Enteroviral protease 2A cleaves dystrophin: Evidence of cytoskeletal disruption in an acquired cardiomyopathy

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Enteroviruses such as Coxsackievirus B3 can cause dilated cardiomyopathy, but the mechanism of this pathology is unknown. Mutations in cytoskeletal proteins such as dystrophin cause hereditary dilated cardiomyopathy, but it is unclear if similar mechanisms underlie acquired forms of heart failure. We demonstrate here that purified Coxsackievirus protease 2A cleaves dystrophin *in vitro* as predicted by computer analysis. Dystrophin is also cleaved during Coxsackievirus infection of cultured myocytes and in infected mouse hearts, leading to impaired dystrophin function. *In vivo*, dystrophin and the dystrophin-associated glycoproteins α-sarcoglycan and β-dystroglycan are morphologically disrupted in infected myocytes. We suggest a molecular mechanism through which enteroviral infection contributes to the pathogenesis of acquired forms of dilated cardiomyopathy.

Heart failure is a leading cause of cardiovascular mortality in the US and Europe[1,2] and is a 'hallmark' of dilated cardiomyopathy, a multifactorial disease in which there is evidence of enteroviral infection in up to 30% of patients[4,5]. Some forms of dilated cardiomyopathy are diseases of the cytoskeleton[6,7]. For example, frameshift mutations in dystrophin that result in premature termination of translation, such as those that occur in Duchenne muscular dystrophy[8], and missense mutations that affect the function of dystrophin in Becker's muscular dystrophy[9] cause cardiomyopathy[7]. X-linked dilated cardiomyopathy can occur because of mutations in the dystrophin gene[10-12]. A mutation in an actin domain that interacts with dystrophin has been shown to occur in a family with hereditary dilated cardiomyopathy[13]. Moreover, metavinculin[14] and α-sarcoglycan[15] are abnormal in patients with dilated cardiomyopathy. Several animal models also indicate cytoskeletal proteins are a common pathway in the etiology of cardiomyopathy[6,7]. For example, a defect in α-sarcoglycan causes cardiomyopathy in the BIO14.6 hamster[16]. Mice deficient in both dystrophin and utrophin have cardiomyopathy[17], as do mice with gene-targeted knockout of the muscle LIM protein[18]. Although these molecular genetic analyses have greatly advanced the understanding of familial dilated cardiomyopathy, it is unclear whether similar mechanisms underlie the more common, acquired forms of dilated cardiomyopathy.

A subset of human dilated cardiomyopathy is associated with an enteroviral infection of the heart, in particular Coxsackie B viruses[4,5]. However, the mechanisms by which enterovirus infection can cause cardiomyopathy are not clear. Enteroviral infection as well as expression of enteroviral proteins in cardiac myocytes induces a direct cytopathic effect[19]. *In vivo*, cardiac-restricted expression of Coxsackieviral proteins in transgenic mice induces dilated cardiomyopathy independent of viral replication[20]. Isolated myocytes from these mice have impaired excitation–contraction coupling[20]. At present, it is unknown how enteroviral proteins interact with and adversely affect myocyte proteins to induce myocyte dysfunction and cardiomyopathy.

Coxsackie B viruses are members of the picornaviridae family, enterovirus genus. Coxsackieviruses have an approximately 7.4-kb, positive-stranded RNA genome that is translated as a monocistronic polyprotein. Two viral proteases, 2A and 3C, co-translationally cleave the viral polyprotein into mature peptides[21]. In addition to the cleavage activity of protease 2A on the viral polyprotein *in cis*, protease 2A cleaves the eukaryotic translation initiation factors 4G-1 and -2 (eIF4G-1 and eIF4G-2) *in trans*[22-24]. Myocyte restricted host cell substrates for the Coxsackievirus protease 2A have not been described yet, but Coxsackievirus may induce dilated cardiomyopathy by protease 2A-mediated cleavage and inactivation of protein(s) essential for myocyte function.

Proteolytic cleavage by protease 2A depends on specific amino-acid sequence motifs. A search of the Swiss protein database using a neural network algorithm, based on the sequence information from known protease 2A cleavage sites of rhino- and enteroviruses, identified dystrophin (among all human proteins) as one of the most likely potential cellular targets of protease 2A (ref. 24).

Given the importance of mutations in dystrophin that cause dilated cardiomyopathy and the known role for enteroviruses in acquired cardiomyopathy, we sought to determine whether enteroviral protease 2A can cleave dystrophin, as predicted by the

