Enteroviral Protease 2A Directly Cleaves Dystrophin and Is Inhibited by a Dystrophin-based Substrate Analogue*

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Cornel Badorff‡, Neil Berkely‡, Sanjiv Mehrotra¶, Jamil W. Talhouk‡, Robert E. Rhoads**,
Kirk U. Knowlton‡ ♦ ♦

From the ♦Department of Medicine, University of California, San Diego, California 92093, the ¶Department of
Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, Shreveport, Louisiana 71130,
and **Enzyme Systems Products, Livermore, California 94550

Enteroviruses such as Coxsackievirus B3 can cause dilated cardiomyopathy through unknown pathological
mechanism(s). Dystrophin is a large extrasarcomeric cytoskeletal protein whose genetic deficiency causes hereditary
dilated cardiomyopathy. In addition, we have recently shown that dystrophin is proteolytically cleaved by the Coxsackievirus protease 2A leading to functional impairment and morphological disruption. However, the mechanism of dystrophin cleavage and the exact cleavage site remained to be identified. Antibody epitope mapping of endogenous dystrophin indicated protease 2A-mediated cleavage at the site in the hinge 3 region predicted by a neural network algorithm (human, amino acid 2434; mouse, amino acid 2427). Using site-directed mutagenesis, peptide sequencing, and fluorescence resonance energy transfer assays with recombinant dystrophin, we demonstrate that this putative site in mouse and human dystrophin is a direct substrate for the Coxsackieviral protease 2A both in vitro and in vivo. The substrate analogue protease inhibitor z-LSTT-fmk was designed based on the dystrophin sequence that interacts with the protease 2A and was found to have an IC_{50} of 550 nM in vitro. Dystrophin is the first cellular substrate of the enteroviral protease 2A that was identified using by a bioinformatic approach and for which the cleavage site was molecularly mapped within living cells.

Dilated cardiomyopathy, one of the leading causes of heart failure in the United States, is a multifactorial disease that includes hereditary and acquired forms (1, 2). Recent experiments have demonstrated that many hereditary forms of dilated cardiomyopathy are caused by defects of the extrasarcomeric myocyte cytoskeleton (3). Dystrophin is a large cytoskeletal protein that connects the internal F-actin-based cytoskeleton to the plasma membrane where it binds to the β-dystroglycan component of the dystrophin-glycoprotein complex (4). Dystrophin is the protein that is defective in Duchenne and Becker muscular dystrophy (5), both of which have a high incidence of dilated cardiomyopathy (6, 7). Additionally, human X-linked dilated cardiomyopathy can be due to dystrophin mutations (8, 9). In mice, deficiency of dystrophin and utrophin causes severe cardiomyopathy (10). These studies and others (11, 12) have collectively lead to the paradigm that familial dilated cardiomyopathy can result from defective transmission of mechanical force generated in the sarcomere (3, 13). Whereas the importance of genetic dystrophin defects in hereditary cardiomyopathy is well established, the role of dystrophin in acquired cardiomyopathy has just begun to be unraveled.

As much as 30% of human acquired dilated cardiomyopathy is associated with an enteroviral infection of the heart, specifically Coxsackie B viruses (14, 15). In mice, transgenic expression of Coxsackie-B3-viral proteins in the heart is sufficient to induce dilated cardiomyopathy (16). Among the Coxsackieviral proteins are two proteases, protease 2A and protease 3C, both of which are essential for the viral life cycle (17). However, the molecular pathogenic mechanism(s) through which enteroviruses can induce dilated cardiomyopathy have long remained elusive.

Based on a bioinformatic approach (18), we have recently identified dystrophin as a novel myocyte substrate for the enteroviral protease 2A. Dystrophin is proteolytically cleaved by the Coxsackieviral protease 2A, functionally impaired, and morphologically disrupted in cultured cardiomyocytes as well as in the intact mouse heart infected with Coxsackievirus B3. Based on these findings, we proposed that the cleavage of dystrophin during Coxsackievirus B3 infection initiates a cascade of events that contributes to the pathogenesis of dilated cardiomyopathy (19).

Whereas it is clear that dystrophin cleavage occurs both in vitro and in vivo, its mechanism and the exact cleavage site are yet unknown. In the case of eIF4GI, another known protease 2A substrate, some experiments suggest direct cleavage (20), whereas others have proposed that the cleavage in living cells during polioviral infection occurs indirectly through activation of a cellular protease (21). Additionally, the cleavage sites of the other known cellular substrates of the enteroviral protease 2A (eIF4GI (20), eIF4GII (22), and PABP (23)) have not been mapped in vivo. We therefore set out to address these questions in relation to protease 2A-mediated cleavage of dystrophin and to determine whether the sequence of the protease 2A cleavage site in dystrophin could be used to generate a protease 2A inhibitor.

