Vascular disease-causing mutation R258C in ACTA2 disrupts actin dynamics and interaction with myosin

Hailong Lu, Patricia M. Fagnant, Carol S. Bookwalter, Peteranne Joel, and Kathleen M. Trybus

Edited by Edward D. Korn, National Heart, Lung, and Blood Institute, Bethesda, MD, and approved June 15, 2015 (received for review April 17, 2015)

Point mutations in vascular smooth muscle α-actin (SM α-actin), encoded by the gene ACTA2, are the most prevalent cause of familial thoracic aortic aneurysms and dissections (TAAD). Here, we provide the first molecular characterization, to our knowledge, of the effect of the R258C mutation in SM α-actin, with expressed the baculovirus system. Smooth muscles are unique in that force generation requires both interaction of stable actin filaments with myosin and polymerization of actin in the subcortical region. Both aspects of R258C function therefore need investigation. Total internal reflection fluorescence (TIRF) microscopy was used to quantify the growth of single actin filaments as a function of time. R258C filaments are less stable than WT and more susceptible to severing by cofillin. Smooth muscle tropomyosin offers little protection from cofillin cleavage, unlike its effect on WT actin. Unexpectedly, profilin binds tighter to the R258C monomer, which will increase the pool of globular actin (G-actin). In an in vitro motility assay, smooth muscle myosin moves R258C filaments more slowly than WT, and the slowing is exacerbated by smooth muscle tropomyosin. Under loaded conditions, small ensembles of myosin are unable to produce force on R258C actin-tropomyosin filaments, suggesting that tropomyosin occupies an inhibitory position on actin. Many of the observed defects cannot be explained by a direct interaction with the mutated residue, and thus the mutation allosterically affects multiple regions of the monomer. Our results align with the hypothesis that defective contractile function contributes to the pathogenesis of TAAD.

Thoracic aortic aneurysms and dissections (TAAD) are the 18th most common cause of death in individuals in the United States (1). The high degree of mortality is partly due to the fact that aneurysms tend to be asymptomatic until a life-threatening acute aortic dissection occurs. Familial TAAD is an autosomal dominant disorder with variable penetrance, which is characterized by enlargement or dissection of the thoracic aorta (reviewed in 2). The most prevalent genetic cause of familial TAAD, responsible for ~15% of all cases, are mutations in vascular smooth muscle α-actin (SM α-actin), encoded by the gene ACTA2. More than 40 mutations in ACTA2 have been identified to date (3–5). Intriguingly, ACTA2 mutations also differentially predispose individuals to occlusive vascular diseases, such as premature coronary artery disease and strokes (6). ACTA2 mutations thus can lead to either dilation of large elastic arteries like the aorta or occlusion of smaller muscular arteries.

SM α-actin is the most abundant protein in vascular smooth muscle cells, constituting ~40% of the total protein and ~70% of the total actin, with the rest composed of β- and γ- cytoplasmic actin. Actin is critical for contraction and force production by smooth muscle cells, as well as for their proliferation and migration. Dissected aortas show several characteristic features, namely, loss and disarray of the smooth muscle cells in the medial layer, loss of elastic fibers, and proteoglycan accumulation in the medial space (reviewed in 2). The compromised integrity of the aortic wall allows progression to dissection. In contrast, the vascular pathology in the occluded arteries of patients with ACTA2 mutations is characterized by enhanced numbers of smooth muscle cells.

Little is known about the underlying biochemical mechanisms by which mutations in SM α-actin trigger pathways that ultimately result in aortic cell loss or in the cell proliferation typical of occlusive diseases in small muscular arteries. Here, we present an in vitro characterization of the defects caused by the R258C mutation in SM α-actin. To our knowledge, we are the first to express the human SM α-actin isoform successfully using the baculovirus/insect cell system, which is a critical aspect of this study because the effect of point mutations can vary depending on the isoform in which they are present. We investigated the effect of the R258C mutation first because of its prevalence in patients (6), its relatively poor prognosis (median life expectancy of ~35 y of age), and high penetrance (5), and because it causes TAAD as well as moyamoya-like disease, an occlusive disease of the cerebral vasculature.

The actin monomer consists of two major domains, with ATP bound in the cleft between them. In the typical view of monomeric globular actin (G-actin), R258 is located in a helix on the backside of subdomain 4 (Fig. 1A). [Note that the R258 mutation corresponds to amino acid R256 in the actin protein, due to post-translational processing that removes both the N-terminal Met and Cys residues (7)]. In filamentous actin (F-actin), the outer domain consists of subdomains 1 and 2, as well as the inner domain of subdomains 3 and 4. The two helical strands that compose the filament are stabilized by interactions between protomers both within a strand and between strands. R258C lies at the interface between the two strands (Fig. 1 B and C). Upon polymerization, the actin protomer undergoes a conformational change: The two major domains, which are twisted in monomeric G-actin, become flatter with respect to one another in F-actin. Another feature of F-actin is that it can adopt multiple states in which the protomers adopt varied twists and tilts with respect to one another, as well as having different loop conformations (8–11).

