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Rescue of cardiac α-actin-deficient mice by enteric smooth muscle γ-actin

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ABSTRACT **The muscle actins in higher vertebrates display highly conserved amino acid sequences, yet they show distinct expression patterns. Thus, cardiac α-actin, skeletal α-actin, vascular smooth muscle α-actin, and enteric smooth muscle γ-actin comprise the major actins in their respective tissues. To assess the functional and developmental significance of cardiac α-actin, the murine (129/SvJ) cardiac α-actin gene was disrupted by homologous recombination. The majority (≈56%) of the mice lacking cardiac α-actin do not survive to term, and the remainder generally die within 2 weeks of birth. Increased expression of vascular smooth muscle and skeletal α-actins is observed in the hearts of newborn homozygous mutants and also heterozygotes but apparently is insufficient to maintain myofibrillar integrity in the homozygous mutants. Mice lacking cardiac α-actin can be rescued to adulthood by the ectopic expression of enteric smooth muscle γ-actin using the cardiac α-myosin heavy chain promoter. However, the hearts of such rescued cardiac α-actin-deficient mice are extremely hypodynamic, considerably enlarged, and hypertrophied. Furthermore, the transgenically expressed enteric smooth muscle γ-actin reduces cardiac contractility in wild-type and heterozygous mice. These results demonstrate that alterations in actin composition in the fetal and adult heart are associated with severe structural and functional perturbations.**

Actin is a multifunctional protein that plays a fundamental role in a wide variety of cellular processes, including contractility, maintenance of the cytoskeleton, cell division, cell motility, and muscle contraction. At least six highly conserved actins are known in higher vertebrates, four tissue-restricted muscle isoforms (cardiac, skeletal, vascular, and enteric) and two ubiquitous nonmuscle isoforms (1). The actins are generally expressed in pairs with one of the two cytoplasmic, striated or smooth muscle isoforms predominating in a given adult tissue.

Cardiac α-actin, a striated muscle isoform, predominates in the adult mouse, pig, bovine, and human heart (2, 3). Similarly, there is a preponderance of cardiac α-actin mRNA in the heart of the adult chicken (4), mouse (2, 5), and rat (6–8). However, the adult human heart has more skeletal than cardiac α-actin mRNA (9). Though the preponderance of cardiac α-actin in the adult rodent heart is the norm, BALB/c mice, which show a perturbation of the cardiac α-actin locus (10), contain almost equivalent amounts of skeletal and cardiac α-actins in their hearts (2).

A more complex pattern of expression is found in embryonic and fetal hearts. There is coexpression of cardiac and skeletal α-actin genes in the developing heart of chicken (4, 11), mouse (12, 13), rat (8), and human (9). Moreover, transient expression of the vascular smooth muscle α-actin gene is observed during early cardiogenesis in *Xenopus* (14), chicken (11, 15), mouse (16), and rat (17, 18). In contrast, the enteric smooth muscle γ-actin (SMGA) gene is not expressed in the myocardium at any stage (8, 16, 17).

The four muscle actins are closely related and vary at only 10 of the 375 amino acid residues, with only 4–6 exchanges relative to cardiac α-actin. All four muscle actins are completely conserved from birds to humans, and they are expressed in a tightly regulated spatiotemporal manner. The remarkable conservation of the muscle actins along with their tissue and developmental specificity strongly argues that the minor variations among these proteins have some developmental and/or physiological relevance (19). Here we have adopted two transgenic strategies to test this idea. First, the murine cardiac α-actin gene was disrupted in mouse embryonic stem (ES) cells by homologous recombination to generate mice lacking cardiac α-actin. The majority of such mice do not survive to term, and the rest generally die within 2 weeks of birth, although they possess a four-chambered heart. Second, we have rescued homozygous mutants to adulthood by ectopically expressing enteric SMGA in their hearts, using the cardiac α-myosin heavy chain (α-MyHC) promoter.

MATERIALS AND METHODS

Generation of the Targeting Fragment. Cardiac α-actin is encoded by a single-copy gene (20, 21). While the BALB/c and DBA2 mouse strains carry a duplication of a part of this gene, C3H, AKR, 129, and C57BL/6 strains do not (11). A murine cardiac α-actin clone was isolated from a λ DASH II 129/SvJ genomic library (22) and a 2.2-kb *Xba*I–*Sph*I fragment carrying exons 1–5 was subcloned into pUC19. The unique *Nae*I site in exon 2, corresponding to amino acids 19 and 20 in the mature protein, was converted to a *Sal*I site by the addition of the appropriate linker. Subsequently, a 6.0-kb *HPRT* (hypoxanthine phosphoribosyltransferase) minigene (23) was ligated into the *Sal*I site to constitute the final construct, from which the targeting fragment was obtained by digestion with *Kpn*I and *Sph*I (Fig. 1).

