New actin mutants allow further characterization of the nucleotide binding cleft and drug binding sites

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INTRODUCTION

Actin filaments are an important structural component of most eukaryotic cells. The ability of actin monomers to assemble into filaments, and then to rapidly disassemble so that new filaments may form from the monomers is essential for dynamic processes such as cytokinesis, cell motility, establishment of cell polarity, and endocytosis. A more complete understanding of the regulation of actin dynamics will be required before the mechanism by which actin dependent cellular processes occur can be fully understood. The dynamic properties of actin are regulated by the ATP cycle of actin itself, and by numerous regulatory proteins. Natural products that inhibit various steps in the polymerization/depolymerization cycle of actin have proven to be useful tools in mechanistic and functional studies of actin.

Mutagenesis of actin has been a very effective approach to the elucidation of actin properties underlying assembly dynamics, and to the identification of binding sites for actin regulatory proteins and drugs. Various studies provide support for the hydrophobic plug model of actin assembly (Kuang and Rubenstein, 1997a,b; Amberg et al., 1995; Chen et al., 1993), and provide insight into the conformational switch that allows actin to depolymerize after ATP hydrolysis and Pi release (Chen and Rubenstein, 1995; Orlova et al., 1997; Belmont et al., 1999). Studies with rabbit muscle actin have demonstrated that the proteolytic removal of the carboxyl-terminal two or three amino acids of actin results in an increase in the critical concentration, the polymerization rate and the lag in ATP hydrolysis after filament assembly (Strzelecka-Golaszewskia et al., 1995). When mutations removing these terminal residues were introduced into the yeast actin gene, the three amino acid truncation was lethal, while removal of the last two amino acids resulted in temperature sensitivity (Johannes and Gallwitz, 1991). Charged to alanine scanning mutagenesis of yeast actin (Wertman et al., 1992) has provided many useful mutants that have allowed the identification of the binding site yeast fimbrin (Honts et al., 1994; Holtzman et al., 1994), Aip1p, and Srv2p (Amberg et al., 1995). Additional mutagenesis has allowed the identification of the myosin binding site (Miller et al., 1995). Finally the actin mutant collections have been used to suggest the binding sites of phalloidin (Drubin et al., 1993) and latrunculin A (Ayscough et al., 1997). Actin binding drugs have been extremely useful research tools in the study of actin based processes, and some show potential for therapeutic applications (Jordan and Wilson, 1998; Patterson and Carmeli, 1992; Smith et al., 1993). Molecular models of monomeric and filamentous actin provide the potential to establish structure-function relationships for actin (Kabsch et al., 1990; Holmes et al., 1990; Lorenz et al., 1993). Mapping the binding sites for drugs onto...
these molecular models is a crucial step in studying their mechanism of action. Chemical cross-linking studies have provided conflicting data on the location of the phalloidin binding site (Vandekerckhove et al., 1985; Faulstich et al., 1993), while the analysis of mutants that disrupt the phalloidin binding site (Drubin et al., 1993) placed phalloidin in a site that is consistent with X-ray fiber diffraction studies (Lorenz et al., 1993) as well as three-dimensional EM reconstructions of actin filaments decorated with gold-conjugated phalloidin (Steinmetz et al., 1998). Therefore, mutational analysis of actin provides a powerful tool with which to identify drug binding sites.

The lack of phalloidin binding could potentially provide a very useful tool with which to distinguish different populations of actins. However, the phalloidin site mutation (R177A, D179A) also results in temperature sensitivity and a loss of actin cables, suggesting that filament assembly is also impaired. We have constructed 9 new mutations in yeast actin. Six of these lie in the ATP binding cleft, and another is a C-terminal deletion of 2 amino acids. We have also separated two mutations found previously in the double mutant to eliminate phalloidin binding, and were able to generate an actin that fails to bind phalloidin, but still exhibits normal assembly properties. We have used these new actin mutants as well as a large existing collection of actin mutants to map the actin properties. We have constructed 9 new mutations in yeast actin.

Materials and Methods

Construction of mutations

Mutations in the Saccharomyces cerevisiae ACT1 gene were constructed as described previously (Wertman et al., 1992) except that we used the Transformer™ mutagenesis kit (Clontech). Mutations were confirmed in the final haploid yeast strain by sequencing a PCR product from the region containing the mutation. The actin allele numbers and their corresponding mutations are as follows: act1-150, G302A; act1-151, C-terminal deletion of 2 amino acids (CTD); act1-152, G15R; act1-153, G15S; act1-154, Q137A; act1-155, R177A; act1-156; D179A; act1-157, D157E; act1-158, G158A.

Strains and cell culture

Standard media and cell culture conditions were used (Guthrie and Fink, 1991). The strains used in this study are listed in Table 1.

ATP exchange assays

Actin was purified by DNasel affinity chromatography as described (Ayscough et al., 1997) and frozen as G-actin in liquid N2. ATP exchange was measured by diluting the actin into e-ATP (1.2% ethenoadenosine-5'-triphosphate, Molecular Probes), with a final concentration of 20 μM ATP, 200 μM e-ATP, 1.6 μM actin, 10 mM Tris-HCl, pH 7.5, 0.2 mM CaCl2, and the appropriate concentration of latrunculin A or tolytoxin. The sample was then immediately placed into a thermostatted cuvette at 18°C, and the change in fluorescence was monitored (excitation = 360 nm, emission = 410 nm). There was a dead time of approximately 4 seconds between addition of e-ATP and recording fluorescence.

