Thoracic Aortic Aneurysm (TAAD)-causing Mutation in Actin Affects Formin Regulation of Polymerization*

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Received for publication, April 17, 2012, and in revised form, June 7, 2012. Published, JBC Papers in Press, June 29, 2012, DOI 10.1074/jbc.M112.371914

Background: The biochemical mechanisms underlying α-smooth muscle actin-mediated vascular disease are unknown. More than 30 mutations in ACTA2, which encodes α-smooth muscle actin, have been identified to cause autosomal dominant thoracic aortic aneurysm and dissection. The mutation R256H is of particular interest because it also causes patent ductus arteriosus and moyamoya disease. R256H is one of the more prevalent mutations and, based on its molecular location near the strand-strand interface in the actin filament, may affect F-actin stability. To understand the molecular ramifications of the R256H mutation, we generated Saccharomyces cerevisiae yeast cells expressing only R256H yeast actin as a model system. These cells displayed abnormal cytoskeletal morphology and increased sensitivity to latrunculin A. After cable disassembly induced by transient exposure to latrunculin A, mutant cells were delayed in reestablishing the actin cytoskeleton. In vitro, mutant actin exhibited a higher than normal critical concentration and a delayed nucleation. Consequently, we investigated regulation of mutant actin by formin, a potent facilitator of nucleation and a protein needed for normal vascular smooth muscle cell development. Mutant actin polymerization was inhibited by the FH1-FH2 fragment of the yeast formin, Bni1. This fragment strongly capped the filament rather than facilitating polymerization. Interestingly, phalloidin or the presence of wild type actin interfered with actin dynamics may have an adverse effect on normal smooth muscle cell function. Little is known, however, about the biochemical mechanisms by which TAAD mutations in actin contribute to the cell loss or hyperplasia seen in diseased arteries.

Vascular smooth muscle cells switch phenotype during development in response to injury and during atherogenesis (9, 11, 12). α-Smooth muscle actin is expressed early in vascular smooth muscle cell differentiation as well as in the quiescent state. Proliferation and migration of vascular smooth muscle cells depend on tight cytoskeletal regulation. Mutations that interfere with actin dynamics may have an adverse effect on normal smooth muscle cell function. Little is known, however, about the biochemical mechanisms by which TAAD mutations in actin contribute to the cell loss or hyperplasia seen in diseased arteries.

Our recent studies have focused on the effects of the R258H mutation on actin dynamics. R258H is one of the most common TAAD actin mutations and is of particular interest because of the range and severity of associated clinical disorders. In addition to TAAD, this mutation causes the congenital cardiovascular defect, patent ductus arteriosus, as well as moyamoya disease, a distinctive pattern of occlusive stroke (2). The clinical findings support the idea that the mutation affects both developmental arterial function and ongoing maintenance. To better understand the biochemical effects of the mutation R258H, we utilized the budding yeast model. Yeast actin is 86% identical and 94% similar to α-smooth muscle actin, and the residues altered by human ACTA2 mutations are conserved between human and yeast actin.
isoforms. The single actin isoform in yeast and ability to correlate in vivo findings with in vitro analyses have allowed us to successfully use this system to understand the biochemical mechanisms underlying not only TAAD (13) but other actin-mediated diseases (14–16).

The residue Arg-258 in /H9251-smooth muscle actin corresponds to Arg-256 in yeast actin. (The yeast numbering system will be used for descriptions from here on.) Recent analyses of filamentous actin structure indicate that Arg-256, located in actin subdomain 4, is involved in cross-strand interactions within the filament (Fig. 1). As such, residue Arg-256 may play an important role in filament stabilization (17). Furthermore, structural abnormalities in the filament induced by the mutation potentially could interfere with actin interaction with the proteins that regulate filament dynamics. In this work, we present our investigations of the effects of the R256H mutation on cytoskeletal function and actin polymerization properties. In addition, we describe analyses of formin regulation of mutant actin filament formation.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNase I (grade D) was purchased from Worthington. DE52 DEAE-cellulose was obtained from Whatman. Micro Bio-Spin P-30 Tris columns and Affi-Gel 10-activated resin were purchased from Bio-Rad. ATP was purchased from Sigma. Rhodamine-phalloidin, FM 4-64, and DAPI were purchased from Molecular Probes. The QuikChange[reg] site-directed mutagenesis kit was from Stratagene, and oligodeoxynucleotides were purchased from Integrated DNA Technologies. Yeast cakes for wild type actin preparations were purchased from a local bakery. All other chemicals were reagent-grade quality.

