Dissociation of Sarcoglycans and the Dystrophin Carboxyl Terminus From the Sarcolemma in Enteroviral Cardiomyopathy

Gil-Hwan Lee,* Cornel Badorff,* Kirk U. Knowlton

Abstract—Enteroviral infection can cause an acquired form of dilated cardiomyopathy. We recently reported that dystrophin is cleaved, functionally impaired, and morphologically disrupted in vitro as well as in vivo during infection with coxsackievirus B3. Genetic dystrophin truncations lead to a marked decrease in dystrophin-associated glycoproteins, whereas expression of only the naturally occurring dystrophin carboxyl terminus, Dp-71, restores the sarcolemmal association of the dystrophin-associated glycoproteins. We sought to determine whether acute cleavage of dystrophin leads to a dissociation of the carboxyl-terminal dystrophin fragment and of the sarcoglycans from the sarcolemma during coxsackievirus B3 infection. We found that in cultured cardiac myocytes and murine hearts infected with coxsackievirus B3, the sarcolemmal localization of the dystrophin carboxyl terminus is lost. The dystrophin-associated glycoproteins α-, β-, γ-, and δ-sarcoglycan and β-dystroglycan were markedly decreased in the membrane fraction of infected cells in culture, and the typical sarcolemmal localization for each of these proteins was lost in coxsackievirus-B3–infected cardiomyocytes in vivo. Furthermore, sucrose gradient ultracentrifugation demonstrated that δ-sarcoglycan was physically dissociated from dystrophin within the membrane fraction. In vivo, the sarcolemmal integrity was functionally impaired with Evans blue dye uptake even though there was no generalized disruption of the sarcolemma of infected myocytes evidenced by intact wheat germ agglutinin staining. In analogy to hereditary sarcoglycanopathies, this disintegration of the sarcoglycan complex may, in addition to the dystrophin cleavage, play an important role in the pathogenesis of enterovirus-induced cardiomyopathy. These results imply a potential role for disruption of the sarcoglycans in an acquired form of heart failure. (Circ Res. 2000;87:489-495.)

Key Words: heart failure ■ cardiomyopathy ■ sarcoglycans ■ myocarditis ■ coxsackievirus

In cardiac muscle, the dystrophin–glycoprotein complex includes dystrophin and the dystrophin-associated glycoproteins α-, β-, γ-, and δ-sarcoglycan; α- and β-dystroglycan; and the recently described sarcospan. This complex is part of the extrasarcomeric cytoskeleton that collectively connects the internal F-actin–based cytoskeleton to laminin-2 of the extracellular matrix. Thereby, it is thought to play an important role in the transmission of mechanical force from the sarcomere to the extracellular matrix. Genetic defects in α-, β-, γ-, or δ-sarcoglycan are the cause of human limb-girdle muscular dystrophy type 2D, 2E, 2C, and 2F, respectively, and can caused dilated cardiomyopathy in humans. A defect in δ-sarcoglycan causes cardiomyopathy in the hamster, and genetic disruption of β-, γ-, and δ-sarcoglycans can cause cardiomyopathy in the mouse. Mutations in dystrophin cause Duchenne and Becker muscular dystrophy, both of which have a high incidence of dilated cardiomyopathy. In addition, dystrophin mutations are a cause of X-linked dilated cardiomyopathy. These studies and others have led to the paradigm that familial dilated cardiomyopathy can result from defective transmission of mechanical force from the sarcomere to the extracellular matrix and that disruption of the dystrophin–glycoprotein complex may be a common mechanism that causes cardiomyopathy. Although the importance of genetic defects of the dystrophin–glycoprotein complex in hereditary cardiomyopathy is well established, little is known about its role in acquired cardiomyopathy.