Significance

Point mutations in vascular smooth muscle α-actin are the most prevalent cause of familial thoracic aortic aneurysms leading to acute dissections, yet the molecular mechanism by which these mutations affect actin function is unknown. An underlying cause of the disease is thought to be contractile dysfunction, which initiates adaptive pathways to repair the defects in the smooth muscle cells. Here, we investigate the effects of the R258C mutation, a prevalent mutation in humans with a poor prognosis. The mutant actin shows multiple defects, including impaired interaction with myosin, formation of less stable filaments, and enhanced levels of monomer. These defects are likely to decrease cellular force production and initiate aberrant mechanosensing pathways that culminate in the disease.

Author contributions: H.L. and K.M.T. designed research; H.L., P.M.F., C.S.B., and P.J. performed research; H.L., P.M.F., and K.M.T. analyzed data; and H.L. and K.M.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

To whom correspondence should be addressed. Email: kathleen.trybus@uvm.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1507587112/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1507587112

PNAS Early Edition | 1 of 10
Here, we compare the biochemical properties of expressed WT and R258C SM α-actin, with the goal of understanding how the mutation affects some of the basic functions of actin, because this initial insult ultimately culminates in vascular disease. In smooth muscle cells, force production requires both proper binding of actin with myosin in the contractile domain and polymerization of actin in the cytoskeletal domain, where it strengthens the membrane for force transmission to the ECM (reviewed in 12). The R258C mutation adversely affects interactions with myosin and weakens filament stability. Our results predict that cells expressing the R258C mutation will have decreased force output and a larger pool of monomeric actin. These dysfunctions could trigger aberrant mechanosensing pathways that culminate in compromised aortic muscle tissue (13). Increasing the monomer/polymer ratio of actin may also have an impact on cellular phenotype, which could have implications for occlusive vascular disease.

Results

Expression of Human Vascular SM α-Actin. Recombinant human SM α-actin was expressed using the baculovirus/SF9 insect cell expression system. Several challenges needed to be overcome to achieve this goal: (i) The purified actin could not retain a tag, because both the N or C terminus was near the myosin binding site; (ii) contamination by endogenous SF9 cell actin must be avoided; and (iii) polymerization of actin during expression in the SF9 cells needed to be prevented because it lowers the protein yield. In addition, expression levels of SM α-actin are considerably lower than what we obtain for β- or γ-actin in smooth muscle. Most of these problems were surmounted by using a method that was designed for expression of toxic actin mutants in Dictostelium (14). The C terminus of actin was fused to a 43-aa actin-monomer sequestering protein, thymosin-β4, followed by a HIS tag. Binding of thymosin-β4 to the cleft between subdomains 1 and 3 renders the expressed actin monomeric in the SF9 cell. The HIS tag allows purification of the expressed actin on a nickel-chelate column. The thymosin-β4–HIS tag is then cleaved from the C terminus of actin by proteolytic digestion with chymotrypsin, whose primary cleavage site is after the last native residue of actin, a Phe. Following ion exchange chromatography to remove the thymosin-β4 and any actin from which the tag was not cleaved, pure actin with no native residues at either the N or C terminus was obtained. This strategy worked equally well for WT and the R258C mutant actin.

SDS gels of the purified WT and R258C actins show the purity of the preparations (Fig. 2B). The WT actin is shown before and after removal of the thymosin-β4–His tag (Fig. 2B, lanes 2 and 3). R258C actin is shown after final purification (Fig. 2B, lane 4). The two actins were run on a 1D isoelectric focusing gel (Fig. 2B). As expected, the R258C migrated faster than WT because the mutation replaced a positively charged residue with an uncharged residue. Their relative mobilities were consistent with the calculated isoelectric points of 5.24 for WT and 5.16 for R258C. Average yields of purified actin were ∼0.5 mg of actin for WT and ∼0.4 mg for R258C actin per 10^9 SF9 cells (200-mL culture).

The WT and mutant SM α-actins were compared with regard to their intrinsic ability to polymerize, their interaction with key actin binding proteins (e.g., smooth muscle tropomyosin, profilin, cofilin), and their ability to be moved by smooth muscle myosin under both unloaded and loaded conditions. The mutation significantly affected most of these properties of actin.

R258C Forms a Less Stable Actin Filament. Polymerization of individual SM α-actin filaments was visualized as a function of time using total internal reflection fluorescence (TIRF) microscopy [10 mM imidazole (pH 7.5), 50 mM KCl, 4 mM MgCl2, 1 mM EGTA, 2 mM MgATP at 37 °C]. The actin filament is polarized such that the two ends differ. The association and dissociation of subunits at the barbed end are intrinsically much faster than at the pointed end. Growth from the barbed end is 10- to 20-fold faster than from the pointed end, and thus we measure total growth from both ends. A series of images at various times illustrate growth of individual filaments (Fig. 3A and Movie S1).
The filaments were not visibly different by eye, and filament breaking was rarely observed for either WT or R258C actin. Neither showed delayed polymerization, suggesting that nucleation was normal for both of them. To extract kinetic parameters, the observed increase in filament length was plotted as a function of actin concentration (Fig. 3B). The slope of each linear plot defines the polymerization rate. The y-intercept is the rate at which actin subunits disassemble from the filament ends. The x-intercept is the critical concentration (i.e., the concentration of monomeric actin in equilibrium with polymer).