Latrunculin A assays

Halo assays to measure sensitivity to latrunculin A, and actin turnover assays were performed at 25°C as described (Ayscough et al., 1997), except that actin structures were observed by immuno-fluorescence with a guinea pig anti-actin antibody (1:2000) (Mulholland et al., 1994) and goat anti-guinea pig secondary conjugated to rhodamine.

Fluorescence staining and microscopy

Phalloidin staining and immuno-fluorescence were performed as described (Ayscough et al., 1997). Images were acquired using a Zeiss Axiophot microscope equipped with a Sony CCD camera controlled by Northern Exposure Software (Phase 3 Imaging).

Endocytosis assay

Yeast cells were grown to an optical density (OD) at 600 nm of 0.25 (5x106 cells/ml). 2 ml of cells were sedimented at low speed in a clinical centrifuge and resuspended in 40 μl of medium. 15 μl of resuspended cells were added to 7.5 μl of 40 mg/ml Lucifer Yellow (Molecular Probes) and incubated at 25°C for 2 hours. Cells were then washed 3 times in ice-cold sucinate/azide buffer (50 mM succinic acid, 20 mM NaNO3, pH adjusted to 5 with NaOH) and resuspended in 6 μl sucinate/azide buffer. 2 μl were placed on a slide for immediate observation. 200 cells were counted to determine the percentage with brightly stained vacuoles.

Tolytoxin assays

Sensitivity to tolytoxin was measured by a halo assay as described (Ayscough et al., 1997), except that the tolytoxin was dissolved in 100% ethanol, and no top agar was used. Tolytoxin Kd measurements were made with a radio-labeled derivative of tolytoxin in which the C-27 ketone was reduced to the corresponding alcohol, yielding 6-hydroxy-7-O-methylscytophycin A (HMSA). To make this ligand, tolytoxin was isolated from the cyanobacterium Scytonema ocellatum by the method described previously (Carmeli et al., 1990). Both synthetic 3H-labeled and unlabelled HMSA were prepared by chemical reduction of tolytoxin using NaBH4 or NaBH₄, respectively. 5 mg of tolytoxin was dissolved in 1.5 ml tetrahydrofuran containing 50 μl isopropanol. 2.5 mg NaBH4 and 113 μg NaBH₄ (50.4 Ci/mmol, New England Nuclear) were added and stirred overnight at room temperature under N2 gas, after which the reaction was terminated by the addition of 1 ml acetone. The crude reaction mixture was washed repeatedly by cycles of drying and resuspension in aceton to quench remaining NaBH₄, then exchanged by repeated dissolution in 1 ml of methanol followed by evaporation under N2. The crude reaction mixtures, labeled or unlabeled, were applied in water to disposable reversed-phase chromatography cartridges (Prep-Sep C18, Fisher Scientific) and eluted with methanol:water (4:1, v/v). The eluate was concentrated and purified by reversed-phase HPLC (1x 25 cm, Alltech Econosil C18, 10 μm) utilizing an isocratic elution solvent of methanol:water (3:1, v/v) and monitoring absorbance at 262 nm. The reaction products were confirmed by 1H- and 13C-NMR spectroscopy. Radioactivity was determined by liquid scintillation techniques. HPLC-purified 3H-HMSA had a specific activity of 11.0 Ci/mmol, or approximately one-fourth the specific activity of the chemical reductant. Aliquots of tritiated HMSA were stored under a nitrogen atmosphere at −20°C in ethanol solution. Tritiated HMSA is stable for approximately 6 months and was re-purified by HPLC as necessary to eliminate radiochemical contaminants.

Binding of 27-3H-HMSA to actin was measured using a DEAE-cellulose filter assay. Each reaction mixture (100 μl total volume) contained 30 μg actin, 10 mM imidazole (pH 8.0), 100 mM KCl, 2 mM MgCl₂, 0.5 mM ATP; 0.2 mM β-mercapto-ethanol, 0.1 mM CaCl₂, 0.005% NaN₃, and 10 μg BSA. Assays were initiated by addition of G-actin to the reaction mixture in 1.5 ml polypropylene microfuge tubes which were then pre-incubated for 20 minutes at 25°C to allow polymerization. Unlabelled ligands and 27-3H-HMSA were added at the start of the indicated incubation period. The final reaction mixtures also contained 0.5% ethanol, the solvent of tolytoxin and HMSA. After mixing and incubating at 25°C for the desired period, samples were transferred to a stack containing four 2.5 cm circular discs of DEAE-cellulose paper (Whatman DE-81). The discs were assembled in a stack on a Millipore microanalysis filter holder. The microfuge tubes were rapidly rinsed two times with 500 μl of ice-cold binding medium which was transferred to the filter...
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stack. The filter stack was incubated for 60 seconds before the total volume was aspirated under weak suction. The stack of filters was rapidly washed in succession with three 3 ml portions of ice-cold binding medium and transferred to a scintillation vial. Data points represent the average of triplicate samples, which were generally within 10% of each other.

