Biochemical basis of human disease-causing actin mutations

Sarah Elizabeth Bergeron

University of Iowa

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BIOCHEMICAL BASIS OF HUMAN DISEASE-CAUSING ACTIN MUTATIONS

by
Sarah E. Bergeron

An Abstract
Of a thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Molecular and Cellular Biology in the Graduate College of The University of Iowa

May 2011

Thesis Supervisor: Professor Peter A. Rubenstein
ABSTRACT

Actin isoform specific mutations have been identified as causes for various human diseases. These include twelve missense mutations in γ-nonmuscle actin leading to early onset autosomal dominate nonsyndromic hearing loss and twenty two missense mutations in α-smooth muscle actin leading to thoracic aortic aneurysms and dissections (TAAD). The molecular mechanisms leading to these human pathologies are mainly unknown, principally due to the inability to isolate pure mutant γ-nonmuscle actin and α-smooth muscle actin in quantities required for biochemical analysis. To begin to address these problems, I have individually expressed the human nonmuscle actin isoforms, β- and γ- nonmuscle actin, in a baculovirus expression system and characterized their biochemical properties. Surprisingly, despite a conserved amino acid difference of only 4 residues at or near the N-terminus, Ca-γ-actin exhibits slower monomeric and filamentous biochemical properties than β-actin. In the Mg-form, the difference between the two is smaller. Mixing experiments with Ca-actins reveal the two will readily co-polymerize. Calcium bound in the high affinity binding site of γ-actin may cause a selective energy barrier relative to β-actin that retards the equilibration between G- and F-monomer conformations resulting in a slower polymerizing actin with greater filament stability. This difference may be particularly important in sites such as the γ-actin-rich cochlear hair cell stereocilium where local mM calcium concentrations may exist.

In hair cells γ-nonmuscle actin seems to play a central role in stereocilia maintenance. To determine how the deafness causing D51N-γ-mutant actin mutation leads to deafness, I expressed and characterized it in the γ-actin background. The D51N mutation, lethal when cloned into yeast, displayed decreased filament stability and polymerization kinetics of an actin more dynamic than γ-actin. This result suggests that the hearing effects of the γ-actin mutations on the hearing apparatus are not simply caused by an inability to polymerize. The observed increased polymerization rates and
decreased filament stability may have major implications for the human disease, as the mutation may alter the ability of the γ-actin to fulfill its maintenance functions.

To address the basis by which TAAD mutations cause vascular dysfunction I introduced two of the know human mutations, N115T and R116Q, into yeast actin, 86% identical to human α-smooth muscle actin. I then generated yeast strains expressing each of these mutations as the sole actin in the cell to assess their effect on actin function in vivo and in vitro. Both mutant strains exhibited reduced ability to grow under a variety of stress conditions, although the N115T cells were more severely affected. In vitro the mutations caused altered thermostability and nucleotide exchange rates indicating effects on monomer conformation with R116Q the most severely affected. The N115T actin demonstrated a biphasic elongation phase during polymerization, while R116Q actin demonstrated a markedly extended nucleation phase. Allele-specific effects were also seen on critical concentration, rate of depolymerization and filament treadmilling. R116Q filaments were hypersensitive to severing by the actin-binding protein cofilin. In contrast, N115T filaments were hyposensitive to cofilin, despite near normal binding affinities of actin for cofilin. The mutant specific effects on actin behavior suggest that individual mechanisms may contribute to TAAD.

Understanding the mechanisms of actin dependent human diseases requires elucidation of the effects of the mutations on the behavior of actin per se, its regulation, and the impact on actin mediated processes within the cell. The work provided in this thesis and future studies will provide the information required to understand the pathways involved in these diseases and form innovative treatments for deafness and TAAD.

Abstract Approved: ________________________________________

Thesis Supervisor

____________________________________

Title and Department

____________________________________

Date
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by

Sarah E. Bergeron

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Molecular and Cellular Biology in the Graduate College of The University of Iowa

May 2011

Thesis Supervisor: Professor Peter A. Rubenstein
CERTIFICATE OF APPROVAL

_________________________________

PH.D. THESIS

This is to certify that the Ph.D. thesis of

Sarah E. Bergeron

has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Molecular and Cellular Biology at the May 2011 graduation.

Thesis Committee: ___________________________________
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Mark Stamnes

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M. Todd Washington
To my loving and understanding husband, Craig. And to my parents, who raised me as a scientist from day one.
The important thing is not to stop questioning. Curiosity has its own reason for existing. The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed.

Albert Einstein
ACKNOWLEDGMENTS

First I would like to thank my mentor, Pete, for pushing the boundaries of what I thought I could accomplish. Your guidance has been unwavering and constantly supportive, thank you. Next I would like to thank my husband, Craig, who always knows how to put a smile on my face in easy and hard times. You have been my rock and my soft place to land.

I would like to give my deepest gratitude to my parents, Kelly and Susan. They told me over and over again “You can do anything you put your mind to” and I have tried to live my life by those words. Thank you so much for always believing in me.

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ABSTRACT

Actin isoform specific mutations have been identified as causes for various human diseases. These include twelve missense mutations in γ-nonmuscle actin leading to early onset autosomal dominate nonsyndromic hearing loss and twenty two missense mutations in α-smooth muscle actin leading to thoracic aortic aneurysms and dissections (TAAD). The molecular mechanisms leading to these human pathologies are mainly unknown, principally due to the inability to isolate pure mutant γ-nonmuscle actin and α-smooth muscle actin in quantities required for biochemical analysis. To begin to address these problems, I have individually expressed the human nonmuscle actin isoforms, β- and γ- nonmuscle actin, in a baculovirus expression system and characterized their biochemical properties. Surprisingly, despite a conserved amino acid difference of only 4 residues at or near the N-terminus, Ca-γ-actin exhibits slower monomeric and filamentous biochemical properties than β-actin. In the Mg-form, the difference between the two is smaller. Mixing experiments with Ca-actins reveal the two will readily co-polymerize. Calcium bound in the high affinity binding site of γ-actin may cause a selective energy barrier relative to β-actin that retards the equilibration between G- and F-monomer conformations resulting in a slower polymerizing actin with greater filament stability. This difference may be particularly important in sites such as the γ-actin-rich cochlear hair cell stereocilium where local mM calcium concentrations may exist.

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decreased filament stability may have major implications for the human disease, as the mutation may alter the ability of the γ-actin to fulfill its maintenance functions.

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Understanding the mechanisms of actin dependent human diseases requires elucidation of the effects of the mutations on the behavior of actin per se, its regulation, and the impact on actin mediated processes within the cell. The work provided in this thesis and future studies will provide the information required to understand the pathways involved in these diseases and form innovative treatments for deafness and TAAD.
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<th>Description</th>
</tr>
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<tr>
<td>ABP(s)</td>
<td>Actin-binding protein(s)</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5’-diphosphate</td>
</tr>
<tr>
<td>ADP-G-actin</td>
<td>ADP bound monomeric actin</td>
</tr>
<tr>
<td>ADP-F-actin</td>
<td>ADP bound filamentous actin</td>
</tr>
<tr>
<td>ADP-P_i-actin</td>
<td>ADP bound actin with phosphate group retained</td>
</tr>
<tr>
<td>Aip1p</td>
<td>Actin-interacting protein 1</td>
</tr>
<tr>
<td>Arp</td>
<td>Actin related protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>ATP-G-actin</td>
<td>ATP bound monomeric actin</td>
</tr>
<tr>
<td>ATP-F-actin</td>
<td>ATP bound filamentous actin</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine 5’-triphosphatase</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>Ca^{2+}-G-actin</td>
<td>Calcium bound monomeric actin</td>
</tr>
<tr>
<td>Ca^{2+}-G-actin</td>
<td>Calcium bound filamentous actin</td>
</tr>
<tr>
<td>Cc</td>
<td>Critical concentration</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’-6-Diaminodino-2-phenylindole</td>
</tr>
<tr>
<td>DE52(DEAE)</td>
<td>Diethyl-aminoethyl-cellulose</td>
</tr>
<tr>
<td>DFNA</td>
<td>Deafness nonsyndromic autosomal dominant</td>
</tr>
<tr>
<td>DFNB</td>
<td>Deafness nonsyndromic autosomal recessive</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase I</td>
<td>Deoxyribonuclease I</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>Etheno (ε)-ATP</td>
<td>1,N^6-ethenoadenosine 5’-triphosphate</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>G-actin</td>
<td>Globular (monomeric) actin</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>H/D</td>
<td>Hydrogen deuterium</td>
</tr>
<tr>
<td>K_d</td>
<td>Equilibrium disassociation constant</td>
</tr>
<tr>
<td>L.S.</td>
<td>Light Scattering</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>Magnesium ion</td>
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<td>Mg^{2+}-G-actin</td>
<td>Magnesium bound monomeric actin</td>
</tr>
<tr>
<td>Mg^{2+}-G-actin</td>
<td>Magnesium bound filamentous actin</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered solution</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>P_i</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein data bank</td>
</tr>
<tr>
<td>Pyrene</td>
<td>N-(1-pyrenel) maleimide</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TAAD</td>
<td>Thoracic aortic aneurism and dissection</td>
</tr>
<tr>
<td>TIRFM</td>
<td>Total internal reflection fluorescence microscopy</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TMR</td>
<td>Tetramethylrhodamine-5-maleimide</td>
</tr>
<tr>
<td>Tris-HCL</td>
<td>[Tris(hydroxymethyl) aminomethane] base adjusted to pH 7.5 using HCL (hydrochloric acid)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YPD</td>
<td>Medium containing yeast extract, peptone, and dextrose</td>
</tr>
<tr>
<td>YPG</td>
<td>Medium containing yeast extract, peptone, and glycerol</td>
</tr>
<tr>
<td>ZBP1</td>
<td>Zipcode-binding protein 1</td>
</tr>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>γ</td>
<td>gamma</td>
</tr>
<tr>
<td>ΔAip1p</td>
<td>Yeast strain where the gene encoding Aip1p has been deleted</td>
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</table>
CHAPTER 1. INTRODUCTION

Actin is a 42kDa protein which can reversibly polymerize into a double-stranded filament. Actin is the main component of the microfilament cytoskeletal system and is present in almost all eukaryotic cells. Actin represents a large proportion of the total protein in most cells, although levels do vary between different cell types. Years of research have provided evidence that actin has many functions in living cells, including but not limited to internal mechanical support, tracks for intracellular transport, and cellular movement (1). Mutations in actin have been shown to cause human disease, although the mechanisms behind the diseased phenotypes are not known. In this thesis I will focus on two classes of these mutations: γ-nonmuscle actin mutations which lead to deafness and α-smooth muscle actin mutations which lead to thoracic aortic aneurysms and dissections. However, before discussing my work on these disease causing mutations I will present background on actin and its regulation as a foundation for my work.

Actin Structure, Function, and Regulation

Actin can exist as a monomer (globular or G-actin) which can reversibly polymerize into a filament (filamentous or F-actin), in a salt dependent fashion. In vivo, the filamentous state is highly favored due to ionic concentrations in the cell. Yet in many cells almost 50% of the actin in the cell is monomeric. This situation calls for the G to F transition to be regulated in vivo, usually through interactions with various actin binding proteins (1). This regulation occurs at multiple levels: the monomeric actin can be sequestered, nucleation of filament formation is regulated, and filament stability is tightly controlled.

Monomer and Filament Structure and Function

Monomeric G-actin is a clam-shaped molecule which can be divided into 4 distinct subdomains (Figure 1-1A). In order to maintain its native state, the actin must be
bound to either ATP or ADP and a divalent cation, either Mg$^{2+}$ or Ca$^{2+}$ (2-6) in the central cleft separating the two halves of the protein. Polymerization of actin monomers leads to a right-handed, double-helical structure (7) (Figure 1-1B). In vitro one can spontaneously induce the G to F transition by placing the actin in physiological concentrations of neutral salts, 50-150 mM KCl and 1-2 mM MgCl$_2$ or CaCl$_2$. In order for the actin to polymerize it must undergo a salt-induced conformational change, making the G-actin polymerization competent. This polymerization can only occur above a minimum concentration of actin monomers, the critical concentration ($C_c$) of actin, which can vary with actin isoforms.

The first step in actin polymerization is the energetically unfavorable nucleation phase, in which actin monomers come together to form a nucleus of ~3 monomers. Next, actin monomers rapidly add to both ends of the forming filament in a process called elongation. This addition continues until the concentration of monomers reaches the critical concentration. At this time the polymers attain a steady state where actin monomers are released from one end of the actin filament at the same rate as they add to the opposing end. The formed filaments have polarity. The actin monomers within the filament all orient with their nucleotide cleft toward the same end of the filament, designated the pointed or minus end. This terminology stems from visualizing actin filaments decorated with myosin. Bound myosin heads cause an appearance of arrowheads in electron micrographs, hence the ends were designated pointed (or minus) and barbed (or plus). The final step in actin polymerization is the steady state or treadmilling phase. This is achieved because the critical concentrations of the ends of the actin filament are not equal (8-10). The barbed end of the actin filament has a ten-fold lower monomer concentration requirement for addition to the filament than the pointed (11).

This filament asymmetry is translated to the monomer itself with the barbed end consisting of actin subdomains 1 and 3 (12). When the concentration of monomers in
solution is between the critical concentrations (Cc) of the ends of the actin filament, steady state is achieved and monomers add to the barbed end, the preferred site for monomer addition, while monomers disassociate from the pointed end. The barbed end of the actin participates in monomer/monomer contacts longitudinally along the actin helix, and it is the region at which a number of proteins, such as cofilin, profilin, and formin, regulate actin polymerization and exert their control (13-16).

The presence of either Mg$^{2+}$ or Ca$^{2+}$ can greatly impact the dynamics of the actin filament. When bound to Mg$^{2+}$, nucleation of G-actin proceeds much more rapidly than when bound to Ca$^{2+}$ (17-19). Once formed, Mg$^{2+}$-G-actin filaments are more flexible than Ca$^{2+}$-G-actin filaments (20-23). Additionally, Mg$^{2+}$-actin has a 3-4 fold decrease in Cc than Ca$^{2+}$-actin which means that since Mg$^{2+}$-actin requires a lower concentration of actin to maintain filament treadmilling it is a more stable form of actin vs. Ca$^{2+}$-actin (18, 24).

The structural and dynamic properties of G- and F-actin at least partially depend on the state of the bound nucleotide (25). Crystallization studies of G-actin have revealed structural differences between ATP- and ADP-actin. The major differences were found primarily in the loops adjacent to bound nucleotide and in the DNase I binding-loop (D-loop) of subdomain 2 of actin (26, 27). For the DNase I loop, there is a conformational change in a 12-residue peptide (His-40 to Asp-51) which converts from an unstructured loop in the ATP- form to an $\alpha$-helix in the ADP- form. This conformational difference has significant impact on actin functions, as ATP-actin can polymerize faster than ADP-actin monomers (28). Also some actin binding proteins associate with ADP-actin with a higher affinity than ATP-actin (cofilin has 2 fold higher affinity) (25). Both pieces of evidence support the model of actin undergoing conformational changes in response to the bound nucleotide; providing another layer to actin regulation.

Polymerization of ATP-G-actin into filaments is associated with hydrolysis of the tightly bound ATP, and it is followed by release of an inorganic phosphate (P$_i$), at a rate determined by the individual actin isoform. Accordingly, monomers of the filament
contain, respectively from the barbed to pointed end, tightly bound ATP, ADP-P_1, or ADP. P_1 release from actin filament subunits subsequent to ATP hydrolysis destabilizes the filament and increases the Cc for assembly. This instability is predominately a result of an alteration in the intermonomer contacts between monomers due to the release of P_1 (29, 30).

Human Actin Isoforms

Multiple isoforms of actin, or isoactins, are found in a number of organisms, although the functional significance of these multiple isoforms is still not well understood. Mammals, birds, and fish synthesize six completely conserved different actin isoforms in a tissue specific manner, clearly suggesting a functional basis for this actin isoform conservation. Isoactin specific function has been demonstrated in the generation of contractile force in muscle and also nonmuscle cell processes such as cell shape determination, cell motility, endo/exocytosis and cytokinesis (1, 31).

While there is only a small degree of residue variability between actin isoforms (no less than 93% identity between mammalian isoactins), one of the divergent regions is the N-terminus (the amino acid sequence alignment of each isoform provided in Figure 1-2). The N-terminus contains a group of acidic residues which can alter the net charge of the actin isoform. The actin isoforms can be identified by this difference in charge which leads to varying isoelectric points, designated α, β, and γ in order of acidic to basic (32-35). Actins can then be further subdivided into muscle and nonmuscle isoactins. Nonmuscle β- and γ-isoactins are present in nonmuscle cells in different proportions depending on the cell type and state of differentiation (35, 36). Additionally these nonmuscle isoactin can be found in some muscle cells in lesser amounts (37-40). The muscle specific actin isoforms include: α-skeletal, α-cardiac, and both an α- and a γ-smooth muscle actin (32). In vascular smooth muscle cells, the predominant isoactin is the α-smooth muscle isoform; alternatively, in enteric smooth muscle tissue γ-smooth
muscle isoactin predominates. Smooth muscle cells also possess a unique actin distribution where roughly half the actin in the cell is comprised of nonmuscle actins.

A detailed study of biochemical differences among the mammalian actin isoforms has been hindered by the difficulty in obtaining large amounts of the individual isoforms in native form. Due to the high degree of similarity between the isoactins, biochemical separation of the isoactins in sufficient yield for protein analysis is not possible. Two of the most similar actin isoforms, β- and γ-nonmuscle actins, vary by only 4 biochemically similar residues with a total difference of 4 carbons at or near the N-terminus. The N-terminal D-D-D- of β-nonmuscle actin is replaced by E-E-E- in γ-nonmuscle actin and V10 in β-nonmuscle actin is converted to I in γ-nonmuscle actin. These differences would be expected to cause little if any divergence in their properties. However, in motile cells, β-nonmuscle actin tends to be concentrated in dynamic areas at or near the leading edge of the cell, whereas γ-nonmuscle actin is found predominantly in stress fibers (41-44).

Multiple hypotheses have been postulated as to how these isoactins are sorted intracellularly. Localization of β-nonmuscle actin in fibroblasts can be partially regulated through a 54-nucleotide sequence found in the 3′ UTR, which interacts with the RNA binding protein ZBP1 (zipcode binding protein 1) to target the β-nonmuscle actin transcript (45-49). In situ hybridization has shown that β-nonmuscle actin mRNA is localized at the leading edge of lamellipodia of motile fibroblasts (44, 50, 51). The localization of β-nonmuscle actin’s mRNA correlates with the localization of β-nonmuscle actin protein to apical cellular structures such as filaments in microvilli of epithelia and auditory hair cells (52) and the leading edge of lamellipodia and filopodia of motile cells (49). No other actin isoform mRNA is specifically localized to a cellular compartment. The specific targeting of β-nonmuscle actin mRNA near these structures suggests that local protein synthesis can contribute to the localization of β-nonmuscle actin protein (45).
Whole-mouse knockouts of each actin isoform lead to specific phenotypes with consequent upregulation of the remaining actin isoforms in some cases. Attempts to rescue the null phenotypes through overexpression of other isoforms (except for α-skeletal actin knockout with α-cardiac rescue (53)) does not completely rescue the null phenotype indicating overlapping but specific functions of the isoactins (54, 55). Interestingly, a β-nonmuscle actin knockout mouse is embryonic lethal (56, 57) while a γ-nonmuscle knockout leads to progressive hearing loss in addition to reduced viability, delayed embryonic cardiac development, small size, and skeletal muscle myopathy (58, 59). The γ-nonmuscle knockout mice that do not survive have respiratory distress within 24 hrs of birth and soon after die, leading to the observed reduced viability (60). A α-skeletal muscle actin knockout mouse presents with a muscle weakness phenotype and is lethal by post-natal day 9 (61). α-Cardiac muscle actin knockout mice are either embryonic lethal or die shortly after birth. These mice present with disorganized myofibrils, bundles of actomyosin filaments that run from one end of a muscle cell to the other important for muscle contraction (55). α-Smooth muscle actin null mice are viable but have defects in vascular contractility and blood pressure regulation (62).

It has been suggested that some actin binding proteins interact preferentially with specific isoactins. Consistent with this hypothesis, the actin binding proteins cofilin (63), ezrin (64), l-plastin (65), βCAP73 (66), thymosin β4 (67), and profilin (68) have been shown to discriminate between muscle and nonmuscle isoactins while Annexin V is reported to bind γ-nonmuscle actin (69), but not β-nonmuscle actin, although unpublished data from our lab suggest otherwise. A final possibility to explain specific isoactin function, as addressed in this thesis work, is that the biochemical differences between the actin isoforms could intrinsically lead to differential properties of the actin filament thus making the isoactins inherently better suited to their specific functions within the cell.
Regulation of Actin Dynamics by Actin Binding Proteins

Actin dynamics are regulated \textit{in vivo} through interactions with various actin binding proteins (ABPs). These proteins are involved in remodeling of the actin cytoskeleton, including forming, destroying, and reforming the vast array of actin-rich structures that exist in eukaryotic cells. All eukaryotic cells contain a core set of ABPs including proteins that are involved in monomer sequestration, actin filament nucleation, filament capping, filament bundling, filament stabilizing, and severing of the filament. Most ABPs have multiple functions in regulating the dynamics and organization of the actin cytoskeleton.

Actin is one of, if not the most, abundant protein in most eukaryotic cells, and its concentration \textit{in vivo} (in most nonmuscle cells 100-500\textmu M (70-73)) is much higher than the Cc of the actin (~0.1 and 0.7\textmu M at the barbed and pointed end respectively (74)). Cells have utilized actin monomer binding proteins, like profilin, to inhibit spontaneous polymerization of actin. Profilin is a small ubiquitously expressed abundant ABP which binds to monomeric actin and has four known biological effects. First, profilin can bind to and sequester actin monomers by sterically hindering association of the monomer-monomer interaction interface required for the formation of actin dimers/trimers (74). Second, binding of profilin to actin (at low profilin:actin ratios) actually promotes barbed end additions of actin filaments (75). Third, it enhances elongation of actin filaments capped with the ABP formin (as described below). Fourth, it accelerates exchange of the bound nucleotide (ATP/ADP) in actin monomers (76). These functions contribute to profilin’s ability to enhance actin turnover \textit{in vivo} and to facilitate the conversion of actin monomers to a polymerization competent state (ATP-bound) (77).

Examples of actin nucleators are the Arp2/3 complex and formin. The Arp2/3 complex nucleates actin filament formation by binding to a preformed filament and mimicking a new filament barbed end, which then elongates at a 70° angle from the
preexisting strand. Dissociation of Arp 2/3 frees the newly formed filament from the mother filament (78-80). Formin nucleates actin by binding to and stabilizing actin dimers and/or trimers, short lived polymerization intermediates (81). Formin also remains associated with the barbed end of the forming filament and guides the addition of monomers to the filament; this activity is called processive capping (82). Formin also works in cooperation with profilin to further enhance elongation. As described above, when profilin is bound to actin, it sequesters actin monomers to prevent polymerization (75, 77, 83); but in the presence of formin, the profilin-actin complex binds to formin at its Formin Homology 1 Domain promoting addition of the actin to the barbed end of the filament (81, 84). The profilin-actin-formin cooperation can enhance filament elongation by up to 5 fold compared to formin alone (15).

Actin bundling proteins cross-link actin filaments either by having multiple actin binding domains (ABDs) or through oligomerization. Examples in the superfamily of actin cross-linking proteins are filamin, spectrin, dystrophin, alpha-actinin, and fimbrin. These ABPs are composed of a pair of actin-binding domains, a variable number of spacer domains, and sometimes a calmodulin-like calcium-binding domain (85). Structurally these ABPs generally contact actin at 1) subdomain two and a part of subdomain one and 2) the bottom of subdomain one in the next actin subunit along the filament (86-89). Some formins, in addition to barbed end binding, can also bind actin filament sides (90) and bundle filaments (91, 92). These bundling proteins are predominantly localized to the leading edge and focal contacts of migrating cells (93-96). Additionally, actin crosslinking proteins frequently function as molecular scaffolds, connecting cytoskeletal networks to extracellular matrix proteins (97-100).