Here, we report the molecular mapping of the enteroviral protease 2A cleavage site in mouse and human dystrophin. The computer-predicted site in the hinge 3 region of dystrophin is directly cleaved in vitro by purified Coxsackieviral protease 2A. In Coxsackievirus B3-infected cells, cleavage occurs at the in vitro cleavage site, thus suggesting direct cleavage in vivo as
The epitopes recognized by anti-dystrophin antibodies rabbit2, Dy4/6D3, and MANDRA-1 are indicated. The putative protease 2A cleavage sites in human and mouse dystrophin are shown as vertical dashed lines including their amino acid (P1 residue) positions. The molecular masses of the fragments that would result from cleavage at these sites are given below. The major fragments are consistent with cleavage in the hinge 3 region. The asterisk indicates the second rod fragment variably detected with antibody Dy4/6D3. C, incubation of human left ventricular membrane fractions with Coxsackievirus protease 2A followed by immunoblotting as in B.

well. Based on the dystrophin sequence that was found to directly interact with the enteroviral protease 2A, we designed a potent substrate analogue inhibitor for this protease.

MATERIALS AND METHODS

Cell Culture and Virus Infection—HeLa cells (a kind gift from Sally A. Huber, University of Vermont) were cultured as described and infected at a multiplicity of infection of 5. Coxsackievirus B3 was derived from the infectious cDNA of the cardiotropic H3 strain of Coxsackievirus B3 and was titered by plaque forming assay (19).

Antibodies—A polyclonal antiserum against the Coxsackievirus protease 2A was generated by immunization of rabbits with recombinant, purified protease 2A from Coxsackievirus B4. Coxsackievirus B4 protease 2A is highly homologous to protease 2A from Coxsackievirus B3 (96% amino acid similarity). Monoclonal dystrophin antibodies recognized the mid-domain (Dy4/6D3, Novocastra) or the C terminus of dystrophin (MANDRA-1 (24), kindly provided by G.E. Morris). A rabbit polyclonal antibody (rabbit 2) against the dystrophin N terminus was obtained from J. M. Ervasti (University of Wisconsin). Anti-Myc and anti-GFP antibodies were from Invitrogen and CLONTECH, respectively. Alkaline phosphatase-labeled goat anti-rabbit IgG and anti-mouse IgG were obtained from Life Technologies, Inc.

Western Blotting—Cytosolic HeLa cell extracts were prepared by hypotonic lysis (25). Membrane fractions from normal mice (C57BL/6) and a human patient undergoing cardiac transplantation were prepared as reported (19). Extracts were separated by SDS-PAGE, transferred to nitrocellulose, and immunooblotted as described (19).

Bacterial Expression Vectors and Protein Purification—Plasmid BTFL-1 contains the full-length human dystrophin cDNA (a kind gift from Dr. L. M. Kunkel). Total mouse heart RNA was isolated with RNAzol B reagent (Tel-Test) and reverse transcribed using the Superscript II pre-amplification system. PCR was performed using the elongase amplification system (Life Technologies, Inc.). A human dystrophin miniprotein (amino acids 2091–2603) containing the hinge 3 region was amplified from plasmid BTFL-1 using a sense primer with an added XhoI site (5'CCCCCTCGAGAATGTACAAGGACCGCAAGG-3') and an antisense primer creating a XhoI site (5'CATGACCTCTGATGGCTCTAG-3'). The corresponding mouse dystrophin sequence (amino acids 2092–2596) was similarly amplified with reverse transcription-PCR (sense, 5'CCCCCTCGAGAATGTACAAGGACCGCAAGG-3'; antisense, 5'CATGACCTCTGATGGCTCTAG-3'). The PCR products were cloned into the XhoI/XbaI sites of pYc3-His2B (Invitrogen) and verified by nucleotide sequencing. After induction with isopropyl-1-thio-beta-D-galactopyranoside, the bacterially expressed His-tagged proteins were purified using nickel-nitriolotriacetic acid agarose (Qiagen) and verified by denaturing conditions. Prior to cleavage reactions, proteins were renatured by dialysis against protease 2A cleavage buffer (100 mM KCl, 50 mM NaCl, 80 mM Tris-HCl, pH 8.0, 1 mM CaCl2, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.1% Nonidet P-40).

