A Potential Yeast Actin Allosteric Conduit Dependent on Hydrophobic Core Residues Val-76 and Trp-79

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Intramolecular allosteric interactions responsible for actin conformational regulation are largely unknown. Previous work demonstrated that replacing yeast actin Val-76 with muscle actin Ile caused decreased nucleotide exchange. Residue 76 abuts Trp-79 in a six-residue linear array beginning with Lys-118 on the surface and ending with His-73 in the nucleotide cleft. To test if altering the degree of packing of these two residues would affect actin dynamics, we constructed V76I, W79F, and W79Y single mutants as well as the Ile-76/Phe-79 and Ile-76/Tyr-79 double mutants. Tyr or Phe should decrease crowding and increase protein flexibility. Subsequent introduction of Ile should restore packing and dampen changes. All mutants showed decreased growth in liquid medium. W79Y alone was severely osmosensitive and exhibited vacuole abnormalities. Both properties were rescued by Ile-76. Phe-79 or Tyr decreased the thermostability of actin and increased its nucleotide exchange rate. These effects, generally greater for Tyr than for Phe, were reversed by introduction of Ile-76. HD exchange showed that the mutations caused propagated conformational changes to all four subdomains. Based on results from phosphate release and light-scattering assays, single mutations affected polymerization in the order of Ile, Phe, and Tyr from least to most. Introduction of Ile-76 partially rescued the polymerization defects caused by either Tyr-79 or Phe-79. Thus, alterations in crowding of the 76–79 residue pair can strongly affect actin conformation and behavior, and these results support the theory that the amino acid array in which they are located may play a central role in actin regulation.

Actin is a major eukaryotic cytoskeletal protein with essential roles in both the cytosol and the nucleus (1, 2). It is a conformationally dynamic molecule (3) that cycles reversibly between monomeric G actin and polymeric F actin (4). The formation of F-actin, which is fundamental to most actin cytoplasmic roles, is carefully regulated both spatially and temporally by a large array of actin-binding proteins (5, 6). Therefore, understanding how this regulation derives from induced conformation changes in actin structure is central to understanding cytoskeletal function.

The actin monomer is a clam-shaped protein (7). Its smaller outer domain consists of subdomains 1 and 2, whereas the larger domain is composed of subdomains 3 and 4. A hinge region spans subdomains 1 and 3 at the bottom of the protein. Another important structural component that is pertinent to the regulation of F-actin formation as well as monomer and filament stability is an adenine nucleotide that resides deep within a cleft separating the two large domains of the protein. G-actin possesses a weak ATPase activity that is activated about 1000-fold during polymerization (8), and the nature of the bound nucleotide, the ATPase activity, and the rate of release of the resulting Pi after ATP hydrolysis are important determinants of actin filament dynamics (9). ATP-G-actin polymerizes more readily than its ADP counterpart, and both ATP- and ADP-Pi F-actin are more stable filaments than ADP-P-actin.

Both the rate of nucleotide exchange involving G-actin and the rate of Pi release from polymerizing F-actin can vary widely between different actins despite their high degree of homology. For example, yeast (Saccharomyces cerevisiae) and mammalian skeletal muscle actins are 87% identical in primary structure (10), and pure yeast actin polymerizes considerably more rapidly than does muscle actin (11, 12) despite the fact that they can readily co-polymerize (13). Yeast actin exchanges its bound nucleotide 10 times faster than does muscle actin (14, 15), and Pi release and ATP hydrolysis are essentially concomitant during yeast actin polymerization (16), whereas with muscle actin, there is a noticeable lag in Pi release (17). This difference between actins in the dynamics of the nucleotide cycle seems to correlate with differences in the roles of these actin isoforms. The yeast actin cytoskeleton must be very dynamic and flexible to meet the demands of a rapidly dividing cell (18, 19), whereas muscle actin is incorporated in relatively stable non-dynamic sarcomeric structures (20–23).