Filament stabilizing ABPs, including tropomyosins, decorate the sides of actin filaments and reduce the rate of subunit dissociation from either the barbed or the pointed end of the actin filament. Actin capping proteins can also stabilize actin filaments. Proteins such as the CapZ/conventional capping protein family tightly associate with and
cap the barbed end of actin filaments. This association limits the local concentration of free actin barbed ends and at the same time increases the rate of formation of new filaments because the actin monomer pool remains preserved (101). Capping proteins can also regulate average filament length; when they associate with free barbed ends they restrict filament growth and maintain short filament length (102, 103).

One example of a severing/disassembling ABP is cofilin which is essential in yeast. Cofilin is a 12-19kDa protein involved in cell growth, differentiation, division, membrane organization and motility (104). It belongs to the actin-depolymerizing factor (ADF)/cofilin family (105, 106) which functions by binding cooperatively to actin filaments (107, 108) and inducing a twist into the actin filament (14, 109). In most cases, this twist induces fragmentation or severing of the actin filament which increases monomer loss from the pointed end (14, 110). While this severing activity seems potentially detrimental, cofilin activity can increase the turnover of actin filaments and also provide new filament ends for polymerization. Cofilin can also bind actin monomer and regulate the G-actin pool by specifically binding ADP-actin monomers and inhibiting nucleotide exchange (111). Therefore, the effect of cofilin on either actin filament growth or reduction depends on the accessibility of actin monomers and the local concentration of cofilin within that distinct subcellular compartment of the cell (112).

Alteration of the interaction between actin and one or more of these ABPs could have significant effects on the regulation of actin filament assembly, organization, and turnover. Analysis of the effects of actin mutations on actin-ABP interactions could provide mechanistic insight into the cause of some actin related human diseases and supply information on normal pathways involved in cytoskeletal network assembly and function.
Actin and Human Disease

Mutations in five of the six actin isoforms have been shown to cause human disease. α-Cardiac actin mutations can cause dilated cardiomyopathy (113) and hypertrophic cardiomyopathy (114). Mutations in β-nonmuscle actin lead to neutrophil dysfunction and recurrent infections (115), and a mutation in β-nonmuscle actin has also been shown to cause autosomal dominant developmental malformations and dystonia (116). α-Skeletal muscle actin mutations lead to nemaline myopathy, intranuclear rod myopathy, and actin myopathy (117-121). Mutations in γ-nonmuscle actin lead to autosomal dominant hearing loss (122, 123). Mutations in α-smooth muscle actin lead to thoracic aortic aneurysms and dissections in combination with allele specific associated cardiovascular defects (124, 125). γ-Smooth muscle actin is the only actin isoform in which no human disease related mutations have been discovered. Additionally, it is known that massive remodeling of the actin cytoskeleton promotes invasive tumor growth (126-128). The discrete disease phenotypes associated with each actin isoform provides further support for their functional specificity.

Hearing and Actin Related Deafness

Hearing loss, the most common sensory defect in America, affects approximately 28 million individuals (~1/10). Additionally, two to three out of every 1,000 children in the United States are born deaf or with a type of hearing impairment (129, 130). Hearing depends on sound-dependent distortion of mechanosensory hair cells within the cochlea of the inner ear. In these cells, staircase arrangements of 20-300 hair-like receptors called stereocilia protrude from the apical surface (Figure 1-3) (131). Mechanical deflection of these structures results in the opening of gated ion channels located on the surface of these protrusions. This opening results in conversion of sound-dependent distortion into the propagation of neuronal signals that are relayed to the brain (132).
Actin within Hair Cells

Hair cell function is contingent upon proper functioning of the actin cytoskeleton which, in this cell, is divided into three distinct compartments (Figure 1-3). Each is organized in different ways and has to function properly for the perception of sound to occur (133-135). Structural integrity and proper function of the hair cell cytoskeleton depend on the interplay between actin filaments and a number of ABPs in order to stabilize the stereocilium, control its length, and help organize the cuticular plate. Examples of a few of these ABPs include cofilin, profilin, and formin which may control filament nucleation and assembly, either in the cuticular plate or in the stereociliary bundle (131). The importance of some of these proteins, such as formin, to hearing is demonstrated by the fact that a formin defect causes deafness (136). Since this mutation most likely causes altered interactions with actin, specific mutations in actin affecting its interaction with each of these ABPs might also lead to a similar or related deafness phenotypes.

Hair cell actin is composed of two different isoforms of nonmuscle actin: $\beta$ and $\gamma$. There are a limited number of cells in the body where the $\gamma$-isoform predominates over the $\beta$ form; hair cells are one of them (70% $\gamma$-nonmuscle and 30% $\beta$-nonmuscle actin) (123). The localization of $\beta$- vs. $\gamma$-nonmuscle actin has been controversial, but the most recent immunolocalization with dye-conjugated primary antibodies has demonstrated that both isoforms are uniformly distributed throughout the hair cell stereocilium (137). The Ervasti lab and colleagues determined that mice with either $\beta$- or $\gamma$-nonmuscle actin null hair cells do form functional, morphologically normal stereocilia and have normal hearing at young ages (56-58). However, in the absence of both nonmuscle actin isoforms, a hair cell specific double $\beta$- and $\gamma$-nonmuscle actin knockout, stereocilia do not form (137). This suggests that a nonmuscle actin isoform is required for hair cell development, however, $\beta$- and $\gamma$-nonmuscle actin are redundant for this phase of hair cell formation. Following development, hair cell stereocilia must be maintained as hair cells
do not regenerate in mammals. During this second maintenance phase of mammalian hair cells, both \( \beta \)- and \( \gamma \)-nonmuscle actin are required as a null mouse for each isoactin develops a specific progressive hearing loss phenotype, along with discrete stereociliary pathology (137).

Loss of \( \beta \)-nonmuscle actin preferentially affects high frequency hearing, while \( \gamma \)-nonmuscle actin null hair cells affect all frequencies and progresses more rapidly. Morphologically \( \gamma \)-nonmuscle actin deficient hair cells randomly lose a large percentage of their stereocilia, but those stereocilia that remain preserve their normal length. In contrast, \( \beta \)-nonmuscle actin deficient hair cells display only slightly fewer stereocilia than control cells, but these stereocilia become uniformly progressively shorter than control stereocilia with age. These different patterns of hearing loss suggest that \( \beta \)- and \( \gamma \)-nonmuscle actin, despite exhibiting nearly identical protein sequences and inner hair cell stereocilia localization patterns, have different and non-redundant functions in the auditory hair cell stereocilia (137).

Review of Biochemical Characterization of Mutations in \( \gamma \)-Nonmuscle Actin Leading to Deafness

Recently it was discovered that ten point mutations in the \( \gamma \)-nonmuscle actin gene cause autosomal dominant non-syndromic hearing loss (DFNA 20/26) (122, 123, 138-141). Each mutation is confined to a single family. Patients with these mutations begin noticing hearing loss in their early teens to late twenties depending on the mutation. Hearing loss begins with high frequency sounds and progresses to overall hearing loss as the patient ages. Each deafness mutation is mapped on the actin structure in Figure 1-4. All, except D51N and E241K, are located in subdomains 1 and 3 of the actin monomer (Figure 1-4) (142).

For many reasons, little is known at the molecular level concerning how these ten mutations result in a malfunctioning sensory cell. First it is extremely difficult to purify
the β-, wild type γ-, and mutant γ-nonmuscle isoactins present in the hair cell apart from one another. Second, even if it were possible to separate the isoactins, one could not obtain quantities sufficient for biochemical analysis. And third, until recently no animal model has been available. At present the only animal model is a γ-nonmuscle knock-out mouse, as described above from the Ervasti lab (58). Because of these obstacles, our lab has initiated analysis of most of the deafness-causing γ-actin mutations in a yeast actin model system (139, 143, 144). Our published data so far suggests the mutations mostly affect regulation by different ABPs and not polymerization per se. Acquisition of such insight is important, as characterization of altered protein-protein interactions resulting from the mutations will provide clues to the molecular basis of both diseased and normal hair cell function and mechanisms relating to actin function in general.

Yeast/Yeast Actin Model System

Yeast has a single essential actin gene, ACT1 (145). Yeast actin has a high degree of homology with the mammalian actin isoforms; the greatest divergence is ~14% between yeast and mammalian muscle actin isoforms (Figure 1-2). All ten of the residues at which the proposed deafness-causing mutations occur in γ-nonmuscle actin are identical in yeast actin. Yeast also contains most of the actin binding proteins found in the human, and there is generally little if any barrier to interactions between actin cytoskeletal proteins from yeast and mammalian cells, although there are quantitative differences (146-149). The wild type (WT) yeast actin gene can easily and quickly be replaced by a mutant actin gene using site-directed mutagenesis and plasmid shuffling. This provides a system whereby the mutant actin is the sole actin expressed by the cell (150), so in vivo phenotypes associated with the mutations are not masked by the presence of wild type actin. I can therefore assess the effect of the mutation on a number of indicators of in vivo actin-dependent function in the yeast: growth under different types of environmental stress, formation of a polarized actin cytoskeleton, and proper
mitochondrial and vacuolar morphology and function using established growth and cytological assays (151).

Mutant actins, purified from these cells by our established protocols (147, 149, 151-155), can then be analyzed biochemically to determine the effects each mutation has on actin function. Finally, one of the main advantages of the yeast system is that one can correlate the effects of the actin mutations observed in vitro with the altered actin function in vivo. As described above, our lab has successfully utilized the yeast model system to characterize the effect of some of the deafness-causing γ-actin mutations on actin function (139, 143, 144).

**Baculovirus Model System**

Yeast actin and human γ-nonmuscle actin have 90% sequence identity. Although this makes yeast a good model system, using human actin found in the inner ear might be more relevant to the actual disease since the 10% difference could alter the phenotype observed in vitro. It had not been possible previously to produce significant quantities of pure human γ-nonmuscle actin, as it is extremely difficult to resolve the structurally similar isoactins found in almost all cell types. One solution to this problem is to express the mammalian actin isoforms in the baculovirus expression system. The first labs to successfully express an actin isoform using the baculovirus system were the Laing lab for α-skeletal actin (156) and the Trybus (157, 158) and Dawson (159) labs for α-cardiac actin. Initially, the characterization of the biochemical properties of each of the human isoforms be required to 1) determine if the human γ-nonmuscle actin and yeast actin have similar biochemical properties, which may validate our previous work in the yeast system, 2) generate a baseline upon which to judge the effect of the deafness mutants in this system, and 3) investigate the biochemical properties of pure individual isoforms of mammalian nonmuscle actins. I have used this system, in **Chapter 2**, to initially characterize the β- and γ-nonmuscle actin isoforms. Using these results as a baseline, in
Chapter 3, I have expressed a deafness causing γ-nonmuscle actin mutant, D51N, and determined the biochemical effects of the mutation on actin function.

Although this system has allowed us to analyze the D51N deafness causing γ-nonmuscle actin mutant in the γ-nonmuscle actin background, it is not without its own problems. As opposed to a yeast model system, one cannot assess the effects of the mutations on actin in the virally infected insect cells producing them, making interpretation of results difficult. Therefore one cannot correlate *in vitro* biochemical properties of the actin with an *in vivo* phenotype/function. Second, there is likely to be a substantial quantity of insect actin present with the purified γ-nonmuscle protein. Tagging the actin with a His tag for further purification is unadvisable because while the tagged actin is able to rescue a null actin cell the cellular phenotype is not normal in yeast (160). Third, production of mutant proteins in the baculovirus system is more time-consuming, labor intensive, and expensive than making the mutant yeast actins.

Thoracic Aortic Aneurysms and Dissections

Another disease associated with mutations in α-smooth muscle actin is thoracic aortic aneurysms and dissections (TAAD). TAAD is the 15th leading cause of death in the United States. Thoracic aortic aneurysms affect 1 per 10,000 individuals and are increasing in prevalence. Aortic aneurysms are frequently asymptomatic prior to dissection or rupture, making identification of individuals at risk difficult (161). Thoracic aneurysms and dissections occur when blood from the aortic lumen enters the wall of the aorta through an intimal tear and dissects along the plane of the aortic wall creating a false lumen. This false lumen fills with blood hence enlarging the diameter of the aorta, creating an aneurysm (Figure 1-5A vs. 1-5B). TAAD is associated with a high level of morbidity and mortality regardless of ongoing progress in surgical techniques (162). Despite having a clear pathological phenotype, aortic medial layer degeneration with loss and fragmentation of elastic fibers and accumulation of proteoglycans (Figure 1-5C), the
underlying mechanistic cause of TAAD is unknown. Medial degeneration could be due to either smooth muscle cell loss or hyperplasia in the aortic media.

Of the 30,000 deaths per year caused by ruptured TAAD, approximately 20% are the result of an inherited disorder (163). Four genes have been identified with familial TAAD and account for approximately 20% of familial TAAD cases. Mutations in the transforming growth factor-β type 1 and type 2 receptors (TGFBR1 and TGFBR2) comprises >5% of TAAD patients, smooth muscle specific myosin heavy chain (MYH11) comprises >1% of TAAD patients, and smooth muscle α-actin (ACTA2) comprises 14% of familial TAAD patients (124, 162, 164, 165). The genetic data collected thus far demonstrates that mutations in α-smooth muscle (α-SM) actin are the leading genetic cause of this disease.

Guo 2007 (124) reported that aortic samples from patients with α-smooth muscle actin mutations displayed two distinct phenotypes. First, within the medial layer of the aorta there were large areas of vascular smooth muscle cell loss, as determined by immunohistology with a monoclonal α-smooth muscle actin antibody. The vascular smooth muscle cells that remained were randomly orientated and had lost their normal parallel orientation to the aortic lumen. The second observed phenotype was an increase in the presence of vascular smooth muscle cells in the vaso vasorum. The vaso vasorum is a network of small blood vessels that supply oxygenated blood to the aorta. Vascular smooth muscle cell proliferation in the vaso vasorum could lead to a decrease in oxygenation (hypoxia) of the aorta and eventually cause the observed vascular smooth muscle loss in the medial aortic layer.

Aortic Function

The aorta is the largest artery in the body, and it functions to transport oxygenated blood from the heart to the body and regulate blood flow and pulse pressure (Figure 1-5A). The length of the aorta can be divided into 3 sections, the ascending aorta
immediately adjacent to the left ventricle of the heart, the aortic arch or the peak of the aorta (looks like an inverted “U”), and the descending aorta extending from the aortic arch to a point in the abdomen where the aorta branches off into smaller arteries. The aorta is comprised of three layers. The tunica intima is the innermost layer followed by the tunica media, a smooth muscle component, and the outer tunica adventitia (Figure 1-5C). The strength and elasticity of the aorta is due to the medial layer composed of concentrically arranged elastic fibers and smooth muscle cells. The smooth muscle cells are longitudinally oriented and dispersed among the circular elastic fibers.

The pathologic hallmark of TAAD is the degeneration of the tunica media with fragmentation and loss of elastin and dysregulation of smooth muscle cells (166). These features led investigators to analyze vascular smooth muscle cells in this disorder and, more specifically, their contractile proteins. Since the majority of the genetic causes of TAAD are due to mutations in contractile proteins, it is important to understand how these proteins function within vascular smooth muscle cells (124, 125).

**Actin Function within Muscle Cells**

The primary physiological function of the contractile unit of the muscle cell is its ability to dynamically change its length and propagate that change into muscle tissue contraction (167, 168). Cellular contraction in striated muscle cells is caused by the sliding of myosin (Type II) and muscle actin (α-skeletal or α-cardiac depending on the cell type) filaments over one another (169). This is accomplished through cross-bridge cycling (Figure 1-6), in which myosin heads from the myosin thick filament interact with the actin thin filament and the myosin head tilts via an ATP dependent process pulling the thin filament in the direction of the myosin tail. The myosin head releases the actin filament, reverses the tilt to its original position, re-associates with the actin filament, and repeats the above actions (170). While it is proposed that a similar actin-myosin interaction is responsible for contraction in smooth muscle cells, there are major
differences in the structure of the contractile unit between skeletal/cardiac (striated) and smooth muscle cells.

In skeletal/cardiac muscle, the sarcomere is the basic functional unit, in which uniform lengths of actin thin filaments are oriented in opposite directions at each end of a sarcomeric unit (Figure 1-7). Myosin thick filaments have myosin heads in bipolar orientations aligned in the middle of sarcomeres (Figure 1-8A). Cardiac and skeletal muscles are striated, where thin and thick filaments are laterally aligned perpendicular to the Z-disks. Thin filaments are oriented in opposite directions at each end of a sarcomeric unit, an orientation that is crucial for production of contractile forces by movement of the myosin to the barbed end of the thin filaments. The barbed ends of thin filaments are anchored to the Z-disks that distinguish each sarcomeric unit. Z-disks are primarily composed of \( \alpha \)-actinin, which crosslinks actin filaments near their barbed and stabilize the polarized orientation of the thin filaments. The pointed ends of actin filaments remain unbound to any particular structure. Yet the ends of the thin filaments are at a defined length, which has been proposed to be regulated through the ABPs nebulin (171), by unknown mechanisms, and tropomyosins (171) which can protect the thin filament from severing.

Smooth Muscle Cell Contractile Unit

In smooth muscle cells, contraction is not under conscious nervous control, and this muscle is referred to as involuntary muscle (vs. skeletal muscle which is voluntary). Unlike in striated muscle, in which the basic contractile unit (sarcomere) is well characterized, the contractile unit in smooth muscle cells has no Z-disk and instead has the end of bundled actin filaments inserted into dense bodies, also primarily composed of \( \alpha \)-actinin. In terms of structure, the smooth muscle contractile unit is poorly defined, although most data suggests that the cross-bridge mechanism is responsible for smooth muscle contraction (168, 172-174). There is a surprising lack of evidence for a repeating
filament lattice structure in smooth muscle tissue. This situation is most likely due to the contractile units being inadequately aligned fashion rather than a lack of their presence. Most smooth muscle cells are connected to one another both in series and in parallel, with longitudinal axes of the cells lying parallel to the direction of force transmission through a bundle of cells.

Based on ultrastructural evidence, the most widely accepted model of a single contractile unit is a side-polar thick filament (Figure 1-8B), vs. bipolar in the sarcomere, and two oppositely oriented thin filaments attached to separate dense bodies (Figure 1-9) (175, 176). Like in sarcomeres the thin filaments on either side of a dense body exhibit opposite polarity in their interaction with myosin heads, i.e. the pointed end of the thin filament is bound to the dense body (177, 178). Based on this model the amount of overlap between the thin and thick filaments should determine the ability of the muscle to generate force.

Smooth muscle cells are spindle-shaped, with a large and centrally located nucleus (Figure 1-10). The thin and thick filaments do not circumvent the nucleus; they attach to the nuclear envelope and use it as a force-transmitting structure (175, 179). To transduce the force generated within the cell, the thin filaments, proposed to be mainly composed of either α- or γ-smooth muscle isoactins depending on the tissue, are anchored to the cell membrane through interactions with adhesion and integrin proteins to link the cytoskeleton with the extra cellular matrix (180, 181). The other actin isoforms found in smooth muscle cells, β- and γ-nonmuscle actin, have been proposed to be primarily involved in 1) forming a network within the cytosol to enhance membrane rigidity and 2) to connect the contractile unit and the cytoskeleton to the cellular membrane in order to transmit tension (181-183). Inhibition of overall actin polymerization in these cells, through either monomer sequestration or capping, demonstrates suppression of tension development and inhibition of constriction (184).
Actin Regulation in Smooth Muscle Cells

Proper cytoskeletal regulation depends on the interplay between actin filaments and a number of ABPs which are essential for vascular smooth muscle cell functions (185). Disruption of insertion of the thin filaments into the dense bodies, altered interactions of actin with cellular adhesion proteins, or loss of tension within the non-contractile cytoskeleton could all lead to decreased tension development in smooth muscle tissues in response to contractile stimuli. Examples of ABPs involved in actin regulation in vascular smooth muscle cells include filament crosslinking proteins, filament stabilizing proteins, filament severing proteins, and proteins which promote nucleation and filament elongation. In this thesis, I will focus on the interaction between the TAAD mutant actin and one protein from these last two groups: cofilin. I have selected this ABP as it binds to the barbed end of the actin monomer near the TAAD-causing α-smooth muscle actin mutations N115T and R116Q. Cofilin is abundantly expressed in vascular smooth muscle cells and has been implicated in the regulation of the size of the pool of monomeric actin available for polymerization during contraction of smooth muscle cells (186-188). The current actin-cofilin model proposes that cofilin makes contact with two longitudinally associated actin monomers within the filament. The cofilin-actin interaction may disrupt the interface between the longitudinally associated actin subunits by increasing the angle of rotation between each actin monomer, thus altering the twist of the actin filament (189). Subdomain 1 of actin is the principal actin-binding interface. Specifically, cofilin has been demonstrated to make contact with the 112–125 helix of actin, which contains the two TAAD mutations N115T and R116Q (Figure 1-11) (13-16).
Patient phenotypes of Selected $\alpha$-Smooth Muscle Actin Mutations Leading to TAAD

Of the 22 missense mutations in $\alpha$-smooth muscle actin which cause TAAD, I will focus on the two described above, N115T and R116Q, for this thesis (Figure 1-12). The $\alpha$-smooth muscle actin mutations are not only associated with TAAD, but additionally they segregate in an allele specific fashion with further cardiovascular defects. The N115T mutation has been found in both $\alpha$-skeletal muscle and $\alpha$-smooth muscle actins and is associated primarily with aneurysms of the thoracic aorta (124, 125, 190). R116Q is the second most common mutation identified in $\alpha$-SM actin, and remarkably, 75% of patients with this mutation have premature coronary artery disease in addition to aortic aneurysms (124, 125). The varied clinical phenotypes suggests that while both of these mutations cause TAAD, the molecular mechanisms leading to aortic disease are allele-specific.

Guo et al. used immunolocalization to visualize $\alpha$-smooth muscle actin filaments in aortic smooth muscle cells derived from two TAAD patients, each of whom had one wild type and one mutant $\alpha$-smooth muscle actin allele. In normal smooth muscle cells, $\alpha$-smooth muscle actin has extensive co-localization with F-actin fibers (stained with phalloidin) extending across the cell and with a small pool of unpolymerized actin in the perinuclear region. Conversely, smooth muscle cells from a patient with the R116Q $\alpha$-smooth muscle actin mutation had an absence of visible $\alpha$-smooth muscle actin filaments and a larger pool of unpolymerized $\alpha$-smooth muscle actin. Smooth muscle cells from a patient with the T351N $\alpha$-smooth muscle actin mutation had diminished $\alpha$-smooth muscle actin-containing fibers, as determined by a decrease in co-localization of $\alpha$-smooth muscle actin specific antibody with total cellular F-actin. Those that remained were present solely at the periphery of the cell and contained large aggregates of $\alpha$-smooth muscle actin (124). In general, both patients demonstrated significantly diminished $\alpha$-smooth muscle actin with a reduction in polymerized actin fibers that did not traverse the
length of the cell. From these data the authors suggest that both R116Q and T351N substitutions perturb α-smooth muscle actin filament assembly or stability (124).

TAAD-causing actin mutations could affect a number of parameters leading ultimately to vascular wall weakening. Mutations in α-smooth muscle actin may impair the ability of the isoactin to properly polymerize. Or, mutations in α-smooth muscle actin may alter the ability of the isoactin to bind to or interact with important ABPs such that the actin is no longer properly regulated. Additionally, they could inhibit the interaction of actin with myosin leading to a diminution of contractile force necessary for the aorta to withstand the pressure exerted by cardiac pumping.