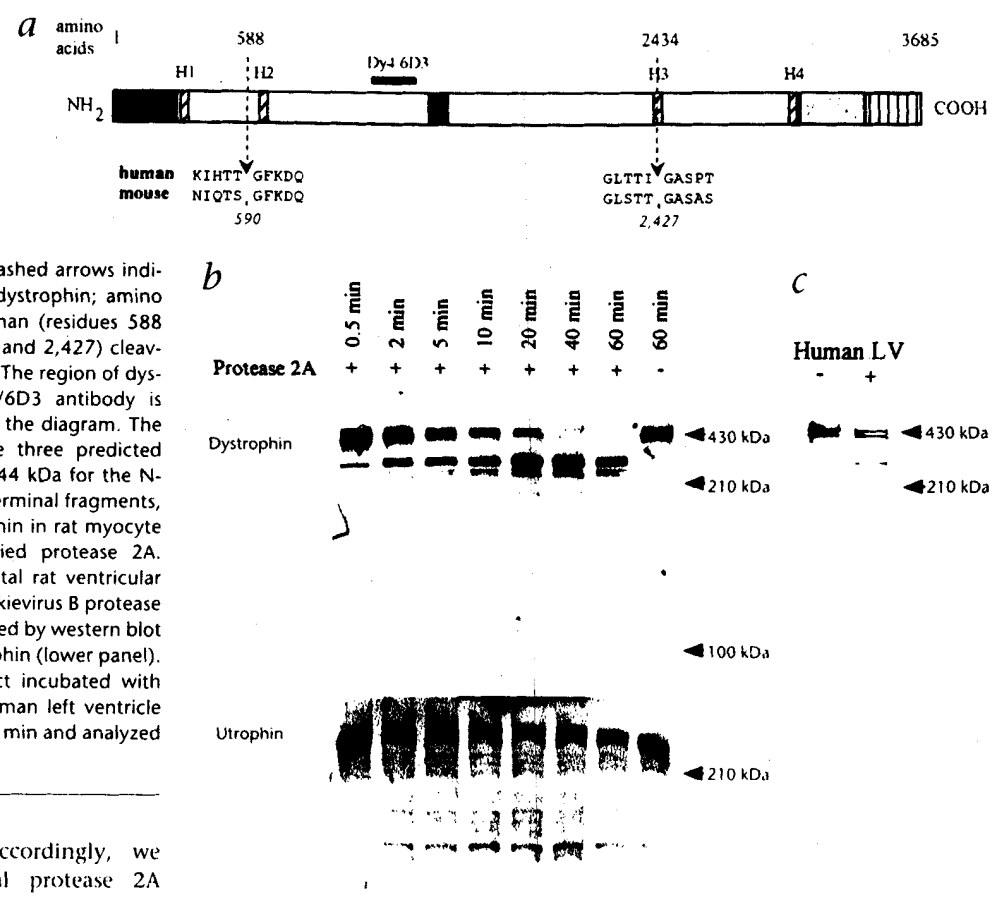


Fig. 1 Cleavage of dystrophin by Coxsackievirus protease 2A. ***a***, Dystrophin contains actin binding domains (■), a rod domain (□) consisting of spectrin-like repeats, a cysteine rich domain (□), and a carboxy-terminal domain (▩). Four hinge segments (H1–H4) that are accessible to proteolytic cleavage are interspersed along the rod domain[35] (diagonally hatched boxes). Dashed arrows indicate the predicted cleavage sites in dystrophin; amino acid sequences of the predicted human (residues 588 and 2,434) and mouse (residues 590 and 2,427) cleavage sites are shown below the arrows. The region of dystrophin used to generate the Dy4/6D3 antibody is shown as a dark horizontal bar above the diagram. The predicted molecular weights for the three predicted cleavage products are 68, 214 and 144 kDa for the N-terminal, mid segment and carboxy-terminal fragments, respectively. ***b***, Dystrophin and utrophin in rat myocyte extracts after the addition of purified protease 2A. Protein extracts from cultured neonatal rat ventricular myocytes were incubated with Coxsackievirus B protease 2A for the times indicated, then analyzed by western blot for dystrophin (upper panel) and utrophin (lower panel). ***c***, Dystrophin in human heart extract incubated with protease 2A. Protein extract from human left ventricle was incubated with protease 2A for 60 min and analyzed by western blot for dystrophin.

neural network algorithm[24]. Accordingly, we demonstrate that Coxsackieviral protease 2A cleaves both human and rat dystrophin *in vitro*. In addition, dystrophin is cleaved during Coxsackievirus B3 infection of cultured myocytes and mice. Unlike full-length dystrophin, dystrophin cleavage products are present in the cytosol, indicating a functional dystrophin impairment by protease 2A cleavage. Staining for dystrophin and the dystrophin-associated glycoproteins α-sarcoglycan and β-dystroglycan is also disrupted in infected myocytes in SCID mouse hearts. These findings indicate a newly discovered molecular mechanism by which enteroviruses contribute to the pathogenesis of cardiomyopathy, and demonstrate a potential role for alterations in cytoskeletal proteins in acquired dilated cardiomyopathy.

Coxsackieviral protease 2A cleaves dystrophin

The cleavage site at residue 588 of dystrophin has a calculated cleavage score of 0.680 (Fig. 1*a*), which is only slightly below that of the 0.697 cleavage score for the known protease 2A substrate eIF4G-1 (ref. 23). We analyzed the remaining sequence of human dystrophin using the NetPicoRNA V1.0 algorithm (http://www.cbs.dtu.dk/services/NetPicoRNA/), and identified a second potential protease 2A cleavage site at residue 2,434 with a score of 0.566. Analysis of the mouse dystrophin sequence demonstrated similar cleavage sites at residues 590 and 2,427.

