Loss of mXinα, an intercalated disk protein, results in cardiac hypertrophy and cardiomyopathy with conduction defects

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Am J Physiol Heart Circ Physiol 293: H2680–H2692, 2007. First published August 31, 2007; doi:10.1152/ajpheart.00806.2007.—The intercalated disk (ID) contains adherens junctions, desmosomes, and gap junctions that maintain the integrity of the myocardium to function in synchrony. The expression and distribution of many of these junctional components are often altered in many types of heart disease (5, 8, 13, 35). However, direct evidence to support a role for these proteins in contributing to cardiomyopathies remains incomplete. The best-characterized example involves the effects of deletion of a key adherens junction component, N-cadherin, on the intercalated disk. N-cadherin functions to mediate Ca2+-dependent homophilic cell-cell adhesion. Conditional deletion of N-cadherin in the adult mouse heart leads to a complete dissolution of the intercalated disk structure and a significant decrease in the expression of the gap junction proteins connexin 43 and connexin 40 as well as the cytoplasmic desmosomal protein plakoglobin. These global perturbations of intercalated disk components subsequently result in dilated cardiomyopathy and in spontaneous ventricular tachycardia and arrhythmic death (12, 15). Although E-cadherin has been shown to restore cardiomyocyte adhesion and cardiac looping in conventional N-cadherin-knockout embryos (18), cardiac-specific expression of E-cadherin results in dilated cardiomyopathy, suggesting that ectopic expression of cadherin in intercalated disks is not sufficient to restore normal cardiac function (7). In addition, mutations in desmosome-associated proteins, including plakoglobin (19), desmoglein-2 (26), and desmoplakin (1), have been identified as the primary cause of arrhythmogenic right ventricular cardiomyopathy, leading to ventricular tachycardia and lethal arrhythmias. Similarly, aberrant desmosome and gap junction organization, with aberrant side-to-side connections, was observed in the hearts of patients with hypertrophic cardiomyopathy (28), providing a first account of the interplay between desmosomal and gap junctional components in human cardiomyopathy. These and other studies demonstrate that the disruption of various intercalated disk components leads to cardiac defects and cardiomyopathies; however, the molecular mechanisms regulating the interplay between these components are complex and remain to be elucidated.

The striated muscle-specific Xin protein was shown to be involved in chicken cardiac morphogenesis by antisense knockdown experiments (33). Subsequent cloning of the mouse homolog identified two Xin genes, mXinα and mXinβ (3). The Xin protein contains a 16-amino acid repeat region (Xin repeat), a putative DNA binding domain, a putative nuclear localization signal, and a proline-rich region. At the present time, neither nuclear localization nor transcriptional activity of mXinα has been detected. mXinα appears to be restricted to the periphery of cardiomyocytes in early embryos and later specifically localizes to the intercalated disk in near-term embryos through adulthood (29). The Xin repeats from the human homolog (hXinα, Cmya1) of mXinα have been shown to bind actin filaments in vitro (23). However, in vivo mXinα...
was not found to associate with thin filaments; rather, mXinα colocalizes with N-cadherin and β-catenin at the intercalated disks (29), suggesting that mXinα interacts with one or more of these molecules. Indeed, Xin communoprecipitates with both β-catenin and N-cadherin from chicken embryonic heart lysates, another indication that Xin is a part of the N-cadherin/β-catenin complex (29). In another study, we have shown (9) the direct interaction between mXina and β-catenin.

To gain insight into mXina function, mXina-knockout mice were created. Viable and fertile knockout mice are observed in Mendelian ratios, and an upregulation of the mXinβ gene was detected. mXina-deficient mouse hearts exhibit cardiac hypertrophy and cardiomyopathy. Ultrastructural examination of mXina-null mouse hearts reveals myofilament disarray and disrupted intercalated disk structure. The expression levels of N-cadherin, β-catenin, p120-catenin (p120ctn), and connexin 43, but not α-catenin or plakoglobin, are decreased significantly in mXina-null mice. These data suggest that mXina plays an important role in regulating the hypertrophic response and maintaining the tightly associated intercalated disk membrane and myofilament organization in normal mice.