Our limited knowledge of molecular mechanisms underlying actin-dependent TAAD is due to the inability to biochemically assess the effects of each of these mutations on actin function for three reasons: 1) difficulty in resolving structurally similar actin isoforms from the mixture found in the vascular smooth muscle cell (191, 192)  2) a lack of mutant actin from patient material for study and 3) our and other lab’s (personal communication) inability to express the α-smooth muscle actin isoform in the baculovirus system. Because of these obstacles, I have instead begun using the budding yeast, *S. cerevisiae*, as a model system for studying these mutations. In this thesis I will investigate the molecular mechanisms of two of the twenty-two mutations that cause TAAD. I have chosen the TAAD-causing mutations N115T and R116Q because these residues are near binding sites for many ABPs, including coflin, formin, and profilin. I have determined the impact of the mutations on inherent biochemical properties of the actin and then determined if these mutations alter the ability of the actin to interact with the ABP coflin. Future studies will be required to determine the ability of the mutant actin to interact with myosin, as α-smooth muscle actin together with myosin compose the contractile machinery in vascular smooth muscle cells (the diseased cell type) (193).

This proposed work will be significant in two ways. It will provide new insight into how actin functions and is regulated in a general sense and more importantly, it will
provide clues as to the manner in which defects caused by these aneurysm mutations lead to vascular wall weakening and pathology.

**Model System for TAAD α-Smooth Muscle Actin Mutations**

The ability to analyze the effects of disease-causing actin mutations biochemically and biophysically requires the ability to make quantities of these proteins in amounts sufficient for the work. Despite our success in using the baculovirus system to characterize the human β- and γ-nonmuscle actin isoforms (Chapter 2) and a deafness-causing γ-nonmuscle actin mutant actin (Chapter 3), our efforts to express α-smooth muscle actin have failed. Thus, in this thesis I have focused on determining the *in vivo* and *in vitro* effects of the selected TAAD-causing α-smooth muscle actin mutations in the yeast background (Chapter 4).

**Focus of Thesis**

The primary goal of this work is to gain insight into how actin mutations affect actin structure and function and lead to human disease, namely deafness and TAAD. My thesis will focus on expression and characterization of the β- and γ-nonmuscle isoactins (Chapter 2). Next, I have begun to characterize the biochemical properties of the yeast lethal deafness mutation, D51N, in the γ-nonmuscle actin background, the isoform in which the mutation naturally occurs (Chapter 3). Finally, I will focus on assessing the effects of two of the TAAD causing α-smooth muscle actin mutations, using yeast as a model system, on actin function *in vivo* and *in vitro* (Chapter 4). I have also assessed the effects of these two TAAD-causing mutations on the ability of the mutant actin to interact with the actin binding protein cofilin (Chapter 4). Exploitation of both systems will provide the maximum understanding of how these mutations affect actin dynamics and eventually lead to deafness and TAAD.
**Figure 1-1. Structure of G- and F-actin.**

Panel A: Front view of the crystal structure of yeast actin modified from PDB: 1YAG (142) using PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC. The four subdomains are numbered and color coded: subdomain 1 grey, subdomain 2 blue, subdomain 3 red, subdomain 4 green. ATP is depicted as an orange stick. Mg\(^{2+}\)/Ca\(^{2+}\) is depicted as a yellow sphere. N and C mark the respective termini. Panel B: Oda 2009 model (194) of 3 actin monomers within an actin filament. The grey and yellow monomers are in one strand of the actin filament. The sand colored monomer corresponds to a monomer in an opposing strand.
Figure 1-2. Comparison of the Protein Sequence of Mammalian and Yeast Actins

BOXSHADE version 3.21 was used to align the amino acid sequences for the six mammalian actins and yeast actin. Each actin is listed below by name, gene designation in italics, and PubMed accession number. The first two are the mammalian nonmuscle actins: γ-nonmuscle actin (ACTG1) NM_001614 and β-nonmuscle actin (ACTB) NM_001101. The next four are the mammalian muscle actins: α-skeletal muscle (ACTA1) NM_001100, α-cardiac (ACTC1) NM_005159, α-smooth muscle (ACTA2) NM_001613, and γ-smooth muscle actin (ACTG2) NM_001615. Finally listed is yeast actin (ACT1) CAA24598.
Actin filaments are denoted by blue arrows. The pointed end of the actin filaments are represented by arrowheads. The three main actin rich subcellular domains are 1) the cuticular plate, containing a randomized meshwork of actin filaments into which actin filaments of the 2) stereocilia are anchored by the highly bundled actin rootlet and 3) the zonula adherens which contain anti-parallel actin filaments and connect hair cells to neighboring supporting cells (not shown).
Figure 1-4. Locations of the Ten Deafness Causing γ-Nonmuscle Actin Mutations.

Figure 1-5. Normal and Patient Aortas.

Figure 1-6. Myosin Cross-bridge Cycling.

Depicted are the steps in ATP-dependent sliding of the actin and myosin filaments, the basic mechanism for muscle contraction. Modified from (195).
1. Myosin head binds to actin filament

2. Myosin head tilts

3. Myosin head releases from the actin filament

4. Myosin head reverses tilt to original position
Figure 1-7. Sarcomeric Structure.

Panel A: Relaxed sarcomere. Panel B: Contracted sarcomere. Schematic representation of the sarcomere, the striated muscle cell contractile unit. Modified from (196).
A. 

Actin Thin Filament

Bi-polar Myosin Thick Filament

M-Line

Filament Sliding

Muscle Contraction

B.
Panel A: Bipolar arrangement of myosin found in striated muscle cells. In this orientation myosin heads all have the same polarity in one half of the filament, and the opposite polarity in the opposite half of the filament. Panel B: Side-polar arrangement of myosin found in smooth muscle cells. In this orientation myosin heads all have the same polarity along the entire length of one side of the filament and the opposite on the opposite side. Brackets indicate myosin heads with similar polarity.
Figure 1-9. Smooth Muscle Cell Contractile Unit.

Schematic drawing of a contractile unit of smooth muscle with a side-polar myosin filament. Striped arrows indicate directions of movement of actin filaments and dense bodies due to cyclic action of myosin cross bridges. Plus sign, barbed end of actin then filament. Minus sign, pointed end of actin thin filament.
Figure 1-10. Schematic Depiction of Contractile Unit Arrangement in a Bundle of Smooth Muscle Cells.

For simplicity the extracellular matrix and organelles (other than the nucleus) are not shown. Double sided arrow is parallel with the axis of force transmission and orientation of contractile units. Modified from (197).
Figure 1-11. Location of Cofilin Binding Site.

Back view of the actin monomer. Crystal structure of yeast actin modified from PDB: 1YAG (142) using PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC. The helix from residue 112 to 125, highlighted in blue, is proposed to be involved in the actin-cofilin interface. TAAD-causing mutations N115T (green) and R116Q (red) are highlighted. ATP is depicted as an orange stick. Mg$^{2+}$/Ca$^{2+}$ is depicted as a yellow sphere. N and C mark the respective termini. Numbers denote actin subdomains.
Figure 1-12. Location of the Twenty-two TAAD Causing α-Smooth Muscle Actin Mutations.

CHAPTER 2. ION-DEPENDENT POLYMERIZATION
DIFFERENCES BETWEEN MAMMALIAN BETA- AND GAMMA- NONMUSCLE ACTIN ISOFORMS

Abstract

β- And γ-nonmuscle actins differ by 4 amino acids at or near the amino-terminus and distant from polymerization interfaces. β-Actin contains an Asp1-Asp2-Asp3 and Val10 whereas γ-actin has a Glu1-Glu2-Glu3 and Ile10. Despite these small changes, conserved across mammals, fish and birds, their differential localization in the same cell suggests they may play different roles reflecting differences in their biochemical properties. To test this hypothesis, we established a baculovirus-driven expression system for producing these actins in isoform-pure populations although contaminated with 20-25% insect actin. Surprisingly, Ca2⁺-γ-actin exhibits a slower monomeric nucleotide exchange rate, a much longer nucleation phase, and a somewhat slower elongation rate than β-actin. In the Mg-form, this difference between the two is much smaller. Ca2⁺-γ-actin depolymerizes half as fast as does β-actin. Mixing experiments with Ca2⁺-actins reveal the two will readily co-polymerize. In the Ca2⁺-form, phosphate release from polymerizing β-actin occurs much more rapidly and extensively than polymerization, whereas phosphate release lags behind polymerization with γ-actin. Phosphate release during treadmilling is twice as fast with β- as with γ-actin. With Mg2⁺-actin in the initial stages, phosphate release for both actins correlates much more closely with polymerization. Ca2⁺ bound in the high affinity binding site of γ-actin may cause a selective energy barrier relative to β-actin that retards the equilibration between G and F- monomer conformations resulting in a slower polymerizing actin with greater filament stability. This difference may be particularly important in sites such as the γ-actin-rich cochlear hair cell stereocilium where local mM Ca2⁺ concentrations may exist. (This

**Introduction**

Birds, fish, and mammals have genes that encode two nonmuscle actin isoforms termed β- and γ-, based on their migration in an isoelectric focusing gel, with β-actin the more acidic of the two. These two nonmuscle isoforms differ at only four positions out of 375 residues, and the divergent residues are very similar between the two proteins. β-actin contains an Asp-Asp-Asp N-terminal tripeptide and a Val at position 10 whereas γ-actin has a Glu-Glu-Glu N-terminal tripeptide and an Ile at position 10 (32) (Figure 2-1).

In nonmuscle cells of mammals, birds, and fish, the β- and γ-nonmuscle actin isoforms exist in varying ratios depending on the cell type, and these ratios are conserved in a cell and tissue-specific way across these three groups of species. Usually they are found in about a 2:1 β- to γ-actin ratio (198). However, there are notable exceptions. For example, in auditory hair cells, the ratio is reversed (52), and in mature skeletal muscle myotubes, γ-nonmuscle actin, which lies in a thin layer just beneath the sarcoplasmic membrane, is the only nonmuscle actin isoform present (38, 199). Despite these small sequence differences, their localization in cultured cells indicates that β- and γ-nonmuscle actins may play very different physiological roles. In motile cells, β-actin tends to accumulate at the membrane–cytoskeleton interface in dynamic ruffling regions near the leading edge of the cell, whereas γ-actin seems to be enriched in stress fibers (200) although this generalization has been challenged in a recent study (201). Spatial and temporal segregation of β- and γ-nonmuscle actins has also been found in gastric parietal cells (202), auditory hair cells (52), osteoblasts (203), and neurons (42, 204).

Despite the apparent differences in physiological behavior of these two isoactins, the molecular basis for this cellular discrimination has been difficult to ascertain. One way for the cell to deal with this problem would be to synthesize the two isoforms in the
different parts of the cells where they predominate. Along these lines, it has been demonstrated that some cells can post-transcriptionally sort β-actin mRNA through its isoform specific 3′-UTR to the leading edge resulting in preferential β-actin synthesis in these regions (205-208). However, streaming of actin away from this area (209, 210) would result in an ultimate mixing of isoforms further toward the cell center. Another possibility is differential recognition of the two isoforms by different actin binding proteins. Toward this end, Namba et al. have demonstrated that L-plastin preferentially binds β- over γ-actin, although the reason for this is unclear (65), especially considering the small differences between the two actins. A third possibility is differential post-translational modifications of the two isoforms. In this vein, it has been reported that β-actin can be N-terminally arginylated following removal of the N-terminal acidic residue (211). Initial estimates based on mass spectroscopic methods suggested that this occurred in 20-40% of the β-actin, although this number may be much lower in different cell types. However, even if a fourth of the β-actin is modified, it is hard to understand how this small fraction could exert its effect over the entire β-actin population. A fourth possibility is that these small inherent structural differences result in propagated allosteric changes that alter distant binding sites for actin binding proteins. However, based on the monomer crystal structure, the three N-terminal actin residues are in an unstructured finger that reaches out into solution from the surface of the protein making this possibility seemingly less likely (16, 194, 212). Position 10, in which the residues differ by only a single methylene carbon, is in the middle of a structural core of subdomain 1, and this difference might result in a conformational change, perhaps via crowding effects.

To date, due to their similar sequences and their presence as mixtures in most cells, a limiting factor in addressing these isoform differences at the biochemical level has been a difficulty in readily obtaining significant amounts of these actins as pure isoform populations. The demonstration that cardiac actin could be successfully expressed in and purified from baculovirus-infected insect cells (158, 159) suggested that
this approach might also be a way to obtain pure preparations of human β- and γ-
nonmuscle isoactins. In this chapter, we demonstrate that we are able to do just that
although endogenous insect actin is present to the extent of ~20-25%. We also show that
this has little effect on the biochemical properties of the individual isoforms. We have
then used these preparations to compare the biochemical properties of these two isoactins
in the monomeric state and in their ability to polymerize. Our data show that despite
their very similar amino acid sequences, the two isoactins display distinct polymerization
characteristics that correlate with their apparently differential roles in motile cells.

Results

Characterization of G-Actin Properties

We first determined if the four amino acid difference between β- and γ-actin
causes differences in the physical behavior of the actin monomers. The apparent melting
temperatures of the actins with calcium bound at the high affinity binding site in the
nucleotide cleft, the form in which they are purified, were determined by following the
change in molar ellipticity as a function of increasing temperature in a CD
spectropolarimeter. The differing residues between β- and γ-actin have no significant
effect on the thermal stabilities (Table 2-1).

We next determined the ability of the calcium G-actins to exchange a bound
fluorescent ATP analog (ε-ATP) as a function of time in a solution with a large excess of
ATP. We also used yeast and muscle actins as reference points since yeast actin has been
shown have one of the fastest exchange rates of all actins and muscle actin one of the
slowest. Table 2-1 shows a slightly but reproducibly faster rate of nucleotide exchange
for yeast actin compared with β-actin. γ-Actin’s nucleotide exchange rate is significantly
slower than β-actin’s but is still much faster than that of muscle actin (213).
Actin Polymerization

Most of actin’s functions within the cell depend on its ability to form filaments, and isoform differences in polymerization and depolymerization kinetics could account for isoform-specific functions. We therefore wanted to determine if the polymerization rates and filament stabilities of the two isoforms differed from one another. Again, yeast and muscle actins were used as standards. Figure 2-2 shows that Ca\(^{2+}\)-β-actin polymerizes nearly as fast as yeast actin. Further, the total light scattering change was almost identical for both β- and γ-actins indicating little if any difference in critical concentration for the isoactins. Surprising, however, was the extremely slow rate of γ-actin polymerization and the extended nucleation phase, especially since the divergent residues between the two non-muscle isoactins would seem to have little effect on the surfaces of the actin involved in monomer-monomer contacts (Figure 2-1). EM analysis of negatively stained samples of filaments of both actins showed no apparent differences between them in filament morphology (Figure 2-10A and B). Insect actin also showed no apparent differences (Table 2-1).

Because of the significant degree to which insect actin was present in our mammalian actin preparations (20-25%) we wished to determine the extent of insect actin influence on γ-actin’s polymerization kinetics. A titration polymerization assay was performed in which the total actin concentration was kept at 4.8 µM with varying ratios of insect actin to γ-actin (Figure 2-3A). The results demonstrated that γ-actin polymerization kinetics remain relatively constant with an additional 10 % pure insect actin and were marginally affected by an additional 25%.

The results from Figure 2-2 suggested that insect and β-actin polymerization kinetics were very similar. We thus studied their relative rates of polymerizations at a lower actin concentration where differences might be more apparent. Fig. 3B shows first that both actins polymerize to the same extent and, second, that they do so with almost
the same rate although the insect actin repeatedly seems to polymerize slightly faster than the \( \beta \)-actin preparation.

As stated before, despite their high degree of chemical identity, \( \beta \)- and \( \gamma \)-nonmuscle isoactins often differentially localize within the cell. We thus wished to determine whether this asymmetry reflected an inherent inability of the two actins to co-polymerize. We first established the polymerization kinetics for 1.2 \( \mu \text{M} \) \( \beta \)- and \( \gamma \)-actins individually. Because this concentration is very near the critical concentration for actin, little if any noticeable polymerization should occur over the course of the experiment as demonstrated in Fig. 4. If the two actins could not co-polymerize, mixing \( \beta \)- and \( \gamma \)-actins with each at 1.2 \( \mu \text{M} \) should produce no polymerization. However, Fig. 4 also shows that such a mixture polymerizes to the same extent as either actin does at double the concentration, 2.4 \( \mu \text{M} \), indicating the two actins are totally copolymerizable. The polymerization rate of the mixture is intermediate between the behavior of the two pure samples reflecting that one isoform is not totally dominant over the other in dictating overall behavior.

Fig. 2 suggests that the \( \gamma \)-actin preparation has a much more protracted nucleation phase than does the \( \beta \)-actin preparation. We thus assessed the ability of increasing amounts of the \( \beta \)-actin preparation to overcome this apparent \( \gamma \)-actin nucleation barrier. Fig. 5 shows that adding increasing amounts of \( \beta \)-actin to \( \gamma \)-actin while holding total actin constant produced a dose-response curve similar to what we saw with insect actin in overcoming the \( \gamma \)-actin nucleation lag.

If, in fact, the delay in \( \gamma \)-actin polymerization relative to that of \( \beta \)-actin was caused by a nucleation barrier, the addition of small amounts of F-actin seeds made from \( \beta \)-actin should largely overcome this observed delay. For this experiment we generated F-actin seeds by sonicating phalloidin stabilized \( \beta \)- and \( \gamma \)-actin filaments and added them at a molar ratio of 1:20 to samples of G \( \beta \)- and \( \gamma \)-actins respectively. Fig. 6 shows that, as predicted, the \( \gamma \)-actin seeds largely overcome the delay seen with G \( \gamma \)-actin alone.
However, the seeded elongation rate of γ-actin is still somewhat faster than that of β-actin suggesting a small difference in their relative elongation rates as well.

One indication of filament stability is its rate of depolymerization. We thus measured the depolymerization rates of Ca\(^{2+}\) β- and γ-F-actins by following the first-order decrease in light scattering as a function of time following addition of super-stoichiometric amounts of DNase I as a sequestering agent for actin monomers as they are generated. Table 1 shows the t\(_{1/2}\) values for this process. Both β- and γ-actins depolymerize slower than yeast actin, and the t\(_{1/2}\) of γ-actin is 1.6 times longer than that of β-actin indicating an enhanced stability for the γ isoform filament.

Phosphate Release Kinetics

Another determinant of filament stability is the rate at which the inorganic phosphate is released from the actin surface once the ATP is hydrolyzed during polymerization since ADP-P\(_i\) F-actin is more stable than ADP F-actin (214). For most polymerizing actins, the P\(_i\) release curve is biphasic: an initial rapid release curve concomitant with polymerization and a slower second phase caused by the treadmilling of monomers through the filament at steady state.

Figures 2-7A and B show the data obtained in the comparison of Ca\(^{2+}\) β-actin vs. γ-actin behavior. For β-actin, the initial P\(_i\) release phase appears to actually proceed at a rate noticeably faster than polymerization as determined by light scattering. In comparison, the first phase P\(_i\) release with yeast actin is much more synchronized with polymerization. This β-actin result suggests some kind of salt-dependent uncoupling of ATPase and polymerization or abortive filament formation and subsequent cycling of monomers. In contrast, the rate of first-phase P\(_i\) release from γ-actin seems to actually lag behind polymerization, similar to what is observed with muscle actin (215, 216). For second-phase treadmilling, the rate of P\(_i\) release is approximately twice as fast for β-actin (2.3x10\(^{-3}\) ± 0.3x10\(^{-3}\) µM P\(_i\)/sec) than for γ-actin (1.1x10\(^{-3}\) ± 0.1x10\(^{-3}\) µM/sec) similar to
the faster depolymerization rate of $\beta$- vs. $\gamma$-actin. Again, this result is suggestive of a more dynamic $\beta$-filament.

**Mg-Actin Behavior**

All of the above assays were performed with calcium initially occupying the high affinity divalent cation binding site on actin. However the predominant actin form in the cell is thought to have bound magnesium due principally to the relative abundance of magnesium vs. calcium in the cytosol. Therefore we wanted to determine if the differences in the behavior of $\text{Ca}^{2+}$-$\beta$- vs. $\gamma$-actin were maintained by the actins in their Mg form. As with $\text{Ca}^{2+}$-actins, no differences in thermostabilities of the Mg form of $\beta$- and $\gamma$-actins were observed (data not shown).

Nucleotide exchange rates for Mg yeast, $\beta$-, and $\gamma$-actins were all accelerated compared with their $\text{Ca}^{2+}$ counterparts, indicative of a much more dynamic monomer in the Mg state (Table 1). Additionally, the two-fold difference in exchange rates for $\beta$- vs. $\gamma$-actin in the $\text{Ca}^{2+}$-form virtually disappeared suggesting the release of selective conformational restraints on the calcium form of $\gamma$-actin accompanying its conversion from the $\text{Ca}^{2+}$- to the $\text{Mg}^{2+}$-form (Table 2-1).

We then repeated the polymerization assay with Mg-actins, using 3.5 $\mu$M actin rather than the higher concentration used previously to allow better differentiation between more rapidly polymerizing actins. Figure 2-8 shows that the rates of both Mg-actin isoforms are faster than their $\text{Ca}^{2+}$ counterparts. Furthermore, as before, the $\beta$-actin polymerizes more rapidly, although the difference between $\text{Mg}^{2+}$-$\beta$- and $\gamma$-actins has been drastically reduced. This result is consistent with less steric restraints on flexibility of the $\text{Mg}^{2+}$-form of the G-actin monomer allowing it to much more easily assume its polymerization-competent monomer conformation. As with $\text{Ca}^{2+}$-actins, EM examination of the $\text{Mg}^{2+}$-actin filaments shows similar filament morphologies for $\beta$- and $\gamma$-actin (Figure 2-10C and D).
The relative rates of $P_i$ release for the two isoactins are also much closer in the
$Mg^{2+}$-form than in the $Ca^{2+}$-form (Figure 2-9A and B). For $\beta$-actin, the first phase $P_i$
release rate appears much more closely synchronized with polymerization suggesting the
absence of substantial abortive monomer cycling (Figure 2-9A). For $\gamma$-actin, there is still
a lag in $P_i$ release relative to the rate of polymerization, although this discrepancy is
smaller than with the $Ca^{2+}$-actin. Finally, as before, with $Mg^{2+}$-actin, the treadmilling rate
for $\beta$-actin ($2.8 \times 10^{-3} \pm 0.2 \times 10^{-3} \, \mu M \, P_i/sec$) is still 1.5 times faster than that for $\gamma$-actin
($1.9 \times 10^{-3} \pm 0.1 \times 10^{-3} \, \mu M \, P_i/sec$).

Discussion

The focus of this work was to determine if, despite their high degree of sequence
identity, the two nonmuscle actins exhibit distinct biochemical properties that might
explain their different cellular roles (198). However, such an assessment requires ready
access to quantities of the individual pure isoforms large enough for biochemical and
biophysical experiments, a goal previously difficult to achieve. The closest model of
pure nonmuscle $\beta$-actin previously established was the scallop adductor muscle $\beta$-like
actin (217). Although scallop adductor actin is similar in sequence to $\beta$-actin, there are
seven amino acid substitutions between the two actins, making scallop adductor actin
more divergent from $\beta$-actin than even $\gamma$-actin (217). Based on our results with only a
four residue divergence, these differences between the actins could cause differences in
their biochemical properties thereby detracting from the usefulness of the scallop actin as
a $\beta$-actin model system (198, 217).

We have addressed this problem by successfully establishing a baculovirus-driven
expression system for each of the individual mammalian nonmuscle isoforms, similar to
what had previously been accomplished for the $\alpha$-cardiac actin by Trybus and colleagues
(159, 218). Others have also used this system to assess the effect of different myopathic
muscle actin mutations in vitro (158). Similarly, our system will allow for the generation
in the nonmuscle actins of pathology-producing mutations such as those associated with autosomal dominant non-syndromic hearing loss (123, 219) to try to gain insight into the biochemical alterations that underlie these pathological states. A problem with this system, however, is the significantly higher amounts of contaminating insect actin compared to what was encountered in the production of a non-polymerizing mutant α-cardiac actin (30). Estimates of contaminating insect actin were not provided for the WT α-cardiac actin preparations. We do not understand the reason for this difference. The presence of the insect actin will complicate one’s ability to use this preparation to study differences in actin-actin binding protein interactions between β- and γ-actins because of the similarities in β- and insect actin behavior. Furthermore, this complication mandates that one include controls with pure insect actin alone in such work. However, the distinct polymerization differences between γ- and insect actins should allow for meaningful comparisons between wild type γ-actin and pathology-producing mutant γ-actins.