To determine whether purified enteroviral protease 2A cleaves dystrophin, we incubated protein extracts from cultured rat neonatal ventricular myocytes with purified recombinant Coxsackieviral protease 2A. We then analyzed the protein extracts by western blot using an antibody against dystrophin, Dy4/6D3, that recognizes the rod domain of dystrophin. There was evidence of the early appearance of a dystrophin cleavage product at 280–300 kDa (Fig. 1*b*). After 10 minutes of incubation with protease 2A, a second dystrophin fragment was detected at 210–230 kDa. After 40–60 minutes, full-length dystrophin was essentially undetectable. Cleavage of human dystrophin in heart extracts by purified protease 2A resulted in two dystrophin fragments of sizes similar to those of rat dystrophin (Fig. 1*c*). The large fragment is comparable to that predicted for protease 2A cleavage at residue 2,434 in the human sequence (282 kDa). The second fragment has a size similar to that predicted for a second cleavage at residue 588 of the human sequence (218 kDa). The structurally related cytoskeletal proteins utrophin (Fig. 1*b*) and α-spectrin (both of which lack predicted protease 2A cleavage sites) as well as two contractile proteins, α-sarcomeric actin and myosin heavy chain (data not shown), were not cleaved after incubation with Coxsackieviral protease 2A. Thus, Coxsackieviral protease 2A cleaves dystrophin with cleavage products that are comparable in size to those that would occur with cleavage at the predicted enteroviral protease 2A-cleavage sites.

Dystrophin function is impaired during virus infection

To determine whether dystrophin is cleaved during Coxsackievirus B3 infection of cardiac myocytes, we infected cultured neonatal rat ventricular myocytes with Coxsackievirus B3. Rat neonatal myocytes, an established myocyte culture model[25], are known to express dystrophin[26] and can be infected with Coxsackievirus B3 (ref. 19). Protein extracts were isolated after infection and analyzed for the presence of dystrophin cleavage products by western blot with the Dy4/6D3 antibody against dystrophin. There was a substantial decrease in the intensity of the full-length dystrophin to very low levels by 36 hours after infec-

