Muscle and yeast actins display distinct behavioral characteristics. To better understand the allosteric interactions that regulate actin function, we created a muscle/yeast hybrid actin containing a muscle-specific outer domain (subdomains 1 and 2) and a yeast inner domain (subdomains 3 and 4). Actin with muscle subdomain 1 and the two yeast N-terminal negative charges supported viability. The four negative charge muscle N terminus in a muscle subdomain 1 background caused death, but in the same background actin with three N-terminal acidic residues (3Ac/Sub1) led to sick but viable cells. Addition of three muscle subdomain 2 residues (3Ac/Sub12) produced no further deleterious effects. These hybrid actins caused depolarized cytoskeletons, abnormal vacuoles, and mitochondrial and endocytosis defects. 3Ac/Sub1 G-actin exchanged bound GTP more slowly than wild type actin, and the exchange rate for 3Ac/Sub12 was even slower, similar to that for muscle actin. The mutant actins polymerized faster and produced less stable and shorter filaments than yeast actin, the opposite of that expected for muscle actin. Unlike wild type actin, in the absence of unbound ATP, polymerization led to ADP-F-actin, which rapidly depolymerized. Like yeast actin, the hybrid actins activated muscle myosin S1 ATPase activity only about one-eighth as well as muscle actin, despite having essentially a muscle actin-specific myosin-binding site. Finally, the hybrid actins behaved abnormally in a yeast Arp2/3-dependent polymerization assay. Our results demonstrate a unique sensitivity of yeast to actin N-terminal negative charge density. They also provide insight into the role of each domain in the control of the various functions of actin.

The yeast _Saccharomyces cerevisiae_ has one essential actin gene (1, 2), _ACT1_, that encodes a protein 87% homologous to skeletal muscle actin. This system has been used widely in the study of actin structure and function because of the ease with which site-specific mutations can be introduced and mutant proteins purified. Because of the high degree of homology between these actins and the ability to genetically manipulate yeast, we and others have used yeast actin to investigate the role of many amino acid residues in various actin functions such as filament formation, nucleotide binding and exchange, and interactions with various actin-binding proteins. There are, however, limitations to this model based on some striking differences in their behavior. Yeast actin polymerizes faster (3, 4), has a faster nucleotide exchange rate (5, 6), a faster P_i release rate (7, 8), and it activates myosin S1 ATPase activity only one-tenth as well as muscle actin (9).

Muscle actin is incompatible with yeast viability, even though yeast and muscle actins differ by only 50 of 375 residues, and most of these differences involve similar replacements (1). These differences may be in binding regions for species-specific actin-binding proteins. Alternatively, they may affect overall filament conformation and dynamics resulting in loss of proper function in the yeast cell. Studies involving specific muscle-like mutations in yeast actin are limited. One region of interest has been the N terminus. Substitution of the N-terminal two acidic residues of yeast actin with four acidic residues from the muscle actin N terminus (4Ac mutant) leads to a phenotypically normal yeast; this modified actin activates skeletal myosin S1 ATPase activity 3-fold better than WT yeast actin (10). Lu _et al._ (11) demonstrated, using a reconstituted cardiac muscle fiber system, that 4Ac yeast actin generated about 80% of the tension seen with muscle actin; WT yeast actin produced tension just greater than base line, and 3Ac actin (three N-terminal acidic residues) produced about 40% of the tension seen with muscle actin. Another study demonstrated that yeast actin possesses a 9-fold difference in affinity for ATP _versus_ GTP in contrast to the 1,000 –2,000 difference for muscle actin. It further showed that this difference is due in large part to a Phe-306 (yeast) to Tyr (muscle) substitution in the nucleotide-binding pocket (12).

In this study, we have addressed three major questions. First, what determinants are important for the unique properties of muscle actin? Second, what is the role of interaction between the large and small domains of actin in controlling actin filament topology and dynamics? Third, what is the molecular basis for the incompatibility between muscle actin and yeast viability considering the high degree of homology between yeast and muscle actins? We reasoned that a yeast/muscle hybrid actin containing one normal yeast and one normal muscle domain would provide insight into answers for these questions. For these studies, we have made a yeast/muscle hybrid actin consisting of subdomains 3 and 4 from yeast actin (large domain) and subdomains 1 and 2 from muscle actin (small
domain. We inserted the muscle substitutions into the small domain because this domain interacts with many different actin-binding proteins and is likely to reflect species-specific differences in its ability to interact with various actin-binding proteins. We have then assessed the effects of the hybrid actin in vitro in yeast and on various parameters of actin function. We used purified proteins. 

MATERIALS AND METHODS

**Mutagenesis and Construction of Mutant Strains**

Mutant actin plasmids were constructed with the QuikChange® site-directed mutagenesis kit from Stratagene (La Jolla, CA) and used to transform yeast essentially as described previously (13). Plasmid shuffling then led to cells producing only the mutant actin. Mutagenic primers were obtained from Integrated DNA Technologies (Coralville, IA). For the 3Ac/sub1 and 3Ac/sub12 actins, the WT plasmid had to be force-selected against 5-fluoroorotic acid (5-FOA), toxic to URA3-containing cells, during the plasmid shuffling. Mutant plasmids rescued from the trp⁺, ura⁻ cells were sequenced to verify the presence of the mutations.

