Experimental Gerontology 49 (2014) 26–34

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Mechanical and non-mechanical functions of *Dystrophin* can prevent cardiac abnormalities in *Drosophila*

Ouarda Taghli-Lamallem [a,b,*], Krzysztof Jagla [b], Jeffrey S. Chamberlain [c], Rolf Bodmer [a,**]

[a] Development and Aging Program, Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Rd, Building 7 Room 7125, La Jolla, CA 92037, USA
[b] GReD, INSERM U1103, CNRS UMR6293-Clermont University, Faculty of Medicine 28, Place Henri Dunant, 63000 Clermont-Ferrand, France
[c] University of Washington School of Medicine, Department of Neurology, Box 357720, Seattle, WA 98195-7720, USA

ARTICLE INFO

Article history:
Received 25 March 2013
Received in revised form 22 October 2013
Accepted 28 October 2013
Available online 12 November 2013

Section Editor: T.E. Johnson

Keywords:
Micro-*Dystrophins*
Heart period
Myofibrils
Nitric oxide synthase
Aging

ABSTRACT

Dystrophin-deficiency causes cardiomyopathies and shortens the life expectancy of Duchenne and Becker muscular dystrophy patients. Restoring *Dystrophin* expression in the heart by gene transfer is a promising avenue to explore as a therapy. Truncated *Dystrophin* gene constructs have been engineered and shown to alleviate dystrophic skeletal muscle disease, but their potential in preventing the development of cardiomyopathy is not fully understood. In the present study, we found that either the mechanical or the signaling functions of *Dystrophin* were able to reduce the dilated heart phenotype of *Dystrophin* mutants in a *Drosophila* model. Our data suggest that *Dystrophin* retains some function in fly cardiomyocytes in the absence of a predicted mechanical link to the cytoskeleton. Interestingly, cardiac-specific manipulation of nitric oxide synthase expression also modulates cardiac function, which can in part be reversed by loss of *Dystrophin* function, further implying a signaling role of *Dystrophin* in the heart. These findings suggest that the signaling functions of *Dystrophin* protein are able to ameliorate the dilated cardiomyopathy, and thus might help to improve heart muscle function in micro-*Dystrophin*-based gene therapy approaches.

1. Introduction

Dystrophinopathies are due to mutations in the *Dystrophin* (*Dys*) gene causing Duchenne and Becker muscular dystrophy (DMD and BMD, respectively) and X-linked dilated cardiomyopathy. In all these pathologies, the heart muscle is affected to different degrees, depending on the type of the mutation and the progression of the disease. Cardiac disease in both DMD and BMD manifests itself as a dilated cardiomyopathy (DCM) and/or cardiac arrhythmia (Corrado et al., 2002). Cardiomyopathy is present in about 90% of DMD/BMD patients, and progressively leads to heart failure, causing the death of 20% DMD and 50% BMD patients (Bushby et al., 2003).

Currently, there are no effective treatments of DCM besides transplantation and pharmacological intervention, such as with angiotensin-converting enzyme inhibitors and beta blockers, which only ameliorate heart symptoms, without correcting the underlying pathology (Kaspar et al., 2009). Inevitably, the DCM worsens as the patient becomes older and as the disease progresses. New therapy alternatives to manage the DCM are thus needed. Viral-based gene therapies, including the adeno-associated virus (AAV) system have recently drawn considerable attention for exploring the potential utility of a modified, but functionally active *Dys* gene in ameliorating the dystrophic skeletal and heart muscle phenotypes (Bostick et al., 2011; Gregorevic et al., 2006).

Two essential functions have been attributed to *Dys* protein. The first one is referred to as a mechanical role: *Dys* links to F-actin via its N-terminal and central actin-binding domains, and to Dystroglycan (Dg) via its WW and cysteine-rich (CR) domains, thus enabling force transduction from the inside to the outside of the cell, and stabilizing the sarcolemma. The second is a signaling role: *Dys* assembles signaling molecules, including neuronal nitric oxide synthase (nNos), growth factor receptor-bound protein 2 (Grb2), Calmodulin, and Calmodulin-dependent kinases (Anderson et al., 1996; Brenman et al., 1996).

Previous work in skeletal muscle of the mouse DMD model ("*mdx*") showed that expressing *Dys* isoforms preserving its mechanical function is beneficial, by improving *mdx* muscle function and preventing dystrophy (Gregorevic et al., 2006; Harper et al., 2002). Other studies suggest that restoring the *Dys*-glycoprotein complex (DGC) by expressing the Dp71 or the Dp116 isoforms in skeletal muscle of *mdx* mice, which lack the mechanical function of *Dys*, does not ameliorate the dystrophic

Abbreviations: Dys, *Dystrophin*; DMD, Duchene muscular dystrophy; BMD, Becker muscular dystrophy; XLDCM, X-linked dilated cardiomyopathy; DCM, Dilated cardiomyopathy; AAV, Adeno-associated virus; Dg, Dystroglycan; CR, Cysteine-rich domain; nNos, Neuronal nitric oxide synthase; Grb2, Growth factor receptor-bound protein 2; DGC, *Dystrophin*-glycoprotein complex; Utrn, Utrophin; Syn, Syntrophin; Dbr, Dystrobrevin; Dg, Dystroglycan; HPs, Heart periods; CT, Carboxy-terminal domain; NO, Nitric oxide; AI, Arrhythmia index; iNos, Inducible Nos; eNos, Endothelial Nos; SR, Sarcoplasmic reticulum.