MATERIALS AND METHODS

Construction of mXina targeting vector and generation of mXina-deficient mice. All animal procedures were performed with the approval of the University of Iowa Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals approved by the National Institutes of Health. To delete the mXina gene, a targeting vector was constructed (Fig. 1A). The targeting vector, cloned into the NotI/EcoRI sites of pBluescript II SK (Stratagene, La Jolla, CA), contains a 311-bp probe used for Southern blot analysis. Germ line transmission of the targeting vector and generation of mXina-null mice were achieved. Viable and fertile knockout mice are observed in Mendelian ratios, and an upregulation of the mXinβ gene was detected. mXina-deficient mouse hearts exhibit cardiac hypertrophy and cardiomyopathy. Ultrastructural examination of mXina-null mouse hearts reveals myofilament disarray and disrupted intercalated disk structure. The expression levels of N-cadherin, β-catenin, p120-catenin (p120ctn), and connexin 43, but not α-catenin or plakoglobin, are decreased significantly in mXina-null mice. These data suggest that mXina plays an important role in regulating the hypertrophic response and maintaining the tightly associated intercalated disk membrane and myofilament organization in normal mice.

Cloning of mXina cDNA and RT-PCR analyses of mXina isoforms and hypertrophy response genes. For mXinβ cDNA cloning, a custom-made cDNA library was prepared from adult mXina-null mouse hearts and used for low-stringency screening with Xin probe (nt 1977–2649) and mXina probe (nt 140–1744) as described previously (33). The composite sequences of the inserts from six positive, overlapping clones contain 4,382 bp, represented by pBKX-2, 3, and 4 (GenBank accession nos. AY775570, AY775571, and AY775572).

Total RNA isolated from mouse hearts was used for cDNA synthesis with random hexamers and SuperScript II reverse transcriptase (Invitrogen, San Diego, CA) as described previously (33). For cloning mXina isoforms, the primer pairs either flanking (Pa-a: nt 3140–3162 and Pa-b: nt 3974–3993) or within intron 2 (Pa-d: nt 3139–3161 and Pa-e: nt 3886–3909, as well as Pa-c: nt 3562–3583 and Pa-b) were designed to specifically amplify mXina isoforms with standard PCR conditions. The resulting PCR products were cloned into pCRITOPO vector (Invitrogen) and sequenced. For the analysis of hypertrophy response genes, previously published primer pairs and PCR conditions for atrial natriuretic factor (ANF), α-myosin heavy chain (MHC), β-MHC, skeletal α-actin, cardiac α-actin, and GAPDH were adapted and carried out (36, 38). Quantitative real-time RT-PCR was performed with the SYBR Green method, with primer pairs designed by ABI software (Applied Biosystems), on the ABI7000 Sequence...

Fig. 1. Targeted deletion of mXina. A: schematic representation of the targeting strategy. The mXina locus contains 3 exons (E1, E2 and E3), with the 5’ boundary of E2 only 4 bp upstream of the translation initiation codon (ATG). The location of the 311-bp EcoRI/KpnI probe used for Southern blot analysis is within E3 but outside the targeting vector. T, targeted locus. B: genotyping of mXina-knockout mouse littermates by Southern blot and PCR analyses. For Southern blot analysis, KpnI-digested genomic DNA was hybridized with a 311-bp probe, which detected 10-kb and 15-kb KpnI fragments from endogenous and targeted loci, respectively. For PCR genotyping, genomic DNAs were amplified with primers designed to amplify the endogenous (E) and targeted (T) loci. The locations of the PCR amplified products for E (573 bp) and T (630 bp) are shown in A. C: Northern and Western blot analyses. For Northern blot analysis, total RNAs were hybridized with the labeled mXina cDNA. A 5.8-kb mXina message was detected in both wild-type and heterozygous RNA lanes, but not in the homozygous RNA lane. The same membrane was reprobed with labeled GAPDH cDNA to show RNA loading. For Western blot analysis, total proteins extracted from age-matched wild-type (lane 1), heterozygous (lane 2), and homozygous (lane 3) hearts were immunoblotted with anti-mXina and anti-GAPDH antibodies. A 150-kDa mXina protein was detected in the wild-type and heterozygous mouse hearts, but not in the homozygous mouse heart. The mXina-null hearts appear to have more GAPDH protein than the wild-type heart (left). Although most protein bands from these heart extracts show equal intensity by Coomassie blue stain, the myosin heavy chains (MHCs; indicated by 194 kDa) are significantly decreased in the mXina-deficient hearts (right). Western blot with anti-α-tubulin antibody shows that wild-type and mXina-deficient mouse hearts contained equal amounts of α-tubulin (bottom left).
Detection System (Center for Comparative Genomics, University of Iowa).

