1 (135 kDa). So, one would expect the hydrodynamics of the two proteins to be essentially indistinguishable on a Superose 6 column. Myosin S1 elutes from the Superose 6 column as a sharp peak centered at 15 ml and its elution position is marked on the profiles in Fig. 2. Most of the newly synthesized S1795GFP protein elutes from the column with a higher apparent molecular weight (Fig. 2A). This behavior is consistent with a less compact structure, an aggregate, or a heterodisperse complex of S1,795GFP with other lysate proteins. A small shoulder of the main S1,795GFP peak elutes from the column at the position of myosin S1, suggesting that perhaps a fraction of the chimera has achieved the compact structure expected of a native myosin motor domain.

The sample in Fig. 2A was depleted of Mg2+-ATP and kept on ice for 40 min before fractionation on the gel filtration column. Previous work has implicated the cytoplasmic molecular chaperonin, CCT, as having a important role in the myosin folding pathway (7). To stimulate chaperonin activity and facilitate the folding of the motor domain, an aliquot of the S1,795GFP was incubated at 30 °C for 1 h with added Mg2+-ATP (Fig. 2B). Instead of improving the recovery of properly folded S1,795GFP, incubation at the elevated temperature shifted the distribution of fluorescence and radioactive protein toward higher molecular weight complexes and aggregates. A fraction of the protein appears to be eluting in the position of CCT. This treatment does not appear to have improved the folding of S1,795GFP. Incubation at 30 °C may be expected to enhance hydrophobic interactions and favor both the interaction with molecular chaperones and off-pathway aggregation.

The sample that was kept on ice without Mg2+-ATP (Fig. 2A) appears less heterogeneous than the sample incubated in folding conditions (Fig. 2B), so we examined the effect of cold incubation on the folding of S1,795GFP. Newly synthesized protein was depleted of Mg2+-ATP with a spin column and incubated at 0 °C for various periods prior to analysis by gel filtration (Fig. 2C). Interestingly, prolonged incubation at low temperatures improved the folding as shown by the increase in

![Fig. 2. Gel filtration chromatography of in vitro synthesized S1795GFP reveals folding intermediates.](http://www.jbc.org/)

The in vitro synthesis of S1795GFP was terminated by passing the expression lysate over a spin column to remove the excess [35S]methionine and Mg2+-ATP, and then aliquots of the lysate were analyzed by Superose 6 gel filtration chromatography (A–C). The elution profile of the S1795GFP was detected via the GFP fluorescence, SDS-PAGE, and autoradiography of the radioactive protein in the column fractions. A and B, the quantitated elution profile of the radioactive S1795GFP (open circles and dashed line) overlaps with the fluorescence profile (solid line). A, analysis of an aliquot that was kept on ice for 40 min after synthesis reveals a major fluorescent peak eluting between 11 and 15 ml that corresponds to the radioactive protein. Note the shoulder eluting in the position of a native papain S1 standard. B, analysis of an aliquot of S1795GFP that was incubated at 30 °C for 1 h after adding back Mg2+-ATP. There is a shift in the elution profile to higher molecular weight forms eluting at or above the molecular weight of native CCT (~900,000) and an increase in the protein eluting in the void volume (V0). C, chromatograms demonstrating the effect of prolonged incubation of newly synthesized S1795GFP at 0 °C without added Mg2+-ATP for 25, 80, and 160 min or with added Mg2+-ATP (140 min + ATP). The elution profiles are offset here for ease of presentation. The column fractions for this experiment were also analyzed for formation of the full-length S1795GFP by SDS-PAGE and autoradiography (data not shown). The fluorescence changes mirror this other method of analysis but provide better resolution of the intermediates. The void volume (V0) of the column, and the elution positions of rabbit reticulocyte CCT (~900 kDa) and papain S1 fragment of myosin (S1) are indicated.
the fluorescent peak eluting at the position of native S1. Several intermediates are evident in the samples from shorter incubations at 0 °C. These intermediates disappear with time as the quantity of S1795GFP eluting at the position of native S1 increases. The addition of Mg2+ ATP during the incubation at 0 °C appears to have little effect on the "cold folding" of S1795GFP. This would suggest that this cold folding pathway is not an energy-dependent chaperone-mediated pathway.

