Effects of Human Deafness γ-Actin Mutations (DFNA20/26) on Actin Function*

Received for publication, February 16, 2006, and in revised form, April 21, 2006 Published, JBC Papers in Press, May 10, 2006, DOI 10.1074/jbc.M601514200

Keith E. Bryan‡, Kuo-Kuang Wen‡, Mei Zhu‡, Nanna Dahl Rendtorff‡, Michael Feldkamp‡, Lisbeth Tranebjaerg¶, Karen H. Friderici§, and Peter A. Rubenstein††

From the ‡Department of Biochemistry, University of Iowa Carver College of Medicine, Iowa City, Iowa 52242, the §Departments of Microbiology & Molecular Genetics and Pediatrics & Human Development, Michigan State University, East Lansing, Michigan 48824, the ¶Wilhem Johannsen Centre for Functional Genomics, Department of Medical Biochemistry and Genetics, University of Copenhagen, DK-2400 Copenhagen K, Denmark, and the ††Department of Audiology, Bispebjerg Hospital, 2400 Copenhagen NV, Denmark

Six point mutations in non-muscle γ-actin at the DFNA20/26 locus cause autosomal dominant nonsyndromic hearing loss. The molecular basis for the hearing loss is unknown. We have engineered each γ-actin mutation into yeast actin to investigate the effects of these mutations on actin function in vivo and in vitro. Cells expressing each of the mutant actins as the sole actin in the cell were viable. Four of the six mutant strains exhibited significant growth deficiencies in complete medium and an inability to grow on glycerol as the sole carbon source, implying a mitochondrial defect(s). These four strains exhibited abnormal mitochondrial morphology, although the mtDNA was retained. All of the mutant cells exhibited an abnormally high percentage of fragmented/non-polarized actin cables or randomly distributed actin patches. Five of the six mutants displayed strain-specific vacuole morphological abnormalities. Two of the purified mutant actins exhibited decreased thermal stability and increased rates of nucleotide exchange, indicative of increased protein flexibility. V370A actin alone polymerized abnormally. It aggregated in low ionic strength buffer and polymerized faster than wild-type actin, probably in part because of enhanced nucleation. Mixtures of wild-type and V370A actins displayed kinetic properties in proportion to the mole fraction of each actin in the mixture. No dominant effect of the mutant actin was observed. Our results suggest that a major factor in the deafness caused by these mutations is an altered ability of the actin filaments to be properly regulated by actin-binding proteins rather than an inability to polymerize.

Hearing depends on sound-dependent distortion of specialized 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. Mechanical deflection of these structures results in the opening of gated ion channels located on the surface of these protrusions. The result is the conversion of sound-dependent distortion into the propagation of neural signals that are relayed to the brain (1).

Hair cell function is contingent upon proper functioning of the actin cytoskeleton. Each mature stereocilium contains a core of actin filaments closely packed into rigid hexagonal bundles cross-linked by several actin-binding proteins (2). These filaments not only regulate the three-dimensional aspects of the stereocilia such as height and diameter, but also provide the rigidity necessary for proper functioning of the stereocilium. The stereocilia are embedded in a dense gel-like meshwork of actin filaments with random polarity called the cuticular plate (3). This structure provides a base in which the stereocilia are anchored and assists in maintaining their erect positions. 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 (4). These rings form focal contacts with neighboring supporting cells and provide tension across the apical hair cell surface, stabilizing the cuticular plate.

Structural integrity and proper function of the cytoskeletal complex depend on the interplay between actin filaments and a number of actin-binding proteins to stabilize the stereocilium, to control its length, and to help organize the cuticular plate. Examples of a few of these actin-binding proteins include spectrin, fimbrin, espin, tropomyosin, harmonin, and formin (5). Additionally, seven different myosins are involved with these structures (MYO1A, MYO3A, MYO6, MYO7A, MYO9, MYO14, and MYO15), and in humans, genetic mutations in all of these myosins cause deafness (6–16). These myosins have the potential to cross-link adjacent filaments, to carry protein cargoes up and down the stereocilium, to organize actin, and to generate tension within these structures. However, the functions of these myosins in the hair cell are only partially elucidated at this time.

