A Mammalian Actin Substitution in Yeast Actin (H372R) Causes a Suppressible Mitochondria/Vacuole Phenotype

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Melissa McKane1, Kuo-Kuang Wen1, Istvan R. Boldogh5, Sharmilee Ramcharan5, Liza A. Pon*, and Peter A. Rubenstein1,2*†‡1,‡2

From the 1Department of Biochemistry, University of Iowa Carver College of Medicine, Iowa City, Iowa 52242 and the 5Department of Anatomy and Cell Biology, Columbia University College of Physicians and Surgeons, New York, New York 10032

To determine the reason for the inviability of Saccharomyces cerevisiae with skeletal muscle actin, we introduced into yeast actin the first variant muscle residue from the C-terminal end, H372R. Arg is also found at this position in non-yeast nonmuscle actins. The substitution caused retarded growth on glucose and an inability to use glycerol as a sole carbon source. The mitochondria were clumped and had lost their DNA, the vacuole appeared hypervesciculated, and the actin cytoskeleton became somewhat depolarized. Introduction of the second muscle actin-specific substitution, S365A, rescued these defects. Suppression was also achieved by introducing the four acidic N-terminal residues of muscle actin in place of the two found in yeast actin. The H372R substitution results in an increase in polymerization-dependent fluorescence of Cys-374 pyrene-labeled actin. H372R actin polymerizes slightly faster than wild-type (WT) actin. Yeast actin-related proteins 2 and 3 (Arp2/3) accelerates the polymerization of H372R actin in the absence of an Arp2/3 complex. In contrast, the S365A substitution dampened the rate of Arp2/3 complex-stimulated H372R actin polymerization, and the addition of the four acidic N-terminal residues caused this rate to decrease below that observed with WT actin in the presence of Arp2/3. Structural analysis of the mutations suggests the presence of stringent steric and ionic requirements for the bottom of actin subdomain 1 and also suggests that there is allosteric communication through subdomain 1 within the actin monomer between the N and C termini.

The actin C-terminal region, consisting of residues 364–375, is located in subdomain 1 on the opposite side of the protein from that of the N terminus and is believed to play an important role in the interaction of actin with different actin-binding proteins such as profilin (1, 2), coflin (3), and gelsolin (4) in the determination of actin filament stability and in the allosteric behavior of the actin filament. The crystal structure of the filament shows that a direct interaction of the N and C termini is virtually impossible because of the intervening mass of the core of subdomain 2 (Fig. 1).

The sequence of the C-terminal peptide is highly conserved throughout the actin family. The sequences for mammalian and avian muscle actins are identical and there are only two differences between yeast and higher eukaryotic actins, a substitution of His in yeast at residue 372 for Arg and a substitution of Ser at residue 365 for Arg just outside of the helix in this peptide. In the first case the His and the Arg carry at least a partial positive charge.

Removal of the C-terminal three residues in yeast actin causes cell inviability, whereas removal of either or both of the first two residues, beginning with the C terminus, shows milder phenotypes including temperature sensitivity and increased cell size (5). The actin C-terminal dipeptide or tripeptide can be removed proteolytically with trypsin (6), and the resulting actin will still polymerize in vitro. However, cleavage results in small increases in the critical concentration of the protein needed for polymerization, a decreased ability to activate myosin ATPase activity, and a large effect on the morphology of actin filaments. Filaments were more highly curved and irregular and exhibited decreased viscosity. Results were also observed on the protease susceptibility of regions of the actin near the nucleotide binding site and in alterations between the C-terminal peptide and the DNase I binding loop at the top of subdomain 2 (7). These data suggest the necessity of an intact C-terminal peptide for the attainment of stable filament formation.

Alteration and modification of Cys-374 alone affect actin function. A C374S mutation in avian nonmuscle β-actin causes adverse changes in critical concentration, the ability to translocate in an in vitro motility assay, and the ability to interact with profilin (8). Modification of Cys-374 with fluorescent reagents such as pyrene maleimide affects both the myosin S1 ATPase activity and sliding velocity using in vitro motility assays, although the specificity of the effect depends on the particular fluorescent probe utilized (7).