Average data from five experiments, performed with three independent protein preparations each of WT and R258C actin, are summarized in Table 1. The assembly rate of R258C actin is ~30% slower than WT (11 ± 16 subunits per μM⁻¹·s⁻¹), whereas the disassembly rate is approximately fourfold faster (2.7 ± 0.7 subunits per s⁻¹), suggesting that mutant filaments are less stable than WT filaments. The critical concentration for polymerization of R258C actin is approximately fivefold higher than for WT (238 nM vs. 48 nM for WT), which will increase the G-actin concentration in the cell. The defects in polymerization are consistent with the fact that R258 is involved in salt-bridge interactions that stabilize cross-strand interactions in the filament, based on the position of this residue in the pseudoatomic model of the actin filament (Fig. 1B and C).

A human heterozygous for the R258C mutation expresses R258C* as a function of time. A TIRF microscopy (A) Growth of 1.5 μM WT G-actin as a function of time. Each panel is separated by 20 s. A yellow arrowhead follows the growth of one filament with time. (B) Rate of polymerization of WT (blue circles) and R258C (red triangles) as a function of actin concentration. Data were obtained from five experiments using three independent protein preparations each of WT and R258C actin. Values (assembly rate, disassembly rate, and critical concentration) obtained from the fits to a line are provided in Table 1. Error bars are SE.

Table 1. Polymerization rates of WT and R258C actin

<table>
<thead>
<tr>
<th>Actin</th>
<th>Assembly rate, subunits per μM⁻¹·s⁻¹</th>
<th>Disassembly rate, subunits per s⁻¹</th>
<th>Critical concentration, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>15.9 ± 3.4</td>
<td>0.7 ± 0.6</td>
<td>47.8 ± 44.2</td>
</tr>
<tr>
<td>R258C</td>
<td>11.2 ± 2.9</td>
<td>2.7 ± 1.0</td>
<td>238.2 ± 49.1</td>
</tr>
<tr>
<td>WT*</td>
<td>6.9 ± 0.7</td>
<td>0.5 ± 0.9</td>
<td>76</td>
</tr>
<tr>
<td>R258C*</td>
<td>6.4 ± 0.8</td>
<td>1.5 ± 1.4</td>
<td>229</td>
</tr>
</tbody>
</table>

Data were obtained from five experiments using three independent protein preparations each of WT and R258C actin. Conditions: 10 mM imidazole (pH 7.5), 50 mM KCl, 4 mM MgCl₂, and 1 mM EGTA (37 °C). TM, tropomyosin.

*Data were obtained from one experiment. Errors are SE of the fit.
Copolymerization of equal amounts of WT and R258C actin. (Fig. 7 and Movie S2): Filaments were severed internally, and they also shortened from either end, with one end much faster than the other, until the filament disappeared. These two behaviors were not mutually exclusive; namely, a given filament could exhibit one or both behaviors. Both processes depended on cofilin concentration and resulted in the destruction of actin filaments.

We first assessed the effect of cofilin on actin filament shortening. As little as 10 nM cofilin induced shortening of R258C filaments (Fig. 7A). At 100 nM cofilin, the process was so fast that no R258C filaments were observed by the time the slide was mounted on the microscope. In contrast, WT filaments only started to slowly shorten at 100 nM cofilin.

WT filaments were also more resistant than R258C filaments to severing by cofilin (Fig. 7B). WT filaments showed virtually no severing at 100 nM cofilin, whereas with 75 nM cofilin, severing events were frequently observed with R258C filaments.

 Binding of cofilin is competitive with tropomyosin (reviewed in 17). Tropomyosin reduced the rate of shortening and strongly suppressed the severing effects of cofilin with WT actin filaments (Fig. 7). Even at 1.2 μM cofilin, there was minimal shortening and almost no severing. In contrast, the R258C filaments were much more vulnerable to attack by cofilin despite the presence of tropomyosin. Smooth muscle tropomyosin binds at least as tightly to R258C filaments as to WT filaments (Fig. 5), so low binding affinity does not explain the result.

Fig. 4. Copolymerization of equal amounts of WT and R258C actin. (A) Rate of polymerization of an equimolar mixture of WT and R258C actin (black circles). Data for the homopolymers (WT, blue circles; R258C, red triangles) were obtained at the same time. Table 2 tabulates the values obtained from the fits (solid lines). Error bars are SE. (B) Schematic to illustrate polymerization of a 50:50 mixture of WT (white circles) and R258C (red circles) actin. A WT monomer can add onto an existing filament that has either a WT or mutant protomer at the end; likewise, a R258C monomer can add onto an existing filament that has either a WT or mutant protomer at the end. The rate of assembly is slower only if the filament end adopts a mutant-like conformation, which skews the curve toward WT values. Details of the model are described in Materials and Methods. Table 2 compares the calculated and experimental values.

filament ends compared with the free actin monomer. The disassembly rate for both WT and R258C was faster in the presence of profilin compared with its absence (increased from 0.7 to 3 subunits per s⁻¹ for WT and from 2.7 to 8.2 subunits per s⁻¹ for R258C) (Table 3), which may be due to the unstable configuration of actin at the end of filament when profilin is still bound. Addition of profilin also increases the critical concentration from ~50 to 330 nM for WT actin because it binds monomeric actin. Unexpectedly, the critical concentration for polymerization of R258C actin increased to a much greater extent in the presence of profilin, from ~240 to 1,680 nM (Table 3). This increase may have cellular implications because the level of free actin monomer is tightly controlled in vivo. The data were fitted to a simple model (Materials and Methods) in which both free actin monomer and profilin-actin can take part in the polymerization process. The parameters obtained from the fit show that profilin binds to R258C actin with ~20-fold higher affinity than to WT (Table 3).