**Construction of Mutant Yeast Strains**—Mutations were introduced into the centromeric plasmid pRS314 (15) containing the yeast actin coding sequence driven by the ACT1 promoter using the QuikChange[reg] mutagenesis kit according to the manufacturer’s instructions. Plasmid containing the desired mutation was introduced into a recipient yeast strain containing a deleted chromosomal ACT1 gene and a plasmid expressing wild type actin (pCENWT) as described previously (18). Plasmid shuffling yielded a viable haploid strain with mutant actin as the sole actin in the cell. The plasmids containing the mutant constructs were re-isolated from these strains and sequenced to confirm the presence of the desired mutation.

**Growth Behavior in Liquid Culture**—Cells were grown in complete liquid YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) with 0.01% adenine (YPAD) at 30 °C on a shaking platform. Growth was determined by diluting an overnight culture of each strain into fresh medium at an initial A_{600} of 0.1 and following growth at 30 °C with agitation. The absorbances of the cultures were recorded as a function of time. The absorbances were back-calculated following the appropriate dilutions to lower the cell density to the linear range of the spectrometer.

**Growth under Stress Conditions**—Temperature sensitivity of mutant actin was examined by plating four serial 10-fold dilutions of the cultures on YPD plates followed by incubation at 24, 30, or 37 °C. Colony size was assessed as a function of time. To assess mitochondrial function, cells were grown on medium containing glycerol as the sole carbon source. Cultures were plated on YPG medium (YPD medium with the dextrose replaced with 2% glycerol) and incubated at 30 °C. To test for...
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hyperosmolar sensitivity, cells were plated on YPD plus 0.5 M and 0.9 M NaCl agar plates and incubated at 30 °C.

Cytology—Cell structures were imaged with an Olympus IX81 microscope and a Hamamatsu camera (Model C10600-10B-H). Camera control and image enhancement were performed using Slidebook5 (3i Inc., Denver, Co.). Vacuoles were imaged following exposure of the cells to the dye FM4-64 as described previously (19). Nuclear and mitochondrial DNA was stained with DAPI as described previously (20). Mitochondria were visualized in living cells by expressing a fusion protein of green fluorescent protein (GFP) conjugated to the mitochondrial signal sequence of citrate synthase kindly provided by Dr. Liza A. Pon (21). Cells expressing the plasmid were grown to an A600 of 0.3–0.5 in YPAD. An aliquot of cells was resuspended and imaged as described below. The actin cytoskeleton was visualized by fluorescence microscopy after staining fixed cells with rhodamine-phalloidin as described previously (20). Cytoskeletal morphology was assessed in budding cells when the daughter was less than half of the size of the mother cell.

To measure cell size, mounted samples were visualized by differential interference contrast microscopy. The long axis of the cell was measured using ImageJ software. For assaying the morphologies of the actin cytoskeleton and mitochondria, images were taken at 0.2-μm intervals in a Z-series through the cell. Out-of-focus light was removed from each section by deconvolution using Slidebook5. The Z-series of deconvolved images were stacked into a two-dimensional image with ImageJ software. Presentation of cell images was done using Adobe Photoshop. All cellular statistical analyses were based on cell counts of >100 for each sample.

Latrunculin A Sensitivity—Sterile filtered discs (0.5 cm in diameter) were presoaked in 2 μl of dimethyl sulfoxide (control), 0.1, 0.5, or 1 mM of latrunculin A (22). Soaked discs were placed on YPD plates containing 100 μl of evenly spread wild type or mutant cells (A600 = 0.1). The plates were incubated at 30 °C for 48 h, and the area of growth inhibition surrounding the drug eluting disc was measured via ImageJ software.