Disruption of the Cardiac α-Actin Gene in ES Cells and Generation of Germ-Line Chimeras. The hypoxanthine phosphoribosyltransferase-negative E14TG2a ES cells derived from 129/Ola blastocysts (24), were grown on feeder layers of mouse embryonic fibroblasts. These cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 15% fetal calf serum in the absence of antibiotics. Electroporation was carried out in 1 ml of the culture medium essentially under conditions described by Shull and co-workers (22), with the targeting fragment at a concentration of 5 nM. After electroporation, ES cell colonies were selected in media supplemented with HAT (120 μM hypoxanthine/0.4 μM aminopterin/20 μM thymidine). HAT-resistant ES cell colonies were expanded for DNA isolation and analysis. Initially the ES cell clones were screened for targeted disruption of the cardiac α-actin gene by PCR (not shown). Subsequently, homologous recombination was con-

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Abbreviations: ES cells, embryonic stem cells; α-MyHC, α-myosin heavy chain; HAT, hypoxanthine/aminopterin/thymidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SMGA, smooth muscle γ-actin.

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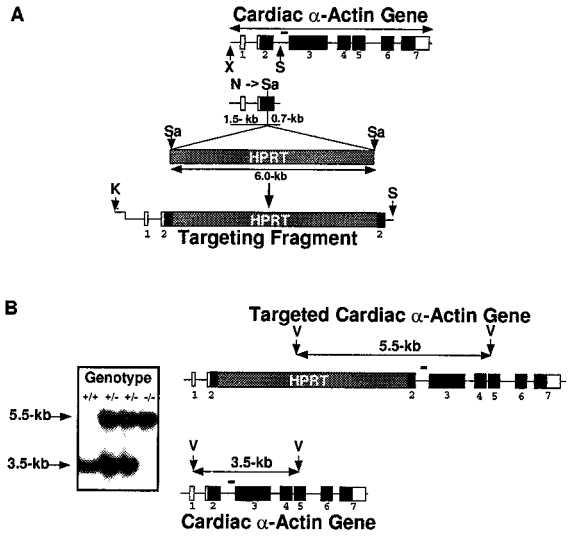

FIG. 1. Targeted disruption of the cardiac α-actin gene. (*A*) Generation of the targeting fragment. Exon–intron organization of the cardiac α-actin gene is shown at the top. Open boxes represent noncoding exons, while dark boxes represent coding exons. Exons (1–7) are numbered. The *Xba*I–*Sph*I fragment used in the targeting construct includes 1.5 kb of sequence upstream and 0.7 kb of sequence downstream of the unique *Nae*I site. The *Nae*I site was converted to a *Sal*I site, and the targeting construct was obtained by the ligation of a *HPRT* minigene *Sal*I cassette. Since the *HPRT* minigene carries an *Xba*I site (not shown) the targeting fragment used for electroporation was released from the targeting construct by digestion with *Kpn*I and *Sph*I. The *Kpn*I site is located in the vector (pUC 19). (*B*) Southern blot pattern of +/+, +/−, and −/− mice. Homologous recombination will lead to the generation of a 5.5-kb band, while the normal allele will give rise to a 3.5-kb band. The illustration to the right shows the location of the *Pvu*II sites in the native and targeted gene. Bar indicates location of the probe used for Southern hybridization. K, *Kpn*I; N, *Nae*I; S, *Sph*I; Sa, *Sal*I; V, *Pvu*II; and X, *Xba*I.

firmed in the candidate clones by Southern hybridization (see below). A precisely targeted clone, designated 3-27, was injected into blastocysts and implanted into pseudopregnant C57BL/6 (Jackson Laboratories) females. Progeny males with a high percentage of coat color chimerism were bred to Black Swiss (Taconic) females to establish germ-line transmission.

DNA Isolation and Analysis. DNA was isolated from ES cells grown on six-well tissue culture plates. The wells were rinsed twice with PBS and the cells were incubated with 0.5 ml of lysis buffer (0.2 M sodium chloride/5 mM EDTA/50 mM Tris·HCl, pH 7.5/0.2% SDS/20 μg/ml proteinase K) for 15 min at 60°C. This was followed by extractions with buffered phenol, phenol/chloroform, and chloroform. Finally, 2.5 vol of cold ethanol was added to the aqueous phase and the DNA was spooled, dried, and resuspended in water. DNA was isolated from mouse tails as described by Hogan and co-workers (25). Southern hybridization was carried out to confirm the targeting event in ES cells and to genotype mice. DNA from ES cells or from tail biopsy samples was digested with *Pvu*II, electrophoresed on 0.7% agarose gels, and transferred to GeneScreen*Plus* nylon membranes (NEN). A 0.5-kb *Sph*I fragment of the cardiac α-actin gene, downstream and contiguous with the 2.2-kb *Xba*I–*Sph*I fragment included in the targeting vector, was used for the generation of the probe. It was radiolabeled with [α-^{32}P]dATP by using a random priming kit (GIBCO/BRL). The blots were hybridized at 68°C, washed under conditions of high stringency, and exposed either to Kodak XAR-5 films or to phosphorimager screens and analyzed on a PhosphorImager (Molecular Dynamics).