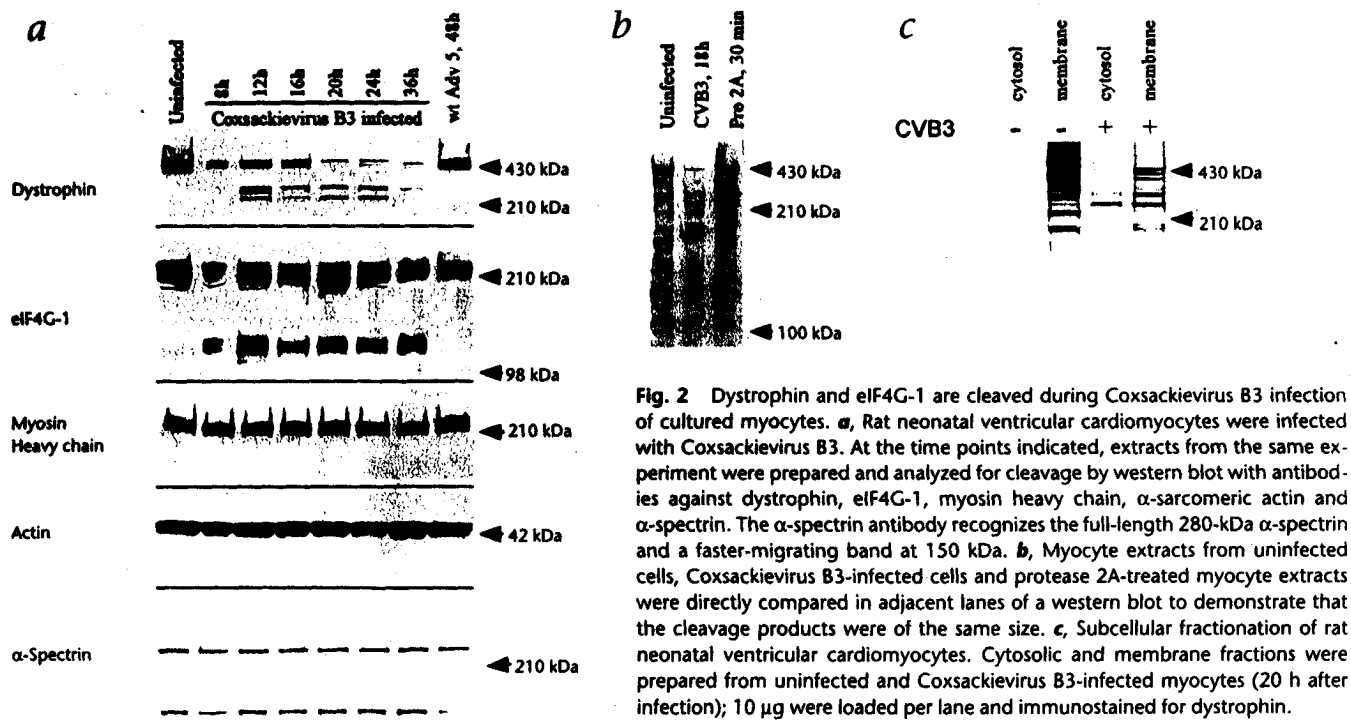


Fig. 2 Dystrophin and eIF4G-1 are cleaved during Coxsackievirus B3 infection of cultured myocytes. *a*, Rat neonatal ventricular cardiomyocytes were infected with Coxsackievirus B3. At the time points indicated, extracts from the same experiment were prepared and analyzed for cleavage by western blot with antibodies against dystrophin, eIF4G-1, myosin heavy chain, α-sarcomeric actin and α-spectrin. The α-spectrin antibody recognizes the full-length 280-kDa α-spectrin and a faster-migrating band at 150 kDa. *b*, Myocyte extracts from uninfected cells, Coxsackievirus B3-infected cells and protease 2A-treated myocyte extracts were directly compared in adjacent lanes of a western blot to demonstrate that the cleavage products were of the same size. *c*, Subcellular fractionation of rat neonatal ventricular cardiomyocytes. Cytosolic and membrane fractions were prepared from uninfected and Coxsackievirus B3-infected myocytes (20 h after infection); 10 μg were loaded per lane and immunostained for dystrophin.

tion (Fig. 2*a*). In addition, dystrophin cleavage products were evident beginning at 12 hours after infection, before the generalized cytopathic effect appeared. The cleavage products were the same size as those seen after recombinant purified protease 2A was added to myocyte extracts (Fig. 2*b*). Western blot for α-spectrin, myosin heavy chain and α-sarcomeric actin (Fig. 2*a*) as well as utrophin (data not shown) demonstrated that cleavage of dystrophin is not secondary to a generalized degradation of proteins, as neither the structurally related proteins utrophin and α-spectrin nor abundant myocyte proteins, myosin heavy chain and α-sarcomeric actin were cleaved in infected cells. Moreover, dystrophin was not cleaved after infection with wild-type adenovirus 5 at a time point associated with a considerable cytopathic effect (Fig. 2*a*).

Appearance of dystrophin cleavage products corresponds with cleavage of the known protease 2A-substrate eIF4G-1. Partial cleavage of eIF4G-1 was detectable as early as 8 hours after infection. As with dystrophin, cleavage of eIF4G-1 was specific to Coxsackievirus infection (Fig. 2*a*).

To investigate the functional relevance of dystrophin cleavage during Coxsackievirus infection, we isolated cytosolic and membrane fractions from infected myocytes. Dystrophin was found only in the membrane fraction in uninfected cells (Fig. 2*c*), as expected[27]. In infected myocytes, the two cleaved dystrophin fragments also appeared in the cytosolic fraction. The control substrate eIF4G-1 was detected at equal concentrations in the cytosolic preparations from both the uninfected and Coxsackievirus B3-infected myocytes (data not shown).

These results demonstrate that dystrophin is proteolytically cleaved after infection of myocytes with Coxsackievirus B3. The dystrophin cleavage products that appear in the cytosolic fraction were the same size as those seen after the addition of purified protease 2A to myocyte extracts. This indicates that protease 2A cleaves dystrophin in Coxsackievirus B3-infected myocytes and that the cleavage functionally impairs dystrophin.

Disruption of dystrophin and sarcolemmal integrity *in vivo*

To determine whether enterovirus-mediated cleavage of dystrophin can occur in the intact heart, we infected both immunocompetent and SCID mice with Coxsackievirus B3. We used SCID mice lacking both T lymphocytes and B lymphocytes to differentiate effects secondary to the host immune response from direct effects of the virus on the cardiac myocyte[28]. Seven days after intraperitoneal injection of 1×10^3 PFU of Coxsackievirus B3, the hearts of both immunocompetent and SCID mice had evidence of dystrophin cleavage (Fig. 3). The main dystrophin cleavage product corresponded with the larger fragment seen after the addition of purified protease 2A to myocyte protein extracts *in vitro*. Again, α-spectrin, myosin heavy chain and α-sarcomeric actin (Fig. 3) as well as utrophin (data not shown) were not cleaved after infection with Coxsackievirus B3. Unlike the complete cleavage of dystrophin that results from Coxsackievirus B3 infection of cultured myocytes, there was not a substantial decrease in full-length dystrophin, as Coxsackievirus B3 does not infect all myocytes *in vivo*.

To determine whether there was morphological disruption of the organized dystrophin staining in infected myocytes in the intact heart, we immunostained hearts of SCID mice with antibody against Coxsackievirus B3 (ref. 29) and the antibody against dystrophin, Dy4/6D3 (Fig. 4*a* and *b*). The typical dystrophin staining was disrupted in most Coxsackievirus B3-infected cells in all hearts ($n = 4$), whereas dystrophin staining was normal, as expected, in uninfected hearts ($n = 4$). Generally, dystrophin was still detectable within infected cells, but there was a loss of the characteristic sarcolemmal localization seen in uninfected cells. Triple-staining with phalloidin, antibody against Coxsackievirus B3 and antibody against dystrophin demonstrated that disruption of dystrophin precedes depolymerization of F-actin in infected myocytes (data not shown).