Previously, to gain insight into the mechanism underlying the difference in nucleotide exchange rates for the highly homologous yeast versus muscle actins, we constructed two yeast/muscle hybrid actins (13). In the first we introduced all of the muscle-specific residues into subdomain 1 of yeast actin (Sub1), and in the second we introduced the three additional residues found in subdomain 2 (Sub12). The Sub1 substitutions decreased the rate of nucleotide exchange for yeast actin to within a factor of two of that of muscle actin. The exchange rate for Sub12 actin was equal to that of muscle actin. Additional work demonstrated that of the three subdomain 2 residues, only the V76I, located in the interface between subdomains 1 and 2 (Fig. 1), was needed for the additional 2-fold retardation observed with Sub12 actin.

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Based on these results, we examined the actin crystal structure to try to determine how the V76I substitution might have exerted such a significant effect on adenine nucleotide exchange rates. For yeast actin, this residue is part of a six-residue linear structure, Lys-118, Trp-79, Val-76, Ile-75, Gly-74, and His-73 (Fig. 1). The sequence is the same for muscle actin except for the Ile-76 substitution. Lys-118 resides on the external surface of the protein at or near Arp2/3 complex (24, 25), cofilin (26, 27), and formin binding sites (28), and His-73 is found on the surface of the nucleotide cleft. This arrangement suggested the six-residue bloc might be a conduit through which the binding of external regulatory proteins could initiate propagated conformational changes through the protein to the nucleotide cleft, thereby altering cleft function and affecting nucleotide exchange. The core of this system appeared to be the closely packed hydrophobic residues at positions 76 and 79. Crowding between these residues could exert a force on His-73, resulting in cleft rearrangement. If this crowding hypothesis was correct, it would predict that substitution of Ile-76 found in muscle actin for the Val found in yeast actin would lead to more crowding against the indole ring of Trp-79, forcing movement of residue 76 toward His-73. Ultimately, His-73 would be forced into the cleft, potentially resulting in its closing around the nucleotide, leading to a more compact and less flexible protein. Such a situation might result in slower nucleotide exchange and changes in filament stability resulting from altered monomer-monomer contacts.

The focus of this paper was 2-fold. First, if our crowding hypothesis was correct, we wanted to determine whether the V76I effects would be seen with this substitution alone in the absence of the subdomain 1 substitutions. Second, we wanted to directly test elements of this crowding hypothesis. To do so, we utilized site-directed mutagenesis to alter crowding by changing residues at positions 76 and 79, both single and together. We then assessed the effects of these mutations in vivo, and after purification of the mutant actins, we assessed the effects of these changes in vitro on actin monomer properties and polymerization.

**EXPERIMENTAL PROCEDURES**

**Construction and Characterization of Yeast Haploid Strain Expressing Mutant Actin—**Yeast haploid strains expressing mutant actins as the only actin were generated as described in Cook et al. (29). Briefly, the yeast centromeric plasmid (pRS314) carrying the yeast actin-coding sequence adjacent to the yeast actin promoter was subjected to site-directed mutagenesis using the QuickChange kit from Stratagene (La Jolla, CA). The primers used for mutagenesis were purchased from Integrated DNA Technologies (Coralville, IA). The plasmid was transformed into haploid cells, in which the only functional WT actin gene is also carried on a centromeric plasmid (pCEN). Transformed cells were subjected to plasmid shuffling using selection for nutritional markers. Mutant plasmids were rescued from the mutant cell and were sequenced to verify the presence of the desired mutation.

To characterize actin-related growth defects, overnight cultures of cells were diluted to $A_{600} \sim 0.1$ in phosphate-buffered saline, and 2 μl of $1 \times, 10 \times, 100 \times, 1,000 \times$-diluted samples were spotted on YPD agar (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) plates followed by incubation at either 30 or 37 °C to test for temperature sensitivity. The same amount and dilutions of cells were spotted on YPG (same contents as YPD but with glycerol substituted for dextrose) to test for mitochondrial function and YPD + 0.9 M NaCl agar plates to test for hyperosmolar sensitivity. Colony size was recorded by
scanning plates after 48 h of growth on an Epson 2450 scanner. The ability to grow in liquid YPD medium was determined by subculturing overnight cultures of cells in YPD to a density with an $A_{600} \sim 0.1$. Cells were grown at 30 °C with agitation, and the extent of growth was followed by the increase in $A_{600}$ over time until stationary phase growth was reached.