RESULTS

Selection of residues for mutagenesis

To genetically probe the actin ATP cycle, we constructed the following mutations in the yeast actin ATP-binding pocket: G15R, G15S, Q137A, D157E, G158A, and G302A. The rationale for construction of these mutations is as follows. The amide groups of D157 and G158 form hydrogen bonds with the γ-phosphate of the bound ATP, making them candidates for residues that participate in ATP hydrolysis, P_i release, or nucleotide exchange. G15, G158, and G302 each contact the nucleoside phosphate and appear to contribute to the linkage of the two major globular actin subdomains. These residues are therefore well positioned to be involved in a conformational change induced by nucleotide hydrolysis (Kabsch et al., 1990; Sheterline et al., 1995). Q137 was selected for mutagenesis on the basis of a mutant in DnaK that abolishes the stimulation of ATP hydrolysis upon substrate binding. DnaK is a member of the hsc70 family of proteins that share structural homology to actin (Flaherty et al., 1991). When E171 in DnaK, the residue that occupies the same position as Q137 in actin, was changed to A, L and K, all of these mutants showed ATPase activity, but this activity was not stimulated by substrate binding (Buchberger et al., 1994). We also constructed a deletion of the two C-terminal amino acids of actin (CTD) in our strain background (this same mutant was made previously by Johannes and Gallwitz, 1991). Previous studies with rabbit muscle actin have demonstrated that the proteolytic removal of the carboxyl-terminal two or three amino acids results in an increased lag in ATP hydrolysis after actin filament assembly (Strzelecka-Golaszewska et al., 1995) and loss of cross strand stabilization (Orlova et al., 1995). Finally, we constructed R177A and D179A single mutations in an attempt to separate loss of phalloidin binding from temperature-sensitivite and actin assembly defects observed for act1-129 which contains both mutations. The residues chosen for mutagenesis are displayed on an atomic model of actin in Fig. 1.

Growth properties and actin distribution in new actin mutants

For each mutant constructed, a fragment of DNA encoding yeast actin carrying a mutation was integrated into the actin locus of diploid yeast as described previously (Wertman et al., 1992). The resulting heterozygous diploids were sporulated to generate haploid yeast expressing either the wild-type or the mutant actin gene.

Diploids heterozygous for either the G15R or the Q137A mutation failed to sporulate. Sporulation of heterozygotes for G15S actin resulted in all nonviable spores. Because we were unable to study the phenotypes of yeast expressing G15R, G15S, or Q137A mutants as the only source of actin, we examined heterozygous diploids expressing one copy of wild-type actin and one copy of either act1-154 (Q137A) or act1-153 (G15R) actin. When examined by immunofluorescence, both strains appeared to have more pronounced actin structures than diploids carrying one wild-type actin gene and an actin deletion (ACT1 hemizygotes). Because ACT1 hemizygotes have less pronounced actin structures than wild-type diploids, this observation suggests that the mutant actins are able to assemble into filaments and that they are not acting as dominant negatives for actin assembly. Consistent with this conclusion, the Q137A heterozygote (ACT1/act1-154) had a latrunculin A sensitivity identical to that of wild-type diploids, while G15R heterozygotes (ACT1/act1-153) were twofold more resistant than wild-type diploids (data not shown).

For the remaining actin mutants, we recovered haploids expressing the mutant actin, and confirmed by PCR that the mutant had integrated at the actin locus. Some general features

Fig. 1. Actin residues chosen for mutagenesis. Standard view of actin and a 180° rotation around the y-axis. Mutagenized residues are shown in color. Characterization of V159N has been reported elsewhere (Belmont and Drubin, 1998; Belmont et al., 1999). D179 is not depicted here, but is highlighted in the space-filling models shown in Fig. 9. The locations of mutations for which we were unable to generate haploids, Q137 and G15, are colored black. The ATP is red.
Only 6% of small budded wild-type cells display depolarized actin patches under these conditions.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
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<tr>
<td>DDY479</td>
<td>a/α ACT1/αCT1::HIS3 his3Δ200 his3Δ200 ura3-52 ade2-1-1 ADE2 ADE4/ade4 TUB2/tub2-201 cry1/CRY1 can1/CAN1</td>
</tr>
<tr>
<td>DDY1104</td>
<td>a/α ACT1/αCT1::LEU2 his3Δ200 his3Δ200 ura3-52 ade2-1-1 ADE2 ade4 TUB2/tub2-201 cry1/CRY1 can1/CAN1</td>
</tr>
<tr>
<td>DDY1531</td>
<td>a/α ACT1/αCT1::Δ1::LEU2 his3Δ200 his3Δ200 ura3-52 ade2-1-1 ADE2 ADE4/ade4 TUB2/tub2-201 cry1/CRY1 can1/CAN1</td>
</tr>
<tr>
<td>DDY1532</td>
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</tr>
<tr>
<td>DDY1533</td>
<td>a/α ACT1/αCT1::Δ1::LEU2 his3Δ200 his3Δ200 ura3-52 ade2-1-1 ADE2 ADE4/ade4 TUB2/tub2-201 cry1/CRY1 can1/CAN1</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>DDY1537</td>
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</tr>
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</tr>
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<td>a/α act1-112::HIS3200 tub2-201 ura3-52</td>
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</tr>
<tr>
<td>DDY1545</td>
<td>a/α act1-119::HIS3200 tub2-201 ura3-52</td>
</tr>
</tbody>
</table>

of haploid strains expressing these six actin alleles are shown in Table 2.