Northern Analysis. Hearts of progeny from heterozygote crosses were excised within 24 h of birth, quick frozen in liquid nitrogen, and stored at −140°C. Tails from such mice were used for genotyping by Southern hybridization (described above). Hearts of littermates (+/+, +/−, and −/−) were used for RNA isolation. Total RNA was extracted from the newborn mouse hearts (both atria and ventricles), with the RNA STAT-60 reagent (Tel-Test; Friendswood, TX) according to the instructions of the manufacturer. A 15-μg sample of RNA was denatured and loaded onto a 2.2 M formaldehyde/1.1% agarose gel, electrophoresed, and transferred to a Biotrans nylon membrane (ICN). Appropriate RNA controls were included in the analysis (not shown). To check the integrity of RNA, aliquots were electrophoresed and stained with ethidium bromide. After transfer the blot was hybridized serially to probes consisting of end-labeled isoform-specific (26) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific oligonucleotides. The compositions of prehybridization and hybridization solutions were as described by Kriegler (27). The sequences of the different oligonucleotides and the corresponding hybridization temperatures are as follows: skeletal α-actin (5′-CGAGTCAATCTATGTACACG-3′), 45°C; vascular smooth muscle α-actin (5′-CACAGTTGTGTGCTAGAGGCAG-3′), 48°C; enteric SMGA (5′-AAGGCTTGAAGGTTTTAATGATCTGTGGCTGGTGACCAAGTCTTGTGGGGATCCCCAGAGAAGG-3′), 55°C; GAPDH (5′-GGCCTTGACTGTGCCGTTGAATTT-3′), 51°C. After overnight hybridization all blots were subjected to two 5-min washes at room temperature with 2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7). Later, the blots were washed with 2× SSC/1% SDS. The temperature and the durations of these washes are as follows: skeletal α-actin, 48°C, once for 20 min and then once for 10 min; vascular α-actin, 48°C, twice for 25 min each; enteric SMGA, 55°C, once for 30 min and then once for 10 min; and GAPDH, 51°C, once for 15 min. In the case of cardiac α-actin a 171-bp 3′ untranslated region (UTR) fragment was isolated from pHMcA-3′UT-DB (28), random primed, and used as a probe. The blot was hybridized at 60°C and washed with 2× SSC twice at room temperature for 5 min each, and once with 2× SSC/1%SDS for 45 min at 60°C. After each of the above hybridizations the blot was exposed to Kodak XAR-5 film. The bound probe was removed by washing the blot with a boiling solution of 0.1% SDS. Removal of probe was confirmed before the next hybridization was carried out.

Western Blotting. Newborn mouse hearts were obtained and stored as above (see *Northern Analysis*). After genotyping, hearts from +/+, +/−, and −/− littermates were used for Western blotting. The hearts were homogenized in 100 μl of PBS, SDS was added to the supernatant to a final concentration of 0.1%, and the tissue debris was removed by centrifugation. Adult hearts were homogenized in 1 ml of Isotris (10 mM Tris·HCl, pH 7.4/0.9% sodium chloride) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and 75 μl of 20% SDS was added to the homogenate. The sample was vortexed for 45 sec, placed in a boiling water bath for 2.5 min, and centrifuged at 10,000 × *g* for 2 min, and the supernatant was transferred to a fresh tube and used for Western blotting. Myofibrils were prepared from newborn mouse hearts essentially by the procedure of Pagani and Solaro (29). Protein was measured by the method of Bradford (30). Total heart protein (5 μg) and myofibrillar protein (2 μg) samples were fractionated by SDS/PAGE and electroblotted to nitrocellulose membranes (Schleicher & Schuell). Total actin can be detected with the monoclonal antibody (mAb) C4 (31), muscle actins with mAb HUC 1–1 (17), vascular smooth muscle α-actin with mAb 1A4 (32), enteric SMGA with mAb B4 (31), and cardiac/skeletal α-actins with mAb 5C5 (33). Antibody binding was revealed by using the Vectastain ABC system (Vector Laboratories).

To estimate the amount of actin in +/+ and −/− hearts, 2 μg of total heart protein was resolved by SDS/PAGE, transferred to nitrocellulose, and stained with the mAb C4 as described above. Control actin aliquots in the linear range (0.1–0.5 μg) were run on the same gel. A digital image of the blot was obtained by using the IS-1000 Digital Imaging System

(Alpha Innotech, San Leandro, CA) and analyzed with the NIH IMAGE 1.43 program. Samples were analyzed in triplicate and the relative amount of actin (−/− compared with +/+) was obtained as an average.

Electron Microscopy. Hearts of progeny from heterozygote crosses were collected within 24 h of birth and the tails were used for genotype analysis. The hearts were immediately minced, fixed in 2% glutaraldehyde for 4 hr, and then transferred to 100 mM cacodylate buffer, pH 7.4. Tissues were postfixed with osmium tetroxide, and thin sections (70–80 Å) were stained with lead acetate. The sections were examined and photographed with a Hitachi H600 microscope. Electron microscopy was carried out "blind" and a correlation of the phenotype with the genotype was observed subsequently.