**Determination of Mutant Phenotypes**

Cell growth in liquid culture at 30 °C was monitored by measuring the cell density (A₆₀₀) with time, and doubling times were determined as described previously (13). Mutant strains were tested for temperature sensitivity, hyperosmotic sensitivity, and the ability to grow with glycerol as a sole carbon source (13).

**Actin Cytoskeleton Observation**

**Mitochondria Visualization**—Mitochondrial morphology was monitored in cells previously transformed with a centromeric plasmid containing the mitochondrial targeting sequence of citrate synthase 1 fused to green fluorescent protein (CS1-GFP) driven by the endogenous citrate synthase promoter as described previously (13, 14).

**Actin Cytoskeleton Observation**—Formaldehyde-fixed cells described above were treated with 0.5 mg/ml saponin (Sigma) in phosphate-buffered saline at room temperature for 30 min and washed three times with phosphate-buffered saline to remove residual detergent. Cells were resuspended in phosphate-buffered saline to which was added rhodamine phalloidin at a final concentration of 1.1 μM. Samples were incubated overnight at 4 °C. Fluorescence images of actin filaments were captured as described below. To establish the normal pattern for the actin cytoskeleton, we only counted budding cells in which the size of the bud was less than 1/3 the size of the mother cells when there is maximum asymmetry of patch distribution and the greatest degree of polarization of actin cables.

**Vacuole Staining**—Vacuoles were stained with MDY-64 from Molecular Probes (Eugene, OR) according to the manufacturer’s protocol with modifications. Overnight cultures were subcultured with agitation at 30 °C from an A₆₀₀ of about 0.1 in YPD to an A₆₀₀ of between 0.3 and 0.4. Cells were resuspended in staining buffer (10 mM HEPES buffer, pH 7.5, and 5% glucose) followed by the addition of 10 μM MDY-64, and the suspensions were incubated at room temperature for 3 min. Cells were resuspended in fresh staining buffer, and vacuole morphology was monitored by fluorescence microscopy as described below. The cells were categorized as containing four or fewer equally sized lobes (normal), greater than four approximately normal to abnormally small sized lobes (hypervesculated), or abnormally large vacuole lobes (greater than half the size of the cell) but fewer than four lobes.

**Endocytosis Assay**—Lucifer yellow internalization was monitored as described previously (15) with modifications. Briefly, 2 ml of cells in early log phase (A₆₀₀ = ~0.3) were collected by centrifugation and resuspended in fresh YPD. Lucifer yellow was added to a final concentration of 13 mg/ml, and the cells were incubated at 30 °C with agitation for 1 h. Cells were then washed three times with ice-cold succinate/azide buffer (50 mM sodium succinate, pH 5, containing 20 μM NaNO₃) and resuspended in a small volume of the same buffer. Labeled vacuoles were observed by fluorescence microscopy.

**Fluorescence Microscopy**

Fluorescence images were collected with a Zeiss Axioplan 2 microscope (Oberkochen, Germany) using a Plan-Apochromat ×100, 1.4 NA objective lens, and a cooled CCD camera (Spot RT monochrome, Diagnostic Instruments, Sterling Heights, MI). Microscope control and image recording/enhancement were performed using Metamorph (Universal Imaging Corp.). Image color manipulation, image overlapping, and cell counting were performed with a combination of ImageJ software package (National Institutes of Health) and Adobe Photoshop version 7.0. For the analysis of actin filament and mitochondrial morphology, 20 z-sections were obtained at 0.2-μm intervals through the entire cell. Out-of-focus light was removed by two-dimensional deconvolution using the Metamorph package, and each series of images was stacked to a two-dimensional image by ImageJ.

**Protein Preparations**

Yeast WT and muscle hybrid actins in the Ca²⁺ globular (G) form were purified as described (16). Actin concentrations were determined by the UV absorbance at 290 nm using the extinction coefficient of ε = 25.6 mM⁻¹·cm⁻¹. G-actin was stored in G-buffer (10 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.2 mM CaCl₂, and 0.1 mM DTT) at 4 °C and used within 5 days following completion of purification. DTT-free F-actin in DTT-free F-buffer (10 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.2 mM CaCl₂, 2 mM MgCl₂, and 50 mM KCl) was labeled at Cys-374 with Oregon Green maleimide (OG; Molecular Probes) at a molar ratio of 1:5 (actin:OG) at room temperature for at least 2 h. The OG-labeled F-actin was collected by centrifugation and depolymerized by dialysis against G-buffer. The extent of labeling was determined by the ratio of the probe concentration determined by UV absorbance at 491 nm (using the extinction coefficient of ε = 8100 M⁻¹·cm⁻¹ for OG) versus the actin concentration determined as described above. The labeling efficiency for each actin was between 65 and 75%. The OG-labeled actins were stored at 4 °C and used within 5 days. Protein A-tagged yeast ActR2/3, N-WASP, and *Listeria monocytogenes* ActA were prepared as described (17). Proteins were aliquoted and stored at −80 °C. Each aliquot of yArp2/3 was used within 2 days after

**Muscularization of Yeast Actin**

The cells were categorized as containing four or fewer equally sized lobes (normal), greater than four approximately normal to abnormally small sized lobes (hypervesculated), or abnormally large vacuole lobes (greater than half the size of the cell) but fewer than four lobes.
thawing. Bovine cardiac tropomyosin was purified as described (18). Characterization of Actin in Vitro

Actin polymerization was monitored by the increase in light scattering or the increase in fluorescence of Cys-374-pyrene-labeled actin following addition of 2 mM MgCl₂ and 50 mM KCl to induce polymerization. Nucleotide exchange was determined by following the displacement of bound etheno-ATP by ATP as a function of time. Thermostability was determined by the change in molar ellipticity of the protein as a function of temperature. These assays and measurement of intrinsic tryptophan fluorescence were performed as described previously (12). For conversion of Ca²⁺-G-actin to Mg²⁺-G-actin, 0.5 mM EGTA, pH 7.5, and 0.2 mM MgCl₂ were added simultaneously to the G-actin solution at 4 °C for 1 h, and the Mg-actin was used within 2 h.