**Cellular Morphology Observations**—All the images were collected with a Zeiss Axioskop 2 Plus microscope using a Plan-Apochromat 100×1.4 numerical aperture objective lens and a Spot RT cooled CCD camera (Diagnostic Instruments, Sterling Heights, MI) controlled by MetaMorph Version 4.5 software (Universal Image Corp., Downingtown, PA). Image enhancement and analysis were done using ImageJ (National Institutes of Health).

To monitor cell morphology at different cell growth stages, cells in early growth ($A_{600} \sim 0.4$) and late growth ($A_{600} \sim 6$) were collected, and images were taken by differential interference contrast microscopy. The length of the long axis of each cell was measured, and at least 100 cells of each strain were counted. To prepare cells for visualization of the actin cytoskeleton and assessment of vacuole morphology, cells cultured overnight were diluted to an $A_{600} \sim 0.1$ and regrown at 30 °C with agitation until the cell $A_{600}$ increased to about 0.4 ~ 0.8. For actin cytoskeleton observation, cells were fixed with 3% formaldehyde and stained with Texas Red-phalloidin as described previously (29). G-actin concentration was determined by UV absorbance at 290 nm using the extinction coefficient $\varepsilon = 25.6 \text{ cm}^{-1} \text{mM}^{-1}$, and the actin was stored in G buffer (10 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.2 mM CaCl$_2$, and 1 mM dithiothreitol). The change in light-scattering intensity was obtained by fitting the fluorescence decrease as described in Wen and Rubenstein (32). A standard curve of actin polymerization, the net change in light scattering for different actin concentrations was measured, and the data were plotted as a function of actin concentration. The critical concentration was obtained by drawing a line through the points and determining its intersection on the x axis. Polymerization data were converted to actin concentration based on the critical concentration determination (32). The phosphate released caused by the hydrolysis of actin-bound ATP after the activation of polymerization dependent actin-ATPase activity was monitored by the EmissionCheck assay kit (Invitrogen) described in Wen and Rubenstein (32). A standard curve allowed conversion of the absorbance change to $P_i$ concentration.

Actin filament morphology and filament length was determined as follows. A 2.5 μM solution of polymerized actin was deposited on a carbon-coated Formvar-layered grid and negatively stained with 1.5% uranyl acetate. Electron micrographs were obtained as described (13). The contour lengths of at least 100 actin filaments from each actin sample were measured using ImageJ, and filament length distribution analyses were carried out using Excel.

**Hydrogen-Deuterium Exchange and Mass Spectrometry**—HD exchange analyzed by mass spectrometry was performed as described previously (35). Briefly, 2 mg/ml G-actin was diluted ~160-fold in deuterated G buffer at room temperature to start the exchange reaction. An aliquot (~300 pmol of protein) was taken at specific time points, mixed with 100 mM phosphate buffer, pH 2.5, to quench the reaction, and stored at ~80 °C for less than 2 days. The labeled proteins were digested on a pepsi column made in-house (36). The digested peptide products were desalted on a peptide macrotrap (Michrom Bioresources, Inc.) and then were separated on a Jupiter Proteo C-12 column. The mass of individual peptides was determined on an LCQ Deca (Thermo Scientific, Waltham, MA) in the University of Iowa High Resolution Mass Spectrometry Facility. Mass spectra were analyzed with Bioworks Browser and Xcalibur Qual Browser Thermo Scientific, Waltham, MA) to identify the peptides. The centroid mass of the peaks was determined with MagTran (36) and plotted as a function of time (supplemental Fig. S1). Data were not adjusted for back exchange, and therefore, all changes in masses were reported as uncorrected changes.