The four residues differentiating β- from γ-actin lie at or near the amino terminus in a position that would not be expected to cause differences in the nucleotide-binding behavior or thermostability of the actin monomer or their ability to polymerize. In terms of thermostability, this is what we observed. For nucleotide exchange, monomers of the two actin isoforms exchanged bound nucleotide more slowly than the rapidly exchanging yeast actin but about 4-6 fold more rapidly than muscle actin, previously shown to have one of the slower nucleotide exchange rates (213). We repeatedly observed, though, that the calcium β-isoform exchanged about 50% more rapidly than the γ-isoform suggesting that one or more of the four residue differences between the two actin isoforms could actually influence the dynamics of the protein around the nucleotide cleft, contrary to our original prediction.

Surprisingly we observed a large difference in polymerization rates for the two actins in the Ca^{2+}-form. The β-actin polymerized much more rapidly than the γ-actin, almost as fast as the rapidly polymerizing yeast actin. The γ-actin polymerized at a rate
much slower than even that of muscle actin, one of the slowest polymerizing actins documented to date. This slower polymerization rate of γ- v.s. β- nonmuscle actin seems to result from both slower nucleation and elongation rates, based on seeded actin polymerization assays. In contrast, in the Mg form which is generally considered to be the more physiologically relevant actin form due to the Ca^{2+}/Mg^{2+} ratios in the cytosol, the difference between the two actin isoforms drastically decreased, although the β-isoactin still polymerized more quickly than the γ-isoform.

Our polymerization-dependent phosphate release assays present a similar picture. Muscle actin retains its phosphate for a substantial time after incorporation of the actin monomer into the filament, whereas with yeast actin, release is essentially simultaneous with polymerization reflecting their relative filament stabilities. In the Ca^{2+}-form, the rapid initial P_i release phase for β-actin appears to occur significantly faster than net polymerization. This observation is consistent with data from Karlsson’s group which demonstrated that P_i release precedes polymerization in both calf thymus actin and β-actin expressed and purified from yeast (220). This relationship may indicate the presence of non-productive or abortive polymerization early in the process. Alternatively in the Ca^{2+}-induced conformation, addition of F-salts may induce a conformational change which selectively activates β-actin’s ATPase without inducing polymerization. In essence, this would cause an uncoupling of these processes. In contrast, γ-actin appears to substantially retain its phosphate in the initial stage and exhibits a much slower treadmilling phase. This observation is consistent with the idea that the γ-filament is an inherently less dynamic and a more stable structure. As with the polymerization studies, conversion of the actin monomers to the Mg form prior to polymerization reduced the difference between the two proteins although P_i release for β-actin was still faster for both phases than for γ-actin.

In summary our baculovirus system-based β- and γ-actin expression system has allowed a rigorous biochemical comparison of these two actins in their isoform-pure
states. Our initial demonstration of an unsuspected ability of four small residue differences to allosterically affect filament conformation provides important new insight into the biochemical basis underlying how these proteins might function differently within the cell.

**Experimental Procedures**

**Materials**

DNase I (grade D) was purchased from Worthington. Affi-Gel 10-activated resin and Micro Bio-Spin P-30 Tris gel filtration chromatography columns were obtained from Bio-Rad. DE52 DEAE-cellulose was acquired from Whatman. N-(1-pyrenyl)maleimide, ATP, ADP, hexokinase, and glucose were acquired from Sigma-Aldrich. Tween-20, enzyme grade, was obtained from Fisher Scientific. Yeast cakes for WT actin preparations were purchased from a local bakery. All other chemicals were reagent-grade quality.

**Cells and Cell Culture**

Low-passage Sf21 cells (BD Biosciences) were cultured in SF900 II medium (Invitrogen) with 1% penicillin/streptomycin. These cells, after being switched to TC-100 Insect Medium with L-glutamine and sodium bicarbonate (Sigma-Aldrich) with 1% penicillin/streptomycin and 10%FBS, were used for recombinant virus production and protein expression.

**Construction of Baculovirus Transfer Vectors**

In conjunction with our collaborator Karen Friderici (Michigan State University), we constructed a donor plasmid with the human β- or γ-nonmuscle actin gene under control of the viral p6.9 promoter. The coding sequences of human β-nonmuscle actin (coding sequence was obtained from pEGFP-Actin BDBiosciences Clontech as PCR template) and γ-nonmuscle actin (coding sequence obtained from IMAGE clone -
ATCC#6875188, ImageID4842665, Genebank accession# BC015695 used as PCR template) were cloned into the pDEST8 donor plasmid. The AcMNpv 6.9 baculovirus promoter was cloned upstream of the actin gene. Our lab transformed *E. coli* cells containing a baculoviral genome with this donor plasmid, using the Invitrogen Bac-to-Bac system. Through transposition a recombinant bacmid DNA was generated containing all essential viral genes and either the human β- or γ-nonmuscle actin gene preceded by the viral p6.9 promoter. This bacmid DNA was then transfected into SF21 insect cells to generate recombinant baculoviral particles. High titer stocks of this recombinant baculovirus are then used to infect insect cells for large scale protein expression. All sequences were confirmed by sequencing of viral DNA from infected cells.

**Purification of Recombinant Actin**

Infected cells (with a multiplicity of infection equal to 2) were harvested 72 hrs post infection by centrifugation, washed in PBS and lysed in a high Tris-buffer [1 M Tris–HCl, pH 7.5, 0.5 mM MgCl₂, 0.5 mM ATP, 4% Triton X-100, 1 mg/mL Tween 20, 1 mM DTT and a protease inhibitor cocktail (benzamidine, leupeptin, aprotinin, antipain, TLCK, TPCK, E-64, each at 1.25 mg/ml)] by sonication. The cell lysate (50 ml from a preparation of approximately 9x10⁸ cells) was cleared by centrifugation at 40,000K for 1 hr using a Beckman L8-70M Ultracentrifuge with a 45Ti rotor. The lysate was then diluted with 1X G-buffer (10mM Tris-HCl, pH 7.5, 0.2mM CaCl₂, and 0.2mM ATP) with the same protease inhibitor cocktail as above. Control insect actin was also purified from uninfected SF21 cells as described for recombinant actin purification.

Muscle actin was purified from rabbit muscle acetone powder, as described by Spudich and Watt (221). Yeast and nonmuscle actins were purified in the calcium form via a combination of DNase I-agarose affinity chromatography, DEAE-cellulose chromatography, and polymerization/depolymerization cycling as described previously.
(222). Purity of the actin preparations were assessed by SDS-PAGE analysis (Figure 2-11). The concentration of G-actin was determined from the absorbance at 290 nm using an extinction coefficient of 0.63 M⁻¹cm⁻¹. The yield was typically 1-2mg of actin per liter of infected cells. Using mass spectroscopic analysis, we determined there to be approximately 20-25% insect actin in our nonmuscle actin protein. Mg-actin was generated by diluting actin in G-Buffer with MgCl₂ for 10 min and then adding EGTA to chelate Ca²⁺ as described previously (223).

Actin Thermal Stability

The apparent melting temperatures of yeast and nonmuscle actins were determined using circular dichroism by following the change in ellipticity of the G-actin sample at 222 nm as a function of temperature between 25 and 90°C as described previously (155). Measurements were made on an Aviv 62 DS spectropolarimeter. Data were fit to a two-state model, and the apparent \( T_m \) value was determined by fitting the data to the Gibbs-Helmholtz equation to approximate the temperature at which 50% of the actin was denatured.

Actin ATP Exchange

The ability of G-actin to exchange its bound nucleotide was assessed by first loading the actin with \( \varepsilon \)-ATP and then following its displacement from the actin in the presence of a large excess of ATP as described previously (155). Exchange rates were determined by fitting the data to a single exponential expression using BioKine Version 3.1.

Actin Polymerization

Polymerization of G-actin in a total volume of 160 µl was induced by the addition of MgCl₂ and KCl to final concentrations of 2 mM and 50 mM respectively (F-salts). Polymerization was monitored at 25°C by following the increase in light scattering of the
sample in a FluoroMax-3 or a Flurolog (model FL3-21) fluorescence spectrometer outfitted with a computer controlled thermostatted four position multi-sample exchanger (HORIBA Jobin Yvon Inc.). Differences in machines can cause variations in the light scattering values; therefore, data from any given graph were obtained from the same fluorescence spectrometer. Both the excitation and emission wavelengths were set to 360 nm with the slit widths for both set at 1 nm.

For seeded actin polymerization assays, 4.8 µM actin was polymerized as above. Then after polymerization was complete, a 1:1 molar ratio of phalloidin was added to the actin. Filaments were sheared creating phalloidin actin seeds (PAS) the seeds were added to G-actin to make up 5% of the total actin in the reaction. F-salts then were added, and polymerization was monitored at 10sec intervals as above.

**P$_i$ Release from Actin**

The rate of P$_i$ release from polymerizing actin samples following ATP hydrolysis was assessed using the commercially available EnzChek$^\text{TM}$ phosphate assay (Invitrogen) at 25 °C. Briefly, this spectrophotometric assay utilizes the purine nucleoside phosphorylase-dependent phosphorolysis of 6-mercapto-7-methylpurine riboside to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine, the latter of which has a characteristic absorbance at 360 nm that is not shared by the nucleotide substrate at pH values greater than 6.5 (224, 225). Following induction of polymerization of 4.8 µM actin, the absorbance was monitored as a function of time, with readings taken automatically at 10-s intervals at 360 nm using a thermostatted cuvette holder set to 25 °C ± 0.1 °C.

**Actin Depolymerization**

Actin was polymerized to steady state levels. DNase I was then added in a 1:5 actin to DNase molar ratio. Depolymerization was monitored as a decrease in light
scattering over time. Depolymerization rates were determined by fitting the data to a single exponential expression using BioKine Version 3.1.

Electron Microscopy

Actin filaments were visualized by depositing 2 µl of a sample containing 4.8 µM F-actin onto carbon-coated Formvar grids. The grids were negatively stained with 1% uranyl acetate, and observed using a JOEL 1230 transmission electron microscope (University of Iowa Central Electron Microscopy Facility). Image J was used to process the images.
Figure 2-1. Locations of the Structural Differences Between β- and γ-Nonmuscle Actins.

Panel A: monomer view of the crystal structure of β-actin, modified from Protein Data Bank code 1HLU (16) using Swiss-PdbViewer Version 3.7. The positions of the differing residues are color-highlighted and labeled: D1, D2, D3 green; V10, pink. Panel B: model of the actin trimer based on the filament model of Oda et al. (194), with the positions of the differing residues color-highlighted and labeled as described above. ATP is depicted as an orange stick and Ca²⁺ or Mg²⁺ ion depicted as red circle.
Figure 2-2. Polymerization Kinetics of Actin Isoforms.

Polymerization of 4.8 μM actin was initiated by the addition of magnesium and potassium chloride as described under “Experimental Procedures,” and the increase in light scattering (L.S.) was monitored as a function of time at 25 °C. Shown are representative plots of experiments performed at least three times with three independent actin preparations. A.U., arbitrary units.
Figure 2-3. Polymerization of Insect and γ-Actin Mixtures.

Polymerization of 3.5 µM total actin was initiated by the addition of magnesium and potassium chloride as described under “Experimental Procedures,” the increase in light scattering (L.S.) was monitored as a function of time at 25 °C. The γ-actin preparation used in this work contains 20-25% insect actin. Panel A: γ-actin preparation, mixtures of γ-actin preparation and insect actin, and pure insect actin were polymerized, numbers behind the isoforms represent the relative percentage of each actin preparation within the reaction. Panel B: Pure insect and β-actin were polymerized. Shown are representative plots of experiments performed at least three times with three independent actin preparations. A.U., arbitrary units.
Polymerization of 2.4 μM or 1.2 μM total actin was initiated by the addition of magnesium and potassium chloride as described under “Experimental Procedures,” and the increase in light scattering (L.S.) was monitored as a function of time at 25 °C. Shown are representative plots of experiments performed four times with two independent actin preparations. A.U., arbitrary units.
Polymerization of 3.5 μM total actin was initiated by the addition of magnesium and potassium chloride as described under “Experimental Procedures,” and the increase in light scattering (L.S.) was monitored as a function of time at 25 °C. Numbers behind the isoforms represent the relative percentage of each actin isoform within the polymerization reaction. Shown are representative plots of experiments performed four times with two independent actin preparations. A.U., arbitrary units.
Figure 2-6. Seeded Polymerization of β- and γ-Actin.

Polymerization of 3.5 µM actin was initiated by the addition of magnesium and potassium chloride as described under “Experimental Procedures,” in the presence or absence of phalloidin stabilized actin seeds (PAS) as indicated above and the increase in light scattering (L.S.) was monitored as a function of time at 25 °C. Shown are representative plots of experiments performed four times with two independent actin preparations. A.U., arbitrary units.
Figure 2-7.  $P_i$ Release Associated with Polymerization of $Ca^{2+}$-Nonmuscle Actin Isoforms.

Panel A: 4.8 µM $Ca^{2+}$-β-nonmuscle actin.  Panel B: $γ$-actin nonmuscle actin was polymerized at 25 °C by the addition of salt. Filament formation was monitored by the change in light scattering and $P_i$ release using the Enz Check Assay. The data was normalized and superimposed as described under "Experimental Procedures." Shown are representative plots of experiments performed at least three times with three independent actin preparations.
Figure 2-8. Polymerization Kinetics of Ca\(^{2+}\) vs. Mg\(^{2+}\)-Nonmuscle Actin Isoforms.

Polymerization of 3.5 µM actin was initiated by the addition of magnesium and potassium chloride as described under “Experimental Procedures,” and the increase in light scattering (L.S.) was monitored as a function of time at 25 °C. Shown are representative plots of experiments performed at least three times with three independent actin preparations. A.U., arbitrary units.
Figure 2-9.  P$_i$ Release Associated with Polymerization of Mg-Nonmuscle Actin Isoforms.

Panel A: 4.8 μM Mg β-nonmuscle actin. Panel B: γ-nonmuscle actin was polymerized at 25 °C by the addition of salt. Filament formation was monitored by the change in light scattering and P$_i$ release using the Enz Check Assay. The data was normalized and were superimposed as described under "Experimental Procedures." Shown are representative plots of experiments performed at least three times with three independent actin preparations.
Figure 2-10. Actin Filaments from Polymerizing Ca$^{2+}$-G-Nonmuscle Actin Isoforms and Mg-G-Nonmuscle Actin Isoforms.

Panel A: Electron micrograph of β-nonmuscle actin filaments in the Ca$^{2+}$-form. Panel B: Electron micrograph of γ-nonmuscle actin filaments in the Ca$^{2+}$-form. Panel C: Electron micrograph of β-nonmuscle actin filaments in the Mg$^{2+}$-form. Panel D: Electron micrograph of γ-nonmuscle actin filaments in the Mg$^{2+}$-form. Actin was polymerized at 4.8 µM and the product was examined by EM as described under "Experimental Procedures." Scale Bar = 100 nm. Shown are representative pictures of three independent actin preparations.
Figure 2-11. SDS-PAGE Analysis of Purified Actin Samples.

10µg of each purified actin sample (as labeled) was run on a 10% SDS-PAGE gel. Shown is a representative gel performed with at least three independent actin preparations.
Table 2-1. Effect of Isoform Residue Differences on Monomer and Filament Characteristics.

<table>
<thead>
<tr>
<th>Cation Bound</th>
<th>Actin Isoform</th>
<th>Monomer Characteristics</th>
<th>Filament Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Thermal Stability, T_m (°C)</td>
<td>Nucleotide Exchange T_{1/2} (sec)</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Yeast</td>
<td>58 ± 1 (9)</td>
<td>35 ± 3 (13)</td>
</tr>
<tr>
<td></td>
<td>β-Nonmuscle</td>
<td>58 ± 1 (6)</td>
<td>66 ± 2 (5)</td>
</tr>
<tr>
<td></td>
<td>γ-Nonmuscle</td>
<td>57 ± 1 (9)</td>
<td>92 ± 15 (9)</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>57 ± 1 (2)</td>
<td>405 ± 41 (3)</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>Yeast</td>
<td>58 ± 1 (2)</td>
<td>4 ± 1 (2)</td>
</tr>
<tr>
<td></td>
<td>β-Nonmuscle</td>
<td>57 ± 1 (2)</td>
<td>10 ± 2 (2)</td>
</tr>
<tr>
<td></td>
<td>γ-Nonmuscle</td>
<td>57 ± 1 (2)</td>
<td>10 ± 4 (2)</td>
</tr>
</tbody>
</table>

Note: Thermal Stability: The T_m, or temperature at which ½ of the actin monomers in solution denature, was determined using circular dichroism, as described in “Experimental Procedures.” Nucleotide Exchange T_{1/2}: The release of G-actin-bound ε-ATP 1 µM actin in G buffer without free ATP was triggered by the addition of 100µM ATP. The decrease in fluorescence caused by ε-ATP exchange was followed over time, and data were fit to a first order reaction mechanism as described under “Materials and Methods.” ½ Time of Depolymerization: The decrease in light scattering of actin filaments was followed as a function of time after the addition of DNase I at 25°C. The t_{1/2} of depolymerization was determined as described under “Experimental Procedures.” The number of experiments performed is indicated in parentheses. ND = Not Determined
CHAPTER 3. BIOCHEMICAL EFFECTS OF DEAFNESS
CAUSING GAMMA-NONMUSCLE ACTIN MUTATION, D51N, IN
THE GAMMA-NONMUSCLE ACTIN BACKGROUND

Abstract

Ten point mutations in γ-nonmuscle actin at the DFNA20/26 locus cause
autosomal dominant nonsyndromic hearing loss. The molecular basis for the hearing loss
is unknown. Previous work demonstrated that the deafness-causing D51N mutation, once
cloned into yeast actin, causes lethality. Thus, we have engineered the mutation into γ-
nonmuscle actin, the isoactin in which the mutation naturally occurs, to investigate the
effects of the mutation on actin function in vitro. The D51N-γ-nonmuscle actin monomer
demonstrated a similar thermal stability and nucleotide exchange rate as wild type γ-
nonmuscle actin. Surprisingly, the mutation caused a dramatic increase in the
polymerization and phosphate release kinetics of the actin. Supporting the possibility
that the mutant actin is a more dynamic actin filament, the mutation also caused an
increased depolymerization rate. The more dynamic filament kinetics of the D51N
mutant actin must be due to alterations in the monomer-monomer interface as the
monomer characteristics were not drastically affected. Our results suggest that the
deafness phenotype caused by this mutation is not due to an inability of the actin to
polymerize, and must therefore be due to an alteration in filament kinetics and/or altered
regulation by actin binding proteins.

Introduction

Hearing results from conversion of sound waves into electrical signals in a
process called mechanotransduction (132). Hearing occurs in the cochlea of the inner
ear, where sound waves create vibrations in the basilar membrane. There, vibrations are
detected and propagated by the hair cells and result in transmission of electrical signals to
the brain. This process depends on actin-based structures called stereocilia which
protrude from the apical surface of cochlear hair cells. They contain a core of cross-linked parallel actin filaments (133) in which the barbed ends of the filaments, the preferred site of actin monomer addition, are oriented toward the stereocilia tips (226). The stereocilia are organized into a staircase like arrangement of 20–300 hair-like receptors per cell (Figure 3-1). Mechanical deflection of these structures, caused by movement of the basilar membrane, triggers opening of gated ion channels located on the surface of the stereocilia (227). Each stereocilium is supported by an array of parallel actin filaments that are packed more densely at the base, the rootlet, which extends into a dense gel-like network of randomly oriented actin filaments, the cuticular plate (134). Encircling each hair cell near its apical surface is the zonula adherens, a circumferential belt of actin filaments that runs parallel to the plasma membrane (135). The integrity of the actin cytoskeleton in all these cellular structures is crucial for hearing.

Hair cell actin is composed of two different nonmuscle actin isoforms: β and γ. Hair cells are one of a limited number of cell types in the body where the γ-isoform predominates (70% γ-nonmuscle and 30% β-nonmuscle actin) (123). These two isoactins differ at only 4 out of the 375 amino acids. First, the N-terminus of β-actin has three Asp residues, whereas that of γ-nonmuscle actin has three Glu residues. Second, at position 10, β-nonmuscle actin has Val while γ-nonmuscle actin has Ile. Localization of the isoactins has revealed that both are uniformly distributed throughout the hair cell stereocilia (137). Ervasti and colleagues determined that mice can use either β- or γ-nonmuscle actin to form normal stereocilia, but loss of either isoactin in the hair cell results in time dependent allele-specific stereociliary defects which lead to different forms of deafness (56-58, 137).

Ten point mutations in the γ-nonmuscle actin gene (ACTG1) cause autosomal dominant nonsyndromic progressive hearing loss designated DNFA20/26 (122, 123, 138, 140). Patients with these mutations have normal hearing until their teens or twenties. At this age, the patients demonstrate noticeable decreases in their hear high frequency
hearing. With age, the patient hearing loss extends over a broader range of frequencies until the hearing loss becomes profound. Eight of the ten point mutations (T89I, K118N, K118M, I122V, P264L, T278I, P332A, and V370A) are located in the barbed end of the actin, the preferred site of monomer addition comprised of subdomains 1 and 3. Only two mutations, D51N and E241K, have been found in the pointed end of actin in subdomains 2 and 4 respectively.

The molecular basis for hearing loss due to these point mutations has been controversial since their discovery. Recently, our lab has investigated the biochemical effects of the mutations on actin structure/function by cloning the original eight of the ten deafness mutations into the yeast actin gene (139, 143, 144). This has allowed us to directly compare the effects of the mutations \textit{in vivo} and \textit{in vitro}. Although this approach has been useful, there is one mutation, D51N, which causes lethality. D51N is located adjacent to the DNase I-binding loop (D-loop) of actin, residues 39-50 (Figure 3-2). This study provides, for the first time, determination of the effect of a deafness-associated $\gamma$-nonmuscle actin mutation in the isoform in which the mutation actually occurs in humans. Our results suggests that the major cause of deafness is not due to an inability to polymerize but more likely to alter actin kinetics and/or an altered ability of the actin filaments to be properly regulated.

\textbf{Results}

Expression of D51N $\gamma$-Nonmuscle Actin

\textit{Sf}21\textit{cells} were infected (at a multiplicity of infection equal to 2) with a baculovirus construct expressing either wild type-$\gamma$- or D51N-$\gamma$-nonmuscle actin under the control of the viral p6.9 promoter. Expression was verified by SDS-PAGE analysis of lysates 72 hours post-infection (Figure 3-3). The nonmuscle isoactins were purified similar to our lab’s standard yeast actin purification scheme, detailed in Materials and Methods (228). SDS-PAGE of the purified actins showed very little contamination by
other proteins (Figure 1A, lane 4). Purity of the actin preparations were assessed by SDS-PAGE analysis (Figure 3-3). Yields were typically 1mg/L cells (2x10^9 cells) for both wild type γ- and D51N-γ-nonmuscle actin, the amount of actin that would be far above that found in uninfected cells. Despite the close proximity of the D51N mutant residue to the D-loop, DNase I binding seemed normal.

**Effect of the D51N Mutation on the Actin Monomer**

The stability and conformational flexibility of the actin monomer are important determinates of the ability of actin to form a functioning cytoskeleton. Since the D51N mutation is lethal in yeast, we sought to determine if the inability of the yeast to survive with the mutant actin was due to a grossly altered conformation of the protein. One indicator of such a change might be an altered thermal stability. We assessed the temperature at which 50% of actin monomers denature using the change in ellipticity at 222nm as a function of temperature. Table 3-1 shows that the D51N-γ-nonmuscle actin has a near normal thermal stability of 60°C, similar to that of wild type γ-nonmuscle actin, 62°C. The lack of an effect was not surprising as D51N is not close to the major elements that are determinates of monomer stability, the nucleotide bridge spanning the two domains of actin near the bottom of the interdomain cleft and the hinge region beneath the base of this cleft.