A subset of human acquired dilated cardiomyopathy is associated with an enteroviral infection of the heart, in particular, coxsackie B viruses. In mice, the transgenic expression of coxsackieviral proteins in the heart is sufficient to induce dilated cardiomyopathy. We recently proposed that cleavage of dystrophin has a role in the molecular pathogenesis of enterovirus-induced cardiomyopathy. Dystrophin is proteolytically cleaved by the coxsackieviral protease 2A in the hinge 3 region and is functionally impaired at the sarcolemma in vivo. Furthermore, sucrose gradient ultracentrifugation demonstrated that δ-sarcoglycan was physically dissociated from dystrophin within the membrane fraction. In vivo, the sarcolemmal integrity was functionally impaired with Evans blue dye uptake even though there was no generalized disruption of the sarcolemma of infected myocytes evidenced by intact wheat germ agglutinin staining. In analogy to hereditary sarcoglycanopathies, this disintegration of the sarcoglycan complex may, in addition to the dystrophin cleavage, play an important role in the pathogenesis of enterovirus-induced cardiomyopathy. These results imply a potential role for disruption of the sarcoglycans in an acquired form of heart failure. (Circ Res. 2000;87:489-495.)

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impaired. Localization of the rod domain of dystrophin is disrupted in cultured cardiomyocytes as well as in the intact mouse heart infected with coxsackievirus B3 (CVB3). We proposed that the cleavage of dystrophin during CVB3 infection initiates a cascade of events that contributes to dilated cardiomyopathy.25 Genetic defects of individual components of the dystrophin–glycoprotein complex can disrupt the assembly and thus the molecular organization of the entire complex. For example, dystrophin frameshift mutations lead to a marked decrease in the other dystrophin-associated glycoproteins in Duchenne muscular dystrophy.27 Interestingly, expression in the heart of a naturally occurring carboxyl-terminal isoform of dystrophin, Dp-71, that contains the β-dystroglycan binding site, is sufficient to restore the sarcolemmal localization of dystrophin–associated glycoproteins in dystrophin-deficient mice but fails to prevent the dystrophic phenotype observed in mdx mice.28,29 This demonstrates that the carboxyl-terminal region of dystrophin is sufficient for the organization of the sarcoglycan complex and that the linkage between actin and dystroglycan is not required for the assembly of the dystrophin–glycoprotein complex. Disruption of δ-sarcoglycan causes markedly decreased sarcolemmal staining for all of the sarcoglycans, whereas disruption of γ-sarcoglycan has a variable effect on individual sarcoglycan components.10,12,30 This indicates that the mechanisms for disruption of the dystrophin–glycoprotein complex determine the pattern of disruption of the sarcoglycan complex. Little is known about how acute cleavage of dystrophin will affect sarcoglycan stability and the integrity of the dystrophin–glycoprotein complex.

Because the viral protease 2A cleaves dystrophin in the hinge 3 region during coxsackievirus-B3 infection, an uncleaved carboxyl terminus of dystrophin may be sufficient to prevent complete dissociation of the sarcoglycans from the dystrophin–glycoprotein complex. For this reason, we sought to determine whether acute dystrophin cleavage by a viral protease has a phenotype that is similar to that observed with genetic dystrophin deficiency or whether it is more like the pattern observed with expression of the carboxyl-terminal dystrophin isoform, Dp71.

Our findings demonstrate that the sarcoglycan complex becomes physically, morphologically, and functionally disrupted with acute cleavage of dystrophin during CVB3 infection. In addition, cleavage of dystrophin results in a phenotype different from Dp71 expression, because the carboxyl-terminal cleavage fragment loses its sarcolemmal localization. Thus, acute cleavage of dystrophin by enteroviral protease 2A disrupts the sarcolemmal dystrophin-associated glycoproteins similar to that observed with genetic dystrophin mutations, which result in translation of a truncated protein. This disruption of the sarcoglycan complex, in addition to the cleavage of dystrophin, may play an important role in the induction of enteroviral cardiomyopathy.

Materials and Methods

Viruses

CVB3 was derived from the infectious cDNA copy of the cardiotropic H3 strain of CVB3.31 Wild-type adenovirus 5 was a kind gift from S. Huang (Scripps Research Institute, La Jolla, Calif.). Virus was titrated with a plaque-forming assay on HeLa cells.31

Mice

Male SCID (C3HSmn.C-Pkrdc−/−J) mice32 were purchased from the Jackson Laboratories. Agammaglobulinemia was verified in all SCID animals.33 Mice (3 to 6 weeks old) were infected with an intraperitoneal injection of 107 plaque-forming units34 of CVB3 and killed at day 7 after infection. In some mice, Evans blue dye was injected intraperitoneally at day 6 after infection, and hearts were harvested 24 hours later.25

Myocyte Culture

Rat neonatal ventricular myocytes were isolated and cultured as described previously. Myocytes were infected at a multiplicity of infection of 100.