The actin isoform distribution in the hair cells is unusual. Both non-muscle β- and γ-actins are found, but unlike the case for most non-muscle cells, the γ-isoform is the predominant isoform by an ~2:1 margin (17, 18). The primary sequence of these two isoforms differs at only four amino acid positions. The most striking difference between these two actin isoforms is at the N terminus of the protein, where β-actin contains three aspartate residues, and γ-actin contains three glutamate resi-
dues (19). The distribution of these isoforms is also nonrandom. γ-Actin is found throughout the hair cell (17, 18). However, there is a divergence of opinion concerning the localization of β-actin. Hofer et al. (18) argued that β-actin is confined to the stereocilium, whereas Furness et al. (17) reported that β-actin is found in both the cuticular plate (~30%) and the stereocilium (~15%).

It was recently discovered that six point mutations in the non-muscle γ-actin gene cause hearing loss (20–22). These mutations are autosomal dominant; one normal and one mutant γ-actin gene are expressed inside the cell. These mutations are T89I, K118M, P264L, T278I, P332A, and V370A, and in each case, the chemical nature of the mutated residue is very different from that of the residue originally present. All are located in subdomains 1 and 3 of actin, as shown in Fig. 1. Two of these (P332A and P264L) fall near the adenine-binding site on the actin and might affect actin monomer dynamics. Four of these (K118M, T278I, P332A, and V370A) are located near the barbed end of the monomer. The actin filament is a polar structure with a “barbed” end and a “pointed” end, and the monomer barbed end, consisting of subdomains 1 and 3, would be located at the barbed end of the filament (23). The filament barbed end is the preferred site for monomer addition during filament elongation. It participates in monomer-monomer contacts longitudinally along the actin helix, and it is the region at which a number of proteins that regulate actin polymerization in the cell exert their control. 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 loss of low frequency sounds, with overall hearing decreasing as the patient ages.

Understanding the effects of these mutations at the molecular level in the context of the hair cell is hampered by the small size of the organ involved and the inability to obtain samples of the structures from the patients for biochemical studies. Even if one were to introduce the mutations into model animals and to establish cultured hair cell preparations, the amount of material available for biochemical analysis would be limited. Additionally, within the hair cell, based on the known isoactin content, the mutant actin would likely not account for more than about one-third of the total actin in the cell. This would make it even more difficult to directly assess the effects of the mutations on actin function.

An attractive model system for studying these mutant actins is the budding yeast Saccharomyces cerevisiae. Yeast actin is 91% identical to non-muscle γ-actin, and it is encoded by a single gene, ACT1 (24). More important, all six of the residues at which the deafness mutations occur in γ-actin are identical in yeast actin. Many of the actin-binding proteins present in mammalian cells are also found in yeast (25, 26), and yeast actin will interact with many of the mammalian isoforms of these actin-binding proteins (27, 28). The wild-type (WT)2 actin gene can

---

2 The abbreviations used are: WT, wild-type; e-ATP, 1,6-ethenoadenosine 5’-triphosphate; DAPI, 4’,6-diamidino-2-phenylindole; GFP, green fluorescent protein.
Effects of Human Deafness Actin Mutations on Actin Function

easily be replaced with a mutant actin gene, generated by site-directed mutagenesis, providing a system in which the mutant actin is the only actin in the cell (29). The effects of these mutations on actin function within the yeast cell can be assessed by a number of cytological assays (30). The actins can then be purified and analyzed biochemically to determine the effects each mutation has on actin function in vitro. Finally, one can try to correlate the effects observed in vitro with the altered actin function in vivo. We have used such a system repeatedly to study actin structure-function relationships to delineate the effects a mutation has on the ability of the actin to polymerize, to interact with actin-binding proteins, and to activate myosin, leading to the generation of contractile force (27, 28, 30, 31, 33–35).

In this study, we cloned each of the deafness alleles into yeast actin and isolated cells expressing each of these mutant actins as the sole actin in the cell. We then assessed the effects of these deafness mutations on cell behavior and their effects on actin monomer stability and polymerizability in vitro using purified proteins.

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 from Sigma. 1,N\(^6\)-ethenoadenosine 5’-triphosphate (\(\epsilon\)-ATP), rhodamine-phalloidin, FM 4-64, and 4’,6-diamidino-2-phenylindole (DAPI) were purchased from Molecular Probes. The QuickChange\textsuperscript{\textregistered} site-directed mutagenesis kit was from Stratagene, and oligodeoxynucleotides were purchased from Integrated DNA Technologies. Yeast cakes for WT actin preparations were purchased from a local bakery. All other chemicals were reagent-grade quality.