Northern and Western blot analyses. Total RNA isolation and Northern blot analysis were performed as previously described (33). The labeled probes included 311-bp mXin, pBKX-2 mXinβ (nt 1–3319), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Whole hearts dissected from ~6- to 9-mo-old wild-type, heterozygous, and homozygous mXinα-knockout littermates were homogenized and used for Western blot analysis as described previously (29, 37). The antibodies used included rabbit anti-mXin (U1013) (29) and mouse monoclonal anti-GAPDH (Research Diagnostics, Flanders, NJ) and anti-α-tubulin (DM1A, a generous gift from Dr. Steven Blose, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Monoclonal anti-β1-integrin, anti-α-catenin, and anti-plakoglobin antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA). Rabbit anti-connexin 43 and monoclonal anti-N-cadherin, anti-β-catenin, and anti-p120ctn antibodies were obtained from Zymed Laboratories (South San Francisco, CA). Rabbit anti-desmoplakin was purchased from Serotec (Oxford, UK). FA3 monoclonal antibody specifically recognizes α-MHC, as characterized previously (10).

Generation of peptide antibodies specific to mXinα or mXinβ. After amino acid sequence comparison between mXinα and mXinβ, a region of specific peptide sequence with high antigenic index was identified by Lasergene Protean software (DNASTar, Madison, WI). Synthetic peptides to these regions [for mXinα, amino acid

Fig. 2. Characterization of mXinα and mXinβ peptide antibodies. A–H: double-label immunofluorescence microscopy was performed on rat neonatal cardiomyocytes with rabbit polyclonal α-peptide-specific antibody (U1697), β-peptide-specific antibody (U1741), or their respective preimmune serum and with mouse monoclonal anti-cardiac troponin T antibody (CT3). Rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated goat anti-mouse IgG were used for the secondary antibody in the indirect immunofluorescence. Arrows point to the cell-cell contacts, in which cardiac troponin T is absent but both mXinα and mXinβ are enriched. Bar, 10 μm. I: Western blot analysis of cardiac protein extract with U1013 and peptide-specific (U1697 and U1741) antibodies. The α-peptide-specific antibody (U1697) recognizes both mXinα (150 kDa) and mXinα-a (~260 kDa) isoform in 7.5% gel blot, whereas the β-peptide-specific antibody reacts with mXinβ band (~320 kDa) in 6.0% gel blot. The U1013 antibody against amino acids 1–532 of mXinα recognizes both mXinα and mXinα-a bands and cross-reacts with mXinβ. J–L: immunofluorescence microscopy was performed on adult mouse frozen heart sections with rabbit polyclonal U1013, U1697, and U1741 and rhodamine-conjugated goat anti-rabbit secondary antibody. Similar to the U1013 staining, both α-peptide-specific (U1697) and β-peptide-specific (U1741) antibodies recognize the antigens localized to the intercalated disks, and their respective preimmune antibodies do not show this staining pattern (data not shown). Bar, 10 μm.
were dissected, rinsed with phosphate-buffered saline to remove 5–11 mo were weighed in grams. The whole hearts from these mice were fixed overnight. The samples were treated sequentially with 1% OsO4 and 2.5% uranyl acetate, dehydrated, and embedded in epon resin. Ultrathin sections were cut, mounted on Formvar-coated grids, poststained, and examined under a Hitachi H-7000 transmission electron microscope (Central Microscopy Research Facility, University of Iowa).

**Electrocardiographic recordings of isolated, perfused hearts.** The intact heart was isolated from ~3- to 4-mo-old wild-type and mXina-knockout mice and mounted on Langendorff apparatus via aortic cannula. Immediately, the heart was retrogradely perfused through the aortic cannula from a heated storage cylinder with 37°C oxygenated Krebs-Henseleit solution to produce an isolated, perfused Langendorff preparation (20, 21). The electrocardiogram (ECG) was recorded by a Hugo Sachs Elektronik-Harvard apparatus. ECG parameters such as P wave, PQ interval, QRS interval, QT interval, and R-R interval (heart rate) were calculated from ECG tracings as described previously by Kirchhoff et al. (11).

**RESULTS**

**Generation and characterization of mXina-knockout mice.** After electroporation of the targeting vector into ES cells and selection in G418, 189 resistant clones were obtained and analyzed by Southern blot. We obtained 20 positive ES clones containing a 15-kb band of targeted locus and a 10-kb band of mXina locus (Fig. 1B). Two independent clones were used to generate chimeric founders. Heterozygous mice were crossed to generate mXina-null mice. The resulting heterozygous crosses showed a ratio of 1:1:1.8:1 for wild-type:heterozygote:homozygote from 189 mice genotyped, providing evidence that mXina-null mice are viable.
Loss of the \(mXin\) message in homozygous mice was confirmed by Northern blot analysis on total RNAs isolated from hearts of each genotype (Fig. 1C). In the heterozygous heart, decreased \(mXin\) message was also seen. The membrane was reprobed with \(GAPDH\) to reveal equal RNA loading.