From these data we conclude that the GFP domain of the S1795GFP chimera folds rapidly producing a readily detected fluorescence signal that corresponds to the newly synthesized protein. The myosin motor domain of the chimera does not fold rapidly and under normal conditions of synthesis (30 °C with Mg2+ ATP) the chimera either aggregates, or associates with lysate components, e.g. molecular chaperones. Incubation of S1795GFP at 0 °C, a temperature that does not favor the hydrophobic interactions necessary for chaperone binding (17), promotes a slow, chaperone-independent folding pathway. The rate of folding under these conditions is very slow, clearly too slow to be of physiological significance. This observation suggests that the myosin motor domain requires folding catalysts in vivo, however, these catalysts are not effectively supplied by the rabbit reticulocyte lysate used here.

**S1795GFP Expression in Muscle and Non-muscle Cells**—The in vitro synthesis assay does not produce enough protein to effectively assess the activity of the myosin motor domain, e.g. ATP-sensitive actin binding, actin-activated ATPase, and motor activity. We decided to express the protein in eukaryotic cells to produce enough for biochemical characterization, and to verify that the slow folding observed in vitro is not a consequence of the design of S1795GFP. The cDNA for the chimeric gene was cloned into a replication deficient adenovirus expression vehicle using the AdEasy expression system (11). Human adenovirus infects a broad range of cell types, thus facilitating the expression of S1795GFP in a variety of cell lines including muscle cells. This permitted us to evaluate if the folding efficiently was related to chaperone availability and/or specificity as well.

We chose to infect two kidney epithelial cell lines (human 293 cells and monkey COS 7 cells) and a mouse myogenic cell line (C2C12) to characterize the expression of S1795GFP in vivo. Fluorescence microscopy of the infected cells was used to follow the expression of the GFP-tagged protein and establish the time course of the infection (Fig. 3). Expression of S1795GFP was evident within 24 h of infection and fluorescence intensity increased for about 4 days. The kinetics of expression depended on both the multiplicity of infection and the specific cell line tested. Human 293 cells were most sensitive to infection with the recombinant human adenovirus. This cell line is used to package the AdEasy adenovirus because it complements the defective virus and supports replication. As a result, expression in 293 cells could be followed for only 3 days before cell death. The COS 7 cells do not support virus replication and were only slightly less sensitive to the adenovirus than the 293 cells. The mouse C2C12 cells were significantly less sensitive than these other two cell lines. This probably reflects the absence of the preferred virus receptor (the αvβ3 integrin) on the muscle cell line. The fluorescent S1795GFP localized to the cytoplasm in all cell lines examined. The multinucleated C2C12 myotubes are particularly well suited for expression of this cytoplasmic protein because of the large cytoplasm:nucleus volume ratio. They also can be maintained for the longest time after infection because they are no longer dividing. A general cytopathic effect from viral infection or from expression and accumulation of the chimeric protein was not observed in muscle cells.

The characteristic GFP fluorescence spectrum of S1795GFP was used to quantify and optimize the expression experiments (Fig. 4). The fluorescence spectrum of the GFP clone used for these experiments was characterized by three excitation peaks at 472, 488, and 492 nm and a single emission peak at 507 nm. Lysates from uninfected cells contain nothing with fluorescence spectra similar to this. To compare the levels of expres-
sion, cells were harvested 48–96 h after infection, lysed, and extracted with an isotonic buffer. Mouse C2C12 myotubes consistently produced the highest levels of the protein and were clearly better suited for expression of S1795GFP despite the fact that these cells are not as easily infected or maintained as the other cell lines tested.

