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The Xin repeat-containing protein, mXinβ, initiates the maturation of the intercalated discs during postnatal heart development

Qinchuan Wang, Jenny Li-Chun Lin, Stephen Y. Chan, Jim Jung-Ching Lin *

Department of Biology, University of Iowa, Iowa City, IA 52242-1324, USA

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ABSTRACT

The intercalated disc (ICD) is a unique structure to the heart and plays vital roles in communication and signaling among cardiomyocytes. ICDs are formed and matured during postnatal development through a profound redistribution of the intercellular junctions, as well as recruitment and assembly of more than 200 proteins at the termini of cardiomyocytes. The molecular mechanism underlying this process is not completely understood. The mouse orthologs (*mXinα* and *mXinβ*) of human cardiomyopathy-associated (*CMYA*)/Xin actin-binding repeat-containing protein (*XIRP*) genes (*CMYA1*/*XIRP1* and *CMYA3*/*XIRP2*, respectively) encode proteins localized to ICDs. Ablation of *mXinα* results in adult late-onset cardiomyopathy with conduction defects and up-regulation of mXinβ. ICD structural defects are found in adult but not juvenile *mXinα*-null hearts. On the other hand, loss of *mXinβ* leads to ICD defects at postnatal day 16.5, a developmental stage when the heart is forming ICDs, suggesting mXinβ is required for ICD formation. Using quantitative Western blot, we showed in this study that mXinβ but not mXinα was uniquely up-regulated during the redistribution of intercellular junction from the lateral membrane of cardiomyocytes to their termini. In the absence of mXinβ, the intercellular junctions failed to be restricted to the termini of the cells, and the onset of such defect correlated with the peak expression of mXinβ. Immunofluorescence staining and subcellular fractionation showed that mXinβ preferentially associated with the forming ICDs, further suggesting that mXinβ functioned locally to promote ICD maturation. In contrast, the spatiotemporal expression profile of mXinα and the lack of more severe ICD defects in *mXinα*−/−;*mXinβ*−/− double knockout hearts than in *mXinβ*−/− hearts suggested that mXinα was not essential for the postnatal formation of ICDs. A two-step model for the development of ICD is proposed where mXinβ is essential for the redistribution of intercellular junction components from the lateral puncta to the cell termini.

Introduction

The integration of the contraction and relaxation of billions of individual cardiomyocytes is essential for the heart to function. To carry out such integration, individual cardiomyocytes must be excited at the right moment so that they have coordinated contractions in each heartbeat, which requires electrical coupling between cardiomyocytes. The contractile forces generated by individual cardiomyocytes in turn must be transmitted to the correct neighbors so that the tiny forces from each cardiomyocyte is added up for the heart to perform mechanical work, which requires mechanical coupling between cardiomyocytes. These essential electrical and mechanical couplings are carried out by a cardiac specific structure, the intercalated discs (ICDs). The functions of ICDs have been classically attributed to three types of intercellular junctions (Forbes and Sperelakis, 1985). The gap junctions that are made of connexin permit ions to flow between cardiomyocytes for electrical coupling, the adherens junctions that are organized by N-cadherin confer continuity for the myofibrils between cardiomyocytes and are central for transmitting contractile forces, and the desmosomes that are organized by the desmosomal cadherins couple the sarcolemma to the intermediate filaments to maintain the mechanical integrity of the cardiomyocytes. In addition to these functions classically assigned to the ICDs, it is now increasingly realized that ICDs with more than 200 proteins (Estigoy et al., 2009) are specialized membrane domains of the cardiomyocytes and also function in chemical and mechanical signaling as well as ion transportation (Noorman et al., 2009).

Given the important roles of ICDs, it is not surprising that mutations in genes encoding ICD components can cause severe heart diseases, such as the arrhythmogenic right ventricular cardiomyopathy (Delmar and McKenna, 2010). Conversely, various heart diseases not directly related to mutations of ICD

* Corresponding author. Fax: +319 353 2275.
E-mail address: jim-lin@uiowa.edu (J.J.-C. Lin).

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components lead to alterations in the ICDs, and such alterations are likely an important hallmark of the pathology of these diseases (Barker et al., 2002; Noorman et al., 2009; Wang and Gerdes, 1999). The importance of the ICDs in the hearts is further supported by the severe cardiac defects of a number of animal models (Ferreira-Cornwell et al., 2002; Kostetskii et al., 2005; Li et al., 2006; Li and Radice, 2010; Wang et al., 2010). An important theme of these cardiac diseases and defects, either originated from mutations of ICD components or from non-ICD related reasons, is that the molecular integrity of the ICDs are disrupted. The manifested phenotypes include altered morphology, abnormal expression levels and localizations of protein components, as well as changed protein–protein interaction profiles of the ICDs. Thus, the precise structure and the organization of the ICDs are vital for their specific functions, and such structure and organization are prone to be disturbed by pathological factors.