In x-ray crystal structures of G-actin, the C terminus points toward the interior of the protein in subdomain 1 (1, 4, 9–11) and, in the context of the filament, it must be accessible to the filament exterior because Cys-374 is readily modifiable by fluorescent sulfhydryl-reactive reagents. However, several pieces of evidence suggest that in the filament this peptide must be extremely flexible. Polymerization causes a substantial increase in fluorescence of such ligands attached to Cys-374, suggesting that it becomes buried within the filament interior. Additionally, Kim et al. (12) demonstrated that Cys-374 could be intermolecularly cross-linked to Cys residues introduced by mutation at residue 265 in a monomer located across the interstrand space in F-actin and at residue 41 in the DNase I loop of an adjacent monomer in the same helical strand. Such reactivity requires that the C-terminal helix move at least 10 Å without disrupting the filament. This result further suggests that this part of the protein plays a significant role in determining actin filament conformation.

Despite the fact that yeast and muscle actins are 87% identical in amino acid sequence (13), yeast cannot survive with mammalian muscle actin as the sole actin in the cell. Yeast is viable, however, with β-non-
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FIGURE 1. Positions of mutations in yeast actin referred to in this study. Panel A, crystal structure of the actin monomer. Panel B, a trimer of monomers from the filament model by Holmes (54–57). Red, His-372; magenta, Ser-365; blue, Glu-364; green, Asp-363; yellow, N-terminal acidic residues. In the monomer, the bound ATP is shown in the cleft separating the two domains.

Muscle actin as the sole actin, although in this case the cells are extremely sick (14). The reason for the incompatibility between mammalian actins and yeast cell viability is not understood, and we reasoned that it might result from the inability of various yeast actin-binding proteins to interact appropriately with the higher eukaryotic actin. Alternatively, the incompatibility might derive from inherent differences between yeast and higher eukaryotic actins in filament morphology and filament dynamics (15).

Two structures associated with the actin cytoskeleton that might be affected by these differences are mitochondria and vacuoles. F-actin and the actin-related proteins 2 and 3 (Arp2/3) complex localize to the sites of vacuole fusion, and actin remodeling has been implicated in vacuole docking prior to fusion as well as in the late stages of vacuole fusion and in the recruitment of phosphoinositides to sites of vacuole fusion (16, 17). Actin plays a role in mitochondrial fusion and/or fission and also in the determination and maintenance of mitochondrial morphology (18–21).

The inheritance of mitochondria and vacuoles, i.e. the transfer of these organelles from mother to daughter cells, is also actin-dependent. Vacuoles bind to a type V myosin (Myo2p) using adapter proteins (Vac8p and Vac17p) and use Myo2p as a motor and actin cables as tracks for anterograde movement from the mother cell to the bud (22–24). Mitochondria move bidirectionally to the cellular poles (the bud tip and mother cell tip) where they become immobilized. Mitochondria, like vacuoles, use actin cables as tracks for anterograde movement from mother cells to buds (18, 19). However, actin for anterograde mitochondrial movement is Arp2/3 complex-mediated actin polymerization and is not myosin-dependent (25, 26). The movement of mitochondria toward the opposite pole is driven by the retrograde flow of actin cables; that is, mitochondria bind to actin cables and use the motile cables as conveyor belts for retrograde movement toward the mother cell tip (19).

Because the interactions between a number of actin-binding proteins and actin involve subdomain 1, the His/Arg difference between yeast and higher eukaryotic actins at residue 372 might contribute to the incompatibility discussed above despite the ionic similarity between these two residues. Here, we use site-directed mutagenesis of yeast actin to assess the effects of these C-terminal differences on the behavior of actin itself and on the behavior of two organelles in yeast, mitochondria, and vacuoles.

EXPERIMENTAL PROCEDURES

Materials

DNase I (grade D) was purchased from Worthington Biochemicals. Affi-Gel 10 activated resin and Bio-Spin® 30 Tris columns were purchased from Bio-Rad. The QuikChange® site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA), and the DNA primers used for site-directed mutagenesis were obtained from Integrated DNA Technologies (Coralville, IA). N-(1-pyrenyl)maleimide was purchased from Sigma-Aldrich. Yeast cakes for the preparation of wild type actin were obtained locally. All other chemicals used were of reagent grade quality.