Western blot analysis of total proteins extracted from hearts of each genotype further verified loss of \(mXin\) protein in homozygotes (Fig. 1C) and reduced levels of \(mXin\) protein in the heterozygote compared with the wild-type mouse (Fig. 1C, Western blot, top left). When this membrane was probed with anti-GAPDH, a significant increase in GAPDH protein was found in the \(mXin\)-null heart. Moreover, the Coomassie blue-stained gel of total extracts revealed a decrease in the amount of MHC band (indicated by 194 kDa) in heterozygous and homozygous hearts (Fig. 1C, Western blot, right). In contrast, similar Western blot analysis showed that there was no significant change in the amount of \(\alpha\)-tubulin in the \(mXin\)-knockout mouse hearts (Fig. 1C, Western blot, bottom left).

Insertion of the \(LacZ\)-Neo cassette at the beginning of exon 2 within the targeted gene allowed the endogenous \(mXin\) promoter to control the expression of the \(LacZ\) gene. To determine the expression pattern of the knockin \(LacZ\) gene, whole-mount embryos at embryonic day (E)8.0, E8.25, E9.0, and E13.5 were dissected and stained for \(\beta\)-galactosidase (\(\beta\)-gal) expression. \(\beta\)-gal was observed throughout the heart tube at E8.0 (Supplemental Fig. 1A) but not in the E7.5 embryo (data not shown), consistent with the first detectable level of \(mXin\) protein expression (29).\(^{1}\) Paraffin sectioning of E8.0–8.25 embryos revealed \(\beta\)-gal expression exclusively in the myocardial layer (Supplemental Fig. 1, B and C). \(\beta\)-gal staining was detected throughout both the looped (E9.0) and the septated (E13.5) hearts in addition to expression within the skeletal muscle of the E13.5 embryo (Supplemental Fig. 1, E–G). A wild-type E9.0 embryo was used as a control and

\(^{1}\) The online version of this article contains supplemental material.
and 675 bp) and a major band (462 bp) were detected (Supplemental Fig. 1A). β-gal expression was continuous within the heart from E9.0 through adulthood (data not shown). Therefore, the β-gal expression in mXin-deficient mice recapitulated endogenous mXin expression and did not interfere with normal cardiac development.

Upregulation of mXinβ in mXinα-null knockout mice. Immunofluorescence microscopy of wild-type hearts with U1013 anti-mXinα antibody revealed that mXinα colocalizes with N-cadherin at the intercalated disk (Supplemental Fig. 2A, a and b). In mXinα-null hearts, some fluorescent signal coinciding with N-cadherin was still detected (Supplemental Fig. 2A, c and d). This suggested the possibility of a second mXin gene, whose protein product is recognized by the U1013 anti-mXinα antibody. Indeed, with low-stringency Southern blot a second band between exon 2 and exon 3. When primer pairs (Pa-a and Pa-b) flanking this intron were used in PCR, two minor bands (853 and 675 bp) and a major band (462 bp) were detected (Supplemental Fig. 3). The 853-bp and 462-bp products are consistent with the presence of at least two mXin isoforms, a minor form of mXinα-a and a major form of mXinα. The nucleotide sequences of these PCR fragments (except 675 bp) further confirmed two isoforms of mXinα in the heart. The 675-bp amplified product was a PCR artifact due to mismatch priming and amplified a fragment from glucose phosphate isomerase 1.

Like mXinα, mXinα-a was significantly decreased in the heterozygous sample and completely absent in the homozygous sample, as detected by Western blot analysis with U1013 (Fig. 3B), consistent with the idea of mXinα-a as an alternatively spliced isoform of the same mXinα gene. In contrast, the mXinβ protein in the same blot was proportionally increased in the mXinα-deficient mouse hearts (Fig. 3B). Therefore, it appears that the upregulation of mXinβ in the mXinα-knockout heart is at both the message and the protein levels. This, together with the same localization of mXinα and mXinβ, suggests that the increased expression of mXinβ likely plays a compensatory role. It should also be noted that the Coomassie blue-stained gel analysis of total protein extracts from each genotype also revealed decreased levels of MHC in the mXinα-deficient hearts (Fig. 3A). However, there was no significant change in α-tubulin levels in the mXinα-deficient mouse heart (Fig. 3C), demonstrating equal sample loading.