The fluorescence was measured and used to follow the fractionation of S1795GFP through ammonium sulfate precipitation. This approach established an immediate difference between the protein expressed in the epithelial cell lines and the mouse myocytes. The S1795GFP extracted from the epithelial cell lines precipitated with ammonium sulfate between 20 and 40% saturation, whereas the protein expressed in myocytes was soluble at 40% saturation and precipitated between 40 and 60% saturation.

This difference became more striking when the concentrated protein fractions were analyzed by gel filtration chromatography (Fig. 5). The protein extracted from COS 7 cells and precipitated with ammonium sulfate elutes in the void volume of the column as a large peak of fluorescence with a broad tail. Identical behavior was found for the S1795GFP expressed in human 293 cells (data not shown). This behavior is inconsistent with a properly folded 116-kDa monomer, but is consistent with either an aggregated protein or a complex of S1795GFP with other cellular components, e.g. chaperones. A peak eluting at a position consistent with a monomer was detected, but this peak accounted for only a small percent of the total protein. Analysis of the fractions by SDS-PAGE and Western blotting with anti-myosin and anti-GFP (data not shown) specific antibodies detected a single band with an apparent molecular weight of 116,000 and no evidence for degradation or loss of the GFP tag (Fig. 5, insets).

In contrast, the protein extracted from C2C12 myotubes and precipitated with ammonium sulfate elutes from the gel filtration column as a monomer with apparent molecular weight of 116,000 (Fig. 5). A small amount (<3%) of the S1795GFP extracted from C2C12 myotubes does precipitate at 40% saturation of ammonium sulfate like the protein extracted from COS 7 cells. This fraction is a mixture of both the high molecular weight complex and monomer when analyzed by gel filtration (data not shown). The expression of S1795GFP in C2C12 myoblasts, the single cell precursor to the multinucleated myotubes, produced a protein that behaved similar to the S1795GFP extracted from the myotubes (data not shown). However, the level of expression in the myoblasts was significantly lower than that achieved with differentiated myotubes (Fig. 4).

The differences in the behavior of the expressed protein isolated from myocytes versus the epithelial cells suggested that the muscle cells may be uniquely suited for expression and purification of functional S1795GFP. We used anion exchange chromatography to fractionate the S1795GFP from both COS 7 cells and C2C12 myotubes after ammonium sulfate precipitation. The protein isolated from the C2C12 myotubes eluted as a sharp peak at about 0.25 M NaCl from a Mono Q column, whereas that protein from the COS 7 cells eluted as a broad peak between 0.25 and 0.4 M NaCl (data not shown). Both fractions were tested for Mg\(^{2+}\) ATP-sensitive binding to F-actin (Fig. 6). The S1795GFP isolated from the C2C12 myotubes binds to F-actin in the absence of Mg\(^{2+}\) ATP and pellets with the actin filaments. The addition of Mg\(^{2+}\) ATP blocks the co-sedimentation of S1795GFP with actin, indicating that it has Mg\(^{2+}\) ATP-sensitive actin binding activity. The protein fraction from the COS 7 cells binds poorly to F-actin in both the presence and absence of Mg\(^{2+}\) ATP. This indicates that S1795GFP isolated from COS 7 cells does not have native actin binding activity.

Isolation of Active S1795GFP from C2C12 Myotubes—To purify the S1795GFP expressed in C2C12 myotubes, the protein was extracted and fractionated with ammonium sulfate before ion-exchange chromatography on a Mono Q column. The fluorescent protein in the column fractions was pooled and recovered by binding to F-actin in the absence of Mg\(^{2+}\) ATP and sedimentation of the actin filaments. S1795GFP was released from F-actin by redissolving the pellet in buffer containing Mg\(^{2+}\) ATP and pelleting the F-actin once again. This final step also removes any inactive protein generated during the various purification steps. The S1795GFP isolated from C2C12 myotubes was purified to near homogeneity (Fig. 7C). The actin added at the last step of the purification was the only major contaminant remaining in the preparation. This purified sample was tested for myosin motor activity.