Congenital dystrophin deficiency leads to increased plasma membrane permeability with uptake of tracers such as Evans

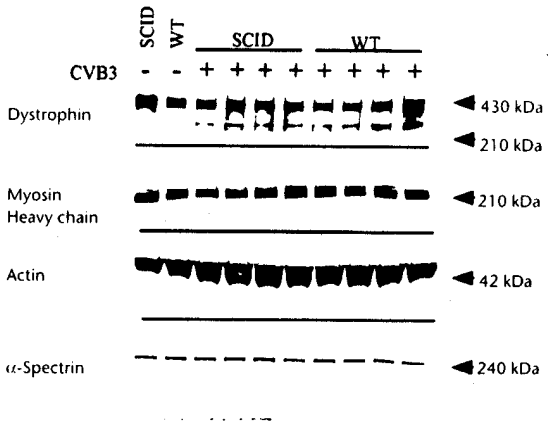


Fig. 3 Dystrophin is cleaved during Coxsackievirus B3 infection *in vivo* in SCID and immunocompetent mice. SCID and immunocompetent mice (C3H background) were infected with Coxsackievirus B3; 7 d later, heart extracts from uninfected and infected mice were analyzed for cleavage of dystrophin, α-sarcomeric actin, α-spectrin and myosin heavy chains by western blot.

blue dye in muscle tissue[40]. Seven days after infection, SCID mice were injected with Evans blue dye. There were many myocytes that stained positive for Evans blue dye in all infected SCID mouse hearts ($n = 4$; Fig. 4*c* and *d*), but none in uninfected control hearts ($n = 2$; data not shown). Simultaneous staining for Coxsackievirus B3 and dystrophin showed that the dye uptake occurred specifically in virally infected myocytes with a disrupted dystrophin staining pattern (Fig. 4*c*). These *in vivo* results demonstrate cleavage and morphological disruption of dystrophin staining as well as a loss of the sarcolemmal integrity in infected cardiomyocytes.

Effects of Coxsackievirus on dystrophin-associated glycoproteins

In Duchenne muscular dystrophy, the primary genetic dystrophin defect leads to secondary alterations of other members of the sarcolemmal dystrophin-glycoprotein-complex. For example, staining of α-sarcoglycan and β-dystroglycan in dystrophin-deficient muscle is profoundly reduced or absent[10,11]. We found a similar result in infected SCID mouse hearts ($n = 4$). Sarcolemmal localization of α-sarcoglycan was disturbed in a subset of infected cells (Fig. 4*e* and *f*) and localization of β-dystroglycan was substantially disrupted (Fig. 4*g* and *h*). Because α-sarcoglycan and β-dystroglycan are not cleaved during Coxsackievirus B3-infection (data not shown), this indicates that this disruption is an indirect effect of dystrophin cleavage.

Discussion

We have shown that enteroviral protease 2A is sufficient to cleave dystrophin. Dystrophin is also cleaved during enterovirus infection both *in vitro* and *in vivo*. Cleavage of dystrophin is not a nonspecific response to cell death in virally infected cells, as dystrophin cleavage does not occur in adenovirus-infected cells. Our results indicate that enteroviral infection of cardiac myocytes leads to disruption of the cytoskeleton through enteroviral protease 2A-mediated cleavage of dystrophin. Cleavage of dystrophin by the enteroviral protease 2A is the first example, to our knowledge, of viral protein-mediated disruption of a myocyte-restricted protein.

The importance of abnormalities in dystrophin and other cytoskeletal proteins has been demonstrated in hereditary forms of dilated cardiomyopathy, both in humans and in rodents[6,7,9,18]. These findings indicate that defective transmission of force through abnormal cytoskeletal structures or loss of the sarcolemmal integrity initiates a cascade of events that leads to dilated cardiomyopathy. Although genetic analyses have greatly advanced our understanding of the mechanisms that underlie familial dilated cardiomyopathy, it is unclear whether similar mechanisms apply to the more common, acquired forms of dilated cardiomyopathy such as viral myocarditis[5].