We next determined the ability of the G-actins to exchange a bound fluorescent ATP analog (ε-ATP) as a function of time in a solution with a large excess of ATP (Table 3-1). The ε-ATP exchange rate seemed unaffected by the mutation, indicating that the mutation does not affect the nucleotide binding region.

**Effect of the D51N Mutation on Actin Polymerization**

A potential cause of deafness associated with the D51N mutation is an adverse effect on actin polymerization, leading to malformed or structurally unstable stereocilia.
Additionally, since this mutation leads to lethality in yeast, we initially hypothesized that it might severely alter actin polymerization kinetics. To test this hypothesis, we compared the ability of the purified wild type γ- and D51N-γ-nonmuscle actins to polymerize. Figure 3-4A demonstrates that the lag normally present in γ-nonmuscle actin polymerization is absent in D51N-γ-nonmuscle actin. Also, the elongation phase is dramatically accelerated in D51N-γ-nonmuscle actin vs. wild type γ-nonmuscle actin, although the extent of the reaction appeared to be the same. These results suggest two possibilities. First, the mutant actin monomers can more easily adopt a F-actin conformation, possibly forming nuclei or oligomers that can act as seeds for polymerization. Second, the filament is unstable and fragments during the polymerization process. This alteration in polymerization kinetics appears to have little to no effect on the gross morphology of the actin filaments, as viewed by electron microscopy (Figure 3-4B and C).

Effect of the D51N Mutation on Actin Filament Dynamics – Pi Release and Depolymerization

Two determinants of filament turnover are the relative rate constants for monomer association with or disassociation from the filament and the rates of ATP hydrolysis and Pi release during polymerization (214). We thus measured the depolymerization rate of wild type γ- and D51N-γ-nonmuscle actins by following the first-order decrease in light scattering as a function of time following addition of super stoichiometric amounts of DNase I as a sequestering agent for actin monomers. Table 3-1 shows the t_{1/2} values for this process. γ-Nonmuscle actin depolymerizes more slowly than yeast actin (t_{1/2} \approx ~70sec (229)). In comparison the D51N-γ-nonmuscle actin depolymerizes ~25% faster. This decrease indicates either an increased off rate of actin monomers from the filament or decreased binding to DNase I due to the mutation. Since purification of wild type and
D51N-γ-nonmuscle actin yield similar amounts of actin, in a DNase I-dependent process, we have assumed near normal binding although this question should be further explored.

Next, we assessed the P_i release kinetics, for most actin isoforms the P_i release curve is biphasic: an initial rapid release curve concomitant with polymerization and a slower second phase caused by the treadmilling of monomers through the filament at steady state. As previously published (229), the first-phase P_i release from γ-nonmuscle actin lags behind polymerization, similar to what is observed with muscle actin (Figure 3-5A) (216). For D51N-γ-nonmuscle actin, the initial P_i release rate appears to be concomitant with polymerization and ~4 times faster than that of wild type γ-nonmuscle actin (γ-D51N-nonmuscle actin = 9.8 x 10^{-3} ± 0.2 x 10^{-3} μm P_i/sec, γ-nonmuscle actin = 2.2 x 10^{-3} ± 0.3 x 10^{-3} μm P_i/sec) (Figure 3-5B). During the treadmilling phase, D51N-γ-nonmuscle actin P_i release rate, 1.4 x 10^{-3} ± 0.2 x 10^{-3} μm P_i/sec, is ~40% faster than that of γ-nonmuscle actin, 1.0 x 10^{-3} ± 0.1 x 10^{-3} μm P_i/sec. The faster P_i release rate during elongation and treadmilling phases suggests that the D51N-γ-nonmuscle actin is a more dynamic actin filament.

Discussion

Impact – 1st Characterization of a Yeast-Lethal γ-
Nonmuscle Actin Mutation

The data presented provides insight into how the D51N mutation in γ-nonmuscle actin leads to altered actin behavior eventually leading to deafness. Our previous data, of the other eight deafness causing γ-nonmuscle actin mutations cloned into yeast, demonstrate that the actin mutations do not abolish the ability of the actin to polymerize (139, 143, 144). Surprisingly, prior work demonstrated that cloning of the D51N mutation into yeast actin, as the sole actin in the cell, prevents cell growth. These data demonstrates that the lack of viability is not due to an inability of the mutant actin to polymerize. This result suggests that the effects of the γ-nonmuscle actin mutations on
the hearing apparatus could be due to the drastic alteration in the polymerization kinetics of the mutant vs. wild type actin.

Interestingly, the deafness-causing D51N-γ-nonmuscle actin displays biochemical properties similar to β-nonmuscle actin. The increased P_i release rate of the D51N-γ-nonmuscle actin correlates with this mutant actin’s faster off rate of actin monomers from the filament. This decreased filament stability may have major implications for the human disease, as the presence of β-nonmuscle as the sole actin in the hair cell of mice, is not sufficient to maintain normal hair cell stereocilia structure or hearing after 6 weeks of age (137). It is possible that the mutation alters the ability of the γ-nonmuscle actin to fulfill its maintenance functions. A complete understanding of how individual deafness causing γ-nonmuscle actin mutations affect hair cell structure and function cannot be carried out until a knock-in animal model is developed. However, the biochemical impact of these mutations on the isoactin in which they occur coupled with future studies on the effects of these mutations on actin regulation should provide crucial clues concerning the molecular perturbation of hair cell architecture which leads to hearing loss.

Role of DNase I Loop in Actin Dynamics

Examination of the actin filament, derived from the model of the Oda F-actin trimer (194), provides clues as to how the D51N mutation may lead to altered actin filament kinetics. Figure 3-6 shows that R37 (purple residue), D51 (green residue), and K84 (blue residue) form a tightly packed element which may help control the DNase I binding-loop (D-loop) structure. The four most mobile and flexible domains within the actin are the DNase I binding loop (D-loop; residues 39–50), the W-loop (residues 165–172), the hydrophobic loop (H-loop; residues 264–273), and the C-terminus (including residues 374 and 375) (230). The D-loop is disordered in most crystal structures of G-actin (27, 231-233) but can adopt specific conformational states. The D-loop was
observed to be unstructured in ATP tetramethylrhodamine-5-maleimide (TMR)-bound actin (27, 234), as a β-strand in the crystal structure of actin complexed to DNase I (212), and as an α-helix in the crystal of ADP TMR-bound actin (26), signifying the conformational dynamics intrinsic to this loop.

From the Holmes model of F-actin (12, 212) the main filament contacts were between subdomain 3 of the upper protomer and subdomain 4 of the lower protomer, supported by additional subdomain 1 and subdomain 2 contacts involving the W-loop, the C-terminus, and D-loop. A recent highest-resolution model from Oda (194), Namba (235), and refinements in the Holmes model (236, 237) support the assumption that the G-to F transition requires two major conformational changes before the monomer structure is fully incorporated into the filament. The first involves a rotation of the two major domains by ~20°, which leads to a flattening of the actin molecule within the filament. Secondly, the D-loop adopts an extended open loop conformation. Once in the F-actin state, residues 40-45 of the D-loop interact with residues 166-169 of a neighboring subunit along the actin helix and with a loop at positions 264-273 of the subunit from the opposite strand. (12, 194, 236, 237). In the Holmes actin filament model one of the main predicted filament contacts is the D-loop residues 40-45 interacting with the W-loop (residues 165-172) of the next highest protomer in the strand (12).

D51 may participate in an ionic interaction with positively charged residues R37 (below) and K84 (above). Mutation of D51 to N would lead to a complete loss of ionic interactions, leaving only potential hydrogen bonding. Additionally, loss of the negatively charged aspartic acid would increase the positive charge density of that structural unit. This combination of effects could exert a substantial destabilizing effect on the domain and lead to the observed altered filament kinetics. Experimental evidence also suggests that the dynamic loops of monomeric actin form important inter-protomer contacts within the filament and probably determine its conformational states (238). The functional importance of the dynamics of the D-loop is further demonstrated by the fact
that modifications or proteolytic cleavage directly affects actin polymerization (239-242). Although we have thus far concentrated on straight polymerization kinetics, future work will have to include ABP interaction studies. We would like to determine the effect of the mutation on the interaction with ABPs that bind between monomers of the actin filament, such as cofilin. Additionally the possible effect of the mutation on the interaction of actin and myosin in the hair cell will be discussed in the final chapter of this thesis.

Experimental Procedures

Materials

DNase I (grade D) was purchased from Worthington. Affi-Gel 10-activated resin and Micro Bio-Spin P-30 Tris gel filtration chromatography columns were obtained from Bio-Rad. DE52 DEAE-cellulose was acquired from Whatman. N-(1-pyrenyl)maleimide, ATP, ADP, hexokinase, and glucose were acquired from Sigma-Aldrich. Tween-20, enzyme grade, was obtained from Fisher Scientific. All other chemicals were reagent-grade quality.

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Construction of Baculovirus Transfer Vectors

The coding sequence of human wild type γ-nonmuscle actin was cloned into pDEST8 with the AcMNpv 6.9 baculovirus promoter upstream of the actin gene. Site directed mutagenesis was used to construct the D51N-γ-nonmuscle actin in the same
pDEST8 vector. The Invitrogen Bac-to-Bac system was then used to generate recombinant bacmids containing either the wild type γ-nonmuscle actin or D51N-γ-nonmuscle actin gene. All sequences were confirmed by sequencing of viral DNA from infected cells.

Purification of Recombinant Actin
Infected cells (with a multiplicity of infection equal to 2) were harvested 72 hrs post infection by centrifugation, washed in PBS and lysed in a high Tris-buffer [1 M Tris–HCl, pH 7.5, 0.5 mM MgCl$_2$, 0.5 mM ATP, 4% Triton X-100, 1 mg/mL Tween 20, 1 mM DTT and a protease inhibitor cocktail (benzamidine, leupeptin, aprotinin, antipain, TLCK, TPCK, E-64, each at 1.25 μg/ml)] by sonication. The cell lysate (50 ml from a preparation of approximately 9x10$^9$ cells) was cleared by centrifugation at 40,000K for 1hr using a Beckman L8-70M Ultracentrifuge with a 45Ti rotor. The lysate was then diluted with 1X G-buffer (10mM Tris-HCl, pH 7.5, 0.2mM CaCl$_2$, and 0.2mM ATP) with the same protease inhibitor cocktail as above. Positive protein expression and purity of the actin preparations were assessed by SDS-PAGE analysis (Figure 3-3). The concentration of G-actin was determined from the absorbance at 290 nm using an extinction coefficient of 0.63 M$^{-1}$cm$^{-1}$. The yield of actin was typically 1mg of actin per liter of infected cells, well above the level obtained from uninfected cells.

Actin Thermal Stability
The apparent melting temperatures of the actins were determined using circular dichroism by following the change in ellipticity of the G-actin sample at 222 nm as a function of temperature between 25 and 90°C as described previously (155). Measurements were made on an Aviv 62 DS spectropolarimeter. Data were fit to a two-state model, and the apparent $T_m$ value was determined by fitting the data to the Gibbs-Helmholtz equation to approximate the temperature at which 50% of the actin was denatured.
Actin ε-ATP Exchange

The ability of G-actin to exchange its bound nucleotide was assessed by first loading the actin with ε-ATP and then following its displacement from the actin in the presence of a large excess of ATP as described previously (155). Exchange rates were determined by fitting the data to a single exponential expression using BioKine Version 3.1.

Actin Polymerization

Polymerization of G-actin in a total volume of 160 µl was induced by the addition of MgCl₂ and KCl to final concentrations of 2 mM and 50 mM respectively (F-salts). Polymerization was monitored at 25°C by following the increase in light scattering of the sample in a FluoroMax-3 or a Flurolog (model FL3-21) fluorescence spectrometer outfitted with a computer controlled thermostatted four position multi-sample exchanger (HORIBA Jobin Yvon Inc.). Differences in machines can cause variations in the light scattering values; therefore, data from any given graph were obtained from the same fluorescence spectrometer. Both the excitation and emission wavelengths were set to 360 nm with the slit widths for both set at 1 nm.

Pᵢ Release from Actin

The rate of Pᵢ release from polymerizing actin samples following ATP hydrolysis was assessed using the commercially available EnzChek™ phosphate assay (Invitrogen) at 25 °C. Briefly, this spectrophotometric assay utilizes the purine nucleoside phosphorylase-dependent phosphorolysis of 6-mercaptop-7-methylpurine riboside to ribose 1-phosphate and 2-amino-6-mercaptop-7-methylpurine, the latter of which has a characteristic absorbance at 360 nm that is not shared by the nucleotide substrate at pH values greater than 6.5 (224, 225). Following induction of polymerization of 4.8 µM actin, the absorbance was monitored as a function of time, with readings taken
automatically at 10-s intervals at 360 nm using a thermostatted cuvette holder set to 25 °C ± 0.1 °C.

Actin Depolymerization

Actin was polymerized to steady state levels. DNase I was then added in a 1:5 actin to DNase molar ratio. Depolymerization was monitored as a decrease in light scattering over time. Depolymerization rates were determined by fitting the data to a single exponential expression using BioKine Version 3.1.

Electron Microscopy

Actin filaments were visualized by depositing 2 µl of a sample containing 4.8 µM F-actin onto carbon-coated Formvar grids. The grids were negatively stained with 1% uranyl acetate, and observed using a JOEL 1230 transmission electron microscope (University of Iowa Central Electron Microscopy Facility). Image J was used to process the images.
Figure 3-1. Hair Cell Morphology.

Panel A: Scanning electron microscopy (SEM) image showing the apical surface of outer hair cells located in the organ of Corti of the mouse. Bar = 5µm. Panel B: Diagram of molecular architecture of the hair cell’s actin cytoskeleton. Actin filaments are denoted by blue arrows. The pointed end of the actin filaments are represented by arrowheads. The three main actin rich subcellular domains are 1) the cuticular plate, containing a randomized meshwork of actin filaments into which actin filaments of the 2) stereocilia are anchored by the highly bundled actin rootlet and 3) the zonula adherens which contain anti-parallel actin filaments and connect hair cells to neighboring supporting cells (not shown).
Figure 3-2. Location of the Ten Deafness-Causing γ-Actin Mutations.

Front view of the crystal structure of actin modified from PBD: 1YAG (142) using PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC. The positions of the mutation are highlighted blue except for D51N which is highlighted yellow. The D-loop (DNase I binding loop) is colored red. ATP is depicted as an orange stick. Mg\textsuperscript{2+}/Ca\textsuperscript{2+} is depicted as a yellow sphere. N and C mark the respective termini. Numbers denote the actin subdomains.
Figure 3-3. Expression and Purification of Wild Type \( \gamma \) and D51N-\( \gamma \)-Nonmuscle Actin Using the Baculovirus System.

Panel A: 12% SDS-PAGE gel, lane one ladder, lane 2 lysate (total protein expression) of Sf21 insect cells infected with D51N-\( \gamma \)-nonmuscle actin. Asterisk at ~42kDa. Panel B: 10% SDS-PAGE gel, lane one ladder, lane 2 purified wild type \( \gamma \)-nonmuscle actin, lane 3 purified D51N-\( \gamma \)-nonmuscle actin. Asterisk at ~42kDa.
Figure 3-4. Polymerization Kinetics of Wild Type and D51N Mutant Actin.

Panel A: Polymerization of 4.8µM actin was initiated by the addition of MgCl₂ and KCl as described under “Experimental Procedures,” and the increase in light scattering (L.S.) was monitored as a function of time at 25°C. Shown is a representative plot of an experiment preformed with three independent actin preparations. A.U., arbitrary units. Panel B: Electron micrograph of 4.8µM wild type γ-nonmuscle actin. Panel C: Electron micrograph of 4.8µM D51N-γ-nonmuscle actin. Scale bar 100nm.
Figure 3-5. P₁ Release Associated with Polymerization of Wild Type and D51N Mutant Actin.

Panel A: 4.8 μM Wild type γ-nonmuscle actin. Panel B: 4.8μM D51N-γ-nonmuscle actin. Each actin was polymerized at 25 °C by the addition of F-salts. Filament formation was monitored by the change in light scattering and P₁ release using the EnzChek Assay. The data were normalized and superimposed as described under “Experimental Procedures.” Shown are representative plots of experiments performed at least three times with three independent actin preparations.
Figure 3-6. Predicted Effect of the D51N Mutation on the Filament.

Panel A: Model of the actin trimer based on the filament model of Oda et al. with monomers in various colors including: cyan, red, and gray (47). Residues are color-highlighted and labeled: in the gray monomer: K84, blue; Y53 orange; S52, pink; D51 green; D51N, yellow; R37, purple. Panel B: depiction of the spatial organization and possible stabilization function of D51 in maintaining the K84 (blue) and R37 (purple) ionic interaction in the actin filament. Panel C: depiction of the change in charge due to modification of the D51 residue to N, resulting in a possible loss of ionic interactions. Models modified using the PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.
Table 3-1. Actin Monomer and Filament Characteristics of Wild Type and D51N-γ-Nonmuscle Actin.

<table>
<thead>
<tr>
<th>Actin</th>
<th>Thermal Stability $T_m$ (°C)</th>
<th>ATP Exchange $t_{\frac{1}{2}}$ (sec)</th>
<th>$\frac{1}{2}$ Time of Depolymerization (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma</td>
<td>62 ± 1 (4)</td>
<td>112 ± 2 (3)</td>
<td>207 ± 4 (2)</td>
</tr>
<tr>
<td>Gamma-D51N</td>
<td>60 ± 0.2 (4)</td>
<td>101 ± 4 (3)</td>
<td>158 ± 15 (2)</td>
</tr>
</tbody>
</table>

Note: Apparent Melting Temperatures: $T_m$ of the listed actins were determined using circular dichroism, as described in “Experimental Procedures.” ATP Exchange: The release of actin-bound -ATP 1 μm actin in G buffer without free ATP was triggered by the addition of 100 μm ATP. The decrease in fluorescence caused by -ATP exchange was followed over time, and data were fit to a first order reaction mechanism as described under “Experimental Procedures.” Depolymerization Rate: The decrease in light scattering of actin filaments was followed as a function of time after the addition of DNase I at 25 °C. The $t_{\frac{1}{2}}$ of depolymerization was determined as described under “Experimental Procedures.” The number of experiments performed is indicated in parentheses.
CHAPTER 4. ALLELE-SPECIFIC EFFECTS OF THORACIC AORTIC ANEURYSM AND DISSECTION (TAAD) ALPHA-SMOOTH MUSCLE ACTIN MUTATIONS ON ACTIN FUNCTION

Abstract

Twenty-two missense mutations in ACTA2, which encodes α-smooth muscle actin, have been identified to cause thoracic aortic aneurysms and dissections (TAAD). Limited access to diseased tissue, the presence of multiple unresolvable actin isoforms in the cell and lack of an animal model has prevented analysis of the biochemical mechanisms underlying this pathology. We have utilized actin from the yeast \textit{S. cerevisiae}, 86% identical to human α-smooth muscle actin, as a model. Two of the known human mutations, N115T and R116Q, were engineered into yeast actin and their effect on actin function \textit{in vivo} and \textit{in vitro} was investigated. Both mutants exhibited reduced ability to grow under a variety of stress conditions, which hampered N115T cells more than R116Q cells. Both strains exhibited abnormal mitochondrial morphology indicative of a faulty actin cytoskeleton. \textit{In vitro} the mutant actins exhibited altered thermostability and nucleotide exchange rates indicating effects of the mutations on monomer conformation with R116Q the most severely affected. N115T demonstrated a biphasic elongation phase during polymerization while R116Q demonstrated a markedly extended nucleation phase. Allele-specific effects were also seen on critical concentration, rate of depolymerization and filament treadmilling. R116Q filaments were hypersensitive to severing by the actin-binding protein cofilin. In contrast, N115T filaments were hyposensitive to cofilin, despite near normal binding affinities of actin for cofilin. The mutant specific effects on actin behavior suggest that individual mechanisms may contribute to TAAD. (This chapter was published in the Journal of Biological Chemistry. 2011 Apr 1;286(13):11356-69. Epub 2011 Feb 2.)
Introduction

Aneurysm and dissection of the thoracic aorta is a major cause of mortality, accounting for 0.5 to 1% of deaths annually in the United States, and the incidence is increasing, affecting 9 – 16 in 100,000 individuals per year (161, 243). Aortic aneurysms tend to be asymptomatic until dissection, contributing to the high degree of morbidity and mortality. Aortic aneurysms can occur in the absence of systemic findings of a connective tissue disorder complicating diagnosis (244). Twenty percent of affected patients inherit the disorder, and the vast majority have an autosomal dominant pattern of inheritance. However, variable penetrance further hobbles patient identification in patients with familial thoracic aortic aneurysm and dissection (TAAD) (245).

Multiple genes and loci have been associated with familial TAAD (246, 247). Mutations in ACTA2, which encodes α-smooth muscle actin, are the most common genetic cause of familial TAAD, responsible for 15% of cases (124). Interestingly, mutations in ACTA2 are associated with an array of cardiovascular diseases from congenital to premature acquired heart disease. Clinical problems include patent ductus arteriosus, occlusive strokes and coronary artery disease in addition to thoracic aortic aneurysms (125). α-Smooth muscle actin is one of six highly homologous isoactins expressed in mammals. Mutations in the actin family have recently been associated with disease, the vast majority of which behave in a dominant fashion. Over the past decade, mutations in α-cardiac (ACTC), α-skeletal (ACTA1), and γ-cytoplasmic actin (ACTG1) have been associated with cardiomyopathies, skeletal myopathies and deafness, respectively. As such, the role for the α-smooth muscle actin in human disease is not surprising.

In vascular smooth muscle cells, α-smooth muscle actin has many roles including maintenance of cell wall integrity, force transduction, and regulation of vascular smooth muscle cell proliferation (248). α-Smooth muscle actin is the most abundant protein in vascular smooth muscle cells making up 40% of total cellular protein and over 70% of
the total actin (249). The other two actin isoforms in the vascular smooth muscle cells are β- and γ-cytoplasmic actin. Histopathology from individuals with α-smooth muscle actin mutations noted loss or disorganization of the vascular smooth muscle cells in the tunica media of the aorta (125). Analysis of aortic vascular smooth muscle cells showed diminished α-smooth muscle actin, with substantially fewer polymerized actin fibers that did not extend completely across the cell body. α-Smooth muscle actin staining did not co-localize with polymerized actin fibers and was found clumped along the cell wall or nuclear wall.

To gain insight into how ACTA2 mutations cause disease, the functional changes in the context of the structure of the actin molecule must be established. The actin monomer can be divided into two large domains. An adenine nucleotide binds between the two halves bridging the cleft to impart stability to the protein and plays a major role in controlling protein dynamics. Each of the domains can be each further divided in two subdomains as shown in Figure 4-1. Actin polymerizes to form a polar filament with what is termed a barbed and a pointed end. The barbed end is the preferred site for monomer addition during filament elongation, and as such, is where much of actin assembly regulation occurs. The asymmetry can be imparted to the actin monomer as well. Subdomains 1 and 3 constitute the monomer barbed end, whereas subdomains 2 and 4 make up the pointed end.

At least 19 autosomal dominant missense mutations in α-smooth muscle actin have now been identified in familial TAAD, and they are distributed across all four subdomains (124, 163, 250). The focus of this paper is on two of these mutations, N117T and R118Q, which are adjacent to one another in a secondary structural element, near the barbed end of the protein. The location is predicted to be involved in intermonomer interactions as shown in Figure 4-1. Of interest, the two mutations have different clinical phenotypes. The N117T mutation has been found in both α-skeletal muscle and α-smooth muscle actins, and is associated primarily with aneurysms of the thoracic aorta.
R118Q is the second most common mutation identified in α-smooth muscle actin, and remarkably 75% of patients with this mutation have premature coronary artery disease in addition to aortic aneurysms. The varied clinical phenotypes suggests that while all 19 of these mutations cause TAAD, the molecular mechanisms by which different mutations lead to aortic disease are allele-specific.