Mutagenesis and Construction of Mutant Strains

The QuikChange® site-directed mutagenesis kit from Stratagene was used to introduce mutations into the yeast actin coding sequence. The starting plasmid for mutagenesis was pRS314WN, a derivative of pRS314 (27) carrying the WT yeast actin coding sequence and promoter region in addition to the TRP1 gene. Subsequent mutations were carried out on the mutagenic plasmids. In each case, the plasmid DNA was sequenced to verify the mutation(s). The mutant plasmids were transformed into a trp1, ura3-S2 haploid cell in which the chromosomal ACT1 gene had been disrupted by replacement of the coding sequence with the LEU2 gene. Wild-type actin was expressed in these cells from another centromeric plasmid, pGENWT, containing the URA3 gene. Transformants were selected on tryptophan-deficient medium and then subjected to plasmid shuffling to eliminate the WT actin gene. The mutant plasmids were rescued from the trp1, ura3 cells and sequenced to verify that the mutations were still intact.

Growth Curves and Doubling Times

Overnight cultures of WT and mutant strains were sub-cultured in YPD (1% yeast extract, 2% peptone, and 2% dextrose) growth media to an OD600 of 0.1 and placed at 30 °C with agitation. The OD600 taken after 1 h of sub-culturing was considered to be the zero time point, and cell optical densities were monitored until reaching a plateau (usually at least 48 h). Growth curves were plotted as OD600 versus time. To determine the doubling times, early and mid-log phase time points were plotted as ln(OD600) versus time, and a linear regression line was fit to

3 The abbreviations used are: Arp2/3, actin-related proteins 2 and 3; CS1, citrate synthase 1; DAPI, 4',6-diamidino-2-phenylindole; 4Ac, four acidic N-terminal amino acid residues of muscle actin; GFP, green fluorescent protein; WT, wild-type.
the data. The doubling time was then determined by the equation doubling time = 0.693/slope

**Temperature Sensitivity, Hyperosmotic Sensitivity, and Growth on Glycerol**

Mutant strains were tested for temperature sensitivity, hyperosmotic sensitivity, and mitochondrial defects in the following manner. Overnight cultures of cells were sub-cultured to an OD_{600} of ~0.1 in YPD. Cultures were grown at 30 °C to an OD_{600} of 0.3 (~3 × 10^6 cells/ml). Then, 3 μl of a 1×, 10×, 100×, and 1000× dilution were spotted on YPD plates placed at 24, 30, and 37 °C to test for temperature sensitivity. The same amount and dilutions were spotted on YPD plus 0.9 M NaCl agar at 30 °C to test for hyperosmolar sensitivity and on YPC complete medium containing 2% glycerol as a sole carbon source at 30 °C to test for mitochondrial defects. Plates were observed at 24, 48, and 72 h.

**Cell Staining**

**Visualization of Mitochondria**—Mitochondria were visualized in living cells using a fusion protein containing the mitochondrial signal sequence of citrate synthase 1 (CS1) fused to a green fluorescent protein (GFP) (CS1-GFP). CS1-GFP was expressed using a centromere-based plasmid under control of the endogenous citrate synthase promoter (28). Yeast cells were transformed using the lithium acetate method (29). CS1-GFP labeling of mitochondria was specific and had no detectable effect on mitochondrial morphology, respiration, or movement under our experimental conditions. Cells expressing CS1-GFP were grown to mid-log phase in synthetic, glucose-based, liquid media at 30 °C. Temperature-sensitive mutants were grown at 23 °C. Samples were mounted on microscope slides and visualized by fluorescence microscopy as described below.

**Actin Visualization**—The actin cytoskeleton was visualized using rhodamine-phalloidin (Invitrogen), a ligand that binds specifically to actin polymers (30). Fixed cells were exposed for 10 min in the dark to rhodamine-phalloidin at a final concentration of 2.5 μM in a solution consisting of a 4:1 ratio of NS (20 mM Tris-HCl, pH 7.6, 0.25 M sucrose, 1 mM EDTA, 1 mM MgCl₂, 0.1 mM ZnCl₂, 0.1 mM CaCl₂, 0.8 mM phenylmethylsulfonyl fluoride, and 0.05% (v/v) 2-mercaptoethanol) to methanol. Stained cells were mounted on microscope slides and visualized by fluorescence microscopy.