Cells were grown in liquid YPD medium to an A600 of 0.3–0.5 and treated with 5 μM latrunculin A for 1 min. Cells were washed three times with fresh YPD medium (YPD with 0.01% adenine) and incubated in 5 ml of medium at room temperature with intermittent shaking. Cells were removed in 250-μl aliquots at designated time points, fixed with formaldehyde, stained with rhodamine-phalloidin, and imaged using an Olympus fluorescence microscope as described previously (20). More than 50 cells were analyzed for each sample, and the percentage of cells with actin cables was quantified.

Protein Purification—Wild type and mutant yeast actin were purified from lysates of frozen yeast cells using a combination of DNase I-agarose affinity chromatography, DEAE-cellulose chromatography, and polymerization/depolymerization cycling as described previously (23). The quality of actin preparations was assessed using SDS-PAGE and Coomassie Blue staining of the gels. The concentration of G-actin was determined from the absorbance at 290 nm using an extinction coefficient of 63 M⁻¹ cm⁻¹. Actin was stored as G-actin in G buffer (10 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.2 mM CaCl₂, and 0.1 mM dithiothreitol). Purified actin was stored at 4 °C and used within 3 days of purification.

The His-tagged Bni1 yeast formin FH1-FH2 C-terminal fragment was expressed in and purified from Saccharomyces cerevisiae strain (BDY502) as described (24, 25). Purified foramin fragments were stored in stock buffer (10 mM Tris, pH 7.5, 150 mM KCl, 0.2 mM MgCl₂, 1 mM dithiothreitol, and 10% glycerol) at a concentration of 5–10 μM at −80 °C. Protein concentration was determined from absorbance at 280 nm with an extinction coefficient of 14650 M⁻¹ cm⁻¹.

Actin Polymerization—Polymerization of G-actin in a total volume of 120 μl was induced by the addition of MgCl₂ and KCl to final concentrations of 2 and 50 mM, respectively. Polymerization was monitored at 25 °C by following the increase in light scattering of the sample using a FluoroMax-3 fluorescence spectrometer outfitted with a computer-controlled thermostatted four position multisample changer (HORIBA Jobin Yvon, Inc.). The excitation and emission wavelengths were both set to 360 nm with the slit widths set at 1 nm.

Actin polymerization experiments involving Bni1 used pyrene-labeled actin and followed the same methods as above with exceptions listed below. N-(1-pyrenyl) maleimide (Sigma) was covalently attached to actin at Cys-374 (pyrene-actin) as described previously (26). Wild type and mutant samples each contained 10% pyrene-labeled actin and were preincubated at room temperature for 15 min prior to induction of polymerization. For admixture experiments, wild type and mutant actin were individually labeled and then combined. The excitation and emission wavelengths were set at 365 and 385 nm, respectively. For most experiments, the designated concentration of the Bni1 fragment was added to the reaction mixture prior to induction of polymerization. Where noted, the Bni1 fragment was added to the reaction mixture midway into the elongation phase of polymerization. For polymerization experiments using phalloidin, unlabeled phalloidin (Sigma p2141) was added to the reaction mixture prior to induction of polymerization, as described previously (27). All polymerization experiments were performed at least three times with at least three different actin preparations.

Critical Concentration Determination—The critical concentration (Cₐ) of each actin was determined from the net change in light scattering of an actin polymerization reaction and 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 in the x axis.

Electron Microscopy—To visualize actin filament morphology, samples of 1.5 μM wild type actin and 2.25 μM mutant actin were deposited onto carbon-coated Formvar grids, negatively stained with 1% uranyl acetate, and imaged with a JOEL 1230 transmission electron microscope (University of Iowa Central Electron Microscopy Facility) equipped with a Gatan UltraScan 1000 CCD 2000/2000-pixel camera. Accelerating voltage of the transmission electron microscope was 100 kV. ImageJ software was used to process the images and measure the individual filament lengths for R256H and wild type actin.
Statistical Analysis—All data are presented as means ± S.D. Results for wild type and mutant actin were compared by using a paired t test with a p value < 0.05 considered significant.