Understanding the effects of α-smooth muscle actin mutations at the molecular level in the context of the aortic wall is hampered by the difficulty in obtaining sufficient samples from patients for biochemical studies. Even if one were to introduce the mutations into model animals to establish cultured vascular smooth muscle cell preparations, the amount of material available for biochemical analysis would be limited. Additionally, the presence of three essentially unresolvable isoforms of actin in the vascular smooth muscle cells would hinder direct assessment of the effects of the mutations on α-smooth muscle actin function.

An attractive model system for studying the effects of actin mutations is the budding yeast, *Saccharomyces cerevisiae*. Yeast actin is 86% identical and 94% similar to α-smooth muscle actin, and is encoded by a single essential gene, *ACT1* (145). More importantly, the two residues at which the TAAD mutations occur in α-smooth muscle actin are identical in yeast actin (N117T and R118Q in α-smooth muscle actin correspond to N115T and R116Q in yeast actin, for this thesis the yeast numbering system is used). In addition, actin regulation is well conserved between species. Many of the actin-binding proteins in mammalian cells are also found in yeast (146, 148), and yeast actin will interact with most of the mammalian isoforms of actin-binding proteins due to the high degree of homology between actins (147, 149). Mutations can be readily introduced into the actin gene by site directed mutagenesis to express mutant actin as the sole actin isoform. The impact of mutations on actin function in the cell can be assessed cytologically, and the actin can be purified for studies of the biochemical effects of the mutations on the molecule. The *in vivo* and *in vitro* data can be analyzed for alterations
in molecular behavior to provide insight into the mechanisms underlying aortic disease. Our lab has previously applied this approach to the analysis of mutations in γ-nonmuscle actin that cause autosomal dominant early onset deafness (139, 143, 144).

In this study, either N115T or R116Q mutant actin was expressed as the sole actin in the cell. The effects of the TAAD mutations on cell behavior and actin related cellular functions were characterized. Actin was purified and the impact of the mutations on actin monomer and polymer function in vitro was assessed.

Results

Effect of Mutations on Yeast Cell Growth

To assess the effects of missense mutations on actin function in vivo, the basic biological functions dependent on actin in yeast were examined including cell growth, cell division, and response to temperature or osmotic stress (251-253). Yeast cells expressing N115T or R116Q actin as the sole actin in the cell were compared to wild type. There were no differences in doubling time (~2hrs) or extent of growth with the mutant isoforms under normal growth conditions (data not shown). Cells expressing mutant actin were 25% larger than wild type cells, however. The diameter of wild type cells was 7.8 ± 0.98 µm while N115T and R116Q cells measured 9.88 ± 1.4 µm and 9.87 ± 1.18 µm respectively (p < 0.001). In addition, the N115T strain exhibited temperature-sensitive growth at 37 °C on YPD medium, and both N115T and R116Q strains had defective growth in hyperosmolar medium indicative of an impaired actin cytoskeleton (Figure 4-2A).

A functional cytoskeleton is required for mitochondrial function and inheritance (254-256). To test for defects in mitochondrial integrity, the ability of mutant cells to grow on glycerol as a sole carbon source was assessed. Yeast requires mitochondrial glycerol-3-phosphate dehydrogenase to utilize glycerol for glycolysis (257, 258). As such, actin-dependent deficiencies in mitochondrial function can lead to impaired growth
on glycerol. Both the N115T and R116Q strains exhibited mildly diminished growth on glycerol medium (Figure 4-2A), indicating relatively preserved mitochondrial function.

Effect of the Mutations on Actin Cytoskeletal Patterns and Organelle Morphology

Alterations in morphology of the cell and actin cytoskeleton could result from mutations in actin. Disruption of the actin cytoskeleton may lead to loss of polarization during budding resulting in aberrant cell size and actin organization (144). Actin filaments in yeast are organized as cables and patches. Cables facilitate movement of organelles to the daughter cell during division. Patches are distributed in a cell-cycle dependent manner and localize at sites of endocytosis (259, 260). Both mutations affected cytoskeletal morphology with high frequency of abnormalities compared to wild type (p < 0.01) (Figure 4-2B and C). Cells expressing N115T had abnormally thick actin cables and inappropriately distributed patches. Cells expressing R116Q demonstrated a dramatic reduction in the presence of actin cables and an increase in the size and number of actin patches (Figure 4-2B and C). Thus, the two neighboring mutations cause different effects on actin cytoskeletal morphology.

Actin regulates organelle movement from the mother cell to the bud along polarized actin cables. Given the abnormalities seen in the actin cytoskeleton, organelle morphology and distribution in the mutant cells were assessed. Vacuole morphology, regulated by actin, was visualized by staining with FM4-64. Both mutations resulted in abnormal vacuole morphology (p < 0.01 compared to wild type), but again in an allele specific manner (Figure 4-2B and C). Wild type cells most commonly have one to four vacuole lobes. Ninety one percent of wild type cells displayed normal vacuole morphology while only 68% of N115T and 64% of R116Q cells exhibited the normal phenotype. Of interest, the abnormal vacuole morphology found in mutant cells differed between alleles. Hypervesiculation with 5-10 small vacuoles was seen in 75% of the
abnormal N115T cells. In contrast, a single oversized lobe filling the cell was the dominant morphology in abnormal R116Q cells (Figure 4-2B).

To assess the effect of mutations on DNA distribution, genomic and mitochondrial DNA were imaged by staining cells with the DNA-intercalating dye DAPI. We found no differences in DNA distribution between cells expressing the mutations and wild type (data not shown). The normal findings of a large single spot consistent with nuclear DNA and multiple diffuse, faint extra nuclear spots consistent with mitochondrial DNA had similar prevalence in all three cell types.

Disorganization of the polarized actin cytoskeleton can lead to abnormal fission/fusion events that regulate mitochondrial morphology and function (261). To visualize the mitochondria, cells were transformed with a plasmid containing GFP fused to the mitochondrial targeting sequence of citrate synthase. Normal mitochondrial tubular structures are arranged in an orderly, polar manner running from the mother cell to bud. This normal mitochondrial pattern was present in 94% of wild type cells compared to only 43% of the N115T cells and 37% of the R116Q cells (p < 0.05) (Figure 4-2B and C). Both the N115T and R116Q cells demonstrated fragmented and aggregated mitochondrial tubules. Despite these morphological effects, mitochondrial function remained relatively normal as assessed by growth behavior as described above.

The cytoskeletal dysfunction in mutant cells prompted evaluation of cytoskeletal stability in vivo. Latrunculin A is a drug that sequesters actin monomers and reversibly promotes rapid depolymerization of actin filaments (262-265). Greater sensitivity of cells to Latrunculin A can indicate an increased monomer off-rate contributing to an unstable actin cytoskeleton. Using a disc diffusion assay, the sensitivity of cells to Latrunculin A was assessed. Discs saturated with a range of drug concentrations were placed on cultures for 48 hours, and the zone of growth inhibition surrounding the disc was compared (Figure 4-3). Both mutants displayed increased growth inhibition
compared with wild type cells with R116Q demonstrating the most sensitivity to Latrunculin-A.

**Effect of the Mutations on In Vivo Phenotype in a ΔAip1 Background**

The *in vivo* findings support overall cytoskeletal instability. If so, decreasing the effects of the machinery in the cell responsible for filament turnover might rescue the phenotype. One such regulator is the F-actin severing protein, cofilin. Cofilin is an essential protein in yeast, however, its activity can be attenuated *in vivo* by eliminating Aip1p, an enhancer of cofilin. The N115T and R116Q mutations were expressed in Δaip1:pCENWT host cells and the above cytological assays were repeated (Figure 4-4). In the absence of Aip1p, the reduction in cofilin activity rescued the mild growth defects in hyperosmolar media seen with the R116Q mutation (compare with Figure 4-2) but the frequency of cytoskeletal abnormalities persisted. In contrast, N115T cells displayed a marked improvement in cytoskeletal morphology (*p < 0.005*). The frequency of normal cytoskeletal structure doubled in the background of attenuated cofilin activity. No differences were seen, however, in actin-dependent vacuole morphology or mitochondrial morphology (Figure 4-4B).

**Effects of the Mutations on the Actin Monomer Characteristics *in Vitro***

To gain insight into the molecular basis for the *in vivo* phenotypes described above, wild type or mutant yeast actin was purified to assess the biochemical effects of the mutations in G- and F-actin function. No difference in the amount of expressed actin was noted in mutant cells compared to wild type based on protein purification yield. Thermal stability of G-actin was determined by quantifying the unfolding of the monomer as a function of temperature as detected by a change in circular dichroism. Both mutants showed decreased thermal stability. The *T*<sub>m</sub> for N115T actin, 55 ± 1°C,
and R116Q, 53 ± 2 °C, were both significantly lower than wild type actin (59 ± 1 °C, p < 0.005 for both) (Table 4-1).

Mutation-dependent effects on G-actin dynamics can also alter the rate of exchange of the adenine nucleotide from deep within the protein. The kinetics of fluorescent ε-ATP exchange from wild type and mutant protein actin was determined. Both mutants had prolonged t½ values as compared to wild type: wild type 36 ± 3 sec, N115T 43 ± 2 sec, R116Q 54 ± 8 sec (p < 0.0001 for both). The delayed exchange implies mutant monomers are more rigid and/or assume a more closed conformation around the nucleotid e than wild type actin.

Effects of Mutations on Actin Polymerization

A potential cause for the abnormal vascular smooth muscle cell morphology found in patients is a mutation-dependent alteration of actin polymerization, leading to delayed or altered dynamic adaptation of the contractile or cytoskeletal apparatus. Polymerization kinetics are a factor in this dynamic response. The location of the two mutations along the barbed end of the monomer, where interactions facilitate monomer addition to the filament, suggests mutations might alter polymerization behavior characterized by changes in nucleation or elongation rates or critical concentration. The effects of the mutations on actin polymerization kinetics were assayed by quantifying the change in light scattering as the actin polymerizes (Figure 4-5A). Light scattering assays were used rather than pyrene fluorescence because of the inability to label the R116Q mutant actin preventing isoactin comparisons (discussed later). Both mutants displayed polymerization defects but with different phenotypes. N115T repeatedly demonstrated a biphasic elongation phase. R116Q had a prolonged nucleation phase and a more rapid elongation phase compared with both wild type and N115T actin. R116Q also displayed a lower final extent of polymerization. Electron microscopic examination confirmed that both mutant actins produced a relatively normal appearing filament, although the average
filament length of N115T actin was 30% smaller than wild type actin (p < 0.0001) (Figure 4-6A). Thus, N115T and R116Q polymerization kinetics support that mutations destabilize monomer-monomer contact without significantly disrupting filament structure.

TAAD actin mutations in patients are autosomal dominant with expression of both a wild type and mutant allele. To determine the effect of wild type actin on the mutant phenotype in vitro, mutant actin was co-polymerized with increasing amounts of wild type actin, keeping the total actin concentration constant (Figure 4-5B and C). Increases in the proportion of wild type actin caused a commensurate normalization of the polymerization profile, suggesting a proportional rather than synergistic effect of the mutant actin on the wild type actin behavior.

Effects of the Mutations on Filament Stability

To further quantify filament instability that might be associated with these mutations, the minimum concentration of actin monomers required for polymerization, the critical concentration (Cc), was determined for N115T and R116Q actin (Table 4-1). The critical concentration for both mutants was higher than wild type actin; wild type was 0.47 µM ± 0.19 compared to 0.86 µM ± 0.37 for N115T and 1.03 µM ± 0.36 for R116Q (p < 0.05 for both). The increase in critical concentration indicates both mutations cause filament instability.

Effects of Mutations on Actin Depolymerization

One determinant of actin filament stability is the relative on-rate of actin monomers to the filament relative to the off-rate of monomers leaving the filament. An unstable actin filament would demonstrate either decreased on-rate or an increased off-rate. To assess if a faster off-rate contributes to the filament instability described above, the depolymerization rate was quantified. DNase I was added to polymerized actin to bind the G-actin that detaches from the filament ends. N115T actin showed an increased
off-rate with a $t_{1/2}$ 3.9 times faster than wild type, while R116Q is 7.8 times faster than wild type ($p < 0.001$ for both) (Figure 4-3B). These findings again support that both mutations result in filament instability.

Alterations in $P_i$ Release

The alterations in mutant filament stability and dynamics can manifest by effects on ATP hydrolysis and $P_i$ release during polymerization. Yeast actin releases inorganic phosphate immediately following ATP hydrolysis. $P_i$ release for the N115T mutation initially followed polymerization, similar to wild type actin, but during the treadmilling phase when monomer attachment at the barbed end is matched by monomer release at the pointed end, $P_i$ release continued more rapidly than wild type actin in a linear manner (Figure 4-7). The phosphate release rate of R116Q is slower than wild type during elongation, corresponding to its nucleation lag phase, but then dramatically increases during treadmilling phase. Increased phosphate release during treadmilling implies increased turnover of actin subunits during treadmilling, or alternatively, increased fragment formation providing more substrate for continuous addition of monomers onto the newly formed fragments. Both explanations are consistent with less stable mutant actin filaments.

Effects of the Mutations on the Actin-Cofilin Interaction

The altered monomer-monomer interactions leading to filament instability may also affect the sensitivity of the mutant filaments to the severing activity of the actin binding protein cofilin. Cofilin is a major regulator of filament dynamics in vivo that binds between two adjacent actin subunits in the filament promoting disruption of inter-monomer bonds. Cofilin also sequesters monomers, preferably when bound to ADP as is the case when the monomer dissociates from the filament (14, 109, 266). We examined the susceptibility of the wild type and mutant actin to increasing amounts of yeast cofilin
Yeast cofilin tends to decorate rather than sever actin filaments at stoichiometric levels, resulting in an increase in light scattering as seen with wild type actin in Figure 4-8A. As cofilin was added to wild type actin filaments, light scattering increased at 2:1 and 1:1 actin:cofilin concentrations attributed to cofilin binding and decoration of the filament (Figure 4-8A). As cofilin concentrations increased beyond 1:1, light scattering decreased consistent with filament severing and monomer sequestration. At the highest concentrations of cofilin studied, little residual filamentous wild type actin can be identified by electron microscopy (Figure 4-8B). The same analyses were done for each mutant. Remarkably, N115T and R116Q showed divergent responses to cofilin (Figure 4-8A). R116Q behaved as initially hypothesized and was hypersensitive to cofilin. At a 3:1 actin:cofilin ratio, R116Q showed a 20% drop in light scattering consistent with filament severing. R116Q light scattering decreased further at increasing concentrations of cofilin, when wild type filaments were abundant, dropping to pre-polymerization baseline values at actin:cofilin ratios greater than 1:1. As an additional control, F-buffer was added instead of cofilin, which verified that changes in light scattering are not due to simple mixing of the samples. In stark contrast, the response of N115T actin to cofilin was nearly opposite that of R116Q. N115T showed an initial decrease in light scattering at lower concentrations of cofilin with a steady drop to 60% of initial light scattering by the benchmark 1:1 concentration (Figure 4-8A). However, as the concentration of cofilin was raised, the light scattering increased as well suggesting cofilin decoration and stabilization of intact filaments. At cofilin concentrations 2.5 times that of actin, well past concentrations inducing total disassembly of both wild type and R116Q filaments, light scattering values were more than 75% of initial light scattering values.

Electron micrographs of wild type, N115T and R116Q actin filaments confirmed the spectrophotometric data (Figure 4-8B). At 1:1 actin:cofilin concentrations, wild type actin filaments were thicker with an irregular surface indicative of decoration. At higher concentrations of cofilin, only small aggregates were observed. R116Q showed
hypersensitivity to cofilin severing demonstrating no recognizable filaments at cofilin concentrations equivalent to or higher than actin. In contrast, N115T filaments persisted at a 1:1 actin:cofilin ratio and beyond. Filament lengths for each actin were measured from electron micrographs at the varied cofilin concentrations. As expected, the average filament length corresponded to the light scattering values (Figure 4-6B).

To ascertain if mutant actin exhibits a dominant effect on the interaction between cofilin and wild type actin, the response of mutant and wild type heteropolymers was assessed, as above. Similar to the isolated polymerization data, the response of R116Q/wild type actin to cofilin was muted in a dose related manner by the addition of wild type actin, indicating a proportional effect of the mutant (Figure 4-8C). Likewise, addition of N115T to wild type actin produced a protective effect proportional to the mutant mole-fraction. Filament morphology as determined by electron microscopy again supported the spectrophotometric findings (data not shown).

Effects of Mutations on Cofilin Binding

To assess the relative contribution of altered cofilin dependant severing versus sequestration, the affinities for cofilin to ATP-G-actin and F-actin were determined for wild type and N115T mutant actins. The binding of cofilin to R116Q actin could not be analyzed, as the elimination of DTT from solution needed for reaction of the actin with pyrene-maleimide resulted in protein denaturation. The $K_d$ for wild type G-actin was 0.8 $\pm$ 0.4 $\mu$M, while the N115T mutant actin $K_d$ was 2.5 times lower at 0.3 $\pm$ 0.1 $\mu$M (Table 1). The increased affinity did not translate to the cofilin F-actin interaction as the $K_d$ of cofilin to wild type actin filaments, 0.9 $\pm$ 0.2 $\mu$M, was similar to N115T, 0.7 $\pm$ 0.2 $\mu$M. Thus, the persistence of N115T filaments did not result from altered affinity of cofilin for mutant F-actin.
Discussion

The data presented begins to elucidate the biochemical basis by which mutations in α-smooth muscle actin lead to thoracic aortic aneurysms and dissections (TAAD). This is the first attempt to address the molecular mechanism of this cardiovascular disorder. Yeast actin was used for analyses given the high homology with α-smooth muscle actin and opportunity for in vivo / in vitro correlation. While it would be desirable to study the effect of these mutations in α-smooth muscle actin per se, we have not yet been able to express this actin isoform in a baculoviral system despite repeated attempts. The two mutations chosen for study, N115T and R116Q, occupy adjacent positions on a secondary structural element in actin thought to be intimately involved in the monomer-monomer interface necessary for actin filament formation (12, 194, 267). Despite their immediate proximity, the mutations have different clinical phenotypes. R116Q is second most common identified mutation in patients with TAAD, and is interestingly associated with premature coronary artery disease. N115T, on the other hand, is a less prevalent mutation and has been associated with stroke but not coronary artery disease (124, 125). Thus, our goal was to not only gain insight into positions 115 and 116 in actin behavior but also understand the mechanisms underlying the distinct clinical phenotypes.

In summary, our data indicate allele-specific effects of mutations on actin behavior both in vivo and in vitro. The N115T mutation produces more deleterious effects in yeast cells, but the R116Q mutation leads to more striking effects in vitro. Growth under stress conditions was generally worse with the N115T actin, as a greater percentage of cells displayed abnormal cytoskeletal morphology. Conversely, the R116Q mutation results in more dramatic effects on actin monomer behavior and polymerization especially relative to filament nucleation and the critical concentration needed for polymerization. While allele-specific differences were observed in vitro, both mutations displayed filament instability based on four criteria. First, both mutant actins exhibit
faster disassembly rates in the presence of DNAse I. Second, in vivo effects of Latrunculin A, a drug that facilitates actin depolymerization, signify decreased cytoskeletal stability in the mutant cells. Third, the rate of treadmilling, based on the rate of P_i was greater for both R116Q and N115T. Finally, both mutant actins have increased critical concentrations.

The differences in clinical phenotypes described above may stem from the role of α-smooth muscle actin in the vascular wall, to transmit force applied by the myosin head. The severe instability of the R116Q actin monomer-monomer interface, as evidenced by marked alterations in polymerization kinetics, critical concentration, and depolymerization rates, would limit the ability of the actin filament to withstand this force leading to disruption of the smooth muscle. The decreased stability of R116Q actin filaments in vitro is consistent with the absence of α-smooth muscle actin-containing filaments and an increased the pool of unpolymerized α-smooth muscle actin in vascular smooth muscle cells from R116Q TAAD patient samples (124). Conversely, the N115T mutation may predominantly effect the interaction of filament regulatory proteins with the actin. A functional cytoskeleton requires rapid, reversible assembly and disassembly of actin filaments in a temporal and spatial pattern. The altered cofilin-N115T actin interactions indicate altered regulation of this dynamic process. Lack of published histopathology involving the N115T mutation makes the correlation between the current model system and the disease unavailable. The differences emphasize that further study of the impact of mutations on actomyosin function is needed.

Vascular smooth muscle cells from TAAD patients should contain a mixture of both wild type and mutant α-smooth muscle actin due to the autosomal dominant nature of the disease. We assessed the effects of mutant actin on wild type actin behavior to determine if the mutant actin exerted a dominant or proportional influence. Actin behavior was altered at all ratios of mutant and wild type actin in a manner proportional to the mole fraction of mutant actin. At a 1:1 ratio of mutant to wild type actin, the
mixture that should be present in TAAD vascular smooth muscle cells, we observed significant effects on filament behavior.

The purification process of the mutant actins exposed that the R116Q mutant actin is more likely to denature than wild type or N115T actins. Further experimentation revealed that the major reason for R116Q destabilization was an increased sensitivity to oxidative damage, as omission of the reducing agent DTT from purification steps led to protein denaturation. This observation suggests that a factor contributing to R116Q disease pathology may be increased sensitivity to oxidative stress, especially if inflammation and the attendant production of reactive oxygen species are involved in the disease process. Another well-documented case for oxidative damage to actin has been seen in patients with sickle cell disease in which erythrocyte actin undergoes oxidative intra-molecular disulfide crosslinking leading to abnormal cytoskeletal behavior (268).

The alteration of the monomer-monomer interface in mutant F-actin can influence interactions with actin regulatory proteins. Our data support that both N115T and R116Q mutations affect actin regulation by the actin-binding protein, cofilin. Cofilin plays a central role in modulating cytoskeletal dynamics in vivo via its ability to bind to and sever F-actin and bind and sequester ADP G-actin. Cofilin has two binding sites on actin, one of which encompasses actin residues 105-132. Furthermore, the 112-125 helix, in which the two mutations are located, interacts strongly with cofilin based on the results of ELISA and fluorescence experiments (269). Unexpectedly, the two mutations had opposite effects on the sensitivity of F-actin to cofilin in vitro. R116Q actin was much more sensitive to yeast cofilin than wild type actin. Conversely, N115T filaments were hyposensitive to severing by cofilin. N115T filaments remained largely intact, even at the highest cofilin concentration tested. Part of cofilin’s ability to sever actin filaments has been attributed to the twist that cofilin binding imparts on the F-actin helix, inducing strain on monomer-monomer interfaces. The cofilin-resistance displayed by the N115T mutation may result from alteration in the interface, producing more flexibility and a
greater ability to accommodate the additional strain. The persistence of N115T filaments at high concentrations of cofilin cannot be explained by a resistance to cofilin sequestration as the binding affinity of cofilin for the N115T mutant G-actin is tighter than that of wild type. We have observed resistance to cofilin previously in the deafness mutant P332A but to a lesser extent. At a actin:cofilin ratio of 1:2.5, approximately half of the original P332A actin filaments persisted (P332A at 55% vs N115T at 80%)(143).

Examination of the actin filament, derived from the model of the actin trimer of Oda et al. (267) based on electron cryomicroscopy, provides clues to the different effects caused by the two mutations. Focusing on N115T effects, Figure 4-9 shows that N115, K113, and P112 of one monomer (red) form a tightly packed element that interacts with the C-terminal end of helix 191-199 on an adjacent monomer (gray). This prediction is supported by recent Hydrogen/Deuterium exchange data from our laboratory, which shows decreased amide proton exchange in the 105-132 peptide following actin polymerization. This indicates that when comparing G- to F-actin new contacts are formed between the 105-132 peptide and monomers of the filament such that this peptide is now protected from the solvent (270). Modeling suggests that substitution of threonine for asparagine at residue 115 decreases the packing density in this element (compare Figure 4-9 Panel A and B), creating a conformational change that may well alter the interaction with the opposing monomer.