The motor activity of S1795GFP was measured using the in vitro motility assay (Fig. 7A). S1795GFP is a very well behaved molecular motor supporting sliding actin filament velocities of 3 μm/s; roughly 85% of the maximum velocity of the intact embryonic muscle myosin from which the motor domain was derived (9). Long actin filaments translocated along the motor-coated surface in smooth and continuous tracks producing a uniform velocity distribution (Fig. 7B). The protein was attached to a nitrocellulose-coated glass surface using an anti-GFP mAb to bind the GFP domain. The location of the epitope within the GFP domain is not known; however, the antibody provides a highly favorable attachment as judged by the degree of motor activity that it supports. Substitution of the anti-GFP mAb with an anti-S2 mAb that reacts with epitope not found on S1795GFP produced no attachment or movement of actin fila-
ments, confirming that motor activity was dependent on the correct attachment of S1 795GFP to the surface. The S1 795GFP protein isolated from C2C12 myotubes is a stable and highly active myosin motor domain fused to GFP.

**DISCUSSION**

**Design of a Motor Domain::GFP Chimera**—To investigate the folding of the striated muscle myosin II motor domain independent of the interaction with the light chains, we created a myosin motor domain that lacked the light chain-binding region and was tagged with GFP. The residues that define the limits of the myosin II motor domain have been determined by truncation studies with *Dictyostelium* myosin (18). Truncation of *Dictyostelium* myosin at Arg-761 yields the shortest myosin motor domain that retains the kinetic properties of myosin. This corresponds to Lys-785 of the embryonic skeletal muscle myosin isoform used here. *Dictyostelium* myosin truncated at this residue and fused to artificial lever arms including GFP produces active motor molecules with motility properties similar to single-headed myosin (19, 20). Chymotrypsin digestion of skeletal muscle myosin after removal of both light chains also yields a functional motor domain (21). This proteolytic fragment ends about 5 residues past the site we have used for fusion to GFP.

We chose to truncate myosin at Ala-795 to disrupt the essential light chain-binding site and to achieve a smooth transition from the COOH-terminal α-helix of the myosin motor domain into an NH2-terminal α-helix of GFP. We have found that fusion of the motor domain of the embryonic skeletal muscle myosin to the fast folding variant of GFP does produce a functionally active chimeric motor when expressed in the correct cellular environment, muscle cells. Thus, our design (S1 795GFP) yields a single-chain myosin that appears to fold properly in the correct context and exhibit myosin motor activity.

Iwane et al. (22) designed a motor domain::GFP fusion protein based on the adult chicken skeletal muscle myosin heavy chain and synthesized it in an *in vitro* transcription-translation assay. They immobilized the fusion protein to a glass surface and demonstrated Mg2+ ATP binding and release in single molecule assays; however, they did not characterize the protein for complete folding or actin binding activity as we have done here. We show here that the *in vitro* synthesized protein folds slowly; thus, Mg2+ ATP binding may be the only activity they could detect.

**Cold Folding in Vitro**—When S1 795GFP is expressed in *in vitro* using a transcription-translation assay, the GFP domain appears to fold rapidly and autoxidize to produce its unique fluorescent signature. This fluorescent domain provides a convenient handle to track the motor domain folding intermediates. The folding of the myosin motor domain at 30 °C is much slower than the GFP domain and is inhibited by off-pathway interactions such as aggregation and/or association with cellular proteins. Physiological temperatures and macromolecular crowding inside cells accentuate hydrophobic interactions that contribute to aggregation. Not surprisingly, these are also the conditions that favor target binding by certain chaperones (17). The absence of either the right set of chaperones or release factors may lead to trapping of S1 795GFP in a partially folded state.
state. Under conditions that minimize hydrophobic interactions, for example, dilute solutions and low temperatures, many proteins are capable of spontaneous folding. The intervention by molecular chaperones is unnecessary under these conditions. This is what we observed for S1795GFP: folding is enhanced by incubating the newly synthesized protein at 0 °C instead of 30 °C and that folding pathway appears to be largely ATP-independent, suggesting that it is chaperone-independent. However, the basal rate of folding at low temperatures is too slow to be physiologically relevant. Thus, to achieve rates of synthesis compatible with the level of expression of myosin in muscle, we conclude that folding of the myosin motor domain must be catalyzed by molecular chaperones.