R258C Actin Filaments Are Propelled at Slower Speeds than WT. An unloaded in vitro motility assay was used to measure the speed at which smooth muscle myosin moves WT vs. R258C actin. We first performed the typical motility assay using filaments that were stabilized and visualized using rhodamine-phalloidin. Mutant and WT filaments were moved at the same speed (Fig. 8A). In the presence of smooth muscle tropomyosin, WT actin moved ~40% faster than bare actin. In contrast, the speed at which myosin moved the R258C filaments was not enhanced by tropomyosin. This increase in speed resulted in WT filaments moving ~1.7-fold faster than R258C filaments in the presence of tropomyosin.

The in vitro motility was repeated in the absence of phalloidin to test if this compound, which stabilizes the filament, moderates the defects caused by the mutation. To visualize the filaments, WT actin was directly labeled with rhodamine and incorporated into the filament as 25% of the total actin. The speed of filament movement slowed as the percentage of R258C actin in the filament decreased from monomers on native gels. Fig. 2C shows the results with DNase, but essentially identical gels were obtained with gelsolin or both behaviors. Both processes depended on cofilin concentration and resulted in the destruction of actin filaments.

Table 2. Polymerization rates of mixtures of WT and R258C

<table>
<thead>
<tr>
<th>Actin</th>
<th>Assembly rate, subunits per μM·s</th>
<th>Disassembly rate, subunits per s</th>
<th>Critical concentration, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>20.4</td>
<td>0.70</td>
<td>34.4</td>
</tr>
<tr>
<td>R258C</td>
<td>11.5</td>
<td>1.91</td>
<td>165.9</td>
</tr>
<tr>
<td>50% WT/50% R258C</td>
<td>18.2</td>
<td>1.48</td>
<td>81.5</td>
</tr>
<tr>
<td>R258C</td>
<td>Calculated</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18.2</td>
<td>1.31</td>
<td>71.9</td>
</tr>
</tbody>
</table>

Conditions: 10 mM imidazole (pH 7.5), 50 mM KCl, 4 mM MgCl₂, and 1 mM EGTA (37 °C).

*Details of the model are described in Discussion.*
increased from 50–75% (Fig. 8B). The slowing of speed by the mutant actin was accentuated in the presence of tropomyosin. In some patients, Arg-258 is mutated to His instead of Cys. We expressed and purified R258H to test if this mutation had similar effects to R258C. In filaments containing 75% mutant actin, speeds were comparable whether the Arg residue was mutated to Cys or His (minus tropomyosin: 0.47 ± 0.13 for R258C vs. 0.50 ± 0.10 for R258H; plus tropomyosin: 0.31 ± 0.08 for R258C vs. 0.32 ± 0.09 for R258H).

To determine if the slowing of speed with the mutant actin filament was due to a change in the rate of release of ADP from myosin, a stopped-flow experiment was performed. Acto-smooth heavy meromyosin (HMM)-ADP was mixed with 2 mM MgATP. Under these conditions, the rate of acto-HMM dissociation is rate-limited by ADP dissociation. The rate of ADP release from the mutant and WT filament was the same (134 ± 1 s⁻¹ for WT actin and 127 ± 1 s⁻¹ for R258C actin).

**Actomyosin Force Generation Is Severely Impaired When Tropomyosin Is Bound to the R258C Filament.** To test if the R258C mutation affects force generation by myosin, we carried out a one-bead laser trap experiment to investigate the force/velocity relationship of a small ensemble of smooth muscle heavy meromyosin with WT and R258C actin filaments (Fig. 9A). When studying actin mutants, an advantage of the one-bead setup is that the actin filaments do not require phalloidin for stabilization because they are attached to the coverslip. In the more traditional three-bead assay, the actin is suspended between two beads and stabilized with rhodamine-phallolidin.

We first compared the force/velocity relationship obtained with bare WT and R258C actin filaments. A force-feedback system is used to clamp the force at five levels between 1 and 5 pN. The velocity at any force level was obtained by fitting the raw data to a line (Fig. 9B). The force/velocity relationship was then fitted to the Hill equation. As expected, velocity slowed as force increased (Fig. 9C). There was virtually no difference between the curves obtained with bare WT vs. bare R258C filaments. In the presence of smooth muscle tropomyosin, the WT filament showed the same force/velocity relationship as in its absence. In contrast, velocity was greatly reduced at all force levels for R258C actin-tropomyosin filaments.

**Discussion**

Multiple facets of SM α-actin function are quite severely affected by the R258C mutation. Defects induced by the R258C mutation include (i) slower and less productive interactions with smooth muscle myosin, particularly in the presence of tropomyosin, that lead to reduced force output; (ii) formation of a less stable filament with greater susceptibility to cleavage by cofolin even in the presence of tropomyosin, which enhances actin filament dynamics; and (iii) higher affinity binding to profilin, which increases the monomeric pool of actin. How these in vitro defects affect smooth muscle cell function must be viewed in light of the many roles that SM α-actin plays in vascular smooth muscle cells. These roles include production of contractile force, maintenance of the integrity of the submembrane cytoskeleton, cellular mechanosensing, and regulation of cell differentiation and proliferation. Genetic mutations that predispose patients to TAAD include mutations in the contractile proteins and their regulators, ECM structural components of the aortic wall, and proteins involved in mechanosensing. It has been proposed that smooth muscle cell contractile dysfunction is one of the primary insults that activates the stress and strain pathways that initiate maladaptive remodeling of the smooth muscle cell, ultimately culminating in an aortic aneurysm (reviewed in 2, 13).