RESULTS

Effect of Mutations on Cytoskeletal Morphology and Cell Growth—Our studies began with analyses of the actin cytoskeleton. The two major components of the cytoskeleton in yeast are actin cables and patches. Cables are organized along the mother-bud axis during polarized cell growth and facilitate movement of organelles to the daughter (28). Endocytic patches are distributed in a cell cycle-dependent manner and localize in the bud early in the cell cycle (29–31). Cytoskeletal morphology of mutant and wild type cells were determined by analysis of 500 cells stained with fluorescent phalloidin by three masked reviewers. In cells expressing R256H actin, patch distribution and staining intensity was normal but cables were less pronounced and difficult to visualize (Fig. 2). Stepwise analyses of Z-series images with contrast enhancement allowed identification of polarized cables in mutant cells, but overall, the cables were faint. These cytoskeletal findings were far more common in R256H mutant cells compared with wild type cells (41% versus 6% respectively, p < 0.01).

Disruption of the cytoskeleton may lead to loss of polarization during budding or growth deficits. Despite the high rate of cytoskeletal abnormalities, we found no differences between wild type and R256H mutant actin on cell size (4.6 ± 0.7 μm versus 4.7 ± 0.7 μm, respectively), doubling time (~ 2.1 h) or extent of growth in liquid medium. To further evaluate cytoskeletal effects, we monitored growth under stress conditions that require rapid physiologic reorganization of the actin cytoskeleton (32, 33). Growth was monitored in hypothermic (24 °C), hyperthermic (37 °C), and hyperosmolar conditions (medium containing 0.9 M NaCl), and no differences were identified between wild type and R256H strains (data not shown).

Thus, despite the morphologic abnormalities, the cytoskeleton conserves sufficient functionality to maintain growth and accommodate environmental stress.

Despite maintenance of cell size and growth, we questioned whether actin function was sufficient to preserve morphology and distribution of organelles. Vacuoles, mitochondria, and nuclei were analyzed to assess morphology and localization in the cell. Vacuoles typically consist of one to four small, evenly sized lobes. Abnormal morphology was infrequently identified in wild type cells (7%). In comparison, nearly half of the R256H cells had abnormal morphology (49%, p < 0.02; Fig. 2). The dominant phenotype was that of a single lobe greater than two-thirds the size of the cell or hypovesiculation, although 12% of abnormal cells were hypervesiculated with more than four lobes of varied size.

Disorganization of the polarized actin cytoskeleton can also lead to abnormal fission/fusion events that affect mitochondrial integrity and function (34). We evaluated the effect of the mutation on both mitochondrial and genomic DNA distribution by staining with DAPI. We found no differences in DNA distribution between wild type and R256H cells (data not shown). Despite the normal mitochondrial DNA distribution, mitochondrial morphology was affected by the actin mutation. Normally, mitochondrial are long, tubular structures aligned along the mother-bud axis, as was identified in 95% of wild type cells. Nearly a third of mutant cells, however, had fragmented and aggregated mitochondrial tubules. Overall, the incidence of abnormal mitochondrial morphology was 5% in wild type cells.
and 34% in R256H cells, p value < 0.01. Regardless, mutant cells were able to maintain growth on glycerol based medium (YPG), indicative of sufficient mitochondrial function (data not shown).

Impact of R256H Mutation on Actin Cytoskeleton Assembly in Vivo—The morphologic abnormalities identified in the actin cytoskeleton led us to further investigate whether the R256H actin causes a defect in actin assembly. To address this question, we examined the sensitivity of wild type and R256H cells to latrunculin A, a drug that sequesters actin monomers and reversibly promotes rapid depolymerization of actin filaments (22, 35, 36). Plate cultures of wild type and mutant cells were exposed to filter discs saturated with a range of concentrations of latrunculin A. The area of growth inhibition surrounding each disc was quantified. Mutant cells were more sensitive to latrunculin A than wild type cells (Fig. 3). For example, the area of growth inhibition around the 1 mM latrunculin A disc was 4.38 ± 0.08 cm² for the R256H cells versus 1.18 ± 0.01 cm² for wild type (p < 0.001).