Although adjacent to N115, the greater extent of filament instability induced by the R116Q mutation suggests a different mechanism may be involved. The C-terminal peptide of actin, ending with residue F374, is involved not only in formation of a monomer-monomer interface but also in allosteric regulation of the dynamics of the actin filament (271). The Oda et al. model shows a packed array of residues in subdomain 1 encompassing E107, R116, V370, and H371 (Figure 4-10). In this array, R116 appears to participate in an ionic bond with E107, providing stability to this structural element. An R116Q alteration would not only interrupt this ionic interaction but the substitution of
the smaller glutamine for arginine would also destroy the packing of the element. This combination of effects could exert a substantial effect on actin C-terminal peptide behavior leading to the altered filament kinetics and stability observed. Our hypothesis is supported by the fact that mutations in V370 in this element give rise to two other diseases in humans. In α-skeletal muscle isoactin, the V370F mutation causes a severe nemaline myopathy (272) while the V370A mutation in gamma-cytoplasmic isoactin leads to early onset autosomal dominant deafness (123). Our attempts to introduce the V370F mutation into yeast actin resulted in lethality while the V370A mutation resulted in an extremely compromised cell with many of the traits identified in the R116Q cells (144).

Establishing the pathophysiological basis for TAAD caused by mutations in α-smooth muscle actin is a step toward improving the identification and treatment of patients. Understanding the mechanisms of the disease requires elucidation of the effects of the mutations on the behavior of actin per se, its regulation, and the impact on actin mediated processes within the cell. Our results demonstrate the utility of using the yeast/yeast actin model system to correlate the effects of the mutation in vitro with the biological changes caused by the mutant actin in vivo as the only actin in the cell. For the two mutants studied, we observed allele-specific effects in actin polymerization and regulation, which may account for the differences in disease phenotypes. Application of this approach to the remaining α-smooth muscle actin TAAD mutations should greatly improve our knowledge of the molecular effects of these mutations as well as provide insight into the dynamics of wild type actin.

**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. 1,N6-ethenoadenosine 5′-triphosphate (ε-ATP), rhodamine-phalloidin, FM 4-64, and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Molecular Probes. The QuikChange® 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 (144) containing the yeast actin coding sequence driven by the ACT1 promoter using the QuikChange® mutagenesis kit according to manufacturer instructions. Plasmids containing the desired mutations were introduced into a recipient yeast strain containing a deleted chromosomal ACT1 gene and a plasmid expressing wild type actin (pCENWT) as described previously (273). Plasmid shuffling yielded viable haploid strains for each of the mutations. 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) at 30 °C on a shaking platform. Growth was determined by diluting an overnight culture of each strain into fresh medium at an initial A600 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 media 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 hyperosmolar sensitivity, cells were plated on YPD plus 0.9 M NaCl agar plates and incubated at 30 °C.

Cytology

Cell structures were imaged with an Olympus IX81 microscope and a Hamamatsu (Model# C10600-10B-H) camera. Camera control and image enhancement were performed using MetaMorph Version 4.5 software (Universal Image Corp., Downingtown, PA). Presentation of cell images was done using CorelDRAW 11. All cellular statistical analysis was based on cell counts of >100 for each sample. To measure cell size, mounted samples were visualized by differential interference contrast microscopy. The long axis of the cell was measured using Image J.

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 (256). Cells expressing the plasmid were grown to an A600 of 0.3–0.5 in Ura– synthetic medium to force retention of the URA3-marked plasmid in the otherwise Ura3– cells. An aliquot of cells was resuspended in VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA), and the cells were then observed by fluorescence microscopy as described above. Z-sections through the cell were obtained at 0.15-μm intervals. Out-of-focus light was removed by deconvolution using MetaMorph software, and each series of deconvolved images was

The actin cytoskeleton was visualized by fluorescence microscopy after staining fixed cells with rhodamine-phalloidin as described previously (151). Vacuoles were imaged following exposure of the cells to the dye FM4-64 as described previously (274). Nuclear and mitochondrial DNA was stained with DAPI as described previously (151).

**Latrunculin A Sensitivity**

Sterile filtered discs (0.5 cm in diameter) were presoaked in 2 µl DMSO (control), 0.1, 0.5, or 1 mM of Latrunculin A (248). Soaked discs were placed on YPD plates containing 100 µl of evenly spread wild type or mutant cells (OD600=0.1). The plates were incubated at 30 °C for 48 hr, and the zone of growth inhibition around the drug eluting Latrunculin A disc was assessed.

**ΔAip1p Deletion Strain Studies**

Using an Δaip1:pCENWT strain as the host (274), pRS314 plasmids containing the promoter region, the TRP1 gene, and the coding sequence for wild type or mutant yeast actin were transformed into this haploid yeast strain (228). Transformants were selected on tryptophan-deficient medium and then subjected to plasmid shuffling to eliminate the wild type actin gene. Growth curves, growth characteristics and cytology were determined as described above for wild type and each mutant in the ΔAip1p deletion strain.

**Protein Purification**

Wild type and mutant yeast actins 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 (228). 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 0.63 M$^{-1}$ cm$^{-1}$. Actin was stored as G-actin in G buffer (5 mM Tris-HCl, pH 7.5, 0.1 mM ATP, pH 7.5, 0.2 mM CaCl$_2$, and 0.2 mM dithiothreitol). All actins were stored at 4 °C and used within four days of purification. Yeast coflin was purified from *Escherichia coli* carrying a recombinant construct for the protein according to Lappalainen et al. (143, 275) and the concentration of the purified coflin was determined by absorption at 280 nm with an extinction coefficient of 14650 M$^{-1}$ cm$^{-1}$.

**Actin Polymerization**

Polymerization of 4.8 µM G-actin in a total volume of 120 µl was induced by the addition of MgCl$_2$ and KCl to final concentrations of 2 and 50 mM, respectively (F-salts). Polymerization at 25 °C was monitored by following the increase in light scattering of the sample in a FluoroMax-3 fluorescence spectrometer outfitted with a computer-controlled thermostatted four-position multi-sample exchanger (HORIBA Jobin Yvon Inc.). The excitation and emission wavelengths were both set to 360 nm with the slit widths set at 1 nm. To determine the effects of coflin on preformed actin filaments, the desired amount of coflin was added to the polymerized actin sample, and the resulting change in light scattering was monitored. For experiments examining the effects of different mole fractions of mutant actin on overall actin behavior, wild type and mutant actin were combined with a final total actin concentration of 4.8 µM before induction of polymerization. All polymerization experiments were performed at least three times with at least three different actin preparations.

**Actin Depolymerization Rates**

Actin was polymerized to steady-state levels followed by the addition of DNase I in a 1:1 actin to DNase molar ratio. Depolymerization was monitored as a decrease in
light scattering over time. Depolymerization rates were determined by fitting the data to a single exponential expression using BioKine Version 3.1.

Circular Dichroism Measurements

The apparent melting temperatures of wild type and mutant actins were determined using circular dichroism by following the change in ellipticity of the G-actin sample at 222 nm as a function of temperature between 25 and 90 °C as previously described (144). Measurements were made on an Aviv 62 DS spectropolarimeter. Data were fit to a two-state model, and the apparent Tm value was determined by fitting the data to the Gibbs-Helmholtz equation to approximate the temperature at which 50% of the actin was denatured.

ε-ATP Exchange

The ability of G-actin to exchange bound nucleotide was assessed by first loading the actin with ε-ATP and quantifying the rate of displacement from actin in the presence of a large excess of ATP as described previously (144). Exchange rates were determined by fitting the data to a single exponential expression using BioKine Version 3.1.

Cofilin Binding Assay - G-Actin

Pyrene-labeled G-actin was made according to Feng et al. (276). Increasing amounts of cofilin were added to a 1.5-ml sample containing 1 µM 100% pyrene-labeled G-actin, and the cofilin-dependent increase in pyrene fluorescence was recorded on a FluoroLog3 fluorescence spectrometer outfitted with a computer-controlled thermostatted sample exchanger with continuous sample mixing (HORIBA Jobin Yvon Inc.). All experiments were performed in G-buffer containing 50 mM KCl. Note that this concentration of KCl will not induce polymerization of yeast actin as polymerization of yeast actin requires Mg^{2+}, unlike muscle actin (277). The excitation and emission wavelengths were 344 and 386 nm, respectively, with the corresponding slit widths of 1
and 2 nm. Using Microsoft Excel, experimental data were fit to the quadratic binding isotherm,

$$\Delta F = F_{\text{max}} \frac{[A] + [C] + K_d - \sqrt{(A + C + K_d)^2 - 4AC}}{2[A]}$$

(Eq. 1)

where $\Delta F$ is the observed fluorescence change of the actin-cofilin complex after the fluorescence of the G-actin alone has been subtracted. $F_{\text{max}}$ is the maximum fluorescence change at complete saturation of actin with coflin. A and C are the concentrations of G-actin and coflin, respectively, and $K_d$ is the observed dissociation constant. The solver function was used to minimize the difference between the experimental data and the best fit to produce the $K_d$.

Cofilin Binding Assay - F-Actin

Pyrene-labeled G-actin was made according to Feng et al. (276). Polymerization of 4.8 μM G-actin, 10% pyrene labeled and 90% unlabeled actin, in a total volume of 120 μl was induced by the addition of F-salts. The change in actin-pyrene fluorescence due to actin polymerization was recorded with a FluoroLog3 fluorescence spectrometer (Jobin Yvon-Spex). The excitation wavelength was 365 nm. The change in fluorescence intensity at emission wavelength 386 nm was recorded over time for kinetics analyses. After steady state was reached, an increasing amount of coflin was added to the sample, and the coflin-dependent quenching of the pyrene fluorescence was recorded. The excitation and emission wavelengths were 344 and 386 nm, respectively, with the corresponding slit widths of 1 and 2 nm. Using Microsoft Excel, experimental data were fit to the quadratic binding isotherm (as above).
Critical Concentration Determination

To measure the critical concentration ($C_c$) of each actin, the net change in light scattering of an actin polymerization reaction was measured as a function of increasing actin concentration. Polymerization of G-actin, at concentrations between 4.8 µM and 1 µM, was induced by the addition of F-salts and monitored by light scattering. The final increase in light scattering for each actin concentration was recorded and plotted versus actin concentration. The critical concentration of actin was obtained by fitting the points to a linear regression trendline and determining its intersection on the x axis.

Electron Microscopy

To visualize actin filament morphology, samples of 4.8 µM F-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 2k × 2k CCD camera. Accelerating voltage of the transmission electron microscope was 100 kV. Image J was used to process the images and measure the individual filament lengths for N115T, R116Q, and wild type actins (143).

Statistical analysis

All data are presented as means ± standard deviation. Results for wild type and mutant actins were compared by using a paired t-test with a p value < 0.05 considered significant.
Figure 4-1. Locations of the Twenty-two α-Smooth Muscle Actin Missense Mutations in Yeast Actin.

Panel A: Back view of yeast actin monomer crystal structure (142), modified from Protein Data Bank code 1YAG using the PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC. The positions of the mutations studied are color-highlighted and labeled: N115T, orange; R116Q, green; remaining 17 TAAD missense mutations, blue. ATP is depicted in red; Mg$^{2+}$ is denoted as a yellow ball. Numbers denote the actin subdomains, and N and C mark the respective termini. Panel B: model of the actin trimer based on the filament model of Oda et al. 2009 (194), with the N115T and R116Q mutations color-highlighted and labeled as described above. The trimer model highlights the location of the mutations along the intermonomer interface. Numbers and symbols denote the actin subdomains of the individual monomers comprising the trimer. Subdomains 1 and 3 constitute the barbed end of the actin molecule, while subdomains 2 and 4 constitute the pointed end; this polarity is also consistent within the filament.
Figure 4-2. Effect of TAAD Mutations on Cell Growth and Actin Dependent Cellular Structures.

Panel A: Comparison of wild type or mutant yeast strain growth on complete solid medium with either 2% dextrose (YPD at 30 °C and 37 °C), in the presence of 0.9M NaCl, or 2% glycerol as the sole carbon source. Cells cultures were diluted and spotted onto YPD- or glycerol containing agar plates and grown at 30 °C unless otherwise specified as described under “Experimental Procedures.” Pictures were taken 48 hrs subsequent to plating the cells. Panel B: Fluorescence microscopy of cells expressing wild type, N115T and R116Q yeast actin. Cells assessed were those in which the bud was less than one-third the size of the mother cell. Results are based on assessment of >100 cells for each sample. The cytoskeleton was visualized after staining fixed cells with rhodamine–phalloidin. Vacuoles were observed following exposure the cells to dye FM4-64. Mitochondria were visualized with GFP as described under “Experimental Procedures.” Scale bar = 10µm. Larger fields of cells for each assay can be found in Figure 4-11. Panel C: The bar height indicates the percentage of the cell population that exhibited normal structures. Error bars indicate standard deviation. Differences were statistically significant as compared to wild type actin, p < 0.05.
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Figure 4-3. Effect of Mutations on Filament Depolymerization *In Vivo and In Vitro.*

Panel A: Sterile filtered discs (0.5 cm in diameter) were presoaked in either 2ul DMSO (control) or 2ul of 0.1, 0.5, or 1mM of Latrunculin A and placed on YPD plates containing 100ul of evenly spread wild type or mutant cells (OD$_{600}$=0.1). The plates were incubated at 30 °C for 48 hr and the zone of growth inhibition was assessed. Panel B: Depolymerization Kinetics: Change in light scattering of pre-formed actin filaments was quantified after the addition of DNase I as a function of time at 25 °C. The $t_{1/2}$ of depolymerization was determined as described under “Experimental Procedures.” The number of experiments performed is indicated in parentheses. Differences were statistically significant as compared to wild type actin, p < 0.0001.
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<td>R116Q</td>
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Figure 4-4. Effect of TAAD Mutations on Cell Growth and Actin Dependent Cellular Structures in ΔAip1 Strain.

Panel A: Comparison of cell growth with wild type or mutant actin expressed in Δaip1::pCENWT host strain on complete solid medium with either 2% dextrose (YPD at 30 °C and 37 °C), in the presence of 0.9M NaCl, or 2% glycerol as the sole carbon source. Cells cultures were diluted and spotted onto YPD- or glycerol containing agar plates and grown at 30 °C unless otherwise specified as described under “Experimental Procedures.” Pictures were taken 48 hrs subsequent to plating the cells. Panel B: Fluorescence microscopy of cells expressing wild type, N115T and R116Q yeast actin. Cells assessed were those in which the bud was less than one-third the size of the mother cell. Results are based on assessment of >100 cells for each sample. The cytoskeleton was visualized after staining fixed cells with rhodamine–phalloidin. Vacuoles were observed following exposure the cells to dye FM4-64. Mitochondria were visualized with GFP as described under “Experimental Procedures.” Scale bar = 10 µm. Larger fields of cells for each assay can be found in Figure 4-12. Panel C: The bar height indicates the percentage of the cell population that exhibited normal structures. Error bars indicate standard deviation. Differences were statistically significant as compared to wild type actin, p < 0.05.
Figure 4-5. Polymerization Kinetics of Wild Type and Mutant Actins.

Panel A: Polymerization of 4.8 µM actin was initiated by the addition of magnesium and potassium chloride as described under “Experimental Procedures,” and the change in light scattering (L.S.) was monitored as a function of time at 25 °C. Shown are representative plots of experiments performed at least three times with three independent actin preparations. Panel B and C: Respectively are mixtures of wild type and either N115T or R116Q mutant actins for a final total actin concentration of 4.8 µM, polymerization was performed as in A.
Figure 4-6. Wild Type and Mutant Filament Length in the Absence and Presence of Cofilin.

Panel A: Filament length was determined for wild type and mutant actins in the absence of cofilin. Aliquots of polymerized actin were examined under the electron microscope following negative staining of the sample with uranyl acetate as described under “Experimental Procedures.” A histogram was plotted to show the percent distribution of filaments (y-axis) in each 0.5 µM size range (x-axis). N115T average length was statistically significant as compared to wild type actin based on Student's t test p < 0.0001. Panel B: Filament length was determined for wild type and mutant actin that were pre-polymerized, mixed with varying amounts of cofilin, and then examined under the electron microscope following staining as above. The average filament length and standard deviation is shown (each data point represents filaments measured at the end of each experiment in Figure 4-9A). All measurements were made of >100 filaments from at least three independent trials using at least two different actin preparations.
Figure 4-7.  $P_i$ Release Associated with Polymerization of Mutant Actins.

Panel A-C:  4.8 µM actin A, wild type B, N115T and C, R116Q was polymerized at 25 °C by the addition of F-salts. Filament formation was monitored by the change in light scattering and $P_i$ release using the EnzChek Assay. The data were normalized and superimposed as described under “Experimental Procedures.” Shown are representative plots of experiments performed at least three times with three independent actin preparations. Panel D: The rate of phosphate release of unpolymerized actin (G-actin), F-actin during the 1st 300sec of polymerization (Elongation), and F-actin during the steady state phase (Treadmilling). Asterisk indicates a $p < 0.001$ as compared to wild type actin.
Figure 4-8. Allele-Specific Effects of Yeast Cofilin on the Light Scattering of Preformed Actin Filaments.

Panel A: Samples containing 4.8 µM concentrations of either wild type or mutant G-actin were polymerized by the addition of F-salts (see “Experimental Procedures”). The polymerization-dependent change in light scattering was monitored as a function of time. Once the samples reached steady state, a range of yeast cofilin concentrations were added to the samples, and the change in light scattering was recorded. Data were normalized to the maximum light scattering (L.S.) value of each sample before cofilin was added (100%). The average light scattering value is plotted relative to the cofilin concentration. Panel B: Electron micrographs of 4.8 µM wild type, N115T, or R116Q actin without cofilin or after the addition of the indicated actin:cofilin ratio. Bar = 100 nm. Panel C, D, and E: Wild type and mutant G-actin for a final concentration of 4.8 µM were mixed and polymerized. Increasing concentrations of yeast cofilin were added to the samples, and the change in light scattering was recorded. The final extent of light scattering after cofilin addition v.s. pre-cofilin addition is plotted versus percentage of mutant actin present in the polymerization reaction. Data in all panels show the average and S.D. from at least three independent trials using at least two different actin preparations. A.U., arbitrary units.
Figure 4-9. Predicted Effect of the N115T Mutation on the Filament.

Model of the actin trimer based on the filament model of Oda et al. with monomers in various colors including: cyan, red, and gray (194). Residues are color-highlighted and labeled: in the red monomer: N115, blue; N115T, green; K113 orange, P112, yellow; in the gray monomer T194, gray; and E195, gray. Panel A: Depiction of the spatial organization and possible stabilization function of N115 in maintaining the P112 (red monomer) interaction with a monomer from the opposing strand (gray monomer) in the actin filament. Panel B: Depiction of the change in spatial organization due to modification of the N115 residue to T, resulting in a loss of packing density as indicated by the red oval. Models modified using the PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.
Figure 4-10. Predicted Effect of the R116Q Mutation on the Monomer.

Model of the crystal structure of yeast actin (142), modified from Protein Data Bank code 1YAG using the PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC. Amino acids pertinent to the regional function of the R116 residue are color-highlighted and labeled: E107, green; R116, red; H371, purple; V370, dark blue. Blowup shown depicts the hypothetical ionic interaction between residues R116 and E107 potentially lost by the R116Q mutation.
Figure 4-11. Effect of TAAD Mutations on Actin Dependent Cellular Structures.

Fluorescence microscopy of cells expressing wild type, N115T and R116Q yeast actin. The cytoskeleton was visualized after staining fixed cells with rhodamine-phalloidin; images were colorized so that the rhodamine-phalloidin staining appears red. Vacuoles were observed following exposure the cells to dye FM4-64; images were colorized so that the FM4-64 staining appears blue. Mitochondria were visualized with GFP as described under “Experimental Procedures,” images were colorized so that the GFP fluorescence appears green. Arrows indicate enlarged cell(s) found in Figure 4-2. Scale bar = 10µm. All image modifications were made using Image J.
Figure 4-12. Effect of TAAD Mutations on Actin Dependent Cellular Structures in ∆Aip1 Strain.

Fluorescence microscopy of cells expressing wild type, N115T and R116Q yeast actin in ∆aip1:pCENWT host strain. The cytoskeleton was visualized after staining fixed cells with rhodamine–phalloidin; images were colorized so that the rhodamine-phalloidin staining appears red. Vacuoles were observed following exposure the cells to dye FM4-64; images were colorized so that the FM4-64 staining appears blue. Mitochondria were visualized with GFP as described under “Experimental Procedures,” images were colorized so that the GFP fluorescence appears green. Arrows indicate enlarged cell(s) found in Figure 4-4. Scale bar = 10µm
Table 4-1. Effect of TAAD Mutations on Actin Monomer and Filament Characteristics.

<table>
<thead>
<tr>
<th>Actin</th>
<th>Thermal Stability Tm (°C)</th>
<th>Nucleotide Exchange Rate $t_{1/2}$ (sec)</th>
<th>Apparent Binding Affinity to Cofilin $K_d$ (µM)</th>
<th>Critical Concentration ($K_d$) (µM)</th>
<th>Apparent Binding Affinity to Cofilin $K_d$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>58 ± 1 (9)</td>
<td>36 ± 3 (11)</td>
<td>0.8 ± 0.4 (3)</td>
<td>0.47 ± 0.19 (4)</td>
<td>0.9 ± 0.2 (3)</td>
</tr>
<tr>
<td>N115T</td>
<td>55 ± 1 (6)</td>
<td>43 ± 2 (6)</td>
<td>0.3 ± 0.1 (3)</td>
<td>0.86 ± 0.37 (3)</td>
<td>0.7 ± 0.2 (3)</td>
</tr>
<tr>
<td>R116Q</td>
<td>53 ± 2 (5)</td>
<td>54 ± 8 (6)</td>
<td>NA</td>
<td>1.03 ± 0.36 (4)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Note: Values with the number of experiments performed are indicated in parentheses. $NA$, results not available due to denaturation of the R116Q mutant in the non-reducing environment required for the experiment. Differences were statistically significant as compared to wild type actin, $p < 0.05$, except for binding of cofilin to N115T F-actin.
CHAPTER 5. GENERAL DISCUSSION

The molecular events by which γ-nonmuscle actin mutations lead to deafness remain unknown. Preliminary work from our lab indicates that allele dependent altered polymerization kinetics, filament stability, and/or cytoskeletal regulation may play a role in the disease process. Cochlear hair cells contain the β- and γ-nonmuscle actin isoforms, which due to an extremely high degree of sequence homology, are virtually impossible to separate. Until the work presented here, it was not possible to obtain isoform pure quantities of either the wild type or mutant γ-nonmuscle actin. I have established a baculovirus protein expression system for the characterization of the individual biochemical properties of β- and γ-nonmuscle actsins. My work in Chapter 2 has ascertained that the two isoactins have surprisingly unique biochemical properties which depend on the bound divalent cation. Using this system in Chapter 3, I have been able to determine that while the deafness causing D51N γ-nonmuscle actin mutation does not prevent the actin from polymerizing, it does have a significant impact on filament dynamics.

Likewise, how mutations in α-smooth muscle actin cause TAAD, is unknown. The mutations may disrupt insertion of actin filaments into the smooth muscle contractile unit, alter actin interactions with smooth muscle cell actin regulatory proteins, or reduce actin filament stability all of which could lead to vascular wall weakening. Despite being unable to express the α-smooth muscle actin isoform in the baculovirus system, in Chapter 4, I was able to use yeast as a model system to assess of the effect of the α-smooth muscle actin mutations on yeast actin function in vivo and in vitro. My results indicate that both of the TAAD-causing mutations studied have allele specific effects on actin dynamics at the protein level and in the cell. The TAAD phenotype in patients may be due to a combination of altered actin filament stability and regulation by an ABP.
Isoactin Specific Biochemical Properties

The molecular basis for \( \beta \)- vs. \( \gamma \)-nonmuscle isoactin specific function and their different involvement in disease has been a mystery. Their almost identical protein sequence provides little insight into their specificity. Based on experiments in Chapter 2, I have demonstrated that these differences are not due to an inherent inability of the two actins to copolymerize. I also unexpectedly demonstrated that \( \beta \)- and \( \gamma \)-nonmuscle actin have distinct biochemical properties. Most surprising was the large difference in polymerization rates for the two actins in the Ca\(^{2+} \) form. This slower polymerization rate of \( \gamma \)- vs. \( \beta \)-nonmuscle actin seems to result from both slower nucleation and elongation rates, based on seeded actin polymerization assays. In contrast, in the Mg\(^{2+} \) form which is generally considered to be the more physiologically relevant actin form due to the Ca\(^{2+}/\text{Mg}\(^{2+} \) ratios in the cytosol, the difference between the two actin isoforms drastically decreased, although the \( \beta \)-isoactin still polymerized more quickly than the \( \gamma \)-isoform. A similar ion-dependent difference in the polymerization of two actins had previously been observed in comparing Dictyostelium discoideum actin with that from skeletal muscle, but these actins were considerably more divergent from one another than the two under consideration here and from different organisms where they play very different roles (278).