Actin is found in two distinct domains in smooth muscle cells. The “contractile” domain of smooth muscle cells consists of interdigitating myosin and stable actin filaments that cyclically interact to generate tension, controlled by phosphorylation of the regulatory light chain of smooth muscle myosin. These filaments are anchored...
mutations in both the myosin heavy chain (MYH11) and myosin light chain kinase (MLCK), which phosphorylates the regulatory light chain of myosin and is required for actomyosin interactions, also cause autosomal dominant inheritance of TAAD (24, 25). Genes encoding proteins involved in either smooth muscle cell contraction or the TGF-β signaling pathway are the predominant causes of familial TAAD.

R258C Destabilizes the Filament. Although R258C was not specifically looked at, immunofluorescence of cultured smooth muscle cells from individuals with ACTA2 mutations showed substantially fewer actin filaments than controls, suggesting that these mutations may interfere with actin assembly (3). Our in vitro results support this idea. R258C actin has a higher critical concentration for assembly, caused by a slower rate of assembly and a faster rate of disassembly. Based on the location of R258 in the model of the actin filament structure (Fig. 1), a polymerization defect was predictable. Recent advances in electron cryomicroscopy have led to the highest resolution structures (3.7–4.7 Å) of the actin filament to date (9, 11). In these structures, R258 (R256 in protein) forms a salt bridge with E197 (E195 in protein) in the same protomer, which, in turn, forms a salt bridge with K115 (K113 in protein) on the cross-strand actin.

Fig. 7. R258C filaments are more susceptible to cofilin-induced shortening and cleavage than WT actin, both in the presence and absence of smooth muscle tropomyosin (TM). (A) Rate of actin filament shortening induced by cofilin (WT, blue bars; R258C, red bars). Lighter shaded bars are in the presence of tropomyosin. Error bars are SE. (B) Frequency of cleavage by cofilin for WT and R258C actin filaments. Frequency is reported as the number of events per 1 min per 1 μm of actin filament. Error bars are SE.
Saccharomyces cerevisiae + TM

(upper, minus tropomyosin) Myosin moved WT actin at a speed of $0.53 \pm 0.18 \mu m/s$ ($n = 2742$) and R258C filaments at a speed of $0.50 \pm 0.16 \mu m/s$ ($n = 3415$). The difference in speeds was statistically significant (Student’s t test, $P < 0.01$) because of the large number of filaments analyzed, but it has no physiological relevance. (lower, plus tropomyosin) In the presence of smooth muscle tropomyosin, myosin moved WT actin at a speed of $0.74 \pm 0.19 \mu m/s$ ($n = 1294$) and R258C filaments at the slower speed of $0.43 \pm 0.15 \mu m/s$ ($n = 3743$) (Student’s t test, $P < 0.01$). Data from five independent actin preparations were pooled.

(b) Speed of movement of WT and R258C actin filaments that were not stabilized with phalloidin. Filaments were visualized by incorporation of 25% WT actin that was directly labeled with rhodamine (solid blue bar, 100% WT; striped red bar, 50% WT and 50% R258C, solid red bar, 25% WT and 75% R258C). (left) Speed of movement of WT filaments ($0.61 \pm 0.13 \mu m/s$; $n = 196$) decreased to $0.53 \pm 0.12 \mu m/s$ ($n = 160$) with 50% R258C and to $0.47 \pm 0.13 \mu m/s$ ($n = 180$) with 75% R258C. The difference between all pairs was statistically significant (Student’s t test, $P < 0.01$). (right) Same comparison in the presence of smooth muscle tropomyosin (TM). The speed of movement of WT filaments ($0.60 \pm 0.10 \mu m/s$; $n = 170$) decreased to $0.43 \pm 0.09 \mu m/s$ ($n = 80$) with 50% R258C and to $0.31 \pm 0.08 \mu m/s$ ($n = 142$) with 75% R258C. The difference between all pairs was statistically significant (Student’s t test, $P < 0.01$). Data from five independent actin preparations were pooled. Filament speed was tracked manually. Temperature, 30 °C.

(1 B and C). This stabilizing salt bridge triad will be disrupted upon mutation of R258 to either Cys or His, which changes both side-chain size and charge. Mutation of R258 to either Cys or His causes similar patient phenotypes. Filament destabilization as a result of an R256H mutation (equivalent to residue Arg-258 in ACTA2) was first observed in the backbone of Saccharomyces cerevisiae actin by Malloy et al. (26). Ionic cross-strand stabilization that involves this residue thus appears to be a property common to SM α-actin and budding yeast actin.

Copolymerization of equal amounts of WT and mutant actin tempers the polymerization defect. The observation that polymerization as a function of actin concentration was skewed toward the WT values could be explained by a simple model if we make the following two assumptions (Fig. 4 and Materials and Methods). First, only when an R258C monomer adds onto a filament that adopts a mutant-like conformation (e.g., a filament with a mutant protomer at the barbed end) will it assemble at a slow rate similar to the rate observed for the R258C homopolymer. In all other scenarios, the monomer adds onto the filament with a faster rate that approximates the WT assembly rate. Second, the disassembly rate depends solely on the actin type (i.e., WT dissociates with the WT disassembly rate, R258C dissociates from filaments with the R258C disassembly rate). As shown in Table 2, the calculated results from this model agree with the experimentally observed values.