Construction of Mutant Yeast Strains and Determination of Growth Characteristics—Mutations were introduced into the centromeric plasmid pRS314 (36) containing the yeast actin coding sequence driven by the ACTI promoter using the QuikChange\textsuperscript{\textregistered} mutagenesis kit according to the manufacturer’s instructions. Plasmids containing the desired mutations were introduced into a recipient yeast strain containing a deleted chromosome containing a deleted chromosomal ACTI gene and a plasmid expressing WT actin (pCENWT) as described previously (37). 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.

The ability to grow in complete liquid YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) was determined from the absorbance of 0.3–0.5 in Ura\textsuperscript{+} synthetic medium to force retention of the URA3-marked plasmid in the otherwise Ura\textsuperscript{–} 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. For mitochondria, ~40 z-sections were obtained at 0.15-\(\mu\)m intervals through the entire cell. Out-of-focus light was removed by deconvolution using MetaMorph software, and each series of deconvolved images was further rendered with NIH Image J.

The actin cytoskeleton was visualized by fluorescence microscopy after staining fixed cells with rhodamine-phalloidin as described previously (30). Vacuoles were observed following exposure of the cells to the dye FM 4-64 as described previously (39). Samples were visualized by fluorescence microscopy as described above. Nuclear and mitochondrial DNAs were visualized following staining of the cells with DAPI as described previously (30). To measure cell size, mounted samples were visualized by differential interference contrast microscopy. The long axis of the cell was measured using Image J. The average length for each strain was compiled from measuring >100 cells for each sample.

Actin Biochemistry—WT and mutant actins were purified from lysates of frozen cells using a combination of DNase I-agarose affinity chromatography, DEAE-cellulose chromatography, and polymerization/depolymerization cycling as described previously (40). Quality of the 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 ml/mg \(\cdot\) cm\(^{-1}\). All actins were used within 4 days following completion of purification.

Actin was polymerized by the addition of 2 mM MgCl\(_2\) and 50 mM KCl to a G-actin sample in a total volume of 120 \(\mu\)l. Polymerization was monitored at 25 °C by following the increase in light scattering of the sample in a thermostatted microcuvette in a FluoroMax-3 fluorescence spectrometer (HORIBA Jobin Yvon Inc.). All polymerization experiments were performed at least three times with different actin preparations.

Dynamic light scattering measurements of the G-actin samples of V370A and WT actins were performed at several differ-
Effects of Human Deafness Actin Mutations on Actin Function

ent concentrations ranging from ~0.25 to 10 μM at 25 °C using a DynaPro dynamic light scattering instrument containing a temperature-controlled microsampler (Protein Solutions, Inc.). Determination of the particle size for each sample was calculated using Dynamic Version 5.26.38 (included with the instrument), and the average particle size at each concentration was based upon measuring three independent samples.

The apparent melting temperatures of WT and mutant acts 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 (35). 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.

The ability of G-actin to exchange its bound nucleotide was assessed by first loading the actin with ε-ATP and following its displacement from the actin in the presence of a large excess of ATP as described previously (35). Exchange rates were determined by fitting the data to a single exponential expression using BioKine Version 3.1.

To visualize actin filaments, samples of 2.4 μM F-actin were deposited onto carbon-coated Formvar grids, negatively stained with 1% uranyl acetate, and observed with a JOEL 1230 transmission electron microscope (University of Iowa Central Electron Microscopy Facility). Lengths of individual filaments for WT and V370A acts were measured using Image J.

**RESULTS**

**Effect of Mutations on the Rate and Extent of Cell Growth**—We first examined the cells for global effects of the mutations on cell behavior by assessing their ability to grow on YPD plates at different temperatures. All six strains were temperature-sensitive for growth at 37 °C (data not shown). We then examined more quantitatively their ability to grow in complete YPD medium at 30 °C. The results revealed that the mutations group themselves into two different growth categories (Fig. 2). Cells carrying the T89I and P264L mutations grew slower than WT cells and to a final density of ~70% that achieved by WT cells. The second group, consisting of K118M, T278I, P332A, and V370A, exhibited significantly slower rates of logarithmic growth and leveled off at a density of ~30% that of WT cells.