Dystrophin is a 427-kDa protein with four main domains: an

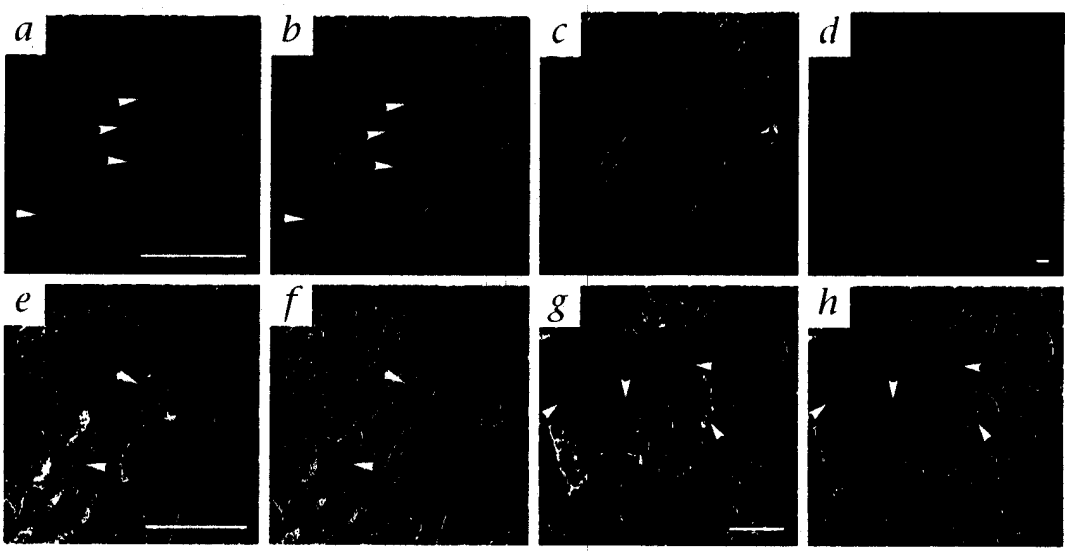


Fig. 4 The sarcolemmal integrity is impaired and dystrophin, α-sarcoglycan and β-dystroglycan staining is disrupted in infected myocytes from SCID mice. *a–d*, SCID mouse heart infected with Coxsackievirus B3 and injected with Evans blue dye 7 d later. *a–c*, Confocal images of the same high-power field. ***a***, Dual stain using an antibody against Coxsackievirus B3 (blue fluorescence) and an antibody against dystrophin (green fluorescence). ***b***, Dystrophin immunostain only. The dystrophin staining pattern is disrupted in the virally-infected myocytes (arrowheads, a and b). ***c***, Superimposed triple fluorescence with Evans blue dye (red) uptake specifically in the virally-infected cells. ***d***, Lower-magnification, non-confocal image showing many cells with Evans dye uptake (bright red) in an infected mouse heart; surrounding uninfected myocytes have a faint background fluorescence. ***e–h***, Hearts from Coxsackievirus B3 infected SCID mice collected 7 d after infection and dual-stained with an antibody against Coxsackievirus B3 (red fluorescence, *e* and *g*) and an antibody against α–sarcoglycan (green fluorescence, *e* and *f*) or an antibody against β-dystroglycan (green fluorescence, *g* and *h*). *e* and *g*, Superimposed red and green fluorescence. *f* and *h*, Only the stain for α–sarcoglycan (*f*) or β-dystroglycan (*h*). The α-sarcoglycan and β-dystroglycan staining pattern is disrupted in virally infected cells (arrowheads, *e–h*). Scale bars represents 50 μm.

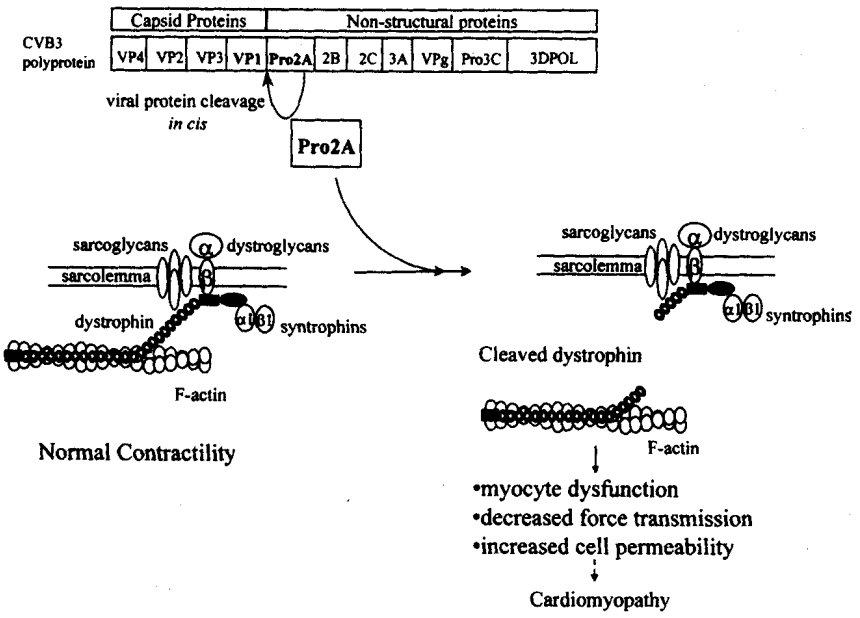


Fig. 5 A model for the role of dystrophin in Coxsackievirus-induced dilated cardiomyopathy. The Coxsackieviral polyprotein consists of the capsid proteins VP1–VP4; the non-structural proteins protease 2A (Pro2A), 2B, 2C, 3A, VPg and protease 3C (Pro3C); and the viral RNA polymerase (3DPOL).