Electron Microscopy and Actin Filament Length Measurement—F-actin, 2.5 μM, was diluted 3-fold with F-buffer and applied to carbon-coated Formvar grids. Following negative staining with 1% (w/v) uranyl acetate, samples were examined using a JEOL JEM-1230 transmission electron microscope. Filaments images were recorded with a Gatan UltraScan 1000 2 × 2k CCD camera (University of Iowa Electron Microscope Facility). We measured the contour lengths of at least 100 filaments of each actin sample, which clearly showed both filament ends, using ImageJ software.

Actin Bead Recruiting Assay—Polystyrene beads, 4.89 μm diameter (Bangs Laboratory, Inc., Fishers, IN), were incubated at room temperature for 1 h in ActA-containing absorption solution (10 mM HEPES, pH 7.5, 150 mM NaCl, and 1 μM ActA) at a final volume ratio of 0.1%. The reaction was stopped by the addition of bovine serum albumin (Sigma) to a final concentration of 10 mg/ml for another 10 min on ice. Beads were stored in storage buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mg/ml bovine serum albumin) at 4 °C for 2 days.

For the actin recruiting assay, the reaction contained 10 mM HEPES, pH 7.0, 0.2 mM ATP, 10 mM DTT, 2.5 mg/ml bovine serum albumin, 2 mM MgCl₂, 50 mM KCl, 200 μM diazabicyclo[2.2.2]octane, 7 μM OG-labeled actin (5% labeled), 0.1 μM yeast Arp2/3, and ActA-absorbed beads at a final concentration of 0.004%. The fluorescent halo formed around the beads, because of actin polymerization triggered by Arp2/3 and ActA, was observed by fluorescence microscopy as described above.

RESULTS

Orientation of the Mutations Used—To make a more muscle-like actin in yeast, we focused our attention on subdomains 1 and 2 (Fig. 1A). These subdomains are on the outside of the filament and serve as the binding surface for a number of actin-binding proteins, and we reasoned they were most likely to exhibit altered interactions with yeast-specific actin-binding proteins. We grouped the variant residues into regional cassettes that we could use to subdivide our study. The names of these cassettes are shown in Fig. 1 and Table 1 and are based on the muscle amino acids introduced. Some differences such as the His-372 in yeast versus the corre-
TABLE 1
Nomenclature and the mutant origin for each cassette of residues used in this report

| Cassette | Origin |
|----------|--------|
| 3Ac      | N (terminus), DED |
| 4Ac      | N (terminus), DEDE |
| TT       | V5T/A6T |
| CLV      | I10C, M16L/C17V |
| TLA      | V103T, M110L/S114A |
| MASTAR   | F132M/S135A, T350S/S358T, T350S/S358T, S365A, H372R |
| VKI (Sub2) | I43V, R68K, V76I |

sponding Arg in muscle actin reflect a difference between yeast and higher eukaryotic actins in general. One significant region, named CLV, reflects a basic difference between muscle actins and nonmuscle actins, including yeast actin as does Thr-103. A third notable difference is the acidic N terminus. Yeast has two acidic residues; β- or γ-nonmuscle actin has three, and muscle actin has four. This set of residues on a totally unstructured finger-like projection from the body of G-actin appears to be a major site of interaction with muscle myosin.

Interestingly, the subdomain 1 mutations appear to largely occupy a column encased in a shell of nonvariant secondary structural elements (Fig. 1). The column runs diagonally through the interior of subdomain 1 connecting the nucleotide-binding site in the inter-domain cleft with the surface of the protein surrounding the C and N termini. This arrangement suggests that this collection of residues may provide a way of transducing information from protein-protein interactions occurring at or near the termini with the nucleotide cleft and hinge regions that control actin dynamics.

Most Subdomain 1 Muscle Substitutions Support Yeast Viability—Beginning with the C terminus, we carried out successive rounds of mutagenesis on the WT yeast actin gene. After we accumulated several muscle-like mutations, we examined the mutant strains for any biologically significant defects. The strains MASTAR, CLV/MASTAR, and TT/CLV/MASTAR (see Table 1) displayed normal growth patterns with respect to growth arrest and doubling time. They also showed no differences from wild-type cells when plated at various temperatures or on media supplemented with either 0.9 M NaCl (resistance to hyperosmolar conditions) or glycerol (functional mitochondria). However, addition of Thr-103, Leu-110, and Ala-114 led to a mutant strain TT/CLV/TLA/MASTAR that has an early growth arrest at about 85% of the level achieved by wild-type cells and a 1.4-fold longer doubling time. It also shows a slight cold sensitivity to 37 °C. All of these mutant cells were obtained by plasmid shuffling in urea−, trp− medium without having to resort to selection on 5-FOA in agreement with our other results demonstrating a lack of significant defects associated with the altered proteins.