**Actin Filament Dynamics Regulated by Binding Proteins.** In nonmuscle cells, the actin cytoskeleton is highly dynamic, and controlled assembly and disassembly of actin play roles both in cell migration

---

**Fig. 8.** Smooth muscle myosin moves R258C filaments more slowly in an in vitro motility assay. (A) Actin filaments were stabilized and visualized with rhodamine-phalloidin. Speeds were determined by a semiautomated tracking program and fitted to a Gaussian distribution. (Upper, minus tropomyosin) Myosin moved WT actin at a speed of $0.53 \pm 0.18 \mu m/s$ ($n = 2742$) and R258C filaments at a speed of $0.50 \pm 0.16 \mu m/s$ ($n = 3415$). The difference in speeds was statistically significant (Student’s t test, $P < 0.01$) because of the large number of filaments analyzed, but it has no physiological relevance. (Lower, plus tropomyosin) In the presence of smooth muscle tropomyosin, myosin moved WT actin at a speed of $0.74 \pm 0.19 \mu m/s$ ($n = 1294$) and R258C filaments at the slower speed of $0.43 \pm 0.15 \mu m/s$ ($n = 3743$) (Student’s t test, $P < 0.01$). Data from five independent actin preparations were pooled. (B) Speed of movement of WT and R258C actin filaments that were not stabilized with phalloidin. Filaments were visualized by incorporation of 25% WT actin that was directly labeled with rhodamine (solid blue bar, 100% WT; striped red bar, 50% WT and 50% R258C, solid red bar, 25% WT and 75% R258C). (Left) Speed of movement of WT filaments ($0.61 \pm 0.13 \mu m/s$; $n = 196$) decreased to $0.53 \pm 0.12 \mu m/s$ ($n = 160$) with 50% R258C and to $0.47 \pm 0.13 \mu m/s$ ($n = 180$) with 75% R258C. The difference between all pairs was statistically significant (Student’s t test, $P < 0.01$). (Right) Same comparison in the presence of smooth muscle tropomyosin (TM). The speed of movement of WT filaments ($0.60 \pm 0.10 \mu m/s$; $n = 170$) decreased to $0.43 \pm 0.09 \mu m/s$ ($n = 80$) with 50% R258C and to $0.31 \pm 0.08 \mu m/s$ ($n = 142$) with 75% R258C. The difference between all pairs was statistically significant (Student’s t test, $P < 0.01$). Data from five independent actin preparations were pooled. Filament speed was tracked manually. Temperature, 30 °C.

**Fig. 9.** Force dependence of smooth muscle myosin HMM interacting with WT or R258C filaments in the presence or absence of smooth muscle tropomyosin. (A) Schematic of experimental setup. Smooth muscle myosin HMM, with a biotin tag at the C terminus, was attached to 1-μm polystyrene beads coated with neutravidin. A myosin-coated bead was then captured by the laser trap and allowed to interact with actin immobilized on the surface. A force-feedback mechanism was used to keep force constant once an interaction was detected. QD, quadrant detector. (b) Sample trace showing the bead motion under different constant loads, varying from 1 to 5 pN. The solid yellow lines are linear fits to the raw data, shown in black. (c) Force-velocity curves for HMM interacting with WT (blue) or R258C (red) filaments in the presence (dashed lines, open symbols) or absence (solid lines, filled symbols) of tropomyosin. The lines are fit to the Hill equation. Error bars are SE.
Fig. 10. Summary of likely differences in cells containing only WT actin vs. cells expressing both WT and R258C actin. The left-hand portion of the schematic cell illustrates that myosin interacts productively with WT actin-tropomyosin to produce force and motion. Most actin is filamentous, with only a small pool of monomeric actin. The filaments that are decorated with tropomyosin are resistant to severing and shortening by cofilin. In contrast, cells expressing both WT and R258C actin would be expected to show slower filament sliding and have a lower force output. Moreover, it is expected that the pool of monomeric actin will increase relative to WT cells, because R258 has a higher critical concentration, a higher affinity for profilin, and is more susceptible to severing and shortening by cofilin.

and plasma membrane invagination during endocytosis and phagocytosis. Assembly is regulated by proteins, including formin and Arp2/3, that control nucleation and polymerization (reviewed in 15), and it is balanced by disassembly of older filaments by proteins in the ADF/cofilin family, which preferentially bind ADP-actin monomers (reviewed in 17). Cofilin accelerates remodeling of the actin network by severing actin filaments, and thus increasing the concentration of ends available for elongation and subunit exchange. Cofilin also depolymerizes filaments from the pointed ends, thereby increasing filament dynamics (27).

Cofilin is, however, also present in striated muscle cells that are considered to have considerably more stable actin filaments. In striated muscle cells, cofilin is thought to be involved in precise length control of the thin filament (28, 29). In vascular smooth muscle cells, increased cofilin activation upon dephosphorylation with slingshot phosphatase 1 has been implicated in vascular smooth muscle cell migration and neointima formation following vascular injury (30). Here, we show that R258C actin filaments are considerably more susceptible to cleavage by cofilin and show increased levels of shortening, compared with WT filaments. In essence, the increased sensitivity of R258C to cofilin should have the same effect as having more activated, unphosphorylated cofilin. Our results suggest that mutation of R258 is likely to result in enhanced actin dynamics and cell migration.