We measured the sensitivity of these new actin mutants to the actin-sequestering drug, latrunculin A, using a halo assay in which various concentrations of this drug were placed on sterile filter discs that were then placed on a nascent lawn of yeast. The D157E mutation confers complete resistance to latrunculin A. Yeast expressing this actin mutant grew directly on the filter even at the highest drug concentrations.

We also found that mutations in the actin alleles whose temperature sensitivity was not lost upon transformation into the nucleus suppress the temperature sensitivity of act1-101, act1-119, act1-120, act1-124, and act1-133. Table 2 shows the in vivo characterization of these mutants.

In order to characterize further the growth properties of the various mutants, we grew them on a variety of plates with specially modified media (Table 3). All of the mutations except D179A displayed increased sensitivity to 0.9 M NaCl. This effect appears to be specific to NaCl, since neither 0.9 M KCl nor 1.3 M sorbitol had this effect. Surprisingly, 0.1 M CaCl₂ rescued the temperature-sensitivity of R177A. The suppression of temperature sensitivity by 0.1 M CaCl₂ turns out to be prevalent among actin mutants. We tested 8 additional temperature sensitive actin mutants and found that five of them (act1-101, act1-119, act1-120, act1-124, and act1-133) were able to grow at 37°C in the presence of 0.1 M CaCl₂, while only three (act1-111, act1-108, and act1-112) were not (data not shown). The mechanism of this suppression is unclear, but might reflect the existence of a calcium activated activity that can directly or indirectly stabilize actin filaments. Interestingly, the three actin alleles whose temperature sensitivity was not suppressed by CaCl₂ are all located on a discreet region of the genome.

<table>
<thead>
<tr>
<th>Actin mutation</th>
<th>dt 25°C (hours)</th>
<th>Growth at 37°C</th>
<th>Relative LAT-A&lt;sup&gt;R&lt;/sup&gt;</th>
<th>Phalloidin binding</th>
<th>LY uptake</th>
<th>IF phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td>+</td>
<td>+</td>
<td>1</td>
<td>+</td>
<td>Normal</td>
</tr>
<tr>
<td>D157E</td>
<td></td>
<td>+</td>
<td>+</td>
<td>Complete</td>
<td>+</td>
<td>Loss of cables (40%) or excess cables (6%)</td>
</tr>
<tr>
<td>G158A</td>
<td>3.3</td>
<td>+</td>
<td>+</td>
<td>0.3</td>
<td>+</td>
<td>89</td>
</tr>
<tr>
<td>R177A</td>
<td>4.2</td>
<td>–</td>
<td>+</td>
<td>0.2</td>
<td>–</td>
<td>23</td>
</tr>
<tr>
<td>D179A</td>
<td>2.9</td>
<td>+</td>
<td>+</td>
<td>0.7</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>G302A</td>
<td>2.7</td>
<td>+</td>
<td>+</td>
<td>0.8</td>
<td>+</td>
<td>64</td>
</tr>
<tr>
<td>CTD</td>
<td>2.7</td>
<td>+</td>
<td>+</td>
<td>2.5</td>
<td>+</td>
<td>54</td>
</tr>
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</table>

Characterization of haploid yeast expressing the indicated actin with the indicated mutation. dt, doubling time; LAT-A<sup>R</sup>, latrunculin A relative resistance; LY, Lucifer Yellow; IF, immunofluorescence; DP, depolarized. LY uptake is a count of the percentage of cells displaying bright vacular staining after incubating cells for two hours in the presence of Lucifer Yellow. Actin polarization was assessed by counting the percentage of small budded cells that have depolarized actin. Only 6% of small budded wild-type cells display depolarized actin patches under these conditions.
Characterization of actin drug binding sites

Table 3. Growth of actin mutants on specialized media

<table>
<thead>
<tr>
<th>Actin mutation</th>
<th>YPD</th>
<th>0.1 M MgCl₂</th>
<th>0.1 M CaCl₂</th>
<th>0.9 M NaCl</th>
<th>0.9 M KCl</th>
<th>1.3 M sorbitol</th>
<th>pH 3.5</th>
<th>pH 7.4</th>
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<td>wt</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>D157E</td>
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<td>+</td>
<td>+/−</td>
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<tr>
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</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G302A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
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25°C

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<th>Actin mutation</th>
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<th>0.1 M MgCl₂</th>
<th>0.1 M CaCl₂</th>
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37°C

Table 3. Growth of actin mutants on specialized media

A dilution series of cells was plated on solid rich medium (YPD) supplemented with salts or sugars at the indicated concentrations. Growth was assessed after three days at 25°C or 37°C. The pH plates were made using minimal medium adjusted to the indicated pH with sodium phosphate (high pH) or sodium succinate (low pH). ND, no data.

subdomain 4, while the other alleles are dispersed over subdomains 1 and 2 and the nucleotide binding cleft.