One possible reason for decreased growth of this magnitude is a disruption of proper mitochondrial function caused by the mutation. Yeast requires actin for mitochondrial inheritance, maintenance of mitochondrial morphology (38, 41–43), and stabilization of mtDNA. A single actin mutation (H372R) we recently studied disrupts all three of these processes (30). Mitochondrial function may be easily assessed by examining the ability of the cells to grow on glycerol, as the sole carbon source. A mitochondrial glycerol-3-phosphate dehydrogenase is required for conversion of glycerol 3-phosphate to dihydroxyacetone phosphate, which can then be further metabolized by glycolysis. Mitochondrial defects often result in elimination of this activity. Fig. 3 shows that, although cells carrying the T89I and P264L mutations grew on glycerol, those carrying the four remaining mutations (K118M, T278I, P332A, and V370A), which exhibited the most severe growth retardation in complete medium, could not.

**Effect of the Mutations on Actin Cytoskeletal Patterns and Mitochondrial Morphology**—Mitochondrial inheritance requires the movement of these organelles from the mother cell to the bud along polarized actin cables running between these cells. The current theory for mitochondrial movement is that anterograde movement is Arp2/3 complex-mediated, whereas the myosin Myo2p is necessary for retention of the organelle in the bud (42, 44–46). A properly polarized actin cytoskeleton is also necessary for control of the fission/fusion events that regulate mitochondrial morphology (42). Besides actin cables, actin patches (a second form of polymerized actin) are also observed. These are sites of endocytosis (47), and their distribution is cell cycle-dependent. At early stages following initial enlargement of the bud, the patches are confined almost entirely to the growing bud, whereas at later times, the patches are evenly distributed between the bud and mother cell (48). Disruption of the actin cytoskeleton by actin mutations may lead to depolarized isotropic growth of the cell, resulting in larger and rounder cells. Decreased cell size is often observed in mitochondrion-deficient strains, reflecting their inability to generate enough energy to support optimal growth. We thus measured the effects of the mutations on cell size by determining cell diameter along the long axis of the cell using differential interference contrast microscopy. Fig. 4A shows that the two milder mutant strains capable of growth on glycerol (T89I and P264L) as well as the K118M mutant strain, appeared to be slightly larger than WT cells. Conversely, three of the strains unable to grow on
glycerol (T278I, P332A, and V370A) appeared to be slightly smaller than WT cells. None of the differences were statistically significant, however. Visualization of the actin cytoskeleton with rhodamine-phalloidin showed that all of the mutations resulted in a higher population of cells with fragmented and/or depolarized cables and uniform distribution of patches in both the mother cell and bud in comparison with WT cells (Fig. 4B).

We next assessed the effects of the mutations on mitochondrial morphology using an endogenously expressed fusion protein containing the citrate synthase mitochondrial targeting sequence fused to GFP to visualize the mitochondria. As shown in Fig. 4C, normal mitochondrial tubular structures roughly arranged in a polar manner running from the mother cell to bud were observed in ~80% of the WT cells that displayed GFP fluorescence. The T89I and P264L mutant cells displaying GFP fluorescence exhibited normal mitochondria ~50 and 35% of the time, respectively. In the remaining four mutant strains (K118M, T278I, P332A, and V370A), 13, 24, 7, and 23% of the cells, respectively, displayed normal or nearly normal mitochondrial structures. Interestingly, only a very low percentage of the K118M, T278I, P332A, and V370A mutant cells exhibited any kind of GFP-containing structures compared with the T89I and P264L mutant cells. This may stem from an inability of these four strains to properly incorporate the GFP fusion protein into the altered or defective mitochondria. Alternatively, the mitochondria may be so unstable that there are no organelles left to visualize.

To further determine the extent of mitochondrial damage in the mutant cells, we visualized the mtDNA and genomic DNA by staining the cells with the DNA-intercalating dye DAPI. Fig. 5 shows that, in addition to the large nuclear DNA spots seen in the WT cells, faint diffuse extranuclear spots representing mtDNA were observed. Similar patterns were also observed with the T89I and P264L mutants, which showed no mitochondrial phenotype. mtDNA was present in the four mutant strains unable to grow on glycerol (K118M, T278I, P332A, and V370A), indicating that the cells do not have the Rho− phenotype. However, these strains had distinctly larger and brighter mtDNA spots, suggesting that, in these cells, the mtDNA may be aggregating.