Addition of a Four Acidic N-terminal Sequence to Modified Yeast Actin—To completely create an entire muscle-like subdomain 1, we attempted to convert the yeast N-terminal DSEV amino acid sequence to DEDE (4Ac) in our longest mutant, TT/CLV/TLA/MASTAR. Although cells containing the 4Ac/TT/CLV/TLA/MASTAR plasmid were viable with WT actin expressed in the same cell, we could not obtain cells in which this mutant was the only actin in the cell using standard plasmid shuffling alone or with 5-FOA to bias the selection toward slower growing cells. This synthetic lethality was particularly surprising considering the 4Ac cassette by itself produced no significant phenotype in vivo (10).

**Effect of Muscle Mutations on Cellular Organization**—To better define the cellular basis for the 3Ac/Sub1 and 3Ac/Sub12 actin cell growth defects, we first examined the actin cytoskeleton by staining formaldehyde-fixed cells with rhodamine phalloidin following mild treatment with saponin to increase exposure of the actin to the stain. Both strains exhibited either randomly distributed actin patches or randomly distributed deposition of actin cables of varying lengths (Fig. 3 and Table 2) signifying a loss in polarity within the cells.

The abnormal cytoskeletal patterns suggested the mutations might have affected mitochondrial morphology and distribution because these parameters have been associated with a
properly functioning actin cytoskeleton (19). Using green fluorescent protein-citrate synthase as a mitochondrial marker (see “Materials and Methods”), we observed no strikingly abnormal mitochondrial phenotype, although the cells seemed to exhibit a slightly more aggregated or randomly distributed mitochondrial pattern compared with normal cells (data not shown). This finding agrees with the ability of the mutant cells to grow on glycerol as a sole carbon source.

We also attempted to assess vacuole morphology that is tightly linked to actin cytoskeleton function (20). Only a very small population of the mutant cells could be stained when we attempted to visualize vacuoles with the commonly used membrane dye FM4-64. This result implied that either vacuole integrity was damaged or, because the dye enters through endocytosis, this process is defective in these cell strains. We then used a membrane-permeable vacuole-specific dye MDY-64 instead. Fig. 4 shows that vacuoles were easily observed in all cells tested. However, vacuole appearance was much more heterogeneous than that observed in WT cells. We sorted the cells into three groups according to vacuole morphology as follows: those with normal vacuoles, those with large vacuoles, and those with larger than normal numbers of vacuolar lobes as described under “Materials and Methods.” Table 2 shows that the distribution among these categories was similar for each of the two mutant cell strains. These observations suggest the mutations that led to altered vacuoles, possibly by affecting the fission/fusion processes that regulate vacuole morphology, were common to all strains examined. Assessment of vacuole inheritance was not possible with the experimental approach we employed.

Because of the FM4-64 results, we further examined the two mutant cell strains for endocytosis defects by following the internalization of lucifer yellow whose uptake depends on endocytosis. Fig. 4 and Table 2 demonstrate that only 2% of

![Figure 3](image-url)
3Ac/Sub12 cells and none of the 3Ac/Sub1 cells contain lucifer yellow, again correlating with an endocytosis defect caused by the mutant actins.

In Vitro Characterization of Hybrid Actin Behavior—The yeast/muscle hybrid actins allowed us to assess the importance of the hinge region and inter-domain allosteric interactions on both G- and F-actin function as well as the importance of interactions between subdomains 1 and 2. It also allowed us to determine which parts of muscle actin are important for its characteristic properties. Previous work demonstrated that muscle G-actin appeared to be conformationally more stable than yeast actin based on its higher denaturation temperature and slower rate of exchange of bound nucleotide. We first examined the effects of muscle subdomains 1 and 2 on thermostability of the monomer by assessing the molar ellipticity of the hybrid actins at 222 nm as a function of temperature. Table 3 shows that both subdomain 1 and 1 + 2 substitutions elevated the denaturation temperature to near that of muscle actin. We assessed nucleotide exchange in the monomer by monitoring the first-order exchange of bound fluorescent ATP in a solution containing a large excess of ATP. Table 3 shows that the $t_{1/2}$ for exchange of the 3Ac/Sub1 hybrid was five times longer than that of yeast actin, and that of 3Ac/Sub12 was 8-fold longer than that for yeast and roughly equivalent to the 420 s measured previously for muscle actin.

Another way to assess actin conformational alterations, especially those in subdomain 1, is to determine the tryptophan fluorescence behavior of the protein. Trp fluorescence is very sensitive to local changes in the electronic environment. Actin contains four tryptophans, all in this subdomain. Muscle G-actin has a greater amount of Trp fluorescence than does WT yeast actin. Polymerization results in the quenching of this fluorescence in both actins. However, the extent of quenching is different, about 15% for yeast actin and 22% for muscle actin (Fig. 5). Table 5 and Table 3 show that as the hybrid nature of the actin increases, the Trp fluorescence of G-actin also increases. The same is true for the extent of quenching following polymerization. If one accounts for the difference in critical concentration of the hybrid and mutant actins (described later), both hybrid actins show a decrease in fluorescence significantly greater than that observed for even muscle actin.