**RESULTS**

We had hypothesized that the extent of interaction between residues 76 and 79, included in a string of six amino acids begin-
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In Vivo Effects—Functional actin is essential for yeast viability. Because viable cell lines were produced with each of the mutant actins described above, the mutations did not result in actin denaturation. We first grew the mutant strains on agar plates on complete YPD medium at elevated temperatures to determine whether the mutations caused temperature-sensitive defects in actin function. We tested for the ability to grow on glycerol as a sole carbon source. This requires functional mitochondria in yeast, which in turn depends on a normal actin cytoskeleton for maintenance of mitochondrial integrity and mitochondrial inheritance. Finally, we assessed growth on a hyperosmolar medium containing 0.9 M NaCl. Such conditions lead to cytoskeletal re-organization, which has been shown to be incompatible with certain actin mutations. With the exception of the VY cells, all strains grew the same as WT in all of the conditions tested. The VY showed a lethal phenotype on hyperosmolar medium and grew slower than the other strains in all of the other stress-inducing media tested.

Because differences in growth rates are often difficult to detect on agar plates, we examined growth curves for these strains propagated with agitation in liquid YPD medium at 30 °C. Fig. 2 shows that, compared with WT cells, all of the mutant strains exhibited retarded growth. Of the mutants, VY cells were not divided, and when the A_{600} was >6 with the cells at stationary phase. Actin malfunction often results in ineffective cell division and larger cells. As shown in Table 1, during the logarithmic phase of growth, only the VY cells were ineffective cell division and larger cells. As shown in Table 1, the cells at stationary phase. Actin malfunction often results in crowding increases over that present in WT yeast actin, nucleotide exchange should slow down and, as crowding decreases, although the difference was largest for the VY cells that were also rounder in appearance, suggesting a loss of cell polarity.

Cytology—The abnormal cell morphology observed at 48 h suggested that actin cytoskeletal patterns, composed of membrane patches and cytoplasmic cables, might be disrupted. However, staining of fixed cells with Texas Red-phalloidin showed this to be apparent only in the VY cells with a general loss of cables (Fig. 3). Mitochondrial morphology, as detected by distribution of green fluorescent protein conjugated to the mitochondrial targeting sequence of citrate synthase (37), was normal for all mutant strains. However, the VY cells alone exhibited abnormal vacuole morphology. Instead of the two-four vacuolar lobes normally seen, these cells, when exposed to the dye FM4-64, were characterized by a single large vacuole that filled almost the entire cell (Fig. 3). Occasionally, smaller lobes were also detected in the same cell with the large vacuole. These results demonstrated that the effects of the mutations on cell growth did not stem from a wholesale disruption of cytoskeletal function but reflected allele-specific effects on different cytoskeletal functions.

G-actin eATP Exchange and Thermostability—Our crowding hypothesis involving residues 76 and 79 predicts that as crowding increases over that present in WT yeast actin, nucleotide exchange should slow down and, as crowding decreases,
exchange should accelerate. To test this hypothesis we measured the rate of exchange of fluorescent eATP from actin as a function of time after the addition of excess ATP as described under “Experimental Procedures.” The exchange curves are shown in Fig. 4 and the \( t_{1/2} \) values calculated from these curves are displayed in Table 2. As predicted, increasing the mass at position 79 from Val to Ile (IW actin) resulted in a doubling of the exchange time. This is the same result caused by this mutation when introduced into Sub1 yeast-hybrid actin, showing that this retardation of exchange rate does not necessitate a completely muscle-like outer domain. Conversely, if we decrease the total mass in these two residues compared with WT actin (VF and VY actins), the exchange rate increases substantially over that of WT actin, also as predicted. Surprisingly, the substitution of Tyr for Trp exerted a much larger effect than did Phe, suggesting that the presence of the phenolic hydroxyl led to a much greater degree of structural perturbation that the decreased mass of the phenyl ring alone. Finally, as predicted, substitution of a smaller residue at position 79 to compensate for the V76I substitution restored exchange rates back toward that of WT actin, and the result was identical regardless of whether Phe or Tyr was used.