**Effect of the Mutations on Vacuole Morphology—**Regulation of vacuole morphology and vacuole inheritance in yeast also depends on a properly functioning actin cytoskeleton (49), which we examined with the dye FM 4-64. WT cells usually had four or fewer observable medium sized vacuolar lobes per cell. Fig. 6 shows that the two milder mutant cells (T89I and P264L) had a similar number of lobes compared with WT cells (four or fewer). However, in the P264L mutant cells, the abnormally large single vacuole occupied virtually the entire volume of the
cell. In the remaining mutants, large numbers of abnormally small lobes were observed. However, among these strains, the morphology of the vacuoles was surprisingly strain-specific. The K118M, P332A, and T278I mutant cells had one large lobe with multiple abnormally smaller lobes, whereas the V370A mutant cell was filled with large numbers of small lobes only. Therefore, it appears that, with the exception of the T89I mutation, all of the other mutations lead to abnormal vacuole morphology.

Effects of the Mutations on the Actin Monomer—The stability and conformational flexibility of the actin monomer depend heavily on two structural elements: the nucleotide bridge spanning the two domains of the protein near the bottom of the interdomain cleft and on the hinge region beneath the base of this cleft (Fig. 1). All of the deafness mutations, with the exception of T89I, reside in a part of the protein that could potentially alter the behavior of either the nucleotide bridge and/or the hinge region. Two parameters that might reflect such structural effects are thermostability and the rate at which the actin monomer can exchange its bound nucleotide.

We first assessed the effects of the mutations on thermal stability by determining the temperature at which 50% of the protein was denatured using the change in ellipticity at 222 nm as a function of temperature in a CD spectropolarimeter. Table 1 shows that T89I, T278I, and V370A actins have thermostabilities similar to that of WT actin. These residues reside near the base of or on the exterior of the protein. In contrast, the K118M, P264L, and P332A mutations, which reside in secondary structural elements near the ATP adenine-binding site, showed decreased melting temperatures of 3, 6, and 5 °C, respectively. Interestingly, the two mutations (P264L and P332A) that caused the most destabilization reside in secondary structural elements nearest this site.

We next determined the ability of the G-actins to exchange a bound fluorescent ATP analog (e-ATP) as a function of time in a solution with a large excess of ATP. Table 1 shows that the exchange rates of K118M and T278I actins were virtually the same as that of WT actin. The T89I mutation actually retarded the rate of exchange. Conversely, P264L and P332A actins exchanged their nucleotides 5 times and 3.5 times faster than WT actin, respectively. For these two mutants, the combination of decreased thermostability and increased rates of nucleotide exchange suggests that these mutations affect the adenine-binding site, leading to either a more flexible or more open conformation of the protein in the nucleotide-binding region.

Effects of the Mutations on Actin Polymerization—A potential cause of the deafness associated with these mutations is an adverse effect on actin polymerization, leading to malformed or structurally unstable stereocilia. To test this hypothesis, we measured the ability of the purified mutant actins to polymerize. Fig. 7 demonstrates that, with the exception of V370A actin, the other mutant actins displayed virtually the same rate and extent of polymerization as did WT actin. The V370A polymerization curve is noteworthy for two reasons. First the initial monomer light scattering signal was higher than that of the other mutants, perhaps signifying that the protein was associating somehow in G-buffer (5 mM Tris-HCl, pH 7.5, 0.2 mM CaCl$_2$, 0.2 mM ATP, 0.1 mM DTT). Second, the rate of filament elongation was much faster than that of WT actin, although the extent of the reaction appeared to be the same. These results suggest that the actin monomers might spontaneously form filament oligomers that act as seeds for polymerization or that
the filament is unstable and fragmented during the polymerization process. Either situation would lead to an increased number of filament ends capable of nucleating filament formation, resulting in an enhanced polymerization rate and the generation of shorter filaments for a given amount of actin.

To determine whether the polymerization kinetics of V370A actin resulted from filament fragmentation, we measured the lengths of individual filaments. The morphology of V370A actin filaments resembled that of WT actin (data not shown). Fig. 8 shows that, based on measurements of >100 filaments for each sample, the mean filament length of V370A actin was roughly one-third that of WT actin and that the distribution of filament lengths was much tighter.

To better distinguish between fragmentation and enhanced nucleation as the cause of the shorter filaments, we compared V370A actin solutions with WT actin solutions under non-polymerizing (G-buffer) conditions by dynamic light scattering. Although WT actin was monomeric over the entire concentration range, V370A actin formed aggregates or oligomers ranging from dimers to perhaps hexamers in roughly a concentration-dependent fashion (Fig. 9). If the aggregates were genuine filament oligomers or seeds, the addition of small amounts of the aggregates to a sample of WT actin would cause a disproportionately large enhancement of polymerization in relation to its relative abundance. However, this result was not observed (data not shown). Thus, enhanced ability of the mutant actin to form nuclei may play a role in the accelerated polymerization we observed.