The analysis of the folding of a heavy meromyosin subfragment co-expressed in vitro with myosin light chains has shown that the light chains bind prior to completion of the folding of the myosin motor domain (7). Dimerization of the heavy chains via folding and assembly of the S2 region of the rod also precedes completion of motor domain folding. An interaction of myosin with the molecular chaperonin, CCT, was demonstrated, but the folding reaction in the reticulocyte lysate was still inefficient. Supplementing the lysate with a cytoplasmic extract from C2C12 myotubes accelerated the folding reaction suggesting that muscle cells contain components that selectively accelerate myosin folding. From these observations and the slow folding of the S1795GFP fusion protein in vitro, we have concluded that folding of the myosin motor domain is indeed rate-limiting and must be catalyzed by chaperones that are not effectively supplied in the reticulocyte lysate system.

**Muscle-specific Folding of S1795GFP**—To test this hypothesis, we developed a replication defective adenovirus vector to express the gene for S1795GFP in a variety of cell types including muscle cells. Using the adenovirus delivery system we characterized the expression of S1795GFP in several epithelial cell lines and the mouse C2C12 muscle cell line. S1795GFP was expressed and accumulated in the cytoplasm in all of the cell lines tested. However, only the muscle cell line was capable of producing a properly folded protein with biochemical activities consistent with a myosin motor domain. The predominant product of expression in the epithelial cell lines was an aggregate or complex that contained a full-length and intact 116-kDa protein but retained no activity except the GFP fluorescence. In contrast, expression of S1795GFP in either C2C12 myoblasts or myotubes produced a soluble, monomeric protein that retained Mg2+ ATP-sensitive actin binding activity. Purification of the protein from C2C12 myotubes demonstrated that it is a stable monomeric myosin::GFP chimera with motor activity.
The motor activity is an unambiguous demonstration of the proper folding of the purified S1\textsubscript{795}GFP. We have achieved a sliding actin filament velocity (3 μm/s) approaching that of the intact embryonic chicken myosin used as the base to construct the motor domain::GFP chimera (9). The level of activity we observed is similar to that reported for a \textit{Dicyostelium} myosin motor domain constructed with a synthetic lever arm supplied by two α-actinin repeats (19, 21). The best motor activity for that chimera was achieved when it was attached to the surface by a mAb to an epitope tag on the distal end of the α-actinin repeat. Similarly, we have achieved excellent movement over surfaces produced with anti-GFP binding of the GFP domain, suggesting that GFP is acting as the lever arm of the chimeric motor.

We previously demonstrated that muscle cell extracts contain factors that enhanced the folding of motor domains of HMM expressed in a reticulocyte lysate, suggesting a role for muscle-specific factors in the folding pathway (7). The ability to retain factors that enhanced the folding of motor domains of a motor domain constructed with a synthetic lever arm supplied by two α-actinin repeats (19, 21). The best motor activity for that chimera was achieved when it was attached to the surface by a mAb to an epitope tag on the distal end of the α-actinin repeat. Similarly, we have achieved excellent movement over surfaces produced with anti-GFP binding of the GFP domain, suggesting that GFP is acting as the lever arm of the chimeric motor.

In vivo sliding actin filament velocity (3 μm/s) approaching that of the intact embryonic chicken myosin used as the base to construct the motor domain::GFP chimera (9). The level of activity we observed is similar to that reported for a \textit{Dicyostelium} myosin motor domain constructed with a synthetic lever arm supplied by two α-actinin repeats (19, 21). The best motor activity for that chimera was achieved when it was attached to the surface by a mAb to an epitope tag on the distal end of the α-actinin repeat. Similarly, we have achieved excellent movement over surfaces produced with anti-GFP binding of the GFP domain, suggesting that GFP is acting as the lever arm of the chimeric motor.

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