Cardiac hypertrophy in mXinα-deficient mice. Heterozygous and mXinα-null hearts are modestly enlarged compared with wild-type littersmates at 9 mo of age (Fig. 4A, a–c). Additionally, H&E-stained frontal heart sections exhibit thickening of the ventricular myocardial wall and increased trabeculation, suggestive of hypertrophic cardiomyopathy (Fig. 4A, d–f).

To assess the extent of hypertrophy, heart weight-to-body weight ratios (HW/BW) were determined from wild-type and mXinα-deficient mice at 3–9 mo of age. The HW/BW (mean ± SE) for wild-type males was 4.89 ± 0.20, compared with 5.31 ± 0.22 and 5.76 ± 0.32 for heterozygous and homozygous males, respectively. The HW/BW for wild-type females was 4.69 ± 0.20, compared with 5.32 ± 0.17 and
5.77 ± 0.30 for heterozygous and homozygous females, respectively (Fig. 4B). These ratios are statistically significant by one-way ANOVA between wild-type and homozygous hearts. Although there was no significant difference between wild-type and heterozygous hearts, a trend of enlarged heterozygous hearts was observed. No significant difference was observed between males and females of the same genotype.

To investigate whether cardiomyocytes from mXin-deficient mice are hypertrophic, we employed differential interference contrast microscopy to compare myofiber width in heart sections from at least three mice of each genotype at 9–11 mo of age. With Leica Openlab software, the widths of longitudinal myofibers of similar orientation from frontal sections of hearts of each genotype were measured. The myofiber width of the wild-type heart averaged 9.83 ± 0.17 μm, whereas the heterozygous and homozygous myofiber widths averaged 11.45 ± 0.27 and 12.11 ± 0.22 μm, respectively (Fig. 4C). These differences are statistically significant by one-way ANOVA.

To further characterize the hypertrophic phenotype observed in the mXin-deficient mice, RT-PCR analysis was used to evaluate the expression of known hypertrophy response genes, including ANF, β-MHC, α-MHC, skeletal α-actin, and cardiac α-actin. The results from this analysis demonstrate that ANF expression is modestly increased in mXin-deficient mice (Fig. 5). In addition, β-MHC transcript levels are increased specifically in mXin-null mice, with no change in α-MHC expression. There is also a slight decrease in cardiac α-actin in mXin-null mice, whereas no difference in skeletal α-actin expression was observed between wild-type and mXin-deficient mice (Fig. 5). The observed increase in ANF expression levels was further confirmed with quantitative real-time RT-PCR analysis of four sets of age-matched littermates. Compared with wild-type ANF levels (set to a relative fold value of 1) 1.648 ± 0.109-fold and 1.674 ± 0.266-fold increases in expression were observed for heterozygotes and homozygotes, respectively, after normalization with GAPDH levels. Quantitative real-time RT-PCR analysis with primers for other hypertrophy genes studied was also performed but did not result in specific amplification of these genes.

Masson’s trichrome stain was used to detect evidence of interstitial fibrosis in the mXin-deficient mice. Several areas dispersed throughout the mXin-deficient heart sections stained positive for collagen, indicative of fibrosis (Fig. 6A). In

![Fig. 6. mXin-deficient mouse hearts show increased interstitial fibrosis. A: Masson’s trichrome-stained myocardial sections demonstrate a significant increase in interstitial fibrosis in heterozygous (b, e, h) and homozygous (c, f, i) hearts compared with wild-type (a, d, g) hearts. B: the amount of interstitial fibrosis increases with increasing age, as shown in the graphical representation of the quantified data. This increased fibrosis in mXin-null hearts compared with wild-type hearts was statistically significant at 3 mo (*P = 0.012), 9 mo (#P < 0.001), and 14 mo (+P < 0.001) by ANOVA. The increase in fibrosis in the heterozygote compared with wild-type at 14 mo was also statistically significant (&P < 0.001).]
contrast, very little, if any, fibrosis was present in the wild-type heart sections (Fig. 6A, a, d, g). The amount of fibrosis detected in mXina-deficient mice increased with age from 3 to 14 mo, while very little fibrosis was observed in older wild-type mice (Fig. 6A). Additionally, the tissue morphology appeared disrupted in the older mXina-deficient mice compared with the older wild-type mice and younger mice of all genotypes. Quantification of fibrosis demonstrated that these differences are statistically significant by one-way ANOVA between wild-type and mXina−/− mouse hearts at 3, 9, and 14 mo of age (Fig. 6B). A significant difference in fibrosis was also observed between wild-type and mXina+/− mouse hearts at 14 mo. These results suggest that mXina-deficient mice are predisposed to hypertrophic cardiomyopathy.