Ectopic Expression of Enteric SMGA in the Heart. Transgenic mice carrying a rat SMGA cDNA (34) under the control of the mouse cardiac α-MyHC promoter (35) were generated (K.C., A.K., L.C., and J.L., unpublished results). One transgenic line produced high levels of enteric SMGA in the adult heart, and this isoform was also found to be incorporated into myofibrils (K.C., A.K., L.C., and J.L., unpublished results). The transgene was introduced into the cardiac α-actin +/− mice by crossbreeding. Such +/− mice carrying the α-MyHC-SMGA transgene were inbred to obtain −/− progeny. To ensure adequate controls for the rescue experiments, +/− mice lacking the transgene were obtained on a similarly mixed genetic background (129 plus Black Swiss plus FVB/N), and these animals were also bred to homozygosity for the disrupted cardiac α-actin allele. Survival of −/− mice was monitored in both instances.

Histology. Histology of adult hearts (control and rescued) was done by standard methods. Briefly, hearts were fixed in 10% neutral buffered formalin, dehydrated through a gradient of alcohols, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Physiological Analysis of Mouse Hearts. As described previously (36), mice were anesthetized with i.p. injections of ketamine (50 μg/g of body weight) and thiobutabarbital (Inactin, 150 μg/g of body weight) and placed on a feedback-controlled, heated surgical table. After tracheostomy, the right femoral artery and vein were cannulated for measurement of arterial blood pressure and infusion of agents. Arterial pressure was measured using a Cobe CDXIII transducer (COBE BCT, Lakewood, CO). Solutions were delivered at a rate of 0.1 μl/min per g of body weight. The right carotid artery was then cannulated with a Millar Mikro-Tip catheter (outside diameter 0.67 μm; Millar Instruments, Houston, TX) and the tip was advanced through the aortic valve and into the left ventricle. Blood pressure, left ventricular pressure, and left ventricular dP/dt (first derivative of ventricular pressure) signals were recorded and analyzed using MacLab hardware and software (A. D. Instruments, Milford, MA) at a sampling rate of 1000 samples per sec. Data analysis was performed using a single-factor ANOVA and individual comparisons using single degree-of-freedom comparison contrasts.

RESULTS

Targeted Disruption of the Cardiac α-Actin Gene. We have used a targeting fragment consisting of 1.5 kb and 0.7 kb of cardiac α-actin gene sequences upstream and downstream of the *HPRT* minigene, respectively (Fig. 1*A*). After electroporation into E14TG2a ES cells and selection in HAT, 164 clones were screened for the targeting event by PCR (not shown). Three clones tested positive, and Southern hybridization confirmed the targeting event. However, subsequent Southern analyses with probes internal to the targeting fragment indicated that two of the three clones carried additional copies of the targeting fragment (not shown). Therefore, the remaining clone (3-27) was used for injection into blastocysts and generation of chimeras. Three male chimeras showed germ-line transmission of the disrupted cardiac α-actin gene. The +/− mice thus obtained were indistinguishable from their +/+ littermates with respect to their size, viability, and behavior. Fig. 1*B* shows a pattern typically obtained for the +/+, +/−, and −/− mice by Southern hybridization, and the diagram to the right illustrates the location of the *Pvu*II sites in the normal and the targeted alleles. From the initial F_2 litters it became apparent that there was a clear deviation from Mendelian inheritance (29 +/+, 60 +/−, 9 −/−). Five of the nine −/− mice were smaller than their littermates. All 9 of these −/− mice died within the first 2 weeks of birth. Also, −/− progeny of subsequent heterozygote crosses generally died within the first 2 weeks of birth (not shown). We chose to focus our analyses on newborn progeny of heterozygote crosses. Genotype distribution of such newborn mice clearly shows that ≈56% of the −/− mice do not survive to birth (Table 1). However, among 55 progeny of heterozygote crosses analyzed at 13.5 days post coitus the genotype distribution (18 +/+, 25 +/−, 12 −/−) approximates a typical Mendelian ratio. Thus the majority, if not all, of the −/− mice that die prenatally must succumb after 13.5 days post coitus. The hearts of all 12 −/− mice appeared normal at the level of gross morphology.

Table 1. The majority of the −/− mice do not survive to birth

Genotype	No.	%
+/+	49	29
+/−	102	60
−/−	18	11
Total	169	100

Newborn mice were genotyped by Southern hybridization. It is apparent that about 56% of the −/− mice do not survive to term.

Cardiac α-Actin Depletion Leads to Increased Expression of Vascular Smooth Muscle and Skeletal α-Actins. The survival of