The components of the complex ICDs are assembled mainly during postnatal development as ICDs mature. Several studies have shown that the maturation of ICDs is characterized by drastic reorganization of the distribution of intercellular junctions (Angst et al., 1997; Hirschy et al., 2006; Peters et al., 1994). In embryos, N-cadherin and associated proteins such as β-catenin and mXinα are localized to almost the entire surface of cardiomyocytes in a rather diffused pattern (Sinn et al., 2002). Gap junction and desmosomal proteins are also distributed on the entire surface of cardiomyocytes where cell–cell contacts exist but show more spotted localization than the components of adherens junctions (Coppen et al., 2003; Pieperhoff and Franke, 2007). During postnatal development, the intercellular junctions undergo reorganization by which all three types of intercellular junctions are eventually localized to the ends of cardiomyocytes. Unique to the ICDs of adult mammalian hearts, the adherens junctions also intermix with desmosomes at the molecular level to form a newly identified structure, *area composita* (Pieperhoff and Franke, 2007, 2008). The molecular mechanisms for the maturation of ICDs are largely unknown. However, since all the classic components of the intercellular junctions are already expressed in the embryonic heart, the postnatal reorganization of the intercellular junctions for the maturation of ICDs must be dictated by additional factors that are expressed/activated during the postnatal life.

One such factor might be the intercalated protein mXinβ, a member of the Xin repeat-containing family of proteins, that is specifically localized to the ICDs in the adult cardiomyocytes (Lin et al., 2005). In mice, the Xin repeat-containing gene family has two members, the *mXinα* and *mXinβ*, which encode the mXinα alternatively splicing variants (mXinα and mXinα-a) and mXinβ alternatively splicing variants (mXinβ and mXinβ-a), respectively (Gustafson-Wagner et al., 2007; Wang et al., 2010). The Xin repeats are conserved protein motifs that interact with actin filaments (Choi et al., 2007; Pacholsky et al., 2004). Within its Xin repeat region, the mXinα variants have a conserved β-catenin-interacting domain (Choi et al., 2007). This β-catenin-interacting domain and its counterpart in mXinβ may be responsible for recruiting both the mXin proteins to the adherens junctions, where the mXin proteins may directly couple the N-cadherin–catenin complex to the underlying actin cytoskeleton (Grosskurth et al., 2008; Wang et al., 2012).

Evidence from our previous study indicates that mXinβ may play important roles in the postnatal formation and maturation of ICDs. We observed an up-regulation of mXinβ protein in the heart from postnatal day 0.5 (P0.5) to P13.5. We also found that in the *mXinβ*−/− hearts at P16.5, the intercellular junctions are punctate, and mature ICD-like structures are sparse (Wang et al., 2010). However, several gaps in our knowledge prevent us from drawing a firm conclusion about mXinβ's roles in ICD formation. First, although previous studies showed that ICDs are formed postnatally, descriptions of the reorganization of intercellular junctions between P0.5 and P16.5 are not detailed enough for us to correlate this process with the expression profile of mXinβ. Second, the spatiotemporal expression profile was not fully characterized for mXinβ; we do not know the expression profile of mXinβ after P13.5 and whether mXinβ is localized to the adherens junctions throughout their reorganization. Third, the nature of the defects of ICDs in *mXinβ*−/− hearts was not addressed in our previous study; we do not know whether the ICDs are not formed at all in the *mXinβ*−/− hearts or are formed but then fail to be maintained. In this study, we asked what specific roles mXinβ plays in the formation of ICDs and answered this question by investigating the quantitative expression profiles of mXinβ and the core protein of adherens junctions, N-cadherin; we also provided a detailed description of the time course of reorganization of the intercellular junctions during ICD formation in wild-type and *mXinβ*−/− hearts. Our results suggest a direct involvement of mXinβ in the postnatal reorganization of intercellular junctions.

In addition to studying mXinβ's roles in the maturation of ICDs, we examined mXinα variants' roles in this process because mXinα variants share many conserved regions with mXinβ but seems incapable of compensating for the loss of *mXinβ* for the formation of ICDs (Wang et al., 2010). This is in contrast with the compensatory roles of mXinβ for the loss of *mXinα* we demonstrated previously (Gustafson-Wagner et al., 2007). We asked whether the inability of mXinα variants to compensate for the loss of *mXinβ* is due to insufficient expression during ICD formation or lack of essential protein function required for this process.

Materials and methods

Generation of mXinα−/−;mXinβ−/− double knockout (DKO) mice

All animal procedures were performed with the approval of the University of Iowa 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. Generation and initial characterization of *mXinα*−/− (Gustafson-Wagner et al., 2007) or *mXinβ*−/− (Wang et al., 2010) single knockout (SKO) mice have been previously reported. Both mouse lines were backcrossed to C57BL/6J for at least eight generations and the heterozygotes were maintained in C57BL/6J background. The *mXinβ*-null mice die before weaning, whereas the *mXinβ*+/− and the *mXinα*-null mice are viable and fertile. Therefore, we crossed *mXinβ* heterozygotes to *mXinα*-null mice to obtain double heterozygotes, which were then used for the experiments to generate the *mXinα*−/−;*mXinβ*−/− DKO mice. Gross morphology, histology and molecular characterization of DKO mice were carried out as previously described for *mXinα*−/− or *mXinβ*−/− SKO mice (Gustafson-Wagner et al., 2007; Wang et al., 2010).

Antibodies

Primary antibodies used for both immunofluorescence microscopy and Western blot analysis included rabbit polyclonal antibody (pAb) U1013 (against both mXinα and mXinβ) (Sinn et al., 2002), pAb U1697 (mXinα-specific) (Gustafson-Wagner et al., 2007), and pAb U1040 (mXinβ-specific) (Wang et al., 2010), mouse monoclonal antibody (mAb) 3B9 anti-N-cadherin (Invitrogen, Life Technologies, Grand Island, NY), rat mAb MNCD2 anti-mouse N-cadherin (Developmental Studies Hybridoma Bank, University of Iowa), rabbit pAb anti-connexin 43 (Cx43) (Zymed Laboratories Inc., Invitrogen), rabbit pAb AHP320