Changes in expression and localization of known intercalated disk proteins in mXina-deficient hearts. To determine whether any changes in the expression and/or distribution of known intercalated disk proteins occur in mXina-deficient hearts, Western blot analysis and immunofluorescence microscopy were carried out. Representative Western blot results are shown in Fig. 7A. Quantitation of the Western blots in various exposures from four sets of samples after normalization with α-tubulin indicated that there was no statistically significant change in the expression of α-catenin, plakoglobin, and β1-integrin (Fig. 7B). However, the expression levels of N-cadherin, β-catenin, p120<sup>ctn</sup>, connexin 43 (both phosphorylated and nonphosphorylated forms, see Fig. 7A), and desmoplakin in mXina-null hearts were significantly lower than the levels in wild-type hearts by ANOVA. The levels of α-MHC were also significantly decreased in mXina-deficient hearts. In contrast, GAPDH protein levels in homozygous hearts were 1.5-fold higher than in wild-type hearts. Immunofluorescence microscopy revealed that most known intercalated disk proteins do not significantly change their localization in mXina-deficient hearts, except p120<sup>ctn</sup> and connexin 43 (Figs. 8 and 9). The localization of p120<sup>ctn</sup> changed from more diffuse distribution in the wild-type heart (Fig. 8, G and J) to discrete band/puncta staining in the mXina-null heart (Fig. 8, I and L). In the wild-type heart, connexin 43 was mainly localized to the intercalated disks at cell termini (arrowheads in Fig. 9D), with few lateral cell-cell connections. In contrast, connexin 43 distributions in the mXina-null heart showed a significant increase in lateral cell-cell connections (arrows in Fig. 9F).

Abnormal intercalated disk ultrastructure and myofiber disarray in mXina-null mice. The specific localization of mXina at the intercalated disk and changes in the expression levels of intercalated disk components prompted a qualitative ultrastructural examination of the intercalated disk region of mXina-deficient mice compared with wild-type littermates. A clear boundary between the A and I bands of sarcomeres near the intercalated disk is observed in wild-type and heterozygous hearts (Fig. 10, A and B). This boundary is absent in the mXina-null heart (Fig. 10C); some thick filaments aberrantly extend into the I band at the intercalated disk of the mXina-null heart (Fig. 10C, arrows). The gap junctions (Fig. 10, A and B) near the adherens junctions are easily identified in wild-type and heterozygous hearts but, while still present, are observed less frequently in mXina-null hearts. The filamentous web underlying the adherens junction is less dense in mXina-deficient hearts than in wild-type hearts. While the adjacent faces of the intercalated disks of the wild-type heart are closely and densely associated, the mXina-deficient intercalated disks are more detached, with greater separation (Fig. 10, A–C).

The ultrastructure of the wild-type sarcomeres is highly organized, with distinct I and A bands and a definitive H zone and M lines (Fig. 10D). The Z disks are well aligned, and of a consistent length throughout the heart. In contrast, the I and A bands are not well defined in the severely affected areas of the mXina-null heart (Fig. 10E). The sarcomeres appear distorted and compacted, as the length from Z line to Z line is decreased compared with the wild-type sarcomeres. The Z lines in mXina-null hearts are also diffuse and thickened. However,
some regions of mXin-null hearts appear to have a more organized sarcomere structure (Fig. 10F).

ECG analysis reveals prolonged QT intervals in mXin-deficient hearts. Previous patch-clamp studies revealed that mXin-deficient mouse cardiomyocytes had abnormal electrophysiological characteristics, including an increase in Na\(^+\) inward currents and decreases in L-type Ca\(^{2+}\) currents, transient outward K\(^+\) currents, and Ba\(^{2+}\)-sensitive inward rectifier K\(^+\) currents (3). These together with the observed changes in connexin 43 expression and localization in the mXin-deficient mouse heart suggest that knockout mouse hearts may also have conduction defects. Therefore, we performed ECG recordings on isolated, perfused hearts (Langendorff preparations) of ~3- to 4-mo-old mice of each genotype. A representative ECG tracing for each genotype is shown in Fig. 11, and Table 1 summarizes the ECG parameters calculated from tracings of multiple samples of each genotype. The P wave represents depolarization of the atrium, the PQ interval represents atrioventricular conduction, the QRS interval represents ventricular depolarization, and the QT interval represents depolarization and repolarization of the ventricle. mXina-null mice displayed an increased P wave duration compared with wild-type and heterozygous mice (P < 0.05, ANOVA). Furthermore, mXina-deficient mice showed significantly prolonged QT intervals but no difference in QRS intervals (Table 1). These data on the prolonged P wave and QT interval suggest a defect in atrial depolarization and ventricular repolarization.