**Light Microscopy**—Images were collected with a Zeiss Axioskop 2 Plus microscope (Oberkochen, Germany) using a Plan-Apochromat 100×, 1.4 numerical aperture objective lens, and a cooled charge-coupled device camera (Orca-100; Hamamatsu, Bridgewater, NJ). Illumination with a 100-watt mercury arc lamp was controlled with a shutter (Uniblitz D122, Vincent Associates, Rochester, NY). Camera control and image enhancement were performed using OpenLab software (Improvision Inc., Coventry, UK).

For analysis of mitochondrial morphology, 25 z-sections were obtained at 0.2-μm intervals through the entire cell. The z-sectioning for three-dimensional imaging was carried out using a piezoelectric focus motor mounted on the objective lens of the microscope (Polytech PI, Auburn, MA). Out-of-focus light was removed by deconvolution, and each series of deconvolved images was projected and rendered with Volocity software (Improvision Inc.).

**Actin Purification and Labeling with Pyrene Maleimide**

Yeast WT actin was purified in the calcium form by a DNase-I affinity chromatography/DEAE ion exchange chromatography protocol as described previously (31) with a modification that consisted of the use of 75 ml of 0.6 M NaCl to wash the DNase-I affinity column bound with yeast cellular proteins in order to eliminate the possible binding of coflin to G-actin (32). The quality of the G-actin preparation was analyzed with SDS-PAGE, and the bands were visualized by Coomassie Blue staining. G-actin was stored in G-buffer (5 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.2 mM CaCl₂, and 0.1 mM dithiothreitol) at 4 °C and used within 3 days. The actin concentration was determined by UV light absorbance at 290 nm using the extinction coefficient: ε = 25.6 cm⁻¹ M⁻¹. Dithiothreitol-free actin, 40 μM, was labeled with 45 μM N-(1-pyrenyl)maleimide as described previously (33). The pyrene-labeled F-actin was collected by centrifugation and depolymerized by dialysis against G buffer. Extent of labeling was determined by UV light absorbance at 344 nm using the extinction coefficient for pyrene, ε = 22 M⁻¹ cm⁻¹.

**Actin Polymerization**

Actin was polymerized by the addition to G-actin of MgCl₂ and KCl to final concentrations of 2 and 50 mM respectively, and the increase in light scattering was followed as a function of time. To measure the critical concentration of actin polymerization, the net change in light scattering was measured as a function of increasing actin concentration. The critical concentration of actin was obtained by drawing a line through the points and determining its intersection on the x-axis. Actin polymerization was also assessed by the increase in fluorescence of pyrene-labeled actin with excitation and emission wavelengths set at 365 and 386 nm respectively. Fluorescence measurements were obtained using a FluoroMax (Jobin Yvon Inc., Edison, NJ) fluorescence spectrometer with a thermostatted water bath attached to the cuvette chamber. Pyrene fluorescence emission spectra were obtained by exciting the sample at 365 nm and recording the fluorescence emission from 375 to 450 nm.

**RESULTS**

**Growth Characteristics and Staining of H372R**—To assess the consequences of a non-yeast residue, Arg, at position 372, we first assessed the effect of the H372R substitution in vivo. The strain expressing H372R actin exhibited a 1.6× slower doubling time in complete liquid medium and reached a stationary phase at a cell density ~40% of that achieved by WT cells (Fig. 2). On solid YPD medium the growth characteristics of the cell were much closer to those of a WT strain (Fig. 3). In addition, there was no significant change in growth on plates containing hyperosmolar conditions or at 37 °C (data not shown). Strikingly, however, yeast cells expressing H372R actin showed no growth on glycerol-based medium (Fig. 3). Because mitochondrial respiratory activity is required for growth on non-fermentable carbon sources like glycerol, this result indicates that the H372R substitution may cause a defect in mitochondrial function.

To determine whether the loss of mitochondrial respiratory activity is due to loss of mitochondrial DNA, cells expressing wild type or mutant actin were stained with the DNA binding dye DAPI (Fig. 4). In wild type cells, mtDNA is resolved as punctate, DAPI-stained cytoplasmic structures. In contrast, in H372R cells, there was no detectable mtDNA (Fig. 4). Consistent with this observation, replacement of H372R actin with plasmid-borne wild type actin does not restore mitochondrial respiratory activity (data not shown). Thus, the irreversible loss of mitochondrial respiratory activity observed in H372R cells most likely stems from the elimination of mtDNA.