Effect of Muscle Substitutions on Actin Polymerization—Muscle actin polymerizes more slowly than yeast actin, probably because of slower nucleation and less filament fragmentation, thereby decreasing the number of barbed ends needed for monomer addition (4). We thus examined the effects of the subdomain 1 and 1 + 2 substitutions on actin polymerization (Fig. 6). 3Ac/Sub1 actin polymerizes with a longer nucleation phase, faster elongation phase, and to a lesser extent than does WT yeast actin. With 3Ac/sub12 actin, the elongation rate was even faster, and the final extent was lower than with just subdomain 1 substitution alone. The critical concentration for

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**TABLE 3**
Comparison of the effects of muscle substitutions on actin thermostability, nucleotide exchange, and polymerization-dependent changes in intrinsic Trp fluorescence

All the experiments were repeated with separate actin preparations with essentially the same results.

| Thermotability | $t_{1/2}$ nucleotide exchange | Decrease of intrinsic fluorescence* |
|----------------|-------------------------------|------------------------------------|
| °C             | s                             | %                                  |
| WT yeast actin | 54                            | 12                                 |
| 3Ac/Sub1       | 57.2                          | 238                                |
| 3Ac/Sub12      | 59                            | 429                                |
| Muscle actin   | 58.5                          | 420 (38)                           |

* Data were corrected for the differences in critical concentration of the individual actins.

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3Ac/Sub1, 0.9 μM, was greater than that of WT actin, 0.6 μM, and that of 3Ac/Sub12 was even higher, 1.2 μM. For comparison sake, the critical concentration value of muscle actins is about 0.1 μM (21).

The elongation and critical concentration results suggested that the hybrid nature of the actins created a less stable and more easily fragmented filament compared with WT yeast actin. To test this possibility, we measured the lengths of individual filaments from electron micrographs of preparations of the different F-actins. Fig. 7 shows that both hybrids form filaments (3Ac/Sub1 = 1.6 μm, 3Ac/Sub12 = 1.36 μm) less than half the length of WT yeast actin (3.6 μm), consistent with fragmentation.

To further explore this fragmentation hypothesis, we assessed the effects of added bovine cardiac tropomyosin on the polymerization kinetics. Tropomyosin, a filament stabilizer, prevents fragmentation and should retard the more rapid elongation phase of the hybrid actins if fragmentation was occurring (22, 23). Fig. 8A shows that this is the case. It further reveals that tropomyosin binding restores the extent of polymerization to that of WT yeast actin. Finally, the length of the nucleation phase lengthens as the extent of muscle substitution increases until, with the 3Ac/sub12 actin, the polymerization kinetics resembles that of muscle actin determined previously.

Mg\(^{2+}\) actin polymerizes more rapidly than Ca\(^{2+}\) actin (24), and addition of Mg\(^{2+}\) and KCl to induce polymerization results in an exchange of the bound Ca\(^{2+}\) for the added Mg\(^{2+}\). The prolonged nucleation phase observed with the hybrid actins, especially in the presence of tropomyosin, may have actually resulted from a slower rate of this metal exchange in the hybrids compared with WT yeast actin. To test this possibility, we exchanged the Ca\(^{2+}\) with Mg\(^{2+}\) in G-actin prior to raising the ionic strength to induce polymerization. Fig. 8B shows that in the presence of tropomyosin we obtained the same nucleation behavior as before, thereby eliminating slower metal exchange as an explanation for the prolonged nucleation phase. The polymerization of the Mg\(^{2+}\) form is more rapid than the Ca\(^{2+}\) form, in agreement with what has been observed previously (24). The critical concentrations for this Mg\(^{2+}\) actin, 0.18 μM for WT, 0.36 μM for 3Ac/sub1, and 0.54 μM for 3Ac/sub12, were slightly lower than when we polymerized Ca\(^{2+}\) actin directly. However, they were 10% or less of the total actin present, so they
should have had little if any bearing on the extended nucleation of the hybrids.

Two potential reasons exist as explanations for the filament instability of the hybrid actins leading to filament fragmentation. First, within the filament, there may be mismatched interfaces between monomers either along or across the filament axis involving direct muscle/nonmuscle residue clashes. Alternatively, mismatches between the muscle and nonmuscle halves of the actin monomer may result in conformational changes that alter surface topology of the protein. The result of these propagated changes might be weakened monomer-monomer contacts.

To determine whether the instability was due primarily to altered interactions between muscle and yeast actin residues along the filament axis, we assessed the ability of equal amounts of yeast and muscle actins to copolymerize. Fig. 9 shows that the 50:50 mixture has polymerization kinetics between that seen for either yeast or muscle actin; neither actin individually dominates the behavior of the mixture. Furthermore, the mixture has the same critical concentration as each of the two individual actins. By using yeast and muscle actins differentially labeled with differently colored fluorescent tags, we also show that there is complete mixing of the two types of actin within the same filament. This is the first demonstration that yeast and muscle actins will totally copolymerize and demonstrates that muscle and yeast actin surfaces can interact along the filament axis. Together, these results suggest that the altered polymerization kinetics observed with the hybrid actins reflect conformational changes induced by the presence of the altered residues within a monomer and not altered contacts between monomers directly involving the muscle-specific substitutions. These changes might result in altered interactions between filament domains or within the hinge region at the bottom of the monomer.