**DISCUSSION**

mXina and mXin\(\beta\) evolve from a single gene, and both have overlapping and unique functions. Although embryonic lethality was expected based on previous antisense knockdown experiments in chicken embryos (33), viable and fertile mXina-null mice were obtained. The presence of mXin\(\beta\) in the intercalated disk of the mouse heart, coupled with the upregulation of mXin\(\beta\) at both the message and protein levels in mXina-knockout mice, provides a reasonable explanation for viable mXina-null mice. Previous antisense results did not completely rule out the possibility of a Xin gene family in the chicken (33). However, database homology searches against the complete chicken genome did not support the existence of such a Xin gene family in the chicken. Moreover, amino acid
sequence comparisons revealed that cXin has 42.8% and 33.4% identity to mXinα and mXinβ, respectively. Similar percent identity (42.7% and 33.3%) is also obtained when comparing cXin to the human homologs, hXinα and hXinβ. The percent identity between mXinα and mXinβ, or between hXinα and hXinβ, is only 28%. These results are consistent with the notion that mXinα and mXinβ have evolved from a single Xin gene, and that cXin may be evolutionarily closer to mXinα. On the other hand, mXinα, mXinβ, and cXin all contain the Xin repeat, which is now known as a novel class of actin-binding domain (4, 16, 23). Interestingly, there are 27 Xin repeats in cXin, which is closer to the 28 repeats present in mXinβ than to the 15 repeats in mXinα. Together, these results support a single Xin gene in the chicken and further imply that mXinα and mXinβ may have evolved to perform distinct functions in cardiomyocytes. However, the fact that both mXinα and mXinβ have similar domain structures, similar tissue expression pattern, and similar intracellular localization (16, 17) suggests that they may also play overlapping roles in cardiac development and function. The upregulation of mXinβ in mXinα-null mice further supports this overlapping role, and argues for compensation by mXinβ in the absence of mXinα.

mXin messages and proteins preferentially localize to intercalated disks. We demonstrated that the mXinβ messages were preferentially localized to what appeared to be the intercalated disks of wild-type and mXinα-deficient hearts. The majority of mXinα messages were also found at this specific structure (data not shown). In contrast, intercalated disk localization was not observed for cardiac troponin T messages (data not shown). The localization of mXin mRNAs to the intercalated disk, where the protein is utilized, may provide an efficient way for mXin localization, given the fact that mXin is also capable of binding to actin filaments. In another study, we have shown (9) that mXinα directly binds to β-catenin, and that aa 535–636 of mXinα, located within the Xin repeats, are both necessary and sufficient for this interaction. The overlap of the β-catenin-binding domain with the actin-binding domain on mXin may provide another way for ensuring mXinα localization exclusively to the intercalated disk and not to the thin filaments.

mXinα-deficient mice represent a novel model of cardiomyopathy with conduction defect. In this study, we have shown that a lack of mXinα in the heart results in abnormal intercalated disk ultrastructure, eventually leading to myofibril disarray, fibrosis, cardiac hypertrophy, and cardiomyopathy. De-
spite these findings, the intercalated disk structure appears relatively intact at the light microscopy level. Thus this animal model of cardiomyopathy differs from that derived from the N-cadherin condition knockout mouse (12), in which a complete dissolution of the intercalated disk structure was observed. In mXin-knockout mouse hearts, decreased expression levels of p120ctn, /H9252-catenin, N-cadherin, and desmoplakin were detected, suggesting that perturbations of the adherens junctions and desmosomes are present. This is in contrast to other mouse models for dilated cardiomyopathy such as the muscle LIM protein (MLP)-knockout mouse (2) and the tropomodulin-overexpressing transgenic (TOT) mouse (31). Both MLP-/- and TOT mice show significant upregulation of adherens junction components but not desmosomal compo-

Table 1. Conduction parameters determined by ECG recording of isolated, perfused hearts (Langendorff preparations) from wild-type and mXin-deficient mice

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<th>mXin+/+</th>
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<td>No. of mice</td>
<td>11</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>P wave</td>
<td>15.3±0.4</td>
<td>15.5±0.6</td>
<td>19.1±0.4*</td>
</tr>
<tr>
<td>PQ interval</td>
<td>38.6±1.9</td>
<td>40.0±2.7</td>
<td>40.6±3.8</td>
</tr>
<tr>
<td>QRS interval</td>
<td>9.0±0.4</td>
<td>10.2±0.6</td>
<td>9.0±0.4</td>
</tr>
<tr>
<td>QT interval</td>
<td>41.7±1.1</td>
<td>54.8±3.2*</td>
<td>73.2±6.6*</td>
</tr>
<tr>
<td>R-R interval</td>
<td>193.6±14.8</td>
<td>176.0±10.3</td>
<td>235.1±17</td>
</tr>
</tbody>
</table>