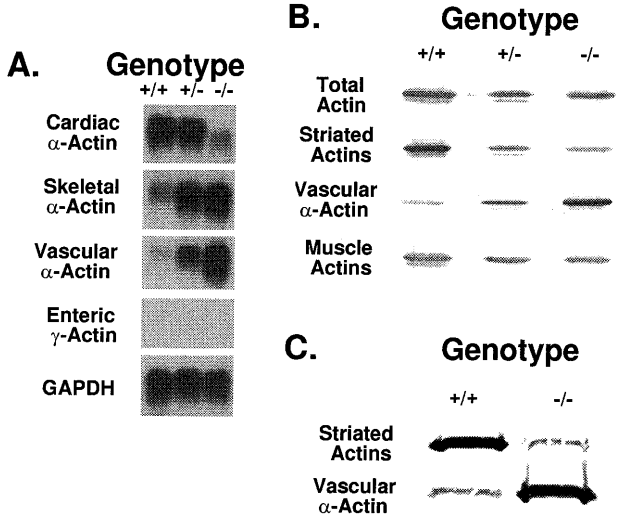

FIG. 2. Expression profiles of actin mRNA and protein from +/+, +/−, and −/− newborn mouse hearts. (*A*) Total heart RNA was analyzed for the expression of the different muscle actins. GAPDH was used as a control for the amount of RNA loaded. The minor band seen in the −/− heart includes transcripts deleted for sequences downstream of the *HPRT* insertion site (not shown). Note the increase in skeletal α-actin and vascular α-actin mRNA in the +/− and −/− hearts compared with the +/+ heart. Enteric SMGA expression is not observed in any of the three newborn hearts. (*B*) Total heart extracts were fractionated on an SDS/12% polyacrylamide gel and transferred to nitrocellulose. The actins were stained with different mAbs (C4 for total actin, 5C5 for the striated actins, 1A4 for vascular smooth muscle α-actin, and HUC 1–1 for muscle actin). Vascular smooth muscle α-actin shows an inverse correlation with cardiac α-actin gene dosage. The minor band seen in the −/− heart must represent skeletal α-actin, since mAb 5C5 recognizes both this isoform and cardiac α-actin. (*C*) Myofibrillar protein was similarly analyzed for its actin composition with the mAbs 1A4 and 5C5.

about 44% of the −/− mice to birth implied compensation with other actin isoforms to maintain adequate cardiac function. Therefore, the actin profiles of the newborn mice were analyzed with respect to RNA and protein. Northern analysis of the −/− hearts shows a minor band of a slightly smaller size on hybridization with a cardiac α-actin 3′ untranslated region probe (Fig. 2*A*). Reverse transcriptase–PCR analyses in combination with Southern hybridization with exon-specific oligonucleotide probes confirmed that this band includes transcripts lacking exon 2 sequences downstream of the *HPRT* insertion site (not shown), which would thus be incapable of producing an actin-like protein. The absence of a smaller polypeptide containing an actin epitope on the Western blots is further evidence of the noncoding nature of the transcripts (not shown). Both vascular smooth muscle α-actin and skeletal α-actin mRNA levels are increased severalfold in the −/− heart relative to the +/+ heart (Fig. 2*A*). Analysis of protein amounts by Western blotting indicated that the total actin content is reduced by about 50% in the −/− hearts compared with the +/+ hearts. However, there was no significant difference in the amount of total heart protein extracted. There also appears to be more vascular smooth muscle α-actin than skeletal α-actin in the −/− heart (Fig. 2*B*). Furthermore, both of these isoforms are incorporated into myofibrils (Fig. 2*C*). Note that while mAb 5C5 stains both skeletal and cardiac α-actins, the actin band observed in homogenates and myofibrils from −/− hearts must represent skeletal α-actin, given the absence of full-length cardiac α-actin mRNA. Enteric SMGA clearly is not involved, since its mRNA cannot be detected (Fig. 2*A*) in hearts from any of the three genotypes (+/+, +/−, and −/−), consistent with earlier reports that it is never expressed in the rodent heart (8, 16, 17).

Interestingly, +/− hearts also display this compensatory response, although to a lesser extent. The hearts of newborn +/− mice demonstrate a reduced level of cardiac α-actin mRNA compared with the +/+ hearts and intermediate levels of skeletal and vascular smooth muscle α-actin mRNAs relative to the +/+ and −/− hearts (Fig. 2*A*). At the protein level vascular smooth muscle α-actin is also increased in +/− hearts (Fig. 2*B*), but it is not possible to estimate the level of skeletal α-actin because mAb 5C5 also binds cardiac α-actin.

Overall, these results indicate that there is a compensatory response to decreased levels of cardiac α-actin in the fetal/neonatal heart which involves increases in the levels of vascular smooth muscle and skeletal α-actins.

Electron Microscopy Reveals Myofilament Disarray in the −/− Hearts. Since there was a depletion in actin content in the hearts of the −/− mice it was important to determine if sarcomeric organization is affected. Electron microscopic analysis shows a variable but extensive loss of thin filaments within the sarcomeres along with myofilament disarray (Fig. 3). The hearts of +/− mice, however, appeared normal by all of these criteria (not shown). Histological examination of the −/− hearts also indicated cardiomyocyte disorganization compared with the control hearts (not shown). Thus, we conclude that the lethal phenotype of −/− mice results from the deficiency in cardiac α-actin which leads to a loss of thin filaments in the sarcomeres and ultimately heart failure. Conversely, the viability of −/− mice, however limited, appears to be the result of a capacity to compensate with other actin isoforms.