Finally, we visualized mitochondria in fixed cells using a fusion protein consisting of the mitochondrial targeting signal sequence of CS1 fused to GFP (CS1-GFP) (19). The cells were counter-stained with rhodamine-phalloidin to allow for comparison of the actin deposition pattern with the mitochondria (Fig. 5A). In wild type cells, F-actin localizes...
to two major structures, actin patches and actin cables. Actin patches
are endosomes that are invested with F-actin and enriched in the bud
(34). Actin cables are bundles of F-actin that align along the mother bud
axis. In the same cells, mitochondria are long tubular structures that are
dispersed through the cell and align roughly along the mother bud axis,
accumulate at the poles of the cell (the bud tip and mother cell tip), and
tend to co-localize with actin cables.

Yeast cells expressing H372R actin exhibit severe defects in mito-
chondrial morphology (Fig. 5 and supplemental Fig. 1, available in the
on-line version of this article). Whereas mitochondria is tubular in yeast
expressing the H372R mutant, >90% of the cells contain abnormally
aggregated mitochondrial structures. This aggregation appears to be
linked to actin disorganization. We also observed defects in the organi-
zation of actin cytoskeleton, i.e. ~50% of the cells analyzed show depo-
larized actin patches and cables.

**Vacuole Defects**—To determine whether the effects of the H372R
mutation extended beyond mitochondria, we also examined the effect
of the mutation on vacuoles, another actin-dependent organelle. Stain-
ing with the vacuole-specific dye FM 4-64 revealed that the vacuole in
the mutant cells was hypervesiculated as opposed to the WT cells in
which generally 2–3 vacuole lobes were observed per cell (Fig. 6). Fur-
thermore, in the mutant cells in which the bud was less than one-third
the diameter of the mother cell, no vacuolar structures were seen in 44
of 87 cells whereas in WT cells this number was 2 of 60 cells. Thus, the
mutation also caused a mild inheritance defect.

**Intragenic Suppressors of the H372R Mutation**—As part of other
research efforts in the laboratory, we introduced two additional muta-
tions in H372R actin. One of these second site mutations is S365A, a
second muscle-specific substitution in the C terminus. The other is
replacement of the two N-terminal acidic amino acids with four acidic
residues (4Ac) found in the N terminus of striated muscle actin. The
positions of these second mutations relative to H372R are depicted in
Fig. 1. Figs. 4–6 show that in both cases the introduction of the second
mutation reverted both the mitochondrial and the vacuolar defects
caused by the H372R mutation as judged by growth on glycerol and the
staining of mtDNA, mitochondria, and vacuoles.

**In Vitro Properties of Mutant Yeast Actins**—Actin polymerization is
often measured by assessing the polymerization-dependent increase in

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**FIGURE 2.** Growth curves of cells carrying WT and three mutant yeast actins. Cells were grown in YPD complete medium. The density of the culture (OD600 nm) is shown as a function of time. The numbers represent the doubling times calculated from the linear part of each growth curve. HR, H372R; SAHR, S365A/H372R; 4AcHR, four acidic residue-N terminus/H372R. The OD values were back-calculated following the appropriate dilutions to lower the cell density to the linear range of the spectrometer.

**FIGURE 3.** Comparison of growth of WT and mutant yeast strains on complete solid medium with either 2% glucose (YPD) or 2% glycerol (YPG) as the sole carbon source. The picture was taken 48 h after plating the cells. Growth was at 30 °C. Mutant notation was as described in Fig. 2. This composite figure was generated by removing lanes of data unrelated to the present work. HR, H372R; SAHR, S365A/H372R; 4AcHR, four acidic residue-N terminus/H372R.