In the hair cell, the mutant actin constitutes about one-third of the total actin, assuming that the wild-type and mutant γ-actins are equally expressed and that the γ/β ratio is 2:1 (18). This situation may mask the effects of the mutation, leading to the relatively mild late onset phenotype these mutations cause in terms of hearing loss. To determine the extent to which this might be true for V370A actin, we co-polymerized V370A actin with increasing amounts of WT actin, keeping the total actin concentration constant. Fig. 10 shows that, in a relatively linear fashion, increases in the amount of mutant actin caused commensurate increases in the rate of polymerization. Furthermore, co-polymerized mixtures of the two actins gave rise to filament lengths intermediate between what was produced separately by each actin (data not shown).

Structural Hypothesis for the Effect of the T278I Mutation—van Wijk et al. (21) postulated that deafness caused by the T278I mutation results from a steric clash between the side chain of the mutant isoleucine with the side chain of the methionine located at position 313 (Fig. 11A). This steric clash would lead to altered nucleotide binding, decreased monomer stability, and decreased polymerizability. van Wijk et al. further predicted that the effect in vivo of the mutation could be eliminated by conversion of Met313 to alanine to alleviate the steric clash (Fig. 11B). An alternative possibility, however, is that the T278I mutation interrupts a hydrogen bond between the side chain hydroxyl of Thr278 and the backbone carbonyl oxygen of Ile274 (Fig. 11, C and D), leading to an altered protein conformation. The proposal that steric effects of the mutation would cause defective actin polymerization does not agree with our results.

**TABLE 1**

Effects of mutations on monomer thermostability, ATP exchange rates, and in vivo phenotypes

The number of experiments performed is indicated in parentheses. For all $T_{m}$ values without a given S.D., multiple determinations were within 0.5°C of the stated $T_{m}$. NA, results not available.

| Strain | Thermostability apparent $T_{m}$ | Nucleotide exchange $t_{1/2}$ | Abnormal actin cytoskeleton | Inability to grow on glycerol | Abnormal vacuole morphology |
|--------|---------------------------------|-------------------------------|-----------------------------|-------------------------------|-----------------------------|
| WT     | 60 ± 1 (7)                      | 52.9 ± 1.3 (8)               | ×                           | ×                             | ×                           |
| T89I   | 61 (1)                          | 75.6 ± 2.1 (3)               | ×                           | ×                             | ×                           |
| K118M  | 57 ± 1 (3)                      | 50 ± 5.8 (2)                 | ×                           | ×                             | ×                           |
| P264L  | 54 (3)                          | 10.1 ± 0.5 (5)               | ×                           | ×                             | ×                           |
| T278I  | 59 (2)                          | 49 ± 1.7 (2)                 | ×                           | ×                             | ×                           |
| P332A  | 55 ± 1 (3)                      | 14.7 ± 1.7 (3)               | ×                           | ×                             | ×                           |
| V370A* | 60 (1)                          | NA                            | ×                           | ×                             | ×                           |

* Altered polymerization kinetics.
because the mutant actin polymerized normally. To test the crowding hypothesis in vivo, we mutated residue 313 in T278I actin to alanine (M313A) as suggested by van Wijk et al. Fig. 11E, clearly shows that introduction of alanine did not restore the ability of the cells to grow on glycerol as the sole carbon source. Thus, the growth phenotype we observed cannot be due primarily to the clash between the side chains of residues 278 and 313.

DISCUSSION

This work is significant for two general reasons. It is the first study to characterize the effect of deafness-related actin mutations in vivo when the mutant actin is the sole actin in the cell. It is also the first study to characterize biochemically the effects of the mutations on actin monomer integrity and the ability to polymerize into filaments in vitro. Actin filaments reside in the hair cell stereocilium and the cuticular plate into which it is embedded. Our results using yeast actin (which is 91% identical to mammalian non-muscle γ-actin) demonstrate that each of the known deafness alleles has significant and allele-specific effects on yeast cell behavior. Furthermore, some of the mutations alter the behavior of the purified actins both at the level of the actin monomer and, in one case, in the ability of the actin to polymerize.