The differences in nucleotide exchange rates suggested that the mutations might also affect the thermostability flexibility of the protein. As a first test of this theory, we assessed the effects of increasing temperature on the circular dichroism of the protein and fit the results to a two-state model. The apparent melting temperature \( (T_m) \) was defined as the temperature at which half the total change in CD occurred (see “Experimental Procedures”). Table 2 shows that the V76I substitution had little if any impact on actin thermostability. Conversely, substituting the smaller Phe or Tyr at position 79 for Trp caused a 4 and 6 degree lowering of \( T_m \), respectively, implying increased thermostability with decreasing mass. As with nucleotide exchange rates, the phenolic hydroxyl increased the magnitude of this effect. The results further show that combining the V76I substitution with the introduction of the smaller residue at position 79 brought the \( T_m \) back toward that of WT actin, although the ability of the larger Ile to reverse the instability was much greater for the IF than the IY actin, again commensurate with the additional disruptive influence of the phenolic hydroxyl of tyrosine.

**Effect of the Mutations on Chymotrypsin Susceptibility**—The nucleotide exchange results coupled with the effects of the mutations on thermostability of the protein suggested mutation-dependent restructuring of the nucleotide cleft. A result of such restructuring could be a reorientation of subdomain 2 at the top of the cleft. Because this region contains cleavage sites for chymotrypsin, trypsin, and subtilisin, such restructuring might be detected by a change in protease susceptibility. We, thus, examined the differential susceptibility of the WT and mutant actins to partial digestion by \( \alpha \)-chymotrypsin. \( \alpha \)-Chymotrypsin cleaves actin between residues 44 and 45 and between 67 and 68 to yield peptides of 35 and 32 kDa. Fig. 5A shows that of all of the actins examined, only WT, IW, and IF actins generated a noticeable band at 35 kDa. To determine if there were quantitative differences among these species, we followed their digestion over time. Quantitation of densitometric scans from mul-
tiple runs (Fig. 5B) shows almost identical kinetics for the appearance of the same peptide fragment for each actin. The middle panel shows that the 35-kDa species accumulates to a greater extent for the IW and IF actins than it does for VW actin, suggesting that the second protease cleavage site in these two mutants is more sequestered. Interestingly, the presence of a smaller residue at 79 alone causes an alteration of the protease cleavage sites, and the presence of Ile-76 is capable of restoring normal susceptibility only if Phe is at position 79. No 35-kDa band is repeatedly seen in either of the constructs where Tyr-79 is present.

Conformational Changes Detected by HD Exchange—The changes in protease susceptibility suggested that the presence of substitute residues at positions 76 and 79 could significantly alter the conformation of actin in at least part of its structure involving the subdomain 2 DNase Ile loop area. To gain insight into just how extensive this conformational change was, we assessed the effects of the different mutations on HD exchange from the amide protons of G-actin as detected by mass spectrometry. The methodology is described under “Experimental Procedures.” The plots for individual peptides showing exchange over time are shown in supplemental Fig. S1, and the results have been collected and summarized via color coding in Fig. 6 and supplemental Fig. S1 and Table S1. We could not calculate rates of exchange because the data were plotted versus log of the time. However, for most of the individual sets of peptides with distinguishable differences, the peptide exchange curves seemed to show differences in total protons exchanged rather than differences in the slopes of the lines over time. Because of scatter in these plots and small apparent differences in slopes if any, we did not actually calculate slope values. We did, however, calculate the percent of actual change compared with total possible amide proton exchange for each peptide at the 10-min time point (supplemental Table S1).

First, for all but one of the peptides depicted, the extent of exchange was greater for WT than for any of the mutants, suggesting that the structure had undergone some type of compaction resulting in decreased solvent exposure. We could not make this comparison for peptides 67–79 or 67–82 because the peptide was not observed in the samples of WT actin peptides. Second, all of the substitutions studied resulted in propagated changes in the core domains of subdomains 1, 3, and 4 as well as the hinge region separating the two halves of the protein. Third, for peptides of comparable length, the amount of exchange can substantially differ (see supplemental Fig. S1). For example, peptide 67–79 exchanges much more completely than does peptide 208–218. Fourth, peptide 67–79, encompassing the mutation sites, is the only peptide that displays a noticeable
change in slope as well as extent among the mutants examined. In this case the slope of the lines representing peptides containing Tyr is greater than those containing Phe at position 79, consistent with our other findings that the Tyr substitution caused greater disruption in actin function than did the Phe.