**FIGURE 4.** Staining of WT and mutant cells DAPI to visualize nucleoid bodies. Cyto-
plasmic dots in the DAPI-stained cells are mitochondrial DNA molecules. The large bright spot in each cell is the nucleus. The mutant notation is as described in the legend to Fig. 2. HR, H372R; SAHR, S365A/H372R.
fluorescence of Cys-374 pyrene-labeled actin. However, the emission spectrum in Fig. 7 demonstrates that the fluorescence increase caused by actin polymerization is substantially higher for H372R, S365A/H372R, and 4Ac/H372R actins than that of labeled WT actin, although the extent of polymerization of all the actins, as measured by light scattering (Fig. 8A), is about the same in each case. Because the Arg substitution for His-372 is a feature shared by these three mutants, it is possible that the residue at this position governs the environment in the monomer/monomer interface between neighboring actins in the filament, which would affect pyrene fluorescence. To evaluate this hypothesis, we examined a mutant that only contains the S365A substitution. The cells containing S365A actin have no aberrant phenotype, and the mutant actin polymerizes slightly faster than WT actin (data not shown). However, pyrene-labeled S365A actin shows an emission spectrum identical to that of WT actin, consistent with the hypothesis. This experiment also provides a molecular basis for the observation that polymerization of pyrene-labeled yeast actin produces a much lower increase than does polymerization of almost any other actin that generally contains an arginine at position 372.

We next compared the polymerization rates of purified H372R actin and the suppressor actins (4Ac/H372R and S365A/H372R) using a light scattering assay. Fig. 8 shows that all three of the actins polymerize slightly faster than the control WT actin with a minor increase in the extent of polymer-
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FIGURE 8. Polymerization of WT and mutant actins in the presence and absence of yeast Arp2/3. Panel A, polymerization of 5 μM actin was initiated by the addition of salts as described under “Experimental Procedures.” Panel B, the polymerization of 1 μM yeast actin in the presence of a 25 mM yeast Arp2/3 complex following the addition of salt to induce polymerization. For both panels the increase in light scattering was followed as a function of time in a fluorometer with excitation and emission wavelengths at 360 nm. Circle (○), WT; square (□), H327R; triangle (△), S365A/H372R; diamond (◇), four acidic residue N terminus. Shown are representative examples of two experiments, each conducted three times with three different actin preparations.

FIGURE 9. The relationship of either His-372 (H372) (A) or Arg-372 (R372) (B) to Glu-364 (E364). The distance between the side chains of residue 372 and Glu-364 is shown by the dotted line. Structures are based on the yeast actin monomer crystal structure (Protein Data Bank code 1YAG) (58) and modified by Swiss-Pdb viewer 3.7 program (www.expasy.ch/spdbv/mainpage.html)

In trying to understand the molecular basis for the H372R phenotype, the act-101 allele results proved to be quite informative when coupled with our own observations. The act-101 actin contains the D363A/E364A double mutation, and the positions of these residues relative to residue 374 are shown in Fig. 9. Glu-364, in one short helix in the C-terminal region, can potentially interact electrostatically with the cationic histidine residue at 372 in the helix adjacent to the C terminus. With an Arg at 372 the likelihood for an ionic interaction should be enhanced because of the increased charge and larger size of the Arg side chain bringing it to within ~6.2 Å of the Glu-364 side chain. The known flexibility of this part of the protein, based on both cross-linking and

surprisingly revealed that substitution of the partially cationic His at residue 372 with the fully basic Arg found in other actins caused a drastic phenotype in yeast, namely aggregated, fragile, nonfunctional mitochondria that had lost their DNA and a hypervesiculated vacuole.

Previous work showed that certain actin mutations lead to mitochondrial defects. Charged-to-alanine scanning mutagenesis of yeast actin resulted in a number of mutant actins that affected mitochondrial organization (41). Because multiple charged residues were simultaneously neutralized, these mutations might be considered much more drastic than the H372R single change that is the focus of our study. Furthermore, in the case of these simultaneous multiple mutations, there is always a question as to which changes are actually responsible for the observed phenotype. Initially, an assessment of vacuole integrity and inheritance was not part of the characterization of the alanine-scanning actin mutants. However, other studies revealed, using yeast actin mutations generated by us (22, 42), that actin was involved in both vacuole morphology determination and vacuole inheritance and that mutations could be found that affected vacuole behavior independent of mitochondria (41).