Antibodies

Rabbit polyclonal antibodies anti-CVB334 (generous gift of Andreas Henke) and anti-δ-sarcoglycan9 (kindly provided by Vincenzo Nigro) were previously described. Monoclonal antibody MANDRA1 is specific for the carboxyl terminus of dystrophin35 (kindly provided by G.E. Morris). Monoclonal antibodies against α-, β-, and γ-sarcoglycans and β-dystroglycan were all from NovoCastra (Newcastle, UK). Rhodamine-labeled wheat germ agglutinin (WGA), biotinylated horse anti-mouse or anti-rabbit IgG, and streptavidin-alkaline phosphatase were from Vector Laboratories. Alkaline phosphatase–labeled goat anti-rabbit IgG and anti-mouse IgG (H+L) were obtained from Life Technologies. FITC-, Rhodamine Red-X-, and Cy5-conjugated anti-rabbit IgG were from Jackson ImmunoResearch Inc.

Myocyte Fractionation

Cytosolic and membrane fractions were prepared with the pyrophosphate variant as reported previously.36 In some experiments, the membrane fraction was layered onto a linear 5% to 20% sucrose density gradient and subjected to ultracentrifugation as described (Sw41Ti rotor, 200,000 rpm for 20 hours at 4°C). The gradient was then fractionated; the fractions were collected and concentrated with Centricon-10 devices (Millipore).

Western Blotting

Proteins were separated on a 6% or 12% SDS–polyacrylamide gel and transferred to nitrocellulose. Blots were then incubated with primary antibodies for 1 hour at room temperature. Bound antibodies were detected with an alkaline phosphatase–conjugated secondary antibody for 1 hour at room temperature, followed by color development with 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium25 (Promega).

Immunofluorescence

Heart tissue was embedded in Tissue-Tek O.C.T. compound (Sakura) and snap-frozen in isopentane chilled in liquid nitrogen. Unfixed 6-μm cryosections were permeabilized with 0.3% Triton X-100 in TBS. Coxsackievirus-infected cells were identified with a rabbit polyclonal anti-CVB3 antibody at 1:200 dilution followed by Rhodamine Red-X– or Cy5-conjugated anti-rabbit IgG antibody (1:100). Dystrophin and the dystrophin–associated glycoproteins were visualized with monoclonal antibodies followed by a biotinylated secondary antibody and streptavidin-FITC (1:100). Cell membrane glycoproteins were visualized with a rhodamine labeled WGA (1:100). Slides were imaged with confocal laser scanning microscopy37 (Bio-Rad).

Results

Dystrophin Cleavage in CVB3-Infected Myocytes Is Associated With Loss of Carboxyl-Terminal Dystrophin and Decreases in Transmembrane Components of the Dystrophin–Glycoprotein Complex

Separation of the amino-terminal dystrophin portion from the carboxyl-terminal region of dystrophin via cleavage in the
hinge 3 region leads to loss of the sarcolemmal localization of the dystrophin rod domain and amino terminus during CVB3 infection. However, it is not known whether cleavage of dystrophin in the rod domain will also result in loss of sarcolemmal localization of the carboxyl terminus of dystrophin and a decrease in membrane concentration of other members of the dystrophin–glycoprotein complex.