Rescue of Cardiac α-Actin-Deficient Mice by Ectopically Expressed Enteric SMGA. On the basis of the above experiments, we hypothesized that the cardiac α-actin-deficient mice could be rescued by expressing an adequate amount of another muscle actin in the heart. Moreover, since the hearts of BALB/c mice, which contain as much as 50% skeletal α-actin, are hyperdynamic (37), we speculated that cardiac function would be altered. We introduced the α-MyHC promoter-driven SMGA transgene into cardiac α-actin +/− mice, which were then bred to homozygosity. At adulthood 17 of the 207 progeny were −/−. Thus about 32% of the −/− mice are rescued. In contrast, among 49 progeny from control crosses, none of the 5 −/− mice that were born survived to adulthood.

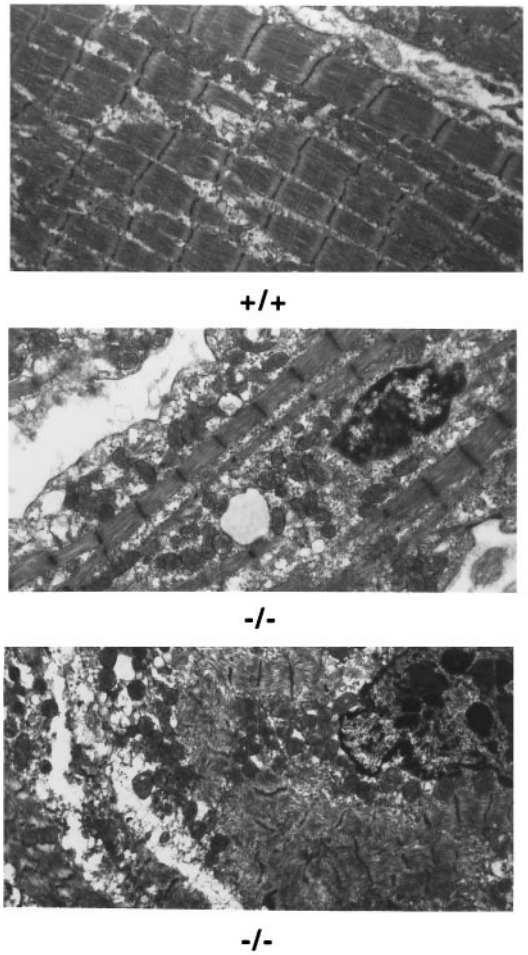

FIG. 3. Electron micrographic analysis of newborn +/+ and −/− hearts. Note the myofibrillar disarray in the −/− heart. (×3,000.)

There Is a Significant Amount of Enteric SMGA in the −/− Rescued Hearts. The actin profile was analyzed in two adult −/− rescued hearts and compared with two adult +/+ hearts (Fig. 4). As expected, the −/− rescued heart extracts show minimal immunostaining with mAb 5C5, which stains the striated actins. The faint reactivity observed in these −/− heart extracts must be due to skeletal α-actin, since a full-length cardiac α-actin transcript is absent (not shown). The total actin content (indicated by staining with mAb C4) of the rescued −/− hearts is similar to that of control +/+ hearts. The transgenically produced enteric SMGA clearly constitutes the major portion of muscle actin in the heart, though minor amounts of vascular smooth muscle and skeletal α-actins are also present. It therefore follows that main-

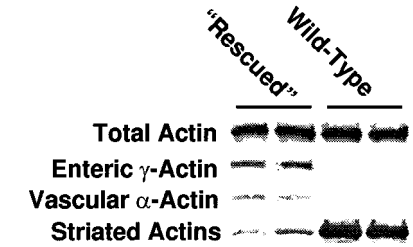

FIG. 4. Extracts from adult hearts of +/+ and −/− rescued mice were electrophoresed on SDS/polyacrylamide gels and the actins were detected by staining with the different mAbs. The −/− rescued hearts have an abundance of enteric SMGA and minor amounts of vascular smooth muscle and striated (skeletal) actins. The total actin content is, however, similar in all the hearts.

Table 2. Cardiac function in mice +/+ or +/− for the cardiac α-actin gene and in mice +/+, +/−, or −/− for this gene and expressing the α-MyHC promoter-driven enteric smooth muscle γ-actin transgene (SMGA$^+$)

Mice	*n*	MAP, mmHg	Systolic LVP, mmHg	Heart rate, bpm	dP/dt_{max}, mmHg/sec	dP/dt_{min}, mmHg/sec
+/+	7	82 ± 5	105 ± 5	423 ± 13	8,817 ± 339	−8,178 ± 657
+/−	7	83 ± 4	105 ± 5	436 ± 47	8,435 ± 864	−7,632 ± 760
+/+ SGMA$^+$	8	75 ± 3	96 ± 4	406 ± 27	6,079 ± 404*	−5,093 ± 474*
+/− SGMA$^+$	7	77 ± 1	91 ± 4*	426 ± 17	5,500 ± 291*	−4,440 ± 320*
−/− SGMA$^+$	3	61 ± 3*	76 ± 6*	393 ± 13	3,653 ± 318*	−3,141 ± 390*

Note that four −/− mice carrying the transgene died upon the administration of anesthesia, while only one mouse among the remaining genotypes died; this is probably indicative of the limited functional capacity of the hearts of the rescued mice. MAP, mean arterial pressure (1 mmHg = 133 Pa); LVP, left ventricular pressure; bpm, beats per minute; dP/dt_{max} = maximal rate of myocardial contraction; dP/dt_{min} = maximal rate of myocardial relaxation.
*$P < 0.05$ compared with +/+.

tenance of cardiac function and the rescue of the −/− mice must be mediated mainly by enteric SMGA.