**Effect of Mutations on Actin Polymerization**—The effects of the mutations on propagated conformational change throughout the protein suggested that polymerization might also be affected in terms of altered monomer-monomer contacts and the conformational changes required to drive actin from its G-monomer to its polymerization-competent F-monomer form. We, thus, assessed the polymerization behavior of the mutant actins compared with their WT counterpart. First of all, Table 2 shows that the mutations, with the exception of V76I, all cause a rise in critical concentration for filament formation. The effects are largest with the substitution of Tyr or Phe for Trp-79; as with the other parameters we have assessed, the presence of the phenolic hydroxyl on Tyr adds significant disruption. In both cases, however, the introduction of Ile at residue 76 (IF and IY versus VF and VY actins) works to restore the critical concentration closer to that of WT actin, apparently correlating with our “crowding hypothesis.”

This apparent rescue, however, is far from complete based on the kinetics of polymerization as shown in Fig. 7. All of the single mutants, compared with WT actin, display an elongated nucleation phase and a faster elongation phase. Both results can arise from altered monomer-monomer contacts due to the mutations. The elongated nucleation would arise from decreased ability to form a nucleus with 3–4 monomers, whereas the more rapid elongation phase would result from increased filament fragmentation leading to the creation of more filament barbed ends for monomer addition. In terms of rescue ability, introduction of the increased mass of Ile at residue 76 to compensate for the decreased mass of Phe at 79 has little effect on polymerization kinetics. However, a significant rescue is observed when Ile is introduced to balance the Tyr at 79.

If the more rapid elongation phases were, in fact, caused by filament fragmentation, an additional hallmark would be decreased filament length. We, therefore, examined samples of the polymerized actins by electron microscopy after negative staining as described under “Experimental Procedures” and measured the lengths of >100 filaments in each sample. No noticeable difference in filament appearance was observed (data not shown). However, Fig. 8 and Table 2 show definite effects on average filament length and the length distributions compared with WT actin. The V76I substitution, with its increased mass, produced a slight redistribution toward a shorter filament, although the distribution was still dispersed as with WT actin. Phe and Tyr individually at residue 79 produced a significant redistribution to shorter lengths. However, contrary to the other parameters we have assessed, W79F produced greater shortening than W79Y. The additional introduction of Ile produced a noticeable rescue of the effect of Tyr toward a more normal distribution, but it had a much more moderate effect on countering the influence of Phe at residue 79. Interestingly, a comparison of the parameters in Table shows no consistent correlation between effects of the mutations on monomer properties versus their effects on the polymer.

The release of P_i from polymerizing actin due to hydrolysis of bound ATP, is usually biphasic. A first relatively rapid phase corresponds to net polymerization, whereas a slower second linear phase corresponds to treadmilling of actin monomers through the filament, although total filament formation is at steady state. For yeast actin, the P_i release occurs concomitantly with polymerization in the first phase (16).
The alterations in filament length and critical concentration produced by the mutants studied suggested that these mutations might also affect filament dynamics as measured by Pi release kinetics (“Experimental Procedures”) as depicted in Fig. 9. For V76I alone, polymerization and Pi release were tightly coupled, as evidenced by the simultaneous lag in both measurements. However, the rate of Pi release after polymerization steady state was significantly faster than that of WT actin, suggesting that more rapid treadmilling occurs. For the Phe and Tyr single mutations, especially in the case of Tyr, Pi release and polymerization appeared strongly uncoupled before the attainment of polymerization steady state with subsequent continued linear Pi release after cessation of net polymerization. This behavior could be explained either by abortive filament formation leading to continuous turnover or by salt activation of the ATPase activity in non-polymerizing monomers. The combination of Ile with Tyr results in a restoration of coupling of Pi release with polymerization and a decrease in the rate of post-polymerization treadmilling. The rescue afforded by combing Ile with Phe was much less apparent as evidenced by a retardation of the onset of Pi release and a partial decrease in Pi release and polymerization uncoupling with virtually no effect on the rate of Pi release during treadmilling. The relative behavior of these two double mutants with respect to Pi release correlates with their behavior in terms of filament lengths discussed above.