* Correspondence to: O. Taghli-Lamallem, GReD, INSERM U1103, CNRS UMR6293-Clermont University, Faculty of Medicine 28, Place Henri Dunant 63000 Clermont-Ferrand, France. Tel.: +33 473 178 181; fax: +33 473 276 132.
** Corresponding author. Tel.: +1 858 795 5295; fax: +1 858 795 5298.
E-mail addresses: ouarda.taghli-lamallem@udamail.fr (O. Taghli-Lamallem), rolf@sanfordburnham.org (R. Bodmer).

0531-5565/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.exger.2013.10.015

phenotypes (Greenberg et al., 1994; Rafael et al., 1996). These data demonstrate that the mechanical role of *Dys* protein is the major contributor to the *mdx* dystrophic pathology. However, Dp116 expression in mouse mutants lacking both *Dys* and Utrophin (*mdx*:*utrn*$^{-/-}$) increases the muscle mass and improves growth, mobility and lifespan (Judge et al., 2011). Dp116 is a non-muscle isoform specifically expressed in Schwann cells of the peripheral nervous system that binds to the DGC components Dg, syntrophin (Syn) and dystrobrevin (Dbr), but does not provide a link to F-actin (it lacks the actin-binding domains). Thus Dp116 allows the assessment of the functional characteristics of the *Dys* 'signaling domain' in the absence of the 'mechanical domain' function.

While many studies have focused on structural–functional analysis of *Dys* in skeletal muscle to develop gene therapy [reviewed by (Blankinship et al., 2006)], little effort has so far been directed to correcting the heart pathology (Bostick et al., 2009, 2011; Hainsey et al., 2003; Townsend et al., 2007; Yue et al., 2003). To differentiate between the 'mechanical' and 'signaling' roles of *Dys* in cardiac muscle function, we generated flies that express either the *Dys* constructs ΔH2-R19/ΔCT and ΔR4-23/ΔCT, which can bind the F-actin, but lack the C-terminal domain that interacts with Syn and Dbr (predicted *Dys* with 'mechanical function') or the Dp116 with the C-terminal domains only, thus lacking the F-actin-binding domains (predicted *Dys* with 'signaling function'). We note that probably none of these *Dys* constructs are involved in nNos signaling, since they lack repeat 16 and 17 of the rod domain implicated in the interaction between nNos and *Dys* (Lai et al., 2009). We provide evidence that both the predicted 'mechanical' (ΔH2-R19/ΔCT, ΔR4-23/ΔCT) and 'signaling' (Dp116) functions of *Dys* are able to ameliorate dilated cardiomyopathy and improve myofibrillar organization. Manipulating *Dp116* and *Nos* in *Dys*$^{-/-}$ mutants provides further evidence of a signaling role of *Dys* in modulating the heart function. We conclude that both 'mechanical' and 'signaling' roles of *Dys* are important for cardiac muscle function.

2. Materials and methods

2.1. Drosophila strains

The flies *Dys*$^{-/-}$ are transheterozygous for *DysExel6184* and *Df(3R) Dl-X43* (Taghli-Lamallem et al., 2008). The *DysEP(3)3397* and the Dystroglycan mutants were a generous gift from R. Ray. GAL4 drivers were: *24B-GAL4* (Brand and Perrimon, 1993) and *Hand-Gal4* (from A. Paululat laboratory) kindly offered by L. Perrin. *UAS-Nos*^{RNAi} from VDRC (transformant ID 27725) and the *UAS-Nos* flies were generously offered by S.A. Davies and P.H. O'Farrell.