With the H372R mutation, the mitochondrial fragility and loss of mtDNA coupled with the hypervesicularity of the vacuole suggest that this mutation may affect a process that is not specific to the direct interaction of either organelle with the actin cytoskeleton. An example of such a general process is that of membrane fission/fusion. Pertinent to this proposition is the recognition that in yeast mitochondrial integrity depends on a carefully regulated fission/fusion cycle which, when interrupted, can result in mitochondrial abnormalities and a loss of mtDNA (43). Hill et al. (22) showed that one of the alanine scanning mutants, act-101, had both a mitochondrial and a vacuolar inheritance phenotype (42). We have recently observed that in this cell the vacuole is also hypervesiculated (data not shown). With this mutant actin, however, the mitochondrial phenotype was much less severe than with that of our H372R actin. Mitochondria were found clumped in 17% of the act-101 cells at 25 °C and in 50% of the cells at 37 °C, whereas our results showed that 90% of the H372R cells had abnormal mitochondria at 30 °C.

In trying to understand the molecular basis for the H372R phenotype, the act-101 allele results proved to be quite informative when coupled with our own observations. The act-101 actin contains the D363A/E364A double mutation, and the positions of these residues relative to residue 374 are shown in Fig. 9. Glu-364, in one short helix in the C-terminal region, can potentially interact electrostatically with the cationic histidine residue at 372 in the helix adjacent to the C terminus. With an Arg at 372 the likelihood for an ionic interaction should be enhanced because of the increased charge and larger size of the Arg side chain bringing it to within ~6.2 Å of the Glu-364 side chain. The known flexibility of this part of the protein, based on both cross-linking and

DISCUSSION

The actin C-terminal peptide plays an important role in regulating actin filament dynamics, and two residues in this region of yeast actin distinguish it from a wide array of actins of other organisms ranging from Schizosaccharomyces pombe (36) and Dictyostelium discoideum (37) to the avian and mammalian muscle and nonmuscle actins (38–40); these residues are a His at position 372 instead of the more usual Arg and a Ser at position 365 instead of the more usual Ala. Our results

The Arp2/3 complex is one of the inducers of actin polymerization in yeast and has been implicated in the control of vacuole morphology and mitochondrial movement (16, 25). We recently demonstrated that yeast Arp2/3 complex nucleated yeast actin polymerization in vitro, independent of the presence of the yeast analogue of the Wiscott-Aldrich syndrome protein (35). We thus assessed the effect of yeast Arp2/3 complex on the polymerization of WT actin, H372R actin, and the two revertant actins. Fig. 8B demonstrates that yeast Arp2/3 stimulates polymerization of H372R actin to a greater extent than WT actin relative to the difference between the rates of polymerization of the two actins in the absence of Arp2/3. The results also show that introduction of S365A brings this degree of stimulation closer to that of Arp2/3-stimulated WT actin polymerization, whereas the addition of the 4Ac mutation actually leads to an Arp2/3-induced retardation of polymerization relative to that observed with WT actin in the presence of yeast Arp2/3.

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chemical modification studies (12, 33), might allow an even smaller inter-residue distance to occur, further accentuating such an interaction. Asp-363 points in the opposite direction and would probably not be part of such an interaction. The position in adjacent helices of the two mutant residues, 364 and 372, both of which lead to mitochondrial and vacuolar defects, seems to specify a single binding domain involved in proper vacuole and mitochondrial behavior. Such behavior in yeast appears to be uniquely sensitive to the balance of positive and negative charge in this area of the actin for two reasons. First, yeast actin has evolved to contain a His at 372, whereas other actins have the more highly charged Arg in this position; second, increasing the positive charge at 372 or decreasing the negative charge at 364 produces an adverse phenotype.

The intragenic suppression of the H372R mutation was surprising, because both suppressor mutations, S365A near the C terminus and the muscle 4-acidic residue stretch at the N terminus on the other side of subdomain 1, simultaneously reverted both the vacuole and mitochondrial phenotypes. This observation suggests that the H372R mutation may cause disruption of the binding of a single protein that functions in regulating both vacuole and mitochondrial behavior. Alternatively, it is possible, although we think less likely, that two independent proteins with the same or overlapping binding sites on actin are also being simultaneously affected by the mutation.