Our data thus far suggest that the cytoskeletal abnormalities have minimal growth effects on a steadily growing population of cells. We questioned whether the abnormalities would manifest in a situation where the reassembly of the cytoskeleton was required. To answer this, we again used latrunculin A to disassemble the cytoskeleton and then monitored reestablishment of the cytoskeleton. Analyses revealed that mutant cells had marked delay in recovery to base-line cable morphology. For wild type cells, half had normal cables 10 min after treatment, and nearly all cells were restored by 50 min. In contrast, R256H cells took 5-fold longer, 50 min, for half of the population to have base-line cable morphology. The difference persisted with mutant cells requiring >90 min for the whole population to reestablish actin cables (Fig. 4).

Effects of R256H mutation on Actin Polymerization—The in vivo data indicate that the mutant actin cytoskeleton is less stable with problems reestablishing the cable network. These anomalies could derive from mutation-mediated defects in actin polymerization. The location of Arg-256 along the inter-strand interface within the filament supports the idea that a change in that the residue might alter polymerization behavior characterized by changes in nucleation or elongation rates or critical concentration. The effects of the R256H mutation on actin polymerization kinetics were assessed by quantifying the change in light scattering during actin polymerization. Wild type and R256H mutant actin were purified from yeast cells, and polymerization was induced and monitored over time. As suspected, the R256H mutation led to polymerization defects; specifically, an extended nucleation phase and a lower final extent of polymerization (Fig. 5A). The lower final extent of polymerization signifies a higher critical concentration for the mutant actin. Additional polymerization studies established a critical concentration for R256H as 1.36 μM compared with 0.60 μM for wild type actin (p < 0.001; Fig. 5B). In verification, electron micrographs of wild type and R256H actin filaments also demonstrated a difference in filament length (electron micrographs not shown). Mutant actin filaments were shorter measuring 3.10 ± 0.89 μm compared with wild type 3.76 ± 1.29
μM (p < 0.01). Together, these results are consistent with filament destabilization due to the mutation.

**Nucleation of Actin Polymerization by Bni1**—The cytoskeletal morphology in R256H cells and the delay in reestablishment of the cytoskeleton suggest a defect in filament nucleation. Formins are proteins that regulate actin filament nucleation. Formins have FH1 and FH2 domains near the C terminus. The FH2 domains of two formins form a collar around the barbed end of the actin filament and facilitate nucleation (37). This formin collar maintains association with the growing barbed end of the filament in a manner termed progressive capping (25). We assessed the effects of formin on actin polymerization using the C-terminal fragment of the yeast formin, Bni1, which contains the FH1 and FH2 domains (38). This Bni1 fragment has been previously shown to cause a concentration dependent increase in the nucleation rate of wild type actin with no observable change in final extent of polymerization (39).

We initially screened 10% pyrene-labeled wild type and R256H actin polymerization with the addition of a range of concentrations of the Bni1 FH1-FH2 fragment. Ten percent of the actin was labeled with pyrene, and the polymerization-dependent increase in fluorescence was quantified over time. To account for the increased critical concentration of R256H mutant actin, actin concentrations with equivalent final extents of polymerization were selected to ensure the same amount of polymerizable actin was used. Accordingly, concentrations for wild type and R256H actin were 1.5 and 2.25 μM, respectively, as described under “Experimental Procedures.” Shown are representative plots of experiments performed at least three times with three independent actin preparations. A.U., arbitrary units.

![Graph A](image1.png)  ![Graph B](image2.png)  ![Graph C](image3.png)  ![Graph D](image4.png)
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Fragment was added prior to induction of polymerization (Fig. 7). These findings support the hypothesis that the R256H mutation in actin alters formin regulation leading to capping rather than normal processive association with the barbed end of the filament.

We hypothesized that the defect in formin regulation of R256H mutant actin was due to changes in conformation of the filament. To answer this question, we repeated the Bni1 polymerization experiments with the addition of stoichiometric amounts of phalloidin to the reaction mixture. Phalloidin binds actin filaments and reduces flexibility with stabilization inter-subunit contacts both along and between the two helical strands. In addition, phalloidin-binding changes the filament conformation causing subdomains 1 and 2 of the actin subunit to rotate outward (40–42). Consistent with our hypothesis, the presence of phalloidin restored polymerization of R256H actin with Bni to wild type kinetics (Fig. 8).