2.2. Dystrophin transgenic flies

The murine *Dys* cDNAs *ΔH2-R19ΔCT*, *ΔR4-23ΔCT* and *Dp116* have already been described (Harper et al., 2002; Judge et al., 2006). The ΔH2-R19ΔCT and ΔR4-23ΔCT lack a portion of the rod domains (spectrin repeats) and the C-terminal region of *Dys*. Dp116 is a short C-terminal isoform, containing two and a half spectrin repeats, the WW domain, the cysteine-rich domain, and the carboxy-terminal domain involved in binding to other DGC proteins like Syn and Dbr (for details of the construct, see Judge et al., 2006). For the generation of transgenic flies, *ΔH2-R19ΔCT*; *ΔR4-23ΔCT*; and *Dp116* cDNA constructs were sub-cloned into the Gal4-inducible vector pUAST at the NotI site, injected into *w*^{1118} embryos, and transgenic lines established. The transgenic flies were crossed to the heterozygous deficient flies *Df(3R) Dl-X43* to generate a stock *UAS-ΔH2-R19ΔCT* (or *UAS-ΔR4-23ΔCT* or *Dp116*)/cyo, *Df(3R)Dl-X43/TM3*. These lines were crossed to *Hand-Gal4*, *DysExel6184/TM3* flies to generate the transgenic rescue flies with the truncated *Dys*.

2.3. Immunostaining of adult Drosophila hearts

Staining of adult flies was performed as described previously (Taghli-Lamallem et al., 2008). Primary antibodies: rabbit anti-Dystroglycan diluted at 1/1000 (gift from A. Wodarz); phalloidin-cy3 diluted at 1/1000, and two rabbit NOS antibodies diluted at 1/400 (gift from P.H. O'Farrell) and 1/100 (Thermo-Scientific, PA1-039). Secondary antibodies: donkey anti-rabbit conjugated to CY3 (Jackson ImmunoResearch) at 1/300. Immunostained preparations were visualized on Olympus FV300 or Zeiss LSM 510 laser scanning confocal microscopes.

2.4. Heart physiological analysis

Flies anesthetized with fly nap (Carolina Biol., Corp.) were aligned on a dish (dorsal down) and dissected to expose the heart for optical recording by previously described protocols (Fink et al., 2009; Ocorr et al., 2007a). Beating heart images were acquired at rate of about 130 frames per second using Simple PCI software (Compix, Sewickley, PA). Cardiac parameter measurements were quantified and generated using the MatLab-based image analysis program (Fink et al., 2009). M-modes illustrate movements of the heart tube edges in the y-axis over time in the x-axis, generated by excising and aligning a single pixel-wide image from successive frames. Heart periods (HPs) are defined as the time between the ends of two consecutive diastolic intervals. Single HPs were plotted in the histograms to see overall distribution and clustering of HPs. We used Prism software, one-way ANOVA analysis and a Tukey test to process statistics on 20 flies for each genotype.

3. Results

3.1. Dystrophin proteins carrying either the 'mechanical' or 'signaling' domains ameliorate Dystrophin-deficient heart dysfunction

The large size of the *Dys* transcript (14 Kb) presents a major challenge for successful gene transfer with viral vectors. This limitation has led to the construction of *mini-* and *micro-Dys* genes (Scott et al., 2002). Among these are the micro-*Dys* constructs ΔH2-R19/ΔCT and ΔR4-23/ΔCT, both maintaining the N-terminal, some of the rod, and cysteine-rich domains, but lacking the C-terminal domain that directly binds to Dbr and Syn, also components of the DGC. These truncated proteins are therefore expected to retain the capacity of force transduction in the sarcolemma, but they may have some 'signaling function' (Fig. 1A) (Scott et al., 2002). By contrast, Dp116 *Dys* contains intact CR and carboxy-terminal (CT) domains, but it lacks the N-terminal and most of the rod domains, preventing its link with the actin cytoskeleton and the mechanical reinforcement at the sarcolemma (Fig. 1A) (Judge et al., 2006).

We had previously found that the heart of *Dys*$^{-/-}$ mutant flies *(Df(3R)Dl-X43/DysExel6184)* was dilated and exhibited abnormal heart performance and contractility, reminiscent of dilated cardiomyopathy in mammals (Taghli-Lamallem et al., 2008). To probe potential differences between *Dys* mechanical and signaling roles, we generated transgenic flies that express *Dys* constructs *ΔH2-R19/ΔCT*, *ΔR4-23/ΔCT*, or *Dp116* in all muscles (24B-Gal4 driver) or specifically in the heart (Hand-Gal4 driver). Our choice of constructs was made to differentiate between portions of *Dys* that may or may not confer the mechanical reinforcement at the cell membrane, based on mammal studies in skeletal muscles. First, we tested the heart- and muscle-specific effects of these micro-*Dys* constructs in a wildtype background, and found that their overexpression in mesodermal tissues does not induce cardiac abnormalities (Fig. S1). We then expressed *ΔH2-R19/ΔCT*, *ΔR4-23/ΔCT*, or *Dp116* in all muscles or heart alone, to attempt to restore heart function in *Dys*$^{-/-}$ flies. Cardiac physiology was assessed by analyzing high-speed optical recordings of beating hearts in young and old flies (1 and 5 weeks old) (Fink et al., 2009). Expression of the truncated *Dys* constructs in *Dys*$^{-/-}$ caused a significant decrease in systolic diameters, and a