As shown in Figure 1A, the cleavage of dystrophin with CVB3 infection led to loss of the dystrophin carboxyl terminus in the membrane fraction. In addition, there was a marked decrease in the amount of α-, β-, γ-, and δ-sarcoglycans in the membrane fraction (Figure 1A). To determine whether each component of dystrophin–glycoprotein complex is cleaved during CVB3 infection, α-, β-, γ-, and δ-sarcoglycans and β-dystroglycan were examined with immunoblotting in cultured rat ventricular cardiac myocytes infected with CVB3. As shown in Figure 1B, cleavage of the 35-kDa δ-sarcoglycan with CVB3 infection could not be detected in either the membrane or cytosolic fractions despite a marked decrease in the amount of intact protein in the membrane fraction. Similarly, cleavage for α-, β-, and γ-sarcoglycans and β-dystroglycan could not be detected in the membrane or cytosolic fractions after CVB3 infection (Figure 1A and data not shown). Adenovirus infection has been implicated in the pathogenesis of human dilated cardiomyopathy22; however, there was no change in the level of the dystrophin-associated glycoproteins after infection with wild-type adenovirus 5 at a time point that had a marked cytopathic effect (Figure 1A). This indicates that the observed sarcoglycan reduction during infection with CVB3 is not a nonspecific response to the virus-induced cytopathic effect. Second, the overall membrane protein composition in CVB3-infected cells was similar to that of uninfected cells as assessed with Coomassie blue staining (Figure 1C). Because the sarcoglycans and β-dystroglycan are transmembrane proteins, they were present only in the membrane and not in the cytosolic fraction.

δ-Sarcoglycan Is Physically Dissociated From Dystrophin During CVB3 Infection of Cultured Cardiomyocytes

Because the sarcoglycans were reduced in but not dissociated from the membrane, we next investigated the physical integrity of the dystrophin–glycoprotein complex within the membrane fraction of virally infected cardiomyocytes. Normally, the sarcoglycans are physically associated with dystrophin and cofractionate together with dystrophin on sucrose density gradient ultracentrifugation.36 Among the sarcoglycans, only δ-sarcoglycan can be cross-linked to β-dystroglycan.38

We analyzed the fractions of a membrane preparation from virally infected myocytes separated with sucrose density gradient ultracentrifugation for the presence of dystrophin and δ-sarcoglycan (Figure 2). In uninfected cells, the intact dystrophin protein and δ-sarcoglycan were physically associated and cofractionated in the lower fractions (5 to 7) of the gradient (Figure 2A). The weak signal seen in fractions 11 and 12 represents an unknown immunoreactive band. In the membrane fraction of infected myocytes, dystrophin was reduced to nonmeasurable levels (Figure 2B). The presence of

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**Figure 1.** Reduction in the dystrophin–glycoprotein complex in the membrane during CVB3 infection of cultured cardiomyocytes. Cardiac myocytes were infected with CVB3 or adenovirus 5 (ADV5). After infection, the cells were harvested and analyzed as described. A, Immunoblotting of membrane fractions for the carboxyl terminus of dystrophin; α-, β-, and γ-sarcoglycans (SG); and β-dystroglycan (DG) demonstrated that all of these proteins are markedly decreased in the membrane fraction of CVB3-infected cells. ADV5 infection did not affect membrane localization of these proteins. B, δ-Sarcoglycan (35 kDa) is not cleaved by CVB3 infection but is reduced in the membrane fraction of CVB3-infected cells. A and B, Arrowheads on the left indicate the size of the molecular weight markers, whereas the arrows on the right identify the location on the blot of the indicated protein. C, Coomassie staining shows a similar amount total protein in the membrane fraction of infected and uninfected myocytes (molecular weight markers on the left).
the immunoreactive bands in fractions 11 and 12 and of \( \delta \)-sarcoglycan in fractions 3 to 8 indicates that the fractions of the 2 gradients were comparable and that there may be a small amount of dystrophin in the membrane of infected cells. As observed previously (Figure 1A), the total amount of \( \delta \)-sarcoglycan was reduced in the membrane fraction from the virally infected myocytes. Most important, however, \( \delta \)-sarcoglycan was found in the upper fractions of the gradient (fractions 9 to 15) dissociated from dystrophin.

These results indicate not only that the level of \( \delta \)-sarcoglycan is decreased but also that the physical integrity of the dystrophin–glycoprotein complex is impaired after CVB3 infection in cultured cardiomyocytes.

Sarcoglycan Complex Is Morphologically Disrupted in CVB3–Infected Mouse Hearts

In addition to the biochemical analysis of the sarcoglycan complex in cultured cells, we morphologically investigated dystrophin and dystrophin-associated glycoproteins in the hearts of SCID mice infected with CVB3 by immunostaining.