The Rescued Hearts Are Extremely Hypodynamic. To assess cardiac function in these transgenically altered hearts, physiological analyses were carried out (Table 2). The results indicate that the ectopic expression of enteric SMGA in the +/+ heart significantly reduces the rates of ventricular contraction and relaxation ($P = 0.006$ and $P = 0.004$, respectively). In wild-type mice the maximal rate of contraction falls from about 8,800 mmHg/sec to about 6,100 mmHg/sec when enteric SMGA is present, and the maximal rate of relaxation is reduced similarly. Moreover, enteric SMGA expression in a −/− background produces hearts that are extremely hypodynamic. The maximal rates of contraction and relaxation are reduced to about 3,700 mmHg/sec and −3100 mmHg/sec, respectively, levels that are only about 40% of those obtained for the +/+ heart ($P < 0.0001$ for both). Blood pressure and intraventricular pressure also are significantly reduced in these mice, but the heart rate does not significantly differ among these experimental groups. Interestingly, cardiac function in +/− hearts is indistinguishable from that of the +/+ heart, but enteric SMGA expression in this background produces a significant reduction in the rate of contraction and relaxation ($P < 0.0001$ for both).

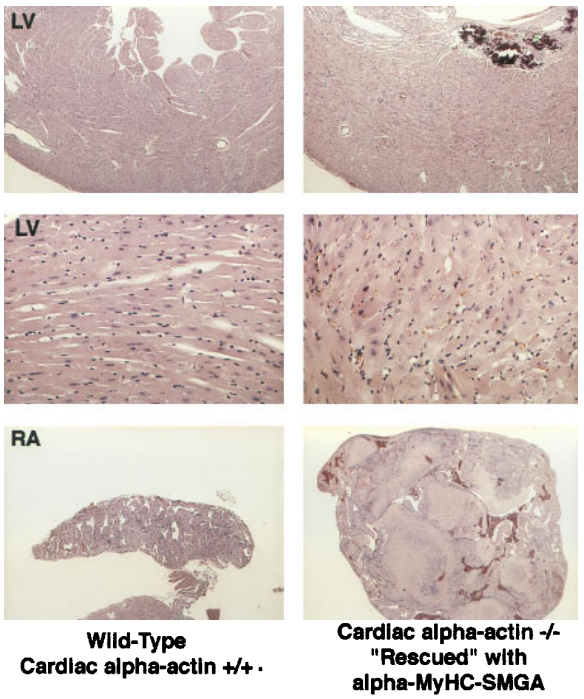

FIG. 5. Histopathology of a −/− rescued heart indicates hypertrophy. Comparison between *Left* and *Right* clearly shows myocyte hypertrophy in the left ventricle of the rescued heart. Also note the thrombi occluding a highly dilated right atrium (*Bottom*). LV, left ventricle; RA, right atrium. (*Top* ×15, *Middle* ×40, and *Bottom* ×4.)

Hypertrophy Is Evident in the Hearts of −/− Mice Rescued to Adulthood by Ectopic Expression of Enteric SMGA. Three adult −/− (ages 4–8 months) rescued hearts were examined for their histopathology. Stained sections of one of the three are presented along with a +/+ control in Fig. 5. All three −/− rescued hearts were significantly enlarged and rounded (not shown). They showed marked hypertrophy of the left ventricular septum and free wall characterized by myocyte hypertrophy, mild fibrosis, and moderate disorganization. There was also a marked dilation of the right ventricular free wall in two of the three hearts examined. Thrombi were evident in the left ventricular septal wall and also occluding a highly dilated right atrium. In one instance the thrombi contained calcium and were epithelialized, and cartilage metaplasia was observed in a thrombus in the atrium (Fig. 5). There was also a thickening of the endothelium of the left ventricle with fibrous tissue.

DISCUSSION

Despite their considerable amino acid sequence homology, the four muscle actin isoforms show a characteristic tissue restriction in their pattern of expression (1). Though there is little direct evidence, the minor sequence variations among the muscle actins have been postulated to be important in the context of unique physiological requirements of each tissue (19, 38). Transgenic strategies now permit us to address the issue of actin structure–function relationships in a whole-body context by disrupting actin genes and ectopically expressing other actins as shown here.