In the clinical disease state, the actin mutations are autosomal dominant with both normal and mutant alleles in the same cell. Incorporation of wild type subunits into the filament may influence conformational changes related to mutant actin. To determine the consequence of wild type actin on R256H actin biochemistry, we examined the polymerization kinetics of admixtures of wild type and mutant actin. Interestingly, wild type actin appeared dominant over the mutant actin. Lesser amounts of wild type actin disproportionately normalized the final extent of polymerization of mutant actin. For example, a 50:50 mixture of wild type and R256H actin had a final extent of polymerization at 80% of wild type actin, nearly twice that of mutant actin alone.

We extended this line of inquiry to the effect of the Bni fragment on polymerization of admixtures of wild type and mutant actin. Consistent with the polymerization experiments, wild type actin had a dominant effect on the interaction of the formin fragment with the mutant actin (Fig. 9). Wild type actin normalized polymerization kinetics of R256H actin with Bni disproportionate to the percentage. Only 25% of wild type actin

FIGURE 7. Effect of late addition of Bni1 on actin polymerization kinetics. A, polymerization of 10% pyrene-labeled wild type and R256H actin was fluorescently monitored over time as in Fig. 6 with the exception that 100 nM Bni was added to the reaction midway during elongation. Time of Bni addition is marked by an arrow. Concentrations for wild type and R256H actin were 1.5 and 2.25 μM, respectively. Shown is a representative plot from experiments performed at least three times with three independent actin preparations. A.U., arbitrary units.

FIGURE 8. Actin polymerization kinetics in the presence of phalloidin. Polymerization of 10% pyrene-labeled actin was quantified by change in fluorescence over time with and without phalloidin and with and without Bni. Actin concentrations were 2.25 μM as was the concentration of phalloidin when included. 100 nM of the FH1-FH2 fragment of Bni1 was used when included. A, wild type actin. B, R256H mutant actin. Shown are representative plots from experiments performed at least two times with two independent actin preparations. A.U., arbitrary units.

FIGURE 9. Polymerization kinetics of admixtures of wild type and mutant actin. A, ratios of wild type and R256H mutant actin were combined with a final concentration of 2.25 μM and induced to polymerize. The polymerization-dependent increase in fluorescence of the 10% pyrene-labeled actin was monitored over time. B, as in A, polymerization kinetics of ratios of wild type and R256H mutant actin was quantified in the presence 100 nM of Bni1. Shown are representative plots of experiments performed at least three times with three independent actin preparations. A.U., arbitrary units.
added to mutant actin led to nearly 50% recovery of the final extent of polymerization.

In summary, the polymerization defects seen with R256H mutant actin are ameliorated by restoration of filament conformation as seen with the addition of phalloidin or wild type actin. These data support that conformation of the barbed end of the actin filament is altered by the R256H mutation leading to flawed filament initiation.

**DISCUSSION**

Our focus was to determine the biochemical effects of the R256H mutation on actin function *in vivo* and *in vitro*. The mutation clearly affected the morphology of actin cables and two organelles whose integrity depends on the cytoskeleton. These alterations, however, did not impact growth of the cells expressing only the mutant actin even under stress conditions. Although mitochondrial patterns are affected, the ability of cells to grow on 2% glycerol indicates that the alterations do not significantly interfere with mitochondrial metabolic capacity. These studies assess growth under conditions where cytoskeletal template alterations do not impact growth of the cells expressing only the mutant actin even under stress conditions.

The R256H actin mutation led to filament instability *in vitro* demonstrated by the increased critical concentration and a delayed nucleation phase during polymerization. Both of these effects could result from an effect of the mutation on inter-strand interactions within the filament leading to altered filament conformation and stability. Models of the actin helix (17, 43) suggest that Arg-256 is involved in interstrand stabilization. Arg-256 and Glu-195 form a triangular unit with the side chain of Lys-113 in the cross-strand monomer (Fig. 10). In this triangle, the guanido group of Arg-256 is 3–4 Å from the side chain of Glu-195 and the Lys-113 side chain amino is within 3 Å of the Glu-195 side chain. Ionic interactions among the three residues would stabilize the unit, and interfering with this complex could lead to filament destabilization. The R256H change could affect this unit via both decreased size and decreased cationic character. Results from another study we are carrying out are in agreement with this hypothesis. The K113E mutation in actin (44) leads to filament destabilization similar to the findings presented here (data not shown).