We examined the distribution of actin in each of the mutants by immuno-fluorescence (Fig. 2, Table 2). Filamentous actin in yeast is found in two types of structures, cortical patches and cytoplasmic cables. In wild-type yeast, the patches are concentrated in the bud during bud growth, and the cables run from the mother to the bud along the mother to bud axis (Fig. 2A). All of the actin mutants, with the exception of D179A, exhibit varying degrees of actin cytoskeleton defects. The quantification of these defects is displayed in Table 2.

Actin filament turnover and fluid phase endocytosis are altered in actin mutants

Actin filament turnover was measured in the actin mutants by observing the rate at which filamentous actin structures disappear after the addition of latrunculin A. This drug sequesters monomer and prevents assembly, but does not actively promote depolymerization (Coue et al., 1987). Therefore, the rate of disappearance of actin structures in the presence of latrunculin A should reflect the rate of actin depolymerization in vivo (Ayscough et al., 1997; Lappalainen and Drubin, 1997). Previously, phalloidin staining was used to determine the number of cells with filamentous actin structures. Because filamentous actin in three of the actin mutants (G158A, R177A, and D179A) considered in this study does not bind to phalloidin, we visualized actin filaments by anti-actin immuno-fluorescence. Fig. 3 shows the percentage of cells with detectable actin structures (either actin patches or cables) after addition of 400 μM latrunculin A. Immunofluorescence is more sensitive than phalloidin staining. Fig. 2 shows the percentage of cells with detectable actin structures (either actin patches or cables) after addition of 400 μM latrunculin A. Immunofluorescence is more sensitive than phalloidin staining. This assay, giving an apparently slower rate of actin turnover than was previously measured with phalloidin staining. In this assay, the D179A mutant had a nearly wild-type rate of loss of actin structures and the CTD mutant had the slowest rate of actin filament turnover. We were not able to test the D157E mutant in this assay because it shows no sensitivity to latrunculin A.

Previously, we suggested that rapid actin filament turnover might be required for fluid-phase endocytosis (Lappalainen et al., 1997; Belmont and Drubin, 1998). We measured fluid-phase endocytosis in all of the new actin mutants by observing the uptake of Lucifer Yellow (LY) from the growth medium into the vacuole. Fig. 4 shows that there is a large variation among the various actin mutants in the amount of LY that is observed in the vacuole after 2 hours. D157E cells appear to have a faster rate of LY uptake than wild-type strains, while all of the other actin mutants have slower rates of fluid phase endocytosis. Table 2 lists the percentage of cells of each strain that exhibit bright vacuolar staining after 2 hours in LY. There is not a tight correlation between rates of actin filament turnover and endocytosis, and it therefore appears that properties of actin in addition to rapid rates of actin filament turnover are important for fluid phase endocytosis.

Latrunculin A does not inhibit ATP exchange on purified D157E actin

Because D157E strains are absolutely resistant to latrunculin A, we postulated that this mutation disrupts the latrunculin A binding site. Latrunculin A inhibits ATP exchange on wild-type yeast actin, but not on a mutant, act1-117, that is resistant to latrunculin A (Ayscough et al., 1997). We purified D157E actin and measured ATP exchange in the presence or absence of latrunculin A (Fig. 5). The rate of ATP exchange was faster for D157E actin, t1/2 of 36 seconds compared to 100 seconds for wild-type yeast actin. 10 μM latrunculin A caused complete inhibition of ATP exchange on wild-type yeast actin, but had no effect on the ATP exchange rate of D157E actin. For comparison, we measured the rate of ATP exchange on purified G158A actin. Although G158A actin has a much slower rate of ATP exchange (t1/2=250 seconds), ATP exchange was inhibited completely by 10 μM latrunculin A. It therefore seems likely that the D157E mutation disrupts the latrunculin A binding site on actin. This conclusion is supported by the observation that D157 lies in close proximity to other residues whose mutation result in latrunculin A resistance.
Identification of the tolytoxin binding site

Tolytoxin and its congeners, a family of macrolides known collectively as scytophycins, are potent cytotoxins produced by the closely related cyanobacterial genera Scytonema and Tolypothrix (Ishibashi et al., 1986; Carmeli et al., 1990). The scytophycins have been shown to induce rapid depolymerization of actin structures in vivo and in vitro (Patterson et al., 1993) and are potentially useful in the treatment of human neoplasia (Patterson and Carmeli, 1992; Smith et al., 1993). We used the actin mutants described here as well as the charged to alanine mutant collection (Wertman et al., 1992) to map the binding site of tolytoxin on the surface of actin. The results of halo assays used to measure relative resistance to tolytoxin are displayed in Fig. 6. There were no alleles that resulted in absolute resistance to tolytoxin. However, three mutant alleles, act1-117 (R183A/D184A), act1-123 (R68A/E72A), and act1-150 (G302A), caused more than a fourfold increase in resistance as compared to wild-type actin. Because none of these mutations causes an increase in actin stability on their own, the increased resistance is not due to a general effect on polymer stability. Two other alleles, act1-159 (V159N) and act1-151 (CTD) were also somewhat resistant. However, these mutants both slow actin filament turnover, and are therefore likely to be resistant to tolytoxin because of this biochemical property rather than specific disruption of the tolytoxin binding site. The mutations that cause more than a fourfold increase in tolytoxin resistance (R68A/E72A, R183A/D184A, and G302A), cluster in a region around nucleotide binding cleft. We therefore propose that these mutations cause increased resistance by disrupting the tolytoxin binding site.