Although the molecular mechanism of severing is not known, it is affected by the structure and mechanical properties of the actin filament (31), which differ when R258 is mutated. Moreover, the binding of smooth muscle tropomyosin protects the WT filament from cofilin cleavage (32), the same is not true for the R258C filaments. This observation supports the idea that the position of tropomyosin on WT vs. R258C filaments differs, consistent with the inhibitory effect of tropomyosin on force and motion generation by myosin when it interacts with the mutant actin.

R258C Increases the Monomeric Pool of Actin by Binding Profilin Tightly. Profilin, one of the most abundant actin binding proteins inside the cell, binds to monomeric actin with high affinity and is the predominant substrate for actin assembly in vivo. Formins accelerate polymerization by increasing the local concentration of actin-profilin at the barbed end (reviewed in 15, 16). Here, we show that the affinity of R258C actin for profilin is ~20-fold higher than to WT. This tight binding decreases formin-mediated actin polymerization. The net result is that in cells containing R258C actin, we predict that the pool of monomeric actin is considerably larger than in WT cells. Another prediction is that more WT monomers will be incorporated into the actin filaments than the mutant in cells expressing both, potentially attenuating the detrimental effect of the mutant actin. The tighter binding to profilin is unexpected because the profilin binding site to actin is far from the location of the R258C mutation. This tight binding indicates the R258C mutation changes not only the actin filament structure but also the conformation of monomeric actin.

A previous study investigated the effect of mutating this same Arg residue to His in the backbone of budding yeast actin, which is only 86% identical to SM α-actin (26). The major defect those investigators observed in vitro was that yeast formin (Bni1) did not enhance polymerization but, instead, strongly capped the filament end. This result was consistent with their cellular results in which budding yeast cells expressing the mutant actin were delayed in their ability to rebuild their actin cytoskeleton following a challenge by the actin-depolymerizing drug latrunculin A. Although we also observed intrinsic polymerization defects with this mutation, and altered interaction of R258C with profilin and cofilin, our results did not show a defect in the mutant actin's interaction with formin for either R258H or R258C. We conclude that the effect of vascular disease-causing mutations is best studied in the SM α-actin backbone rather than a heterologous system.

A potential downstream effect of increasing the amount of monomeric actin in the cell is altering smooth muscle cell phenotype. The subcellular localization of the actin binding transcriptional cofactor myocardin-related transcription factor (MRTF-A) depends on the relative amount of monomeric vs. F-actin. When G-actin concentration in the cytoplasm increases, it binds to MRTF-A and retains it in the cytoplasm. Conversely, upon Rho-A-dependent actin polymerization, MRTF-A accumulates in the nucleus. Once in the nucleus, it interacts with serum response factor to control transcription of smooth muscle-specific genes (reviewed in 33, 34). Thus, a potential outcome is that cells containing R258C actin may lead to the smooth muscle cell developing a less contractile and more proliferative phenotype, because the increased monomeric pool may sequester MRTF-A in the cytoplasm.

Allosteric Effects of the R258C Mutation. A number of the observed defects in R258C were unexpected based solely on its location in actin. These defects include altered binding of smooth muscle tropomyosin and tighter binding of the mutant actin to profilin. The mutant residue is not directly part of the myosin, tropomyosin, or profilin binding site. These latter unexpected changes suggest new pathways of allosteric communication within the actin monomer that have been disturbed by the mutation. An allosteric connection between the profilin binding site and R258 is consistent with results from a hydrogen/deuterium exchange and MS study showing that Arg-258 and residues at the profilin binding site change when SM α-actin is exchanged with formin for either R258H or R258C. We conclude that the R258C mutation changes not only the actin filament structure but also the conformation of monomeric actin.
observed defects can be correlated with vascular disease outcomes and with patient survival rates. This study is the first step toward that goal. For example, both R258C and R391H lead to TAAD and moyamoya-like cerebrovascular disease, whereas other mutations lead only to TAAD (e.g., R288S, R316H; and still others act on TAAD and coronary artery disease (e.g., R149C, R118Q). “Loss-of-function” mutations that lead to smooth muscle cell weakness, a failure of tension sensing, and degeneration are thought to lead to thoracic aneurysms and aortic dissections. With R258C, we have evidence for loss of contractile function. In contrast, “gain-of-function” mutations that lead to cell proliferation and increased motility, or hyperplastic arterial smooth muscle cell growth, might lead to occlusive vascular diseases. With R258C, pathways that lead to cell proliferation could be mediated via the observed altered interactions of R258C filaments with cofillin and profilin, leading to increased pools of monomeric actin, and by the intrinsic lability of the mutant filament. The R258C mutation affects essentially all aspects of actin function that we measured in vitro. Cellular studies with fibroblasts and smooth muscle cells derived from patients with the R258C mutation, or mouse models, will establish how these defects are manifested in a cellular context.

Materials and Methods

Details of cloning, expression, and purification of proteins used in this study; the tropomyosin binding assay, actin labeling and filament formation for motility; the in vitro motility assay; measurement of ADP dissociation rates; and force data acquisition and analysis are provided in SI Materials and Methods.