In vivo, the mildest growth phenotypes were caused by the T89I and P264L substitutions, whereas the other four mutations (K118M, T278I, P332A, and V370A) resulted in severe stunting of cell growth. In terms of deafness, however, no such segregation has yet been observed. The mutations had allele-specific effects on both mitochondria and vacuole structures, showing that the phenotypes were not simply caused by a wholesale disassembly of the actin cytoskeleton. These allele-specific effects may prove useful in determining factors that control the morphology of these two organelles.

Although each of the two milder alleles (P264L and T89I) caused abnormally appearing mitochondria in ~40% of the cells, these cells maintained functional mitochondria, and the T89I mutation was the only one of the six that did not cause an apparent vacuole phenotype. The P264L-associated vacuole phenotype presented as a large single-lobe structure that occupied a majority of the cell volume. This specific allele may interfere with the regulation of vacuole fission, leading to the result observed.

The positions of the T89I and P264L mutations in the actin structure suggest an explanation for the relatively mild effect of the mutations in vivo and on actin polymerization in vitro. Thr<sup>89</sup> resides near the subdomain 1/2 interface near the part of the protein on the exterior of the actin filament (Fig. 1). It may compose part of a binding site for actin-binding proteins without regulating monomer-monomer interactions involved in polymerization. Pro<sup>264</sup> lies at the base of a subdomain 3/4 loop originally believed to be important in cross-strand stabilization within the actin filament (23), and subsequent mutagenesis and cross-linking studies demonstrated the importance of loop flexibility in polymerization (50–55). Near the pivot point of this loop, the P264L mutation may affect loop flexibility enough in the context of a structure such as the stereocilium to affect the long-term stability of actin bundle structures such as the stereocilium.

P332A is very near Arg<sup>335</sup>, a pivot point for the hinge at the bottom of the protein that controls the scissors-like opening and closing of the nucleotide cleft (56). Alterations at this residue may thus alter monomer dynamics, which is consistent with our thermostability and nucleotide exchange results. The P332A mutation had essentially no effect on actin polymerization, indicating that monomer-monomer contacts along the
filament axis or between filament strands were not substantially altered. However, because of its position near the barbed end of the monomer, it might exert an effect on barbed end actin-binding proteins that regulate polymerization without directly affecting polymerization per se. Interestingly, a P332S mutation has been identified in human α-skeletal muscle actin and leads to congenital fiber-type disproportion (57). We have introduced this myopathy substitution into yeast actin, and preliminary results suggest that this P332S change causes the same phenotype in vivo as the P332A deafness allele.

Of the six actin mutations we studied, only V370A had a significant effect on polymerization. In a sample of pure mutant actin, this change caused accelerated polymerization. The enhancement of polymerization cannot result from seeding due to the presence of true F-actin nuclei or oligomers because small amounts of the mutant actin did not accelerate polymerization of WT actin. One possible explanation is that the acceleration results from filament fragmentation during polymerization, creating new barbed ends. Another possibility is that the aggregates in low salt solutions that we observed cause locally elevated monomer concentrations. Either the monomers themselves are inherently more able to form nuclei than WT actin, or the elevated local concentrations could result in enhanced formation of nuclei, thereby accelerating filament formation. Our experiments favor enhanced nucleation as a major factor in the accelerated polymerization, although we cannot exclude increased fragmentation as part of this effect at this time. We have further demonstrated that a mixture of WT and V370A actins, as found in the hair cell, produces polymerization behavior in proportion to the amount of mutant protein in the mixture. Such a change may affect the initial formation of or recycling in hair cell structures enough to cause a gradual erosion of hair cell structural integrity, culminating in hearing loss.

A similar mutation (V370F) has arisen independently in the human α-skeletal muscle actin gene (58). Like its P332S counterpart, it also causes a myopathy, in this case, nemaline myopathy. Examination of the actin structure shows that the alanine mutation leaves a hole in an otherwise compact hydrophobic pocket near the barbed end of the protein near the monomer-monomer interface. This change could affect monomer-monomer interactions and the regulation of filament dynamics by proteins such as cofilin, which interacts with actin near the actin C terminus (59, 60). Conversely, substitution of phenylalanine for the valine would cause severe steric clashes within...
the pocket between the phenyl ring and the other hydrophobic residues that might literally prevent the monomer from folding, leading to the severe pathology observed. Consistent with this hypothesis is our failure to generate yeast cells expressing V370F actin as the sole actin. Val370 is also very near His372. An H372R mutation in yeast actin causes a severe phenotype in yeast characterized by loss of mitochondrial function, disruption of the normal mitochondrial morphology, and, in this case, loss of mtDNA (30). Vacuole morphology is also significantly affected. These phenotypes are very similar to those caused by the V370A mutation, underscoring the importance of this region for regulation of mitochondrial and vacuolar function.