We tested this hypothesis in two ways. First, we measured the effect of tolytoxin on nucleotide exchange. Second, we directly measured the affinity of tolytoxin binding for one of the actin mutants that conferred increased resistance. We measured ATP exchange by monitoring the increase of fluorescence of ε-ATP upon binding to actin. Increasing amounts of tolytoxin increased the t_1/2 of ATP exchange by 2.4-fold (from 100 seconds to 240 seconds) (Fig. 7). Increasing the tolytoxin concentration above 200 μM had no further effect on the rate of ATP exchange. These data are consistent with the tolytoxin binding site being near the ATP binding site.
Because measurements of ATP exchange do not provide a direct measure of drug binding, we purified actin from the act1-117 strain and directly measured the affinity of tolytoxin binding to this actin. We chose this strain because it is relatively healthy, and it is therefore possible to purify sufficient amounts of actin for biochemical assays. The relative $K_d$ values of a radio-labeled tolytoxin derivative were measured using a filter binding assay. Tolytoxin binds to rabbit muscle actin with a $K_d$ of 6 mM and to wild-type yeast actin with a $K_d$ of 8.6 mM. In contrast, the $K_d$ of tolytoxin for act1-117 actin is approximately 17.5 mM (Fig. 8). These data support the hypothesis that tolytoxin binds near the ATP binding pocket and that the resistant alleles disrupt the binding site of tolytoxin on actin.

**DISCUSSION**

**Actin structure/function relationships and actin roles in yeast**

Analysis of actin mutants constructed for this study revealed further insights into structure/function relationships as well as insights into the in vivo functions and regulation of actin. The actin mutants varied in the severity of their effects on actin function. Mutations to G15 and Q137 had the most severe effects, demonstrated by our inability to construct strains expressing only actin with the G15R, G15S, or Q137A mutations. Hsp70 heat shock proteins, sugar kinases, and actin all share structural homology and can therefore be considered members of a superfamily (Flaherty et al., 1991; Bork et al., 1992). In this superfamily, the amino acid in the equivalent position to Q137 of actin is usually E or D, although it is A in *E. coli FtsA* and Q in *B. subtilis FtsA* (Bork et al., 1992). Therefore, we reasoned that a Q to A mutation at this position would be unlikely to disrupt overall protein folding of actin. When the corresponding residue (E171) in DnaK (a member of the hsp70 family) was mutated to A, the mutant protein exhibited no stimulation of ATPase upon substrate binding (Buchberger et al., 1994). Because we could not construct a strain expressing only Q137A actin, we were unable to purify this mutant to determine whether this mutation inhibited assembly-stimulated ATPase activity. Therefore, less disruptive mutations may be required to study the role of Q137 in this process. The amide group of G15 makes a hydrogen bond to the $\beta$-phosphate of ATP (Kabsch et al., 1990). Moreover, G15 is highly conserved among actins from many species (Sheterline et al., 1995). The equivalent position in the hsp70/sugar kinase family is often S or T (Bork et al., 1992). We therefore thought that the change to S might not cause a large disruption of the structure. These strains, however, were unable to sporulate, suggesting a defect in actin function. The heterozygous diploids containing one G15S mutant and one wild-type actin gene were slightly more resistant than wild-type diploids to latrunculin A. We speculate that this mutant might make actin filaments with increased stability, as was seen previously with
the act1-159 mutation (Belmont et al., 1999; Belmont and Drubin, 1998). The failure of the G15 and Q137A mutants to sporulate despite their robust growth when expressed with wild-type actin in heterozygotes in diploids suggests that there may be a specialized role for actin in meiosis that is distinct from its roles in vegetative growth.