Actin Cloning, Expression, and Purification. A chimeric construct composed of human SM α-actin (ACTA2 gene; accession number NP_001604) and human SM α-actin (accession number AA41977.1) was cloned into pcDNA2V for expression in the baculovirus/insect cell system. The Cys residue of actin was not included in the construct because it is not present in mature, processed protein. The thymosin-γ4 gene was separated from the actin gene by a 14 aa linker (ASSGGSSGGSSGAGA) to allow the thymosin-γ4 enough flexibility to occupy the barbed end binding site on the actin monomer, thus preventing the actin from polymerizing with itself or native SM α9 cell actin. A His6 tag was added at the C terminus for purification on a nickel affinity column. The final native residue of actin is Phe, which provides a site for chymotrypsin cleavage to remove the linker, thymosin-γ4, and the His6 tag completely. The native codons for both actin and thymosin-γ4 were replaced with Drasophila preferred codons.

For expression of human SM α-actin, infected SF9 cells (4 × 10⁶) were harvested 3 d after infection and lysed in 160 mL of 1 M Tris HCl (pH 7.5 at 4 °C), 0.6 M NaCl, 0.5 mM MgCl₂, 0.5 mM Na₂ATP, 1 mM DTT, 4% (v/vol) Triton X-100, 1 mM Tween-20, and protease inhibitors [0.5 mM 4-(2-aminoethyl)benzene-sulfonfluoride hydrochloride, 5 μM leupeptin, 0.5 mM tosyllysine chloromethyl ketone, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and then the insoluble fraction was dialyzed overnight against 10 mM Heps (pH 7.5), 0.25 mM CaCl₂, 0.3 M NaCl, 7 mM β-mercaptoethanol, 0.25 mM Na₂ATP, and 1 μM leupeptin. Following dialysis, the supernatant was applied to 5–10 mL of a nickel affinity column (HIS-Select; Sigma–Aldrich). Nonspecifically bound contaminating proteins were washed off with dialysis buffer containing 10 mM imidazole (pH 7.5). Actin was eluted with 100 mM imidazole, 10 mM Heps (pH 7.5), 0.25 mM CaCl₂, 0.3 M NaCl, 7 mM β-mercaptoethanol, 0.25 mM Na₂ATP, and 1 μM leupeptin. The peak fractions were pooled and dialyzed overnight against G-buffer [5 mM Tris (pH 8.26 at 4 °C), 0.2 mM CaCl₂, 0.1 mM Na₂O, 0.5 mM DTT, 0.2 mM Na₂ATP, 1 μM leupeptin]. The protein was clarified by centrifugation, and the thymosin-His tag was cleaved from the actin with chymotrypsin (chymotrypsininclot in a 1:80 weight ratio). The actin was separated from the thymosin-His tag by using a Mono Q 5 × 50 GL column (GE Healthcare) with a gradient of 0.3 M NaCl in 5 mM Tris (pH 8.26 at 4 °C), 0.2 mM CaCl₂, 0.1 mM Na₂O, 0.5 mM DTT, 0.2 mM Na₂ATP, and 1 μM leupeptin, followed by a step to 0.5 M NaCl. Peak fractions were pooled, concentrated, and dialyzed against 5 mM Tris (pH 8.26 at 4 °C), 0.2 mM CaCl₂, 0.1 mM Na₂O, 0.5 mM DTT, 0.2 mM Na₂ATP (pH 7), and 1 μM leupeptin.

Actin Polymerization Visualized by TIRF Microscopy. The 2x polymerization buffer consists of 20 mM imidazole (pH 7.5), 100 mM KCl, 8 mM MgCl₂, 2 mM EGTA, 0.5% (w/vol) bovine serum albumin, 0.26 mg/mL mouse oxidase, 0.10 mg/mL catalase, 6 mM glucose, and 20 mM DTT. The rinse buffer is composed of equal volumes of G-buffer [5 mM Tris (pH 8.26 at 4 °C), 0.2 mM CaCl₂, 0.2 mM Na₂ATP, 0.5 mM DTT] and 2x polymerization buffer.
For depolymerization, if the end protofilament in F-actin is WT, it dissociates with a rate of \( r_{\text{diss, WT}} \) (WT dissociation rate), but if it is R258C, it dissociates with a rate of \( r_{\text{diss, R258C}} \) (R258C mutant dissociation rate). The following two equations describe the dissociation reaction:

\[
\begin{align*}
\text{f} - \text{actin} (n) \cdot \text{WT} &+ \text{WT} \xrightleftharpoons{r_{\text{diss, WT}}} \text{f} - \text{actin} (n+1) \\
\text{f} - \text{actin} (n) \cdot \text{R258C} + \text{WT} &\xrightleftharpoons{r_{\text{diss, R258C}}} \text{f} - \text{actin} (n+1)
\end{align*}
\]

\[
\begin{align*}
\text{f} - \text{actin} (n) \cdot \text{WT} \xrightleftharpoons{r_{\text{diss, WT}} + r_{\text{diss, R258C}}} \text{f} - \text{actin} (n-1) \\
\text{f} - \text{actin} (n) \cdot \text{R258C} \xrightleftharpoons{r_{\text{diss, R258C}}} \text{f} - \text{actin} (n-1)
\end{align*}
\]

ACKNOWLEDGMENTS. We thank Dianna Milewicz, Kristine Kamm, and Susan Lowey for critical reading of the manuscript. We thank Jason Stumpf for the use of his microscope, and Alex Hodges and Elena Kremenshtova for their early contributions to this project. This work was supported by National Institutes of Health Grant P01 HL110869.