As mentioned previously, the four severe mutations, including V370A, lie near to what is referred to as the barbed end of the monomer. Because much of the regulation governing where and how actin filaments assemble within the cell is mediated by proteins that bind either at or near the barbed ends of newly formed filaments, these mutations might significantly affect regulated polymerization in the cell.

One such protein is the Arp2/3 complex, which can nucleate formation of new branched actin filaments preferentially from other newly formed actin filaments (61, 62). Such regions are found most frequently at or near the barbed end of the filament. Arp2/3 has been reported to be required for the movement of mitochondria along actin tracks in yeast (38). The four severe mutations (K118M, T278I, P332A, and V370A) result in a disruption of normal mitochondrial function, and we have demonstrated that the H372R mutation causes an altered interaction with the Arp2/3 complex (30). Thus, altered actin-Arp2/3 complex interaction caused by these mutations may constitute a molecular basis for the deafness they cause.

Formin is another barbed end binding protein whose interaction with actin may be affected by one or more of these deafness mutations. A recently derived x-ray structure of the complex of actin with the FH2 (formin homology 2) domain of formin shows that the formin lies directly across the region of actin in which the four severe deafness mutations are located (63). Formin can either nucleate filament formation or facilitate the elongation of actin filaments (64–67). In yeast, formin is important for the formation of actin cables in a polarized fashion along the mother-bud axis (68, 69), suggesting that the yeast phenotypes associated with these mutations may derive from altered actin-formin interactions. The yeast formins Bni1 and Bnr1 are members of the mDia class of formins, the same type found in the hair cell (70). A mutation in the hair cell formin has also been reported to cause deafness in humans (71). The occurrence of a formin-related pathology supports the hypothesis that the four severe mutations may affect actin-formin interactions by interfering with actin barbed end topology. In further support of this hypothesis, Minin et al. (72) recently demonstrated that formins are necessary for proper mitochondrial distribution in mammalian CV-1 cells. A defect in this distribution in hair cells could alter energy availability, leading to a slow disruption of hair cell organization and function.

Our use of yeast as a model system to assess the effects of human deafness γ-actin mutations on actin function has revealed allele-specific in vivo phenotypes and alterations in vitro using homogeneous purified mutant actins not available in the hair cell. Clearly, we cannot use this system to directly assess the effects of these mutations on hearing per se. However, mechanistic insight gained from yeast in this study along with future studies evaluating the effects of these mutations on actin regulation should provide valuable clues concerning the molecular disruptions in hair cell architecture leading to hearing loss. They should also identify targets for more direct studies in genetically altered animals and hair cell cultures derived from them.

Acknowledgments—We are grateful to Drs. Richard J. Smith and Thomas B. Friedman for comments and suggestions.

REFERENCES

1. Hudspeth, A. J. (1989) Nature 341, 397–404
2. Tilney, L. G., Egelman, E. H., DeRosier, D. J., and Sanders, J. C. (1983) J. Cell Biol. 96, 822–834
3. DeRosier, D. J., and Tilney, L. G. (1989) J. Cell Biol. 109, 2853–2867
4. Hirokawa, N., and Tilney, L. G. (1982) J. Cell Biol. 95, 249–261
5. Muller, U., and Littlewood-Evans, A. (2001) Trends Cell Biol. 11, 334–342
6. Weil, D., Blanchard, S., Kaplan, J., Guildford, P., Gibson, F., Walsh, J., Mburu, P., Varela, A., Levilliers, I., Weston, M. D., Kelley, P. M., Kimberling, W. J., Wagenaar, M., Levi-Acobas, F., Larteg-Piet, D., Munnich, A., Steels, K. P., Brown, S. D. M., and Petit, C. (1995) Nature 374, 60–61
7. Avraham, K. B., Hasson, T., Sohe, T., Balsara, B., Testa, J. R., Skvorak, A. B., Morton, C. C., Copeland, N. G., and Jenkins, N. A. (1997) Hum. Mol. Genet