DISCUSSION

Our previous studies (13) with our yeast-muscle hybrid actins demonstrated that substitution of the yeast actin Val-76 with an Ile found in muscle actin, in the context of a muscle-like subdomain 1 of actin, played a large role in retarding the yeast actin nucleotide exchange rate to that of muscle actin. A re-examination of the G-actin crystal structure led to the idea that residue 76 was part of a linear array of residues extending from the actin outer surface to His-73 within the nucleotide cleft in the interior of the protein that could act as a conduit for intra-monomer allosteric change. We termed this idea our “steric crowding” hypothesis because residue 76 abuts Trp-79 on one side of this array and Ile-75 on the other, creating a hydrophobic core that could transmit conformational perturbations. Exertion of force on the outside of this core would then result in forcing His-73 further into the cleft, resulting in either a cleft closure or structural alteration. Such a sequence of events might easily explain an alteration of nucleotide exchange rates and possibly affect polymerization behavior as well. The efficiency of the system would depend on the extent of contact between these three core residues. The more they were crowded together, the less compliant would be the core and the more efficient the transfer of applied force.

In this study we tested this steric crowding hypothesis by using site-directed mutagenesis to alter the extent of crowding in this central core in yeast actin and to assess the effects of these mutations in vivo and in vitro. In accordance with our hypothesis, each of the single mutants produced deleterious effects in vivo that could be substantially rescued by compensating second mutations. The W79Y mutation, apart from the others, produced a noted vacuolar defect. Normal vacuolar morphology and inheritance requires a properly functioning actin cytoskeleton, and there is evidence that normal vacuolar function is necessary for proper osmoregulation (38). The res-
cure of the ability of the cell to grow in hyperosmolar medium after introduction of the second V76I mutation was accompanied by a restoration of normal vacuole morphology, consistent with this idea.

Our work with these mutant actins as purified proteins in vitro further supported elements of our crowding hypothesis involving residues 76 and 79. First, simply increasing the mass at residue 76 from a Val to Ile resulted in a 50% reduction in nucleotide exchange rate, consistent with closing or altering the nucleotide cleft. This mutation, however, produced no effect on thermostability. Thermal stability measures the global response of the protein to increased heat. It appears that the increased mass of the Ile at residue 76 does not cause enough of a disturbance in the secondary and tertiary structure interactions within the protein to measurably alter thermostability. However, changes in nucleotide exchange rate apparently are much more sensitive to small changes in structure affecting the interior of the nucleotide binding cleft.

Decreasing contact between these residues by replacing the Trp-79 indole ring with either the smaller Phe or Tyr, as predicted, appeared to lead to a more flexible or functionally disrupted actin monomer as evidence by decreased thermostability and increased nucleotide exchange rates. In contrast to the case with the V76I mutation, decreasing the mass at residue 79 must introduce enough play into the protein structure to result in subsequent destabilization of secondary structure elements, leading to the decreased thermostability. Again, consistent with our hypothesis, increasing the size of the residue at position 76 with Ile seemed to compensate for the decreased mass associate with Phe or Tyr at residue 79. Evidence supporting this assertion was a subsequent retardation of nucleotide exchange rates to values that were slower than that of WT but still faster than that of V76I alone. Full or partial rescue of thermostability was also observed depending on the mutation.