**Effect of Actin-bound Nucleotide on Filament Formation and Stability**—The core of muscle-specific residues in subdomain 1 connects with the inter-domain cleft at or near the part of the nucleotide-binding site that interacts with the β- and γ-phosphates. Actin filament stability is determined by the state of the nucleotide with ATP and ADP-P_i F-actins being more stable than ADP-F-actin (24). Thus, the rate of nucleotide hydrolysis and release of P_i are important determinants of actin polymerization. Hydrolysis for both yeast and muscle actins occurs almost concurrently with polymerization at the concentrations used here. However, the rate of P_i release from the two actins is very different. For yeast actin, P_i release is immediate, whereas for muscle actin, there is a substantial lag in release, perhaps leading to increased filament stability (7). We monitored the P_i release rates for the two hybrids, and the results show that for both, P_i release is immediate like WT yeast actin (data not shown). There is no lag, even though, in the case of the subdomain 1 + 2 hybrid, the entire outer domain is muscle-like.

We next wanted to determine how filament stability of the hybrids in the ADP form compared with that of WT yeast or muscle actin. If the experiment is performed in the presence of equal amounts of actin and ATP, the sample should be ADP actin at the end of polymerization. Under such conditions, at 5 μM actin, there should be no significant depolymerization for either yeast (Fig. 10A) or muscle actin (8). When we repeated the experiment with 3Ac/Sub1 actin, it initially polymerized.

**FIGURE 7.** Electron microscopic analysis of filament morphology and length of the hybrid actins. A, after the reaction in Fig. 5 reached steady state, an aliquot from each reaction was diluted 3-fold with F-buffer and examined by EM as described. B, filament lengths were determined from A using at least 100 filaments/sample with ImageJ, and the mean length and standard deviation are depicted. Open bars, WT actin; gray bars, 3Ac/Sub1 actin; black bars, 3Ac/Sub12 actin.
Muscularization of Yeast Actin

FIGURE 8. Polymerization of muscle hybrid actins in the presence of bovine cardiac tropomyosin. A 5 μM sample of either WT (○), 3Ac/Sub1 (■), or 3Ac/Sub12 (△) actin starting with either the Ca2+ (A) or Mg2+ (B) form of actin was mixed with bovine cardiac tropomyosin at a mole ratio of 2:1 (actin:bovine cardiac tropomyosin), and polymerization was initiated by the addition of 2 mM MgCl2 and 50 mM KCl. The increase in light scattering was monitored as function of time. The experiments were repeated with separate actin preparations with essentially the same results. The Mg2+ form of actin was generated from the Ca2+ form of actin as described under “Materials and Methods.” The experiments were repeated with two separate actin preparations with essentially identical results. LS, light scattering; a.u., arbitrary units.

Muscularization of Yeast Actin

is likely to be a significant factor in the increased propensity of the hybrid actin filaments to fragment.

Effect of Muscle Substitutions on the Interaction with Muscle Myosin—Most of the proposed interaction of myosin with actin occurs on actin subdomains 1 and 2 (26). Therefore, with the two-hybrid actins we constructed, we predicted that their interaction with muscle myosin would be much more like muscle actin than yeast actin. We examined this interaction with both the solution actin-activated myosin S1 ATPase activity assay (27) and a sliding filament motility assay (28, 29). V_{max} values for the ATPase assay were 2.2 ± 0.5 s^{-1} (WT), 2.0 ± 0.5 s^{-1} (subdomain 1), and 2.0 ± 0.6 s^{-1} (subdomain 1 + 2). For all three, the K_{m} was between 50 and 60 μM, although the standard error, about 30 μM, was high because of the weak affinity of yeast actin for muscle myosin. In comparison, using rabbit muscle actin, values determined previously were V_{max} = 8.8 s^{-1} and K_{m} = 11.3 μM (10). For the sliding filament assay, the WT and two hybrid actins all gave values of between 1.9−2.0 microns/s. Previous work showed that typical sliding velocities for muscle actin were about 4 microns/s (30). All mean values for both assays were based on triplicate determinations.

Effect of Muscle Substitutions on the Acceleration of Actin Polymerization by Arp2/3—The endocytic defect of the mutant cells suggested that the muscle substitutions might interfere with the ability of Arp2/3 to nucleate filament formation at the site of the endocytic patch (31). We demonstrated previously (32) and showed again in Fig. 11A that yeast Arp2/3 complex, alone, could substantially activate actin filament formation. Addition of an Arp2/3-activating protein further enhanced polymerization. With muscle actin and yeast Arp2/3 complex, however, an Arp-activating protein was needed to induce polymerization. Fig. 11B shows that the polymerization of 3Ac/Sub1 actin was identical in the absence or presence of Arp2/3, and a small stimulation was observed with the further addition of an Arp2/3-activating protein. With 3Ac/Sub12 (Fig. 11C), all three curves were identical; there was no observable stimulation of polymerization with the Arp2/3 system.