Examination of the position of Ser-365 relative to His-372 suggests that the proper function of this part of the yeast actin structure may also have a severe structural as well as an electrostatic requirement. Increasing the size of the side chain of residue 372 from the His imidazole to the Arg guanidinium residue can be compensated for by decreasing the size of the Ser-365 to the single methyl group of the alanine. In fact, examination of the residues in the model indicated that in the presence of Arg-372 the side chain of Ser-365 needed to be rotated ~180° to allow Glu-364 to swing to a position to maximize interaction with Arg-372. With His-372 and the decreased likelihood of an ionic interaction, such rotation would be unnecessary. Removal of the Ser OH group would also decrease the possibility of a hydrogen bond forming with the Arg side chain. Formation of such a bond might lead to increased rigidity in this part of the protein with subsequent deleterious effects stemming from a restriction of accessible conformations. Our observed lack of phenotype associated with the S365A mutation alone would be consistent with either of these possibilities.

The ability of increased N-terminal negative charge density to suppress the H372R mutation is harder to explain. The actin N-terminal finger appears unstructured and is separated from the C-terminal peptide by the body of subdomain 1, precluding interaction of the side chain of residue 372 with the N terminus directly either in the context of the monomer or in the context of the filament based on the Holmes filament model. The alternative explanation is that important allosteric communication occurs through subdomain 1 between these sites. Such communication, previously unreported, might affect the accessibility of actin-binding proteins such as myosin (44, 45) to the N-terminal negative charges. Conversely, N-terminal negative charge density might regulate the conformation of actin in the vicinity of H372R. In the Holmes filament model this region is in the groove between two adjacent actin monomers in one strand of the filament, and this groove is the site for binding of a number of proteins that control filament dynamics.

The potential importance of this region of actin in regulation of filament dynamics is illustrated by our in vitro results pertaining to polymerization of the H372R actin. In vivo, the mutation appears to cause a hyperpolymerization phenotype. Yet, the mutation makes very little difference in the polymerization kinetics of the actin per se. However, the yeast Arp2/3 complex shows a markedly enhanced ability to promote polymerization of the mutant actin relative to the WT protein. The ability of the two suppressor mutations to reverse the in vivo polymerization defect, coupled with the enhanced Arp2/3-dependent polymerization in vitro, supports the notion of allosteric regulation in subdomain 1 as outlined above. The binding site for the Arp2/3 complex on actin has not yet been defined, and we cannot discern whether our results are due to alternations in a localized Arp2/3 binding site or arise from the effect of the mutations on the general surface topology of the actin filament.

Another potential cause for the mild actin phenotype in these cells might be an altered interaction between the actin and either or both of the yeast formins Bni and Bni1 (46) as a result of the H372R mutation. The formins nucleate filament formation or bind to the filament barbed end and/or promote filament elongation (47), and in yeast they are involved in regulating the assembly of actin cables in a polarized fashion extending between the mother and the bud cell (48). Some formins work by promoting actin filament formation as a processive capper. They continually associate with and partially cap the barbed end in a manner that allows the continued addition of new actin monomers but prevents elongation termination by a capping protein (49). Other formins, which enhance the rate of filament elongation per se by modulating the rates of nucleotide hydrolysis on the actin surface (50), require the binding of profilin to the FH1 domain of the formin to produce a complex that then causes acceleration of elongation. Models for both of these modes of action have been proposed (50–53).

Clues to the molecular basis of formin action are provided by the recent elucidation of the x-ray structure of a formin/actin complex (51). The structure shows that the two ends of the formin bridge an actin dimer. One end binds to actin subdomain 1 near the actin N terminus on the front face of the protein. The other appears to bind near the subdomain 1–subdomain 2 interface on the back face of the actin in the region of the C terminus, the site of the H372R mutation. The mild actin defect we detect in vivo in the H372R cells, namely the disorganization of the actin cable system, might result from altered activity of the endogenous formins and/or Arp2/3 due to the effects of the mutation. Use of this and related mutant actins should enable us to obtain valuable insight into the mechanism of formin, the Arp2/3-dependent regulation of actin dynamics, and the role that these regulatory proteins might play in organelle function in yeast.

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Melissa McKane, Kuo-Kuang Wen, Istvan R. Boldogh, Sharmilee Ramcharan, Liza A. Pon and Peter A. Rubenstein

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