Interestingly, Lys-113 is part of a helix, Lys-113-Thr-125, that contains two other TAAD mutations we have previously characterized, N115T and R116Q, in addition to Lys-118, the site of two deafness-causing mutations (14, 45). If our hypothesis is correct, the mutations become part of a pathogenic module that, as a unit, can affect F-actin conformation. The nature and site of a particular mutation in this unit would create a potential spectrum of effects on actin filament regulation, which could translate ultimately into the allele-specific symptoms seen in TAAD patients. In this context, the R256H mutation might cause a propagated conformation change to the filament surface leading to an altered interaction with an actin-binding protein such as formin. Two other pieces of evidence correlate with this helix destabilization idea. First, stoichiomet-
overcome this deficiency and restore nucleation facilitated by formin.

The molar ratio of Bni1p to actin in the cell is ~1 to 500 (51). The concentrations of the Bni fragment used in our experiments are higher than the known cellular concentration of Bni1p. However, Bni1p is highly localized within the cell at the neck or the bud tip so the local concentration relative to actin will be much higher (52). Therefore, the higher concentrations of Bni relative to actin we used for our studies should not present a concern on the grounds of biological significance.

The model of the actin-formin collar proposed by Rosen (49) suggests a molecular explanation for our results. In this model, residue 113 is part of a helix that extends to residue 125 on the actin filament surface, near the formin binding site (Fig. 11). The connection of this helix with residues 256 and Glu-195 of opposing monomer would provide a means for propagating an allosteric signal between the filament exterior and interior. Such an interaction would allow for regulation of filament dynamics by formin binding.

Our results provide new insight into possible mechanisms by which the R256H mutation leads to TAAD. Histological examination of aortic tissue from patients with this mutation show decreased numbers of actin filaments arranged in a disorganized pattern. This scenario is the end result of a long disease process and leads to the question of how this state arises. Two broad reasons could account for the histological findings. First, the mutation may cause a defect in the initial assembly of the contractile apparatus. Our observations concerning the effects of the R256H mutation on formin function are particularly relevant to this concept. FHOD1, a formin protein, is essential for proper deposition of the actin cytoskeleton. The formin FHOD1 plays an important role in formation and organization of the vascular smooth muscle cell contractile apparatus and a lack of FHOD1 results in fewer and grossly disorganized actin filaments (53). The second potential reason for the arterial pathology is that the mutation may cause defects in maintaining the contractile apparatus in a functional organized state once assembly has occurred. The weakened intermonomer interactions caused by the mutation could result in an inherently less stable structure that is more susceptible to turnover. Alternatively, this weakening could lead to an inability of the actin apparatus to withstand the forces imposed on it by

\[ \text{FIGURE 11. Model of Bni interaction with the actin oligomer.} \]

*A*, model of the actin oligomer based on the filament model of Oda et al. (43) and the Bni1 FH1-FH2 fragment from Rosen et al. (49). The actin monomer colors are blue and gray. Residues are color highlighted: orange, Lys-113; marine blue, Lys-118; yellow, Glu-195; red, Arg-256; and pink, helix containing residues 113–125. The FH1-FH2 domain colors are gold and green. The red rectangle frames this unit of interaction. Models were generated using the PyMOL Molecular Graphics System (version 1.5; Schrödinger, LLC). The two models are a side view and a pointed end view of the structure. *B*, schematic drawing demonstrating the formin/actin complex colored as above. The post site of the green bridge is free and accessible to recruit an incoming actin subunit.
smooth muscle myosin in the generation of contractile force needed for setting and maintaining vascular tone. Similar observations have been made in cases where actin mutations affect striated muscle function (54).

Ultimately, delineating the molecular mechanisms leading to arterial wall weakening will require longitudinally following the process over the course of the disease from birth. Animal models to do this are currently being developed. However, understanding the effects of the mutations on actin function per se and its subsequent regulation at the biochemical level with studies as we have reported here will be required to assess how the mutation leads to cellular changes that cause the pathology observed.

Acknowledgment—We thank Rose Lee for participation in the early aspects of this work.

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