The G158A mutation resulted in slow ATP exchange on monomeric actin. While we do not know the specific reason for this, one explanation is that the added side chain may inhibit release of ATP from the pocket due to steric hindrance. It is also worth noting that G158 is in a bend (see Fig. 1). Because A is much more constrained that G (Ramachandran and Sasisekharan, 1968) this substitution is likely to change the conformation of this bend. Such a change in conformation could have two possible consequences that might result in slow ATP exchange. (1) The substitution may have altered the position of the amino acid such that hydrogen bond between the backbone amide and the $\gamma$-phosphate is stronger, thus lowering the off rate of nucleotide from the actin monomer, or (2) the substitution may have altered the conformation of this turn such that the nucleotide binding cleft is narrowed, thus restricting nucleotide exchange. Although yeast expressing G158A actin were able to assemble fairly normal actin structures as evaluated by immuno-fluorescence, purified G158A actin failed to assemble into filaments that we could identify by electron microscopy or light scattering (data not shown). The relatively normal growth of this strain despite a pronounced defect in exchange rates suggests that in vivo, the intrinsic rate of nucleotide exchange may not be rate limiting for the proper assembly of actin structures. D157E actin, in contrast, exhibits fast nucleotide exchange on monomeric actin. The purified D157E actin assembles into filaments that appear normal by electron microscopy and that exhibit normal polymerization and depolymerization properties (data not shown). Actin organization in cells expressing D157E actin is fairly normal, except that some cells appear to have fewer
Characterization of actin drug binding sites

Based on the study of the G158A and D157E mutants, it appears that intrinsic ATP exchange rates may be more important for assembly of purified actin than for assembly of actin in vivo, perhaps because ATP exchange in vivo can be regulated by actin binding proteins such as profilin. Also, it remains unclear to what extent the polymerization defects observed for G158A actin are due to slow nucleotide exchange.

Actin in which the two C-terminal amino acids have been proteolytically removed has been reported to have an increased lag in ATP hydrolysis after actin assembly (Strzelecka-Golaszewska et al., 1995). If this lag is sufficiently long relative to the normal lifetime of an actin filament in vivo, the mutation should result in slower actin filament depolymerization, consistent with our observation of slow actin filament turnover in our latrunculin A depolymerization assays. In contrast, this modified actin also appears more sensitive to mechanical shearing (Mossakowska et al., 1993), perhaps due to the decrease in connectivity between the two long-pitch strands of actin filaments that has been observed in three-dimensional reconstructions of electron micrographs (Orlova et al., 1995). This increased fragility might explain the loss of cables in this mutant. If the cables are composed of actin filaments that are longer than those found in cortical patches, as has recently been suggested (Karpova et al., 1998), or are less protected by actin binding proteins, they might be more sensitive to mechanical shearing. Therefore, cables would be lost from breakage, while the actin filaments in the cortical patches would depolymerize more slowly due to slow ATPase activity.

Previous studies have suggested rapid actin dynamics are required for endocytosis (Lappalainen and Drubin, 1997; Belmont and Drubin, 1998). In this study, some actin mutants showed only slight decreases in actin turnover rates yet most mutants displayed severe endocytosis defects. This result suggested that other properties of actin are also important for fluid phase endocytosis.

**Mapping toxin binding sites on the surface of actin**

Actin mutants have been used to map the binding sites of phalloidin and latrunculin A on actin (Drubin et al., 1993; Ayscough et al., 1997). Here we extend these studies and provide the first identification of a likely binding site for tolytoxin. This approach to mapping binding sites relies on the assumption that the mutations inhibit protein drug interactions by altering the chemical properties of an amino acid that directly contacts the drug. It is possible that a mutation could disrupt binding by changing the overall conformation of actin. In fact, one mapping study using Sac6p (yeast fimbrin) fused to GFP (Doyle and Botstein, 1996) identified three mutations that directly disrupted Sac6p binding and an additional mutation that was located far away from the interaction site (see Belmont and Drubin, 1999). The fourth mutation is thought to inhibit a conformational change that occurs upon Sac6p binding. Therefore caution must be

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**Fig. 7.** Tolytoxin inhibits ATP exchange on monomeric actin. ATP exchange was measured by monitoring the increase in the fluorescence of e-ATP upon binding to monomeric actin in the presence of increasing amounts of tolytoxin. The half time of ATP exchange increased from 100 seconds to 240 seconds upon the addition of 200 μM tolytoxin. No further increases were observed with the addition of more tolytoxin.

**Fig. 8.** Binding of the radio-labeled tolytoxin derivative, 27-[3H]-HMSA, to actin. Binding was measured as described in Materials and Methods. Each data point represents the mean of three replicate measurements. (A) Saturation isotherm for 27-[3H]-HMSA binding. The amount of specific binding has been normalized and expressed as a percentage. For comparison, data for binding of this ligand to rabbit skeletal muscle ($K_d = 6.2 \mu M$) is included. (B) Rosenthal transformation of the specific binding data for wild-type actin yielding a $K_d$ of 8.6 μM. (C) Rosenthal transformation of the specific binding data for act1-117 actin, yielding a $K_d$ of 17.5 μM.
used in the interpretation of mapping with actin mutants. However, all of the drug resistant alleles identified in this study map close to each other for any given drug, making this alternative interpretation less likely. Furthermore, there is no particular phenotype from the actin mutations that correlates with resistance to a given compound. We therefore