Values (in ms) are means ± SE. ECG traces were recorded at 37°C. *P < 0.05, significant difference between wild-type (mXin+/+) and mXin-deficient (mXin+/+ or mXin-/-) preparations.
ponents (6, 24). In addition to decreases in the expression of N-cadherin, β-catenin, p120ctn, and desmoplakin, mXin-knockout mouse hearts consistently show not only a significant decrease in the expression of connexin 43 but also an alteration in its localization. These data are similar to previous results first demonstrating abnormal localization of desmosomal and gap junctional components in human hypertrophic cardiomyopathy hearts, where the authors suggest that this remodeled gap junction localization may contribute to the cardiac arrhythmias associated with hypertrophic cardiomyopathy (28). Indeed, ECG analyses of isolated, perfused hearts show that mXin-deficient mice have a significantly prolonged QT interval but no change in QRS interval, providing evidence for a conduction defect. In a patch-clamp study of ventricular myocytes prepared from wild-type and mXin-deficient mice, we have observed significant reductions in transient outward K+ currents (I\textsubscript{to}) and Ba\textsuperscript{2+}-sensitive inward rectifier K+ currents (I\textsubscript{Kr}) in mXin\textsuperscript{−/−} myocytes (3), which are important components for repolarizing cardiomyocytes. An optical mapping technique has been used to compare the conduction velocity on the front surface of ventricles of wild-type and mXin-null hearts with the MED64 recording system (MED-P515A, Panasonic). The results revealed that the conduction velocity in mXin-null ventricles was significantly slower than that in wild-type ventricles (14). This slower conduction velocity together with the depressed I\textsubscript{to} and I\textsubscript{Kr} detected in ventricular myocytes is consistent with a prolonged QT period in mXin-null ECG. Although the mechanism causing a wider P wave in mXin-null ECG remains unclear, MED64 recording studies on left atrial-pulmonary vein myocardium preparations and connexin 40 studies on its expression and localization may provide evidence to support a wider P wave in the mXin-null heart. Together, these data demonstrate that mXin-knockout mice represent a novel model for the study of cardiomyopathy with conduction defects.

Molecular mechanisms underlying cardiomyopathy in mXin-null mice. In response to abnormal external or internal load/agonists, the adult heart can undergo hypertrophic growth, which often progresses to dilated cardiomyopathy, or can alternatively undergo dilation directly. This pathological hypertrophy reactivates many fetal cardiac genes and represses their corresponding adult counterparts. For example, in the adult mouse heart, cardiomyopathy and pressure overload increase embryonic β-MHC and decrease adult α-MHC (32). This isoform transition correlates well with the observed decrease in cardiac contractility. Consistent with such general gene expression changes associated with cardiac hypertrophy, mXin-deficient hearts exhibit a significant reduction in α-MHC protein as well as cardiac α-actin but not skeletal α-actin message levels. However, the degree of increased α-skeletal actin expression has been shown to be directly related to the severity of the cardiac hypertrophy phenotype, with definite changes only observed when the cardiac mass was increased by >20% (30). Additionally, increased ANF and β-MHC messages were observed, consistent with the hypertrophic phenotype. In contrast, GAPDH, a major enzyme in the glycolytic pathway, was found to be increased in mXin-deficient hearts compared with wild-type hearts, although the GAPDH message level did not change significantly. This is also consistent with the fact that hypertrophic hearts generally exhibit a transition from an oxidative to a more anaerobic metabolism, such as glycolysis, which involves GAPDH (22).

In the present study, we have shown that cardiac hypertrophy in mXin\textsuperscript{−/−} mice begins with abnormal intercalated disk ultrastructure as early as 3 mo of age. This structural alteration is accompanied by a significant decrease in the expression of N-cadherin, β-catenin, and p120ctn, suggesting that the hypertrophy that results in mice around 9–11 mo may be due to impaired organization of the intercalated disk and instability of adhesions. In concert with this suggestion, mXin has been shown to interact directly with β-catenin and actin filaments (9, 16). Through these interactions, mXin may orchestrate the structural integrity of the N-cadherin cellular interface and its connection to the actin cytoskeleton.

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