Correlation Between Isoactin Cellular Function and Biochemical Behavior

We have demonstrated that \( \beta \)-nonmuscle actin treadmills at a faster rate than \( \gamma \)-nonmuscle actin, loss of the \( \beta \)-isoactin in hair cells make the stereocilia dependant on the slower rate of \( \gamma \)-isoactin treadmilling which could account for the observed, slowly developing morphological defects in maintenance of stereocilia length. Loss of \( \gamma \)-nonmuscle actin in hair cells results in decreased number of stereocilia per hair cell and random deterioration of individual stereocilia. The combination of these data and our
results showing an increased stability of the γ-nonmuscle actin implies that in hair cells γ-nonmuscle actin functions in some sort of repair mechanism. Stereocilia loss may result from noise-damaged structures which, in the absence of γ-nonmuscle actin, are unable to maintain structural integrity. My observation of isoform specific differences in nonmuscle isoactin biochemical behavior correlates with their hypothesized function in cochlear hair cells. A more complete understanding of the effects of the deafness causing mutations on stereocilia function awaits the construction of a knock-in mouse model. This model would allow for determination of allele specific actin mutation effects on the hair cell in terms of stereociliary development and degeneration at the cellular level. Ultimately, the combination of biochemical knowledge I provide with the results of cellular studies will be needed to understand the disease mechanism.

Possible Physiological Relevance of the Ion-Dependent Polymerization Differences

One might question the significance of the differential polymerization behaviors of the two nonmuscle actins in the calcium form since the predominant form in the cell should be Mg\(^{2+}\) actin. However, Ca\(^{2+}\) bound in the nucleotide cleft may simply be one of a number of ligands that stabilize an energetically accessible conformation that can significantly differentiate the behavior of the two isoactins. In the cell, actin interacts with a large array of proteins both in the monomeric and polymeric states, and it is entirely possible that the conformational bias observed as a result of calcium binding could also be achieved through the interaction of actin with one or more of its binding partners. The result would be the creation of two populations of dynamically different filaments similar to what is seen in a number of cells.

Cochlear hair cells are at least one situation where the amount of calcium actin might actually be high enough to directly influence the behavior of these two actin isoforms. It has recently been demonstrated that high concentrations of calcium binding
proteins parvalbumin (279) and calbindin (280), approaching 300 µM and 970 µM respectively, are found in the stereocilia along with significant concentrations of two other calcium binding proteins (calretinin (281, 282), and oncomodulin (283-285)). Additionally, stereocilia have the Ca\(^{2+}\) ATPase pump PMAC2 at a concentration of \(~2000\) molecules/\(\mu\)m\(^2\) on the plasma membrane (286). The result could well be locally high persistent calcium concentrations sufficient to create a significant amount of calcium actin since actin actually binds calcium with a \(K_d\) in the nanomolar range, tighter than it binds magnesium (287). This is conjecture since to our knowledge it has not yet been possible to actually measure the calcium concentrations in the stereocilia. Another mode of regulation which cannot be dismissed is that the calcium is working directly on actin-binding proteins which regulate actin dynamics.

Possible Allosteric Interactions Involving the N-Terminal Divergent Residues

The picture painted by the overall set of differing biochemical properties of the nonmuscle isoactins is one in which the binding of Ca\(^{2+}\) at the high affinity binding site in the nucleotide cleft sets up an energy barrier. This barrier must be intensified by the \(\gamma\)-specific residues, N-terminal E-E-E and I10, which inhibit the conversion of the monomer conformation from the non-polymerizable to the polymerizable state. In essence there must be allosteric communication between these residues through subdomain 1 to the rest of the protein. It is tempting to speculate that the difference in behavior can be attributed to residue 10 within subdomain 1 where there is a Val/Ile substitution since this is in a core of secondary structural elements that form part of the nucleotide binding cleft. The alternative candidate would be that the other three N-terminal acidic residues that extend from the surface of subdomain 1 on its exterior might interact with other surface residues leading to a propagated change. However, in the monomer, at least, these residues are so unstructured they cannot be observed (16).
Results from previous work with yeast actin not only hint at such allostery but also make the role of the N-terminal acidic residues in this process more likely. In the monomer, the C-terminal peptide is on the opposite face of the planar actin structure in subdomain 1 than is the N-terminal peptide. There can be no direct spatial contact between them. However, our lab previously demonstrated that the effects of a mutation at residue 372 at the C-terminus of yeast actin could be reversed by increasing the number of acidic residues at the N-terminus from two to four (151). Second, we demonstrated that substitution of the normal two acidic N-terminal residues of yeast actin with the four found in muscle actin by themselves had no effect in vivo or in vitro (288). Introduction of all but the four N-terminal residues of subdomain 1 and 2 muscle specific residues cumulatively into yeast actin was tolerated well by the cell (213). However, replacement of the two yeast N-terminal acidic residues with the four muscle acidic residues in this subdomain 1/2 hybrid protein resulted in cell death. Replacement with only three acidic residues, on the other hand, was compatible with cell viability (213). Clearly, in this case the effect of the unstructured N-terminal acidic residues is determined by the context of the rest of subdomain 1 in the protein, and this linkage has to work in both directions.

Determination of Residues Responsible for Biochemical Differences Between β- and γ- Nonmuscle Actins

To understand the molecular basis for the polymerization differences between β and γ-nonmuscle actins it is necessary to know whether the acidic N-terminal block of residues, the difference at position 10, or possibly both are needed to dictate the behavioral differences. Such insight can ultimately lead to knowledge of how conformational changes caused by these alterations result in differential functional behavior.
To address this problem, one would make two hybrid actins and compare their properties with those of the parental β- or γ-isoactin. The first hybrid would be γ-isoactin with an exchange of its N-terminal E-E-E for the β-actin D-D-D. The second hybrid would involve the exchange of γ-actin I10 for the β-actin V10. Since both parental constructs express well in the baculovirus system, and the planned amino acid substitutions are so similar to the wild type actin residues. To date I have successfully constructed the above proposed hybrid actins. In small scale studies, both constructs demonstrate high levels of actin expression. An unanticipated problem arose during construction of these actins, I was unable successfully express a hybrid derived from β-isoactin with the V10I conversion. Interestingly a similar hybrid derived from the parental γ-isoactin with the I10V conversion was successful. This result prompted analysis of the same residue in yeast actin, I10. The lab was able to demonstrate (personal communication with Kou-Kuang Wen) that small alterations at this location drastically altered yeast growth properties and altered normal cytoskeletal arrangements within the cell, highlighting the significance of this residue in regulation of actin structure/function. Additionally work with yeast/muscle hybrid actins revealed that minor changes can have major effects on overall protein conformation and resulting behavior (270), so examination of the effect of these substitutions on ABP interactions would be necessary.

D51N

Evaluation of D51N-γ-Nonmuscle Actin Mutation

The recently established ability of our lab to express nonmuscle actin isoforms has opened the door to expression of human disease-causing mutant actin in the actin isoform in which they occur. This method, as compared to the yeast model system, may provide a more physiologically relevant assessment of the effects of the mutations on the biochemical properties of the actin and on the ability of the actin to interact with ABPs.
Yeast cells containing actin with the D51N mutation are nonviable, but our isolation of the D51N-γ-nonmuscle actin proves that this lethality is not due to an inability of the actin to polymerize. The mutation dramatically affects the dynamics of the actin; including leading to faster polymerization and a less stable actin filament. The deafness caused by this mutation may be due to the inability of the mutant actin to respond properly to changes in to Ca$^{2+}$ flux, interact properly with ABP(s), or even a combination of the two. The possibility of altered actin-ABP interactions is highly likely, as in the yeast background other deafness-causing actin mutations lead to altered actin-cofilin interactions. Potential ABPs and the rationales for their study are listed later in this chapter. Although it is important to mention that any ABP interaction studies preformed using actin expressed in the baculovirus system should take into account the amount of endogenous insect actin present in the actin preparations, and should contain the appropriate controls.

Role of the D-Loop in Actin-Myosin Interaction

Alteration of the actin-myosin interaction may be a potential cause for deafness, as myosins have many functions within the stereocilia. Multiple myosin isoforms have been localized to specific subcellular compartments. In the hair cell myosins XVα and IIIα both localize at the stereocilia tip, but myosin XVα localizes to the extreme tip region only. Myosin VI localizes to the stereocilia base, suggesting a possible role in regulating the minus ends of actin filaments. Myosins VIIα and Ic localize along the shaft of the stereocilia, implicating a possible role in regulating the retrograde flow of the stereocilia core and interact with interstereociliary links, which tether neighboring stereocilia actin cores. Mutations in any of these motor proteins, which bind to actin, also cause deafness (289-294). The γ-nonmuscle actin mutations may lead to alterations in the ability of actin and myosin to properly interact and thus cause deafness. In the future, the impact of the deafness mutations on the interaction between actin and myosin should be analyzed.
Analysis of the effect of the D51N-γ-nonmuscle actin mutation on the ability to actin to interact with myosin is particularly important as the D-loop has also been suggested to be important for the interaction of actin and myosin. Proteolytic digestion of the D-loop inhibits the actin-activated ATPase activity of myosin II-subfragment 1 (S1) (295) and decreases the velocity of actin filament movement on myosin II in an in vitro motility assay (296).

In the future it will also be important to use this baculovirus system to verify the results obtained from the yeast model system. Should the deafness causing γ-nonmuscle actin mutations exhibit similar biochemical effects on the γ-nonmuscle actin as the yeast actin (139, 143, 144), it will validate using yeast as a model system. Since γ-nonmuscle actin and yeast actin have substantially different biochemical properties (Chapter 2 and (229)) it may be necessary to completely switch to the mammalian background for future studies. Additionally, since most (8 out of 10) of the deafness causing γ-nonmuscle actin mutations are located on the barbed end of the actin, the predominant area of the filament for various ABPs to bind and regulate where and how actin filaments assemble, these mutations might significantly affect regulated polymerization in the cell. Establishing the biochemical basis for deafness caused by mutations in γ-nonmuscle actin can lead to insight into not only the disease mechanism but also into normal actin function within hair cells.

Smooth Muscle Actin TAAD Causing Mutations

While all of the 22 α-smooth muscle actin mutations lead to TAAD, in an allele specific fashion some also produce allele specific additional patient phenotypes one of which is premature coronary artery disease (CAD) (125). CAD is caused by atherosclerosis of the arteries that supply nutrients to the myocardium. In this disease the arterial lining becomes hardened, and swollen with calcium deposits, fatty deposits, and excess inflammatory cells (297). Inflammation causes accumulation of plaque
(extracellular lipids, calcium deposits, fatty deposits, and excess inflammatory cells) which becomes covered by a fibrous cap of smooth muscle cells and connective tissue. Plaque accumulation can restrict and even completely block the supply of oxygen and nutrients to heart muscles causing permanent cell death. It has been determined that, like TAAD, abnormal vascular smooth muscle cell proliferation plays an important role in CAD (297). One of the mutant alleles which cause TAAD, R116Q, also causes premature CAD. In Chapter 4 I have demonstrated that the R116Q mutation has allele specific phenotypes in yeast actin, which validates use of this model system as it may provide insight into the variability of the individual mutations.

Proper regulation of the vascular smooth muscle cell thin filament structure is essential for smooth muscle cell contraction; this regulation is carried out by numerous ABPs. In Chapter 4 I establish that the N115T and R116Q mutations have altered interactions with cofilin. The ABP caldesmon competes with cofilin for binding to F-actin (298-300). Caldesmon localizes to the actomyosin contractile structure in smooth muscle cells and is an integral component of the thin filaments (301-303). Since caldesmon also binds on the same region of the actin it is possible that this mutation contributes to premature CAD through an altered actin-caldesmon/cofilin interaction and translates to reduced forces transduction in vascular smooth muscle cells.

In mutant vascular smooth muscle cells, there is a mixture of actins including: α-smooth muscle (30%), mutant-α-smooth muscle (30%), γ-smooth muscle (10%), and a mixture of β- and γ-nonmuscle actin (30%) (191, 192). The cellular actin may be dominated by the effect of the mutant actin. Alternatively, it is possible that the presence of wild type actin will suppress effects caused by the mutant actin. The latter situation may mask the effects of the mutation, leading to the delayed onset phenotype and lack of symptoms prior to aortic rupture. To determine the extent to which the mutant actin exerts a dominant affect on actin mixtures, it will be important to determine the effect of increasing mole fractions of mutant actin on wild type actin behavior. These results may
provide a clue into the biochemical basis for the autosomal dominant nature of the mutations.

The yeast model system allows for observation of the full effect of the mutation on actin, but in patients only ~30% of the total actin in the cell is mutant (50% of the α-smooth muscle actin is mutant actin). This means the full effect of the mutation may be dampened, accounting for the delayed onset phenotype. Future mixture studies will need to be performed to determine to what extent the mutant actin can affect wild type actin.

Potential Animal Model

There is no good animal model available for this disease, although there is an α-smooth muscle actin knockout mouse it does not display TAAD. In fact these mice have very minor cardiovascular phenotypes including: compromised vascular contractility, lowered blood pressure, and reduced blood flow (62). Additionally, the myosin thick filaments are less dense and disorganized in the α-smooth muscle actin null mouse. The inconsistency between the human disease and the knockout mouse may be because the lack of α-smooth muscle isoactin creates a different phenotype than does the presence of mutant. In the absence of α-smooth muscle actin, the aortic cells compensate by expressing α-skeletal muscle actin. The presence of the wild type α-skeletal actin may have some overlapping functions with α-smooth muscle actin which would explain why the null mice are less severely affected. There are no reports to date of up-regulation of any other actin isoform in TAAD causing α-smooth muscle actin mutation patients. Therefore this mouse may be a good base to introduce the mutant alleles, this may negate the up-regulation of the other actin isoforms and provide an accurate model.

Characterization of Remaining TAAD Causing α-
Smooth Muscle Actin Mutations

In the future the effects of more of the remaining TAAD causing α-smooth muscle actin mutations will have to be addressed. In order to deal with the vast number
of mutations, the best course of action would be to study them in terms of how they cluster on the actin molecule. For example the N115T, R116Q, Y133H, and T351N mutations lie in a possible allosteric conduit, where alterations in one of these residues could cause propagated allosteric changes through the actin molecule and affect barbed-end function. Although within the actin molecule, all four residues are structurally close they cause varying phenotypes in human patients. Mutations R116Q and T351N both lead primarily to CAD, while mutations N115T and Y133H were not associated with CAD (124). This observation indicates that these mutations may affect a number of parameters but they all ultimately lead to aortic vascular wall weakening. The second set of mutations I would study are the R256C and R256H mutations, which lie near the subdomain 3/4 loop, necessary for proper polymerization (304-307). They are the second most prevalent mutations and are found in 15 affected families (124).

Determination of Effects of α-Smooth Muscle Actin Mutations on the Actin-Myosin Interaction

Mutations in two of the major components of the vascular smooth muscle cell contractile unit have been identified as causes of familial TAAD, α-smooth muscle actin and vascular smooth muscle cell-specific β-myosin (124, 207). This situation suggests that the smooth muscle cell contractile unit functions to regulate the structural integrity of the aorta. These α-smooth muscle actin and β-myosin mutations may disrupt smooth muscle cell contractile function leading to TAAD.

The aortic wall of patients with α-smooth muscle actin mutations and vascular smooth muscle cell-specific β-myosin mutations display a similar pathogenic morphology of medial degeneration. Analysis of the aortic tissue from β-myosin mutation patients reveals proteoglycan accumulation, fragmentation of elastic fibers, and a decreased number of vascular smooth muscle cells. There were also localized areas of increased vascular smooth muscle cells in a random distribution, lacking proper parallel
orientation to the lumen of the aorta (124, 207). This proliferation occurs in conjunction with occlusion of the vaso vasorum (smaller blood vessels feeding the aorta), as with α-smooth muscle actin mutation patients (124, 207).

An additional symptom associated with β-myosin TAAD is patent ductus arteriosus (PDA). This is a condition in which a blood vessel called the ductus arteriosus fails to close normally in an infant soon after birth. In the fetus, the ductus arteriosus connects the pulmonary arteries (which supply blood to the lungs) with the aorta (which supplies blood to the body). This allows blood to bypass the fetus' lungs while in the womb. Soon after birth, the lungs fill with air, the vessel is no longer required and closes within days. Failure to close allows abnormal blood circulation between the heart and lungs. Closing requires that changes in the structure and composition of the ductus arteriosus occur before and after delivery, and these changes require smooth muscle cells to migrate, proliferate, differentiate and contract (308). β-Myosin null mice display a delay in closure of the ductus arteriosus. The presence of PDA in this knockout model and human patients with either β-myosin mutations or some α-smooth muscle actin mutations demonstrates the involvement of the contractile unit in this phenomenon. In the future it will be important to assess the interaction of the TAAD causing α-smooth muscle mutant actins with myosin, especially those near the D-loop (V43L) and the N-terminal myosin binding site (T351N, Y133H) and those that also cause PDA (R256C, R256H).

Most myosins have very a very similar actin binding head, therefore as an initial screening I would assay the ability of the mutations cloned into yeast actin to bind to and activate skeletal muscle myosin S1 fragment’s ATPase activity (309, 310). I will use this myosin isoform instead of myosin 15a because it can be readily prepared in quantities necessary for the planned biochemical and biophysical work. If alterations in activity by the mutations are observed, I would examine the actin/myosin interaction by assessing the ability of filaments labeled with fluorescent phalloidin to move in an ATP-dependent
fashion over muscle myosin S1 heads tethered to a glass slide using the standard filament motility assay (311).

**Joint Future Directions**

**Determine the Effects of Pathogenic Actin Mutations on Actin Regulation by Barbed End Actin Binding Proteins**

An array of ABPs including: cofilin, profilin, formin, and Arp2/3, control the time and place of actin polymerization in the cell (13-16, 149, 312). The possibility that the deafness and TAAD mutations affect the ability of the actin to be properly regulated by these proteins should be evaluated. Profilin is an ABP that regulates actin dynamics, and the organization of actin filaments. To determine if the disease causing mutations cause altered actin-profilin interactions that might affect hair cell or vascular smooth muscle cell function, profilin’s ability to increase nucleotide exchange, and bind to each of the mutants should be examined. The disease causing mutations could potentially affect barbed end properties that might interfere with or alter the actin-formin interactions.

Recent work has demonstrated that an important factor in the nucleation and deposition of new filaments is the Arp2/3 complex (74). In smooth muscle cells, contractile stimulation leads to activation of the Arp2/3 complex, thereby initiating actin polymerization and branching mediated by the Arp2/3 complex (185). In vascular smooth muscle cells formation of focal contacts requires Arp2/3-dependent actin polymerization (313). The ability of Arp2/3 to enhance mutant actin polymerization in a bulk light scattering assay should be compared to Arp2/3’s effect on wild type actin (149). Total Internal Reflection Fluorescence (TIRF) microscopy, a method that allows for visualization of single filament formation, should be utilized to quantitatively assess growth rates and branching rates of individual mutant filaments in the presence of Arp2/3 as these parameters cannot be measured by bulk solution assays.
Determine the Effects of Selected Disease Causing Mutations in Yeast Actin on Actin Structure Using HD/Mass Spectrometry

The extent to which point mutations in actin alter the overall actin structure can be examined by using hydrogen/deuterium (H/D) exchange of backbone amide protons detected by mass spectrometry (270, 314). Measurement of the rate and extent that proteins incorporate deuteriums from deuterated water into the amide positions provide insight into the relative protected surface area of a particular protein species and the dynamics of the protein. This technique requires very little protein and is well established in our lab. The results from this technique are twofold. First, this technique provides a look at the structural changes in the actin monomer and filament caused by the deafness/TAAD mutants. Second, the H/D exchange experiments can be performed in the presence of ABPs, which allows for analysis of the structural effect of the TAAD mutations on the actin/ABP interactions. The structural changes can then be mapped on the actin crystal structure to provide a structural explanation for the altered behavior observed with the mutant proteins. Such an analysis may help explain why neighboring mutations in the same structural element can produce effects in opposite directions as we observe with the TAAD N115T and R116Q mutations, in the presence of cofilin (Chapter 4).

The overall goal is to develop therapeutics to these human diseases, but in order to accomplish this one must identify targets for therapeutic agents. To identify targets one must understand the molecular and biochemical basis for the defects caused by these actin mutations similar to the kind of studies I’ve conducted in this thesis. This work and future studies will provide the information required to understand the pathways involved in these diseases and form innovative treatments for deafness and TAAD.
APPENDIX A. BACULOVIRUS PROTEIN EXPRESSION SYSTEM

Baculoviruses are a group of viruses known to infect arthropod hosts, and are named such that *baculo* refers to the rod-shaped capsid of the viral particles. The DNA genome used in this work is that of the double-stranded, covalently closed, circular genome of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV), sized at approximately 130 kbp. The viral infection cycle in cell culture can be divided into three phases: early, late, and very late. The infection cycle described here is based on using an AcMNPV viral genome and the *Spodoptera frugiperda* (fall armyworm) cell line SF21, derived from *S. frugiperda* pupal ovarian tissue at the USDA Insect Pathology Laboratory at Beltsville, Maryland (315).

During the early phase, the first 6 hours of infection, the mature viral particles enter the SF21 cells by adsorptive endocytosis. Nucleocapsids migrate through the cytoplasm to enter the nucleus. Once in the nucleus the core, composed of viral DNA plus p6.9 (a basic protamine-like protein encoded by the *cor* gene) and other tightly associated proteins, is released and production of early viral transcripts begins within 30 min. This phase is also associated with dramatic changes in the host cells, including cytoskeletal rearrangements, chromatin dispersion, and nucleus enlargement.

The late phase ranges from 6 hours post-infection to 20-24 hours post-infection, and is a period of viral DNA replication, late gene expression, and budded virus production. Nucleocapsids are made in the nucleus of infected cells, exit the nucleus, and then bud through the plasma membrane of the infected cells. The budded virus is then released into the extracellular fluid with a loosely fitting membrane envelope. Host gene expression is shut off, with steady state levels of cellular mRNA declining by approximately 12 hours post-infection, and complete host protein synthesis halted by 24 hours post infection.
The very late phase extends from ~20 hours to ~2 days post-infection and correlates with further expansion of cellular nuclear membrane and envelopment of nucleocapsids (vial rod shaped protein shell surrounding the viral DNA/protein core). Additionally, at this stage large arrays of fibrous material, made up of a 10kDa polypeptide known as p10, begin to accumulate in the nucleus. Infected cells will eventually lyse, and release enveloped viral particles into the extracellular fluid.

One of the greatest advantages of using the baculovirus system is the potential for strong expression of a heterologous gene. Some reports indicate that when target protein expression is under the control of a viral promoter, 25-50% of the total cellular protein in the infected cell is the target protein. For the studies reported here, we have utilized the viral promoter p6.9 instead of the more commonly used p10 promoter because the p10 promoter resulted in lower yields of the nonmuscle actin isoforms. The p6.9 promoter controls the expression of the viral core protein cor, a major-viral structural protein (316, 317). It is a very late phase promoter (active at >24 hrs post infection) and when target proteins are harvested after 36 hours post-infection the uniformity of post-translational modifications improves, as this allows time for the proteins to move through the ER and Golgi. The difference in p10 and p6.9 promoter dependent expression patterns of nonmuscle isoactins may be due to the nonmuscle actins interfering with normal cellular/viral processes, and using the very late p6.9 promoter prevents this.
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