Two possible explanations exist for these hybrid actin results. One is that the hybrid actins have a decreased ability to interact with the Arp2/3. Alternatively, the rapid rate of polymerization of the hybrid actins alone might be so fast that an additional acceleration by the Arp2/3 complex is not detectable. We therefore examined the ability of Oregon Green-labeled WT and hybrid actins to be recruited to a polystyrene bead coated with the Arp2/3-activating protein ActA from L. monocytogenes in the presence of exogenous yeast Arp2/3 complex. The results (Fig. 12) clearly show that for both the subdomain 1 and 1 + 2 hybrids, the actins were recruited to the bead in an Arp2/3-dependent fashion as evidenced by the halo surrounding the bead.

DISCUSSION

Muscle and yeast actins share a high degree of identity (87%), and modeling studies carried out by R. Dominguez5 demonstrated no significant effect of the substitution of the muscle-specific residues into yeast actin in terms of alterations of the

5 R. Dominguez, personal communication.
G-actin structure. Despite the high degree of homology between yeast and muscle actins, these differences lead to very different characteristics \textit{in vitro} and incompatibility of muscle actin with yeast viability. Our ability to produce subdomain 1 and subdomain 1/H11001 muscle/yeast hybrid actins has provided significant new insight into the features of the two proteins responsible for a number of these differences. It has also uncovered the importance of conformational interactions within the actin monomer not previously recognized.

\textit{In vivo}, the hybrid actins caused depolarized cytoskeletons, altered vacuole morphology, and defective endocytosis. This situation is very different from the results caused by simply the substitution of muscle-specific Arg in place of His-372, two residues from the C terminus of the protein (13). There, mitochondrial morphology was altered, and mitochondrial DNA was lost. Also lost was the ability of the cell to utilize nonfermentable substrates requiring mitochondrial function.

Particularly interesting was the unique sensitivity of yeast to the negative charge density in the unstructured N-terminal finger of the protein. This section of unstructured polypeptide, in all crystal structures of actin reported to date, extends from the surface of the protein and does not directly interact with the C-terminal peptide. In a yeast actin background, substitution of the 4 negative charged muscle N terminus for the 2 negative charged yeast N terminus produced no observable deleterious cell phenotype (10). The 4 negative charges could actually rescue the phenotype of the H372R mutation discussed above (13). We show here that in the context of a muscle subdomain 1, the 4 negative N-terminal charges cause cell death, whereas an N terminus with 3 negative charges is compatible with cell viability. In summary, the difference between life and death for yeast is one negative charge in an unstructured part of the protein depending on the constitution of the rest of subdomain 1. These results are consistent with our original hypothesis that the core of muscle substitutions forms an information conduit connecting the two termini with each other and with the inter-domain cleft. These substitutions must result in a change in overall protein conformation. This can occur either through the hinge region that connects subdomains 1 and 3 or across the cleft or possibly through the nucleotide bridge, which affects the manner in which these N-terminal negative charges are perceived. Perhaps this happens by alteration of the accessibility of these negative charges to different actin-binding proteins. However, the absence of any structural information concerning the N terminus makes this possibility impossible to test at this time.

The hybrid actins, with muscle-specific residues on the external filament face and yeast on the inside, allowed us to explore which parameters of actin function were controlled by which actin domain. With respect to nucleotide exchange and thermal stability, muscle subdomains 1 and 2 resulted in a more muscle-like behavior as follows: slower nucleotide exchange rates approaching that seen with muscle actin alone and increased thermal stability. The combination of muscle-specific residues in subdomain 1 may alter the interaction of the protein with the nucleotide phosphates resulting in a tighter interaction that leads to a conformationally less flexible protein. The addition of the three subdomain 2 residues produced even more drastic effects than the subdomain 1 residues alone. With respect to these two parameters, then, the muscle nature of the small outer domain appears to dictate the behavior of monomeric actin.

It is generally thought that the surface residues that form the actual binding site for an actin-binding protein dictate

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9.png}
\caption{Copolymerization of yeast and muscle actin. A, 5 \textmu{}M sample of yeast actin (○), muscle actin (□), or a mixed actin sample at a 1:1 mole ratio (△) was polymerized individually, and the increase in light scattering (L.S.) was monitored as a function of time. a.u., arbitrary units. B, 5 \textmu{}M mixed actin sample (1:1 mole ratio) containing 7.5\% Oregon Green-labeled yeast actin and 7.5\% Texas Red-labeled muscle actin was polymerized until steady state. An aliquot of the 50\% diluted sample was examined with a Zeiss fluorescence microscope with TRITC (panel I) and fluorescein isothiocyanate (panel II) filters. The images were taken and modified with Metamorph and Image J for the overlaid image (panel III).}
\end{figure}
the characteristics of the interaction. In the case of the hybrid actin/skeletal myosin S1 interaction, however, this was not true. The 3Ac/sub12 hybrid actin contains what is thought to be the entire muscle actin-specific surface for interaction with muscle myosin. Yet the actomyosin behavior for both hybrid actins was identical to yeast actin in terms of solution actin-activated myosin ATPase activity. In this case, the yeast-specific inner domain (subdomain 3 + 4) somehow affects the topology of the outer muscle-like domain (subdomain 1 + 2) resulting in an overall yeast actin-like behavior. Precedent exists for this type of control. A change in a helix in actin subdomain 4 altered the calcium-regulatory properties of the troponin-tropomyosin complex interacting with the outer actin small domain (33). We demonstrated previously a similar effect in terms of the ability of the same mutation to alter the calcium-regulated tropomyosin-troponin-dependent change in the fluorescence of a pyrene attached to residue 235 in actin subdomain 4 (34). Another possible explanation for the results that we cannot exclude at this time is that the myosin activation caused by the hybrid actins reflects the filament instability of