SCID mice (n=4) were chosen to demonstrate that any potential alterations were a direct viral effect rather than an immune-mediated event. As previously described, the staining pattern for the dystrophin rod domain is disrupted in infected cardiomyocytes in the intact mouse heart with a loss of the typical sarcolemmal localization that is normally seen in uninfected cells. Immunostaining for the carboxyl terminus of dystrophin was performed to determine whether it retained its physiological localization in the absence of a functional rod domain. As shown in Figures 3A and 3B, the sarcolemmal localization of the dystrophin carboxyl terminus was disrupted in infected cardiac myocytes, similar to the result obtained for the dystrophin rod domain.

Triple-color staining with WGA, a plasma membrane marker; \( \beta \)-sarcoglycan; and CVB3 demonstrated that cleavage of dystrophin during CVB3 infection was associated with
a loss of $\beta$-sarcoglycan staining in infected cells. However, the loss of the dystrophin-associated glycoproteins in the sarcolemma was not due to a general disintegration of the plasma membrane, as evidenced by preserved WGA stain in cells with a disrupted $\beta$-sarcoglycan (Figures 3E through 3H). In the absence of the dystrophin carboxyl-terminus, the dystrophin-associated glycoproteins are not localized to the sarcolemma in Duchenne muscular dystrophy.27 Since the Dystrophin carboxy-terminus was absent from the plasma membrane in infected myocytes in vivo, we investigated whether a similar finding would occur in CVB3-infected SCID mouse myocytes ($n=4$). The sarcolemmal localization of $\alpha$-, $\beta$-, $\gamma$- and $\delta$-sarcoglycans and of $\beta$-dystroglycan was disrupted in infected cardiomyocytes in the intact heart to varying degrees (Figure 4).

These results demonstrate a morphological disruption of all components of the sarcoglycan complex tested and $\beta$-dystroglycan in the mouse heart on infection with CVB3.

**Functional Disruption of the Dystrophin-Glycoprotein Complex In Vivo by CVB3**

Genetic sarcoglycan12,39 or dystrophin40 deficiency leads to increased sarcolemmal permeability with uptake of the tracer dye Evans blue. Six days after infection, SCID mice were injected with Evans blue to assess whether dye uptake would also occur in virally infected cardiomyocytes and hearts were harvested after 24 hours.

Immunostaining of Evans blue dye–injected mouse hearts for carboxyl terminus of dystrophin or for $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-sarcoglycans showed that the dye uptake specifically occurred in virally infected myocytes with a disrupted dystrophin staining pattern (Figures 3C and 3D) or in virally infected myocytes with a disrupted sarcoglycan staining patterns (Figure 4).

These data demonstrate a functional impairment of the dystrophin–glycoprotein complex in virally infected cardiomyocytes in vivo.

**Discussion**

The main finding of the present study was that CVB3 infection leads to disruption of the association between dystrophin and the sarcoglycan complex. This was demonstrated by a marked reduction in membrane localization of the carboxyl terminus of dystrophin, each of the sarcoglycans, and $\beta$-dystroglycan in infected cardiac myocytes. In addition, increased sarcolemmal permeability indicates functional impairment of the dystrophin–glycoprotein complex in virally infected cells. Because proteolytic cleavage of the sarcoglycans and $\beta$-dystroglycan was not detected during viral infection, these effects appear to be indirect and secondary to dystrophin cleavage.
The sarcoglycans ($\alpha$, $\beta$, $\gamma$, and $\delta$) form a complex of 4 single-pass transmembrane glycoproteins. Within the multiprotein dystrophin–glycoprotein complex, they form a distinct subcomplex. The physiological role of this subcomplex is not well understood, and the sarcoglycans may have functions beyond stabilization of the sarcolemma. That the sarcoglycans function as a complex is based on the finding that a defect in any one sarcoglycan causes alterations in other components of the sarcoglycan complex. The $\delta$-sarcoglycan component of the dystroglycan complex binds to the carboxyl terminus of dystrophin. The correct sarcolemmal localization of $\beta$-dystroglycan as well as the sarcoglycans depends on a functional dystrophin carboxyl terminus. Consequently, in Duchenne muscular dystrophy patients with dystrophin mutations that result in a truncated protein, the dystrophin-associated proteins are in large part absent from the sarcolemma.