In all cases substitution of Tyr for Trp-79 produced more drastic effects than substitution of Phe. The only difference is the Tyr phenolic hydroxyl group. Molecular modeling shows that there is a cavity into which this group can easily fit, so increased crowding is likely not the explanation for this additional disruptive effect. One possible explanation for this enhanced effect is the establishment of a hydrophilic group in the vicinity of a hydrophobic environment represented by the core of our relay system. A second possible explanation is that the phenolic hydroxyl forms a hydrogen bond with the amide core of our relay system. A second possible explanation is that the phenolic hydroxyl forms a hydrogen bond with the amide side chain of Asn-115 (Fig. 10). These groups are well within the distance required for their strong association, and such a bond could stabilize the actin monomer into an abnormal conformation made more accessible by the decreased contact between residues 76 and 79. Interestingly, a mutation in Asn-115 in smooth muscle α-isoactin leads to dissecting thoracic aortic aneurysms, demonstrating the importance of the conformation of this peptide segment in proper actin function (39). The rescue observed in the IY mutant suggests an equilibrium between two relatively stable states that is pulled back toward the more normal one by the increased mass of Ile-76.

To better understand the consequences of the 76/79 mutations, we assessed their effects both locally and distally on actin conformation. The mutations are at the interface of subdomain 1 and 2. The alterations in protease susceptibility indicated that there were propagated changes to the top of subdomain 2, where the chymotrypsin cleavage sites are. Our HD exchange experiments revealed, surprisingly, that these mutations also caused propagated changes across the hinge region and/or the nucleotide bridge to subdomains 3 and 4 as well in a way that resulted in decreased exchange implying a compaction of the protein. In one sense these results are similar to ones we obtained studying the binding of profilin to yeast actin in that here, too, HD exchange for a number of peptides decreased. However, the propagated effects of profilin binding were much less extensive than we observed with the mutants in this work, especially in the interior of the cleft region, underlining the importance of this core of residues in the overall structural integrity of actin.

This scenario and the location of the structural alterations we observed suggests that monomer-monomer interfaces in F-actin might also be affected by these mutations resulting in alterations in polymerization kinetics. Again, these predictions are consistent with our results. The Tyr and Phe mutations led to significant filament destabilization based on higher critical concentrations, decreased average filament length, an elongated nucleation phase, and a more rapid rate of polymerization due presumably to filament fragmentation. Mutation–dependence filament destabilization was further substantiated by our P_i release results that showed an uncoupling of salt-induced ATP hydrolysis/P_i release from productive polymerization and more rapid post-polymerization nucleotide cycling once polymerization reached a steady state. As before, the effect of Tyr-79 on polymerization was more drastic than the effect of Phe-79. The compensatory V76I mutation rescued P_i release behavior of the Tyr mutant to a greater extent than it did the Phe mutant, and similar results were observed in terms of filament length and polymerization kinetics. Increasing crowding with the V76I mutation alone also affected polymerization kinetics. Filament length was shortened, nucleation was inhibi-

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...teins may exert their effects on actin filament dynamics. In summary, we believe we have identified an important stretch of amino acids that can act as a conduit for propagated conformational change from the exterior of actin to its nucleotide binding core and that this conduit may be coupled with an area between subdomains 1 and 3 that has been identified as a hot spot for the binding of regulatory proteins that affects actin filament dynamics. The governing force of this conduit is the degree of steric contact especially involving hydrophobic residues at positions 76 and 79, as alterations in the extent of contact either in the positive or negative direction can alter actin behavior as a result of propagated conformational change throughout the protein.

Our in vitro results to a large extent mirror our observations in vivo. Alteration of the steric packing of this system in either direction alters cytoskeletal function leading to altered cellular behavior. The situation is the worst for the W76Y mutation both in vivo and in vitro. In this case not only is the packing of the local system altered, but the additional effect of the tyrosine hydroxyl on the Asn-115-containing helix leads to a more severe growth phenotype that reflects the more drastic disturbance of protein function.

This system, then, is an example where maximized efficiency is achieved at a level of contact that does not produce excessive flexibility in one direction or spatial distortion due to overcrowding in the other. Finally, because the external end of this conduit is near the binding sites of proteins that regulate actin polymerization, our work provides insight into how these proteins may exert their effects on actin filament dynamics.

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