FIGURE 10. Effect of actin-bound nucleotide on WT and 3Ac/Sub1 actin filament formation and stability. Unbound ATP was removed from 3Ac/Sub1 G-actins by gel filtration. A, 5 μM yeast WT actin sample was induced to polymerize in the absence of free ATP, and the change in light scattering (L.S.) was monitored. a.u., arbitrary units. B, 5 μM 3Ac/Sub1 actin sample was induced to polymerize as above. At the time signified by the arrow, either ATP (○, 0.2 mM final concentration), phalloidin (□, 5 μM final concentration), ADP (△, 0.2 mM final concentration), or F-buffer without free ATP (◇) was added. The experiment was repeated with two different actin preparations with essentially the same results.

FIGURE 11. The polymerization of hybrid actins in the presence of yeast Arp2/3 and N-WASP VCA fragment. A 2.5 μM sample of WT (A), 3Ac/Sub1 (B), or 3Ac/Sub12 (C) actin was polymerized as above, and the change in light scattering (L.S.) was monitored as a function of time. a.u., arbitrary units. ○, actin alone; □, actin with 25 nM yeast Arp2/3; △, actin with 25 nM yeast Arp2/3 plus 250 nM GST-VCA fragment of N-WASP. The experiment was repeated with two different actin preparations with essentially the same results.
the F-actin rather than the surface-binding site of the protein per se.

The polymerization results of the hybrid mutant actins were unexpected. As the muscle nature of the hybrid increased, the overall rate of polymerization increased to the extent that it was even faster than yeast actin alone. The hybrid actins nucleated more slowly, like muscle actin but elongated much more rapidly than even yeast actin, apparently as a result of accentuated fragmentation of the filaments during polymerization. This conclusion is based on the polymerization kinetics of the actins alone and the apparent elongation of nucleation phase and retardation of elongation in the presence of tropomyosin. In previous studies of actins with similar behavior, the authors (23) have interpreted the results as indicative of an enhanced likelihood of the actin to fragment. Such fragmentation would produce new ends that accelerate elongation leading to smaller, less stable actin filaments with higher critical concentrations than either yeast or muscle actin, which we observe. This behavior occurs even though within each half of the molecule the structures are normal in terms of their respective parent actins.

This same mismatch was reflected in the effect of nucleotide state on polymerization. Under conditions of single nucleotide turnover, ADP muscle and yeast actin filaments separately remained stable. However, with the hybrid actins, the mismatched domains caused disassembly following production of the ADP filament form. The disassembly phase followed first-order kinetics with a $k = 0.0045 \text{s}^{-1}$. This is the same time constant we reported earlier for a conformational rearrangement of an ADP*-F-actin generated following release of the P$_i$ produced by nucleotide hydrolysis (35). Evidently, the conversion of ADP*-F-actin to the mature ADP-F-actin conformation, which normally occurs with WT actin, places the hybrid actin filament in an unstable state leading to depolymerization. Rescue of polymerization by re-introduction of ATP proved that the disassembly resulted from a very large increase in the critical concentration of the hybrid ADP-F-actins relative to their parental counterparts ($\equiv 7 \mu\text{m}$). These experiments show that although individual domain integrity is important for proper actin behavior, the nature of these domains, by influencing their interaction with one another, dictates the behavior of the entire filament.

Cells carrying the hybrid actins have endocytosis defects. Arp2/3-dependent actin filament formation is important in the formation and invagination of the endocytic plaques in yeast, and our solution data indicated a decreased ability of the Arp2/3 complex in the presence and/or absence of an Arp2/3-activating protein to affect the polymerization kinetics of the hybrid actins. Subsequent studies using an Arp2/3-dependent bead assay showed that both hybrid actins could be recruited to the bead surface. Perhaps the endocytosis defect seen in the mutant cells reflects in part an inefficiency in the way Arp2/3 recruits to and/or assembles actin at the endocytic patch because of spontaneous polymerization of the mutant actin as we observed in this study.

At least three different states of actin within the filament have been proposed, depending on the space between the domains and the degree of twist relative to one another (36, 37),

FIGURE 12. Effects of muscle substitutions on the behavior of yeast actin in the ActA/yeast Arp2/3-dependent bead assay. A 6.9 $\mu\text{M}$ sample of WT (A), 3Ac/Sub1 (B), or 3Ac/Sub12 actin (C) (5% Oregon Green-labeled actin) was incubated with ActA-coated polystyrene beads (4.9 $\mu\text{m}$ diameter) and 100 nM yeast Arp2/3 in F-buffer as described under "Materials and Methods." All images were taken 10 min after combination of all reaction components. The experiment was executed with two different actin preparations with essentially the same results.
and the population distribution of these states must reflect the interplay of these two actin domains. Coevolution of these domains within a given cell type must have allowed the achievement of the proper balance between the domains necessary to produce a conformation most beneficial to the needs of that particular cell. However, the extent this mismatch of two otherwise normal domains could have on actin function was surprising to us. Examination of the actins we made as intermediates in achieving the final hybrids, especially those groups of residues in the “information conduit” in subdomain 1, should provide valuable insight into the threshold that must be breeched to create this mismatch or hinge malfunction.

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