We previously reported that the viral protease 2A cleaves dystrophin during CVB3 infection in the hinge 3 region. Because dystrophin cleavage functionally impairs dystrophin with dissociation of the rod domain from the plasma membrane, we initially investigated the effects of protease 2A–mediated cleavage on the dystrophin carboxyl terminus and found that it lost its sarcolemmal localization in virally infected myocytes. In analogy to findings in Duchenne muscular dystrophy, the absence of an intact dystrophin carboxyl terminus led to a severe reduction in its binding partner, $\beta$-dystroglycan, in the membrane fraction in cultured myocytes and to a loss of its sarcolemmal localization in the intact heart. In this regard, the carboxyl-terminal dystrophin cleavage fragment is different from the naturally occurring carboxyl-terminal isoform of dystrophin, Dp71. Dp71 has been shown to be able to restore the dystrophin–glycoprotein complex in dystrophin-deficient mice even though it does not prevent the muscular dystrophy observed in the mdx mice. It is notable that Dp71 has a 7-residue amino terminus that results from an alternative promoter site upstream of exon 63 and that it lacks the amino acids encoded by exons 50 to 62 that are present in the carboxyl-terminal fragment from cleavage by protease 2A. It is, therefore, possible that the protease 2A–generated carboxyl-terminal dystrophin fragment is susceptible to further degradation, whereas the Dp71 molecule is stable. Alternatively, it is possible that the conformational change in the carboxyl-terminal dystrophin cleavage fragment facilitates further degradation by other proteases, although such cleavage fragments were not detected.

Because $\beta$-dystroglycan itself is not proteolytically cleaved during CVB3 infection, its reduction in the membrane fraction appears to be due to functional disruption of dystrophin and loss of membrane localization of the dystrophin carboxyl terminus. Because $\beta$-dystroglycan is a component of the dystrophin–glycoprotein complex and the dystroglycans can be cross-linked to $\delta$-sarcoglycan, a reduction in $\beta$-dystroglycan was predicted to also affect the sarcoglycan complex. Indeed, $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-sarcoglycans were, similar to $\beta$-dystroglycan, reduced in the membrane of virally infected myocytes in cell culture and in the intact mouse heart. Again, this effect on the sarcoglycans appears to be indirect in the absence of any detectable cleavage fragments. Not only were the members of the sarcoglycan complex reduced in the membrane fraction, but also $\delta$-sarcoglycan was partially dissociated from dystrophin, indicating physical disintegration of the dystrophin–glycoprotein complex.

To test the functional relevance of these perturbations, we assessed the sarcolemmal integrity in vivo by injection of Evans blue dye. Only cells that have lost their membrane integrity take up this tracer dye. Genetic sarcoglycan deficiency causes Evans blue dye uptake, as do dystrophin defects. During CVB3 infection of the mouse heart, we found that Evans blue dye was specifically taken up by virally infected cardiomyocytes with a disrupted sarcoglycan staining pattern. This association suggests that the sarcoglycan complex deficiency may play an important role in the observed increase of sarcolemmal permeability.

Based on these results and the known role of sarcoglycan defects in hereditary dilated cardiomyopathy, we conclude that the disruption of the sarcoglycan complex during CVB3 infection may participate in a cascade of events that ultimately lead to enteroviral cardiomyopathy. Consequently, we significantly extended our previous molecular model that exemplifies this cascade. We propose that the initial cleavage of dystrophin by the enteroviral protease 2A triggers loss of the sarcolemmal dystrophin carboxyl terminus and $\beta$-dystroglycan, as well as a disruption of the sarcoglycan complex (Figure 5). It is notable, however, that the sarcoglycan complex is disrupted before total loss of the sarcolemma, as assessed with WGA staining. Because sarcoglycan defects cause human dilated cardiomyopathy, this mechanism is potentially relevant to human disease.

In summary, the sarcoglycan complex is physically, morphologically, and functionally impaired during CVB3 infection. These perturbations appear to be secondary to the dystrophin cleavage and may play an important role in the induction of enteroviral cardiomyopathy.

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