In this report we have shown that the majority of the mice lacking cardiac α-actin do not survive to birth, and those that do succumb shortly afterward. Clearly, the compensatory response to the deprivation of cardiac α-actin in the fetal/neonatal heart involves the two isoactins which are normally expressed in the developing heart: vascular smooth muscle and skeletal α-actins. It is, however, difficult to estimate increases in the amount of skeletal α-actin in the +/− and −/− hearts relative to the +/+ hearts because mAb 5C5 stains both this isoform and cardiac α-actin (33). In contrast to the increases observed in vascular smooth muscle and skeletal α-actin transcripts in the +/− and −/− mice (Fig. 2*A*), there is no change in the level of either of the cytoplasmic actin mRNAs in the hearts of −/− and +/− mice compared with +/+ controls (not shown). Compensatory expression of skeletal α-actin is also seen in adult BALB/c mice, which have a mutant cardiac α-actin locus and decreased cardiac α-actin levels (2). Skeletal and vascular smooth muscle α-actin mRNAs accumulate in the adult rat heart, the former transiently, following hypertrophy induced by pressure overload (7, 39, 40). While the pattern of compensation in the newborn +/− and −/− hearts is in some ways reminescent of the expression seen

in the hypertrophied hearts, there are no morphological changes in the newborn +/− hearts that would suggest hypertrophy (not shown). Yet the heterozygotes show intermediate levels of both vascular smooth muscle and skeletal α-actin mRNA relative to their +/+ and −/− littermates. Therefore we conclude that the observed compensation in cardiac α-actin-deficient mice is not secondary to hypertrophy but may reflect subtle changes in the heart due to a reduced capacity to make cardiac α-actin. We also have analyzed hearts of +/+, +/−, and −/− mice at 13.5 days post coitus and have observed a pattern of expression similar to that seen in the newborn (not shown). It appears that vascular smooth muscle α-actin mRNA levels do not decline in the developing heart of +/− and −/− mice, as is the case in wild-type mice (16), but instead are increased.

All four muscle actins can incorporate into the thin filaments of cultured adult rat cardiomyocytes (41), and vascular smooth muscle α-actin is normally present in the sarcomeres of the developing heart (15, 17). Similarly, both vascular smooth muscle and skeletal muscle α-actins can be detected in myofibrils (Fig. 2*C*) and within the sarcomeres of newborn −/− hearts by immunocytochemistry (not shown). The vascular smooth muscle α-actin seen in myofibrils from the +/+ heart is presumably due to the low level of expression of this isoform in the developing heart, which can be detected as late as 17 days post coitus (16). In any event, the intensity of immunostaining seen in myofibrils from the −/− heart is considerably higher than that seen in the +/+ control. While it is difficult to assess the functional potential of vascular smooth muscle and skeletal α-actins relative to cardiac α-actin, these isoforms obviously provide at least the minimal level of contractility required to sustain the −/− mice to birth. The compensatory expression of actins observed in the hearts of newborn −/− mice, though fairly significant, apparently does not match the levels attained by expression from the wild-type cardiac α-actin gene, as the actin content is decreased by half. Individual variations among −/− mice may account for the extent of viability, with differential activation of the "compensatory" actin genes being directly correlated with the ability to survive.

Since the actin content of the −/− hearts is reduced by half compared with the +/+ hearts, we surmised that the lethal effects of cardiac α-actin deprivation could be due to a general insufficiency of actin. This idea is supported by the fact that ectopic expression of enteric SMGA can rescue about a third (based on a Mendelian frequency) of the −/− mice to adulthood. Clearly the extent of rescue (17 of 207) is only slightly less than the fraction of −/− mice surviving to birth in control crosses (5 of 49). The loss of the rest of the −/− mice is likely to be due to differences in the temporal pattern of expression of the SMGA transgene compared with the endogenous cardiac α-actin gene. During gestation the α-MyHC promoter drives expression of a linked cDNA continuously in the atria with low levels of expression in the ventricles, and it is not until shortly after birth that this promoter drives very high levels of expression in both the atria and ventricles (35). On the other hand, cardiac α-actin transcripts first appear in the heart tube at 7.5–7.8 days post coitus (12) and high levels of expression are attained soon afterward in the presumptive atria and ventricles (12, 13). It appears that only those −/− mice that would otherwise survive to birth can be rescued to adulthood by the SMGA transgene.

The rescued −/− hearts are extremely hypodynamic. The reduced contractility of hearts containing enteric SMGA is consistent with a reduced ability of SMGA to activate skeletal myosin ATPase compared with skeletal muscle α-actin (42). In contrast, the BALB/c heart, which contains significant amounts of skeletal α-actin, is hyperdynamic, and the maximal rate of contraction has been shown to be proportional to the level of mRNA for this protein (37). Thus, cardiac function is altered by varying the types of actin in the heart. Interestingly, the hearts of +/+ mice with the SMGA transgene also show a significant reduction in contractility, and the dynamic properties of the transgenic +/− hearts are intermediate to those of the transgenic +/+ and the −/− hearts. Importantly, while the presence of enteric SMGA in +/+ and +/− hearts results in reduced rates of contraction and relaxation and a slight enlargement, only the hearts of the rescued −/− mice are considerably enlarged. Likewise, the most dramatic reduction in contraction and relaxation is seen in the rescued −/− hearts, where enteric SMGA is expressed in the total absence of cardiac α-actin. The absence of cardiac α-actin probably exacerbates the physiological disorder, ultimately leading to hypertrophy. In any event, the physiological changes associated with the replacement of cardiac α-actin with enteric SMGA are consistent with the hypothesis that the highly conserved muscle actins are functionally specialized for the tissues in which they predominate.

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