![Location on the surface of actin of mutations conferring resistance to tolytoxin, latrunculin A, and phalloidin. ‘Front’ and ‘back’ views of space-filling models of rabbit muscle actin (Kabsch et al., 1990). The subdomains are labeled in Roman numerals. (A) The location of the amino acids mutated in the three actin mutants most resistant to tolytoxin are highlighted in orange. The amino acid residues predicted to be involved in actin subunit interactions in the filament (Holmes et al., 1990) are highlighted in blue, purple, and green. ATP is red. (B) The location of the amino acids mutated in all of the tolytoxin, latrunculin A, and phalloidin resistant mutations identified. The tolytoxin resistant alleles (blue and purple), a new phalloidin binding defective allele (G158A), and a new latrunculin A resistant allele (D157E) were identified in this study. The other latrunculin A resistant and phalloidin binding defective alleles were identified in previous studies (Drubin et al., 1993; Ayscough et al., 1997). The act1-117 allele (R183A, D184A) is resistant to latrunculin A and exhibits increased resistance to tolytoxin as well. ATP is black.

\[\text{Fig. 9. Location on the surface of actin of mutations conferring resistance to tolytoxin, latrunculin A, and phalloidin. ‘Front’ and ‘back’ views of space-filling models of rabbit muscle actin (Kabsch et al., 1990). The subdomains are labeled in Roman numerals. (A) The location of the amino acids mutated in the three actin mutants most resistant to tolytoxin are highlighted in orange. The amino acid residues predicted to be involved in actin subunit interactions in the filament (Holmes et al., 1990) are highlighted in blue, purple, and green. ATP is red. (B) The location of the amino acids mutated in all of the tolytoxin, latrunculin A, and phalloidin resistant mutations identified. The tolytoxin resistant alleles (blue and purple), a new phalloidin binding defective allele (G158A), and a new latrunculin A resistant allele (D157E) were identified in this study. The other latrunculin A resistant and phalloidin binding defective alleles were identified in previous studies (Drubin et al., 1993; Ayscough et al., 1997). The act1-117 allele (R183A, D184A) is resistant to latrunculin A and exhibits increased resistance to tolytoxin as well. ATP is black.}\]
think it likely that the mutations identify a site of drug interaction.

None of the actin mutants tested caused complete resistance to tolytoxin. The two most likely explanations for this result are: (1) none of the mutations result in complete inhibition of tolytoxin binding to actin, or (2) there is a second target of tolytoxin that is contributing to cellular toxicity. While we cannot rule out the possibility of a second target site for tolytoxin, we think it likely that the mutations map the binding site on actin because the resistant alleles cluster to a discrete site on the surface of actin. The residues mutated in these alleles, highlighted in orange in Fig. 9A, are located on the back face of subdomains 2 and 4 near the ATP binding pocket. Tolytoxin has been shown to bind to actin dimers (G. Patterson, unpublished observations). We therefore have highlighted the amino acids that are predicted to be involved in contacts with other actin monomers in the actin filament. The tolytoxin resistant alleles lie close to the ‘hydrophobic plug’ (blue) that is proposed to insert into the hydrophobic pocket (green) to stabilize actin filaments (Holmes et al., 1990). This places the tolytoxin binding site in a location that is consistent with its ability to bind two actin monomers.

Previous reports have identified actin mutations defective in binding phalloidin and latrunculin A (Drubin et al., 1993; Ayscough et al., 1997). In this work, we identified a new latrunculin A resistant allele and an allele defective in phalloidin binding. Fig. 9B illustrates the location of amino acid residues that have been implicated in drug binding by mutational analysis. The newly identified phalloidin (G158A) and latrunculin A (D157E) resistant alleles lie very close to the previously identified binding sites for these toxins. The phalloidin binding site identified by the analysis of actin mutants is consistent with the placement of the phalloidin binding site as determined by X-ray fiber diffraction (Lorenz et al., 1993) as well as three-dimensional reconstructions of electron micrographs of filamentous actin bound to 16kDa tagged phalloidin (Steinmetz et al., 1998). In the latter study, the authors demonstrate that the phalloidin derivative used in a chemical cross-linking study (Vanderkarkhove et al., 1985) is positioned such that it could react with E117. However, E117 does not appear to comprise part of the phalloidin binding site of actin, and this is supported by the observation that a mutation of E117 to A does not inhibit phalloidin binding (Drubin et al., 1993). Therefore, the use of actin mutants to map the phalloidin site was more accurate than the use of chemical cross-linkers.

All of the toxin-binding sites cluster near the ATP binding pocket. Because actins are highly conserved along their entire sequence, it seems unlikely that these three natural products all bind near the same region because of high sequence conservation. It seems more likely that these compounds bind near the nucleotide binding cleft because this is a site where a small molecule can dramatically affect actin function. For example, since nucleotide hydrolysis and exchange are required for actin regulation, toxins that interfere with these reactions are expected to be cytotoxic compounds. An alternative explanation is that this region may be more accessible, since most of the rest of the surface of actin is covered by protein binding sites.

One additional aim of this study was to separate the loss of phalloidin binding from temperature sensitivity and defects in actin organization that were characteristic of the original R177A/D179A double mutant. By separating these two mutations, we demonstrated that both R177 and D179 contribute to phalloidin binding. Moreover, the D179A mutant was completely defective for rhodamine phalloidin binding, but grew nearly as well as wild-type strains under all conditions tested. In addition, its actin structures appear normal by anti-actin immunofluorescence. This mutant may therefore be a useful tool with which to distinguish different populations of actin filaments in the cell.

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