N-terminal domain, a spectrin-like repeat rod domain, a cysteine-rich domain, and a C-terminal domain[12] (Fig. 1). The N-terminal domain as well as an epitope in the rod domain bind actin[13], whereas the cysteine-rich domain and C terminus contribute to binding of β-dystroglycan[12,14]. In the rod domain of dystrophin, four hinge segments (H1–H4) have been identified that are accessible to proteolytic cleavage[15]. The predicted protease 2A cleavage site at residue 2,434 lies within the H3 region between repeats 19 and 20 (ref. 35). Cleavage of dystrophin only at the predicted protease 2A cleavage site at residue 2,434 would result in a fragment of 282 kDa with immunoblotting using the Dy4/6D3 antibody against dystrophin. The main cleavage fragment detected after the addition of protease 2A to myocyte protein extracts is consistent with cleavage at this site. Additional cleavage at residue 588 would shorten the fragment detected by Dy4/6D3 to 214 kDa. This molecular weight is comparable to the second dystrophin fragment seen. Accessibility of a cleavage site in the H3 region of dystrophin to protease 2A may account for the rapid appearance of the 280- to 300-kDa dystrophin cleavage product. The experiments described here do not identify the exact protease 2A cleavage sites, nor do they exclude possible dystrophin cleavage by a protease 2A activated cellular protease.

Cleavage by protease 2A in the region of residue 2,434 would lead to abnormalities in myocyte function similar to those in Duchenne muscular dystrophy with frame-shift mutations in this region of dystrophin[5]. Accordingly, cleavage of dystrophin by protease 2A would separate the actin-binding N-terminal and rod domains from the β-dystroglycan-binding domain. This would disconnect the internal actin-based cytoskeletal and sarcomeric structures from the membrane-bound dystrophin-associated glycoproteins and the external basement membrane. Indeed, the presence of both dystrophin fragments in the cytosolic fraction after infection demonstrates that the cleaved fragments dissociate from the plasma membrane.

The typical dystrophin-staining pattern is disrupted in infected myocytes in the intact mouse heart. The dystrophin-associated glycoproteins α-sarcoglycan and β-dystroglycan are no longer localized to the sarcolemma and the sarcolemmal integrity is disrupted in the infected cells. Because alterations of the dystrophin-associated glycoproteins and increased plasma membrane permeability are also 'hallmarks' of Duchenne muscular dystrophy[11,12], our findings indicate that there is a link between the pathogenic mechanisms of virally induced dystrophin abnormalities and genetic dystrophin deficiencies. A molecular model that illustrates this mechanism is shown in Fig. 5. Enteroviral protease 2A cleaves dystrophin *in trans* during infection, adversely affecting myocytes by impairing transmission of mechanical force and increasing cell permeability. Abnormal localization of other components of the dystrophin–glycoprotein complex may also be involved in the 'cascade' of events that ultimately leads to dilated cardiomyopathy. There are, however, important distinctions between viral-mediated cardiomyopathy and the congenital absence of dystrophin. These include the loss of dystrophin function in a previously normal heart and the focal nature of the infection in viral-mediated cardiomyopathy. Nevertheless, our results add an acquired form of dilated cardiomyopathy to the previously described hereditary cardiomyopathies that are associated with abnormalities in cytoskeletal proteins[9,18]. This extends the paradigm that abnormalities in cytoskeletal proteins are a common pathogenic mechanism leading to many forms of dilated cardiomyopathy[9,17]. This is distinct from hypertrophic cardiomyopathy that is often caused by abnormalities in contractile proteins. Because protease 2A cleaves human dystrophin, this mechanism is potentially relevant to human disease.

Although disruption of dystrophin occurs in the absence of T cells and B cells *in vivo*, our results do not exclude the possibility that the immune system contributes to the pathogenesis of dilated cardiomyopathy. For instance, protease 2A-mediated cleavage of dystrophin might make viral particles and myocyte contents accessible to the immune system, thus contributing to the activation of an immune response in viral cardiomyopathy.

Why should Coxsackievirus cleave dystrophin? Enteroviruses are typically released from the cell by disruption of the cell membrane[21]. Lack of dystrophin weakens the cytoskeleton and increases cell membrane permeability, as has been shown in mdx mice[10] and mice that lack both dystrophin and utrophin[11]. Thus, Coxsackievirus may facilitate its propagation through proteolysis of host cell structural molecules such as dystrophin. This strategy might enhance viral spread in the heart and contribute to the pathogenesis of viral heart disease. Other viruses, such as adenovirus and human immunodeficiency virus, also cleave cytoskeletal proteins[16,17], a mechanism that seems to occur among diverse classes of viruses.

Here we have demonstrated that dystrophin is a cellular target for the enteroviral protease 2A. Dystrophin is cleaved and its staining pattern is disrupted during Coxsackievirus B3 infection. Because enteroviral infections can cause dilated cardiomy-

opathy, and dystrophin abnormalities underlie hereditary cardiomyopathies, our results identify a molecular mechanism by which enteroviral infection may contribute to dilated cardiomyopathy.

Methods

Viruses. The Coxsackievirus B3 used here was derived from the infectious cDNA copy of the cardiotropic H3 strain of Coxsackievirus B3 (ref. 38). Wild-type adenovirus 5 was a gift from S. Huang (Scripps Research Institute, La Jolla, California). Viruses were titered by plaque-forming assay, and myocytes were infected at a multiplicity of infection of 100. Mice were infected by an intraperitoneal injection of 1×10^3 PFU in 0.2 ml PBS (ref 28).

Mice. Male SCID mice (C3HSmn.C-Prkdc^{scid}/J) and non-SCID control mice (C3H/HeJ) were purchased from the Jackson Laboratory (Bar Harbor, Maine) and were maintained in a specified pathogen-free barrier facility at the Animal Resources Facility, University of California, San Diego. Agammaglobulinaemia was verified in all SCID animals[39]. Mice were 5–6 weeks of age at the time of infection and were killed on day 7 after infection[28]. In some experiments, mice were intraperitoneally injected with Evans blue dye[30] on day 7 after infection and their hearts were collected 24 h later.

Myocyte culture and heart tissue. Rat neonatal ventricular myocytes were isolated and cultured as described[25]. Human left ventricular tissue was obtained at the time of heart–lung transplantation from a patient with an atrial septal defect.

Antibodies. Rabbit polyclonal antibodies, antibody against Coxsackievirus B3 (a gift from A. Henke)(ref. 29) and antibody against eIF4G-1 (pp7)(ref. 23) have been described. Antibody MF20 reacts with all sarcomere myosin heavy chains[40]. Antibodies Dy4/6D3, Ad1/20A6 and 43DAG/8D5 react with the mid-rod domain of dystrophin[41], α-sarcoglycan and β-dystroglycan, respectively (all NovoCastra, Newcastle, UK). MANCHO3 is specific for the carboxy-terminus of utrophin and was provided by G.E. Morris[42]. 5C5 (Sigma) is specific for α-sarcomeric actin. Mab1622 (Chemicon, Temecula, California) recognizes α-spectrin. Unconjugated goat antibody Fab-fragments against mouse IgG (heavy and light chains) and antibody against mouse IgM and rhodamine- and Cy5-conjugated antibody against rabbit IgG were obtained from Jackson Immunoresearch (West Grove, Pennsylvania). Biotinylated horse antibody against mouse IgG, and streptavidin-FITC were obtained from Vector Laboratories (Burlingame, California). Alkaline phosphatase-labeled goat antibody against rabbit IgG, antibody against mouse IgG (heavy and light chains) and mouse IgM were obtained from Life Technologies.

Western blots. Myocyte and heart extracts were prepared for immunoblotting as described[43]. Cytosolic and membrane fractions were prepared using the pyrophosphate variant, as reported[27]. Extracts were separated by 6% SDS–PAGE and transferred to nitrocellulose using the NOVEX X-Cell II apparatus (Novex, San Diego, California). Immunoblots of mouse heart extracts were pre-incubated with unconjugated Fab fragments at a dilution of 1:1,000 for 1 h at room temperature to block endogenous immunoglobulins. Blots were then incubated with antibodies Dy4/6D3 (1:100 dilution), MANCHO3 (1:100 dilution), MF20 (1:20 dilution), 5C5 (1:2,000 dilution), Mab1622 (1:1,000 dilution) or antibody against pp7 (1:1,000 dilution) for 1 h at room temperature. Bound antibodies were detected with alkaline phosphatase-conjugated antibody against mouse IgG, antibody against mouse IgM (5C5) or antibody against rabbit IgG (antibody against eIF4G) for 1 h at room temperature, followed by color development using BCIP/NBT (Promega).

Protease 2A cleavage assay. Rat neonatal cardiomyocyte extracts were prepared after 5 d of cell culture as described[44]. Human left ventricular extract was prepared by dounce homogenization in a buffer of 1% Triton X-100, 0.5% sodium deoxycholate, 50 mM NaCl, 80 mM Tris-HCl, pH 8.0, 5 mM DTT, 0.1 mM PMSF and 0.75 mM benzamidine. Extracts were dialyzed overnight against a buffer of 0.1% Triton X-100, 100 mM KCl, 50 mM NaCl, 80 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, 0.1 mM EDTA and 1 mM DTT to remove protease inhibitors and to change the buffer to protease 2A cleavage buffer.

Recombinant, purified protease 2A from Coxsackievirus B4 was prepared as described[23]. Coxsackievirus B4 protease 2A is very homologous to protease 2A from Coxsackievirus B3 (96% amino-acid similarity). In a total volume of 15 µl, 15 µg of protein extract were incubated at 30 °C with 1 µg of protease 2A using the following buffer: 100 mM KCl, 50 mM NaCl, 80 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, 0.1 mM EDTA, 10% glycerol, 2 mM DTT and 0.1% NP-40 (ref. 27). The reaction was stopped with 1 mM Elastinal (Sigma) and samples were analyzed by SDS–PAGE and western blot.

Immunofluorescence. Heart tissue was embedded in OCT Tissue Tek and 'snap-frozen' in isopentane chilled in liquid nitrogen. Unfixed cryosections 6 µm in thickness were permeabilized with 0.3% Triton X-100 in TBS. Coxsackievirus-infected cells were identified with a rabbit polyclonal antibody against Coxsackievirus B3 at a dilution of 1:200, followed by Rhodamine- or Cy5-conjugated antibody against rabbit IgG (1:100 dilution). Dystrophin and the dystrophin-associated glycoproteins were visualized by monoclonal antibodies followed by biotinylated horse antibody against mouse IgG (1:150 dilution) and streptavidin-FITC (1:100 dilution). For triple staining, F-actin and plasma membranes were assessed by FITC-conjugated Phalloidin at a dilution of 1:1,000 (Sigma). Slides were imaged as described[45].

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