In Vitro Translation—Overlapping mouse dystrophin fragments consisting of complete spectrin-like repeats were amplified with sense primers containing an appropriate restriction site and a consensus eukaryotic translation initiation sequence. The antisense primers had a stop codon and a restriction site downstream of the annealing portion. All constructs were cloned into pBluescript SKII (Stratagene). The mouse hinge 3 region was mutated (G9E) by overlap extension PCR (Mouse GE-sense, 5'-ACTACCTCAAGCTTGCGCA-3'; Mouse GE-antisense, 5'-TTGCCAGACGGTGCTAGT-3'). In vitro transcription/translation was performed with [35S]-methionine and the TnT T7 kit (Promega). Prior to digestion by protease 2A, translation products were dialyzed as above.

Mammalian Expression Vectors and Transfection—The 798-base pair XhoI/XbaI fragment of pEYFP-N1 was cloned into the corresponding sites of pECFP-C1 (both CLONTECH) yielding pEFRET. The complete hinge 3 region (26) of human dystrophin (amino acids 2423–2470) was amplified from plasmid BTFL-1 with a sense primer containing an EcoRI site (5'-ACCCGAAATTCATAGCTGACCTAGTCTC-3') and an antisense primer downstream of the endogenous KpnI site (5'-
GTGAAAATCTGCCAGACGACG-3'). The mouse dystrophin hinge 3 region (amino acids 2415–2463) was amplified by reverse transcription-PCR from mouse heart (sense, 5'-ACCGGAAATCTCAGGACGCTG-3'; antisense, 5'-GGTGAGTCGCCAGCTGC-3'). The complete spectrin-like repeats 3 of human (amino acids 549–672) and mouse (amino acids 540–674) dystrophin were amplified using the same primer pair (sense, 5'-ACCGGAAATCTCAGGACGCTG-3'; antisense, 5'-TTAGGTACGTTGGTGTTGACAGCTGGT-3'). The human and mouse hinge 3 regions were mutated (G→E) as for the in vitro translation constructs (Human GE-sense, 5'-CCACTATTGAGCCCTCTCT-3'; Human GE-antisense: 5'-AGGAGGCGCTAAATG-3'). PCR products were cloned into the EcoRI/KpnI sites of pFRET. The constructs were transiently transfected into HeLa cells using calcium phosphate. Cells were virally infected for 8 h at 38 °C after transfection.

Protease 2A Cleavage Assays—Recombinant protease 2A from Coxsackievirus B4 was purified as described (20). In 15 µl, 10 µg of LV membranes were incubated at 30 °C with 1 µg of protease 2A in cleavage buffer. Samples were analyzed by SDS-PAGE and Western blots. Purified bacterial fusion proteins (2 µg) were incubated at 30 °C with 1 µg of protease 2A using the same conditions. N-terminal peptide sequencing (Edman degradation) was performed as reported (23). Fluorescence resonance energy transfer (FRET) between the two tandem-fused GFP variants was assayed in a FluoroMax-2 fluorometer (Johin Yvon-Spex) with an excitation wavelength of 400 nm and a slit width of 2 nm. The emission spectrum from 450 to 600 nm was acquired at 1-nm intervals.

Peptide Synthesis and Peptide Modifications—An octapeptide (RAPGLSTT) encompassing P1–P8 of the mouse dystrophin cleavage motif was custom synthesized (Research Genetics). The tetrapeptide derivatives z-LSTL-fmk and z-LSTT-fmk were obtained in collaboration with Enzyme System Products.

**RESULTS**

Epitope Mapping of Coxsackieviral Protease 2A-mediated Dystrophin Cleavage Fragments—Based on the predictions from a neural network algorithm (18), human and mouse dystrophin both have two putative cleavage sites for the enteroviral protease 2A, located in the spectrin-like repeat 3 and the hinge 3 region (Fig. 1A). Using antibody Dy4/6D3 directed against the mid-rod domain of dystrophin, we previously demonstrated that Coxsackieviral protease 2A cleaves rat dystrophin, yielding a predominant fragment compatible with cleavage in the hinge 3 region. Additionally, a weaker second fragment suggested cleavage at the other putative site (19).

For more detailed analysis, we analyzed human and mouse dystrophin because of their known protein sequence and the experimental models of enteroviral myocarditis/cardiomyopathy (16, 27). When mouse (Fig. 1B) or human (Fig. 1C) cardiac membrane fractions are incubated with recombinant Coxsackieviral protease 2A followed by SDS-PAGE and immunoblotting, the major rod domain fragment (~280 kDa, arrow) consistently observed with antibody Dy4/6D3 is compatible with cleavage in the hinge 3 region. This indicates that this dystrophin cleavage site is conserved in mouse and human. Significantly lesser in signal intensity and reproducibility, a second fragment (asterisk) was detected with mouse dystrophin. When the immunoblotting was performed with antibody MANDRA1 (directed against the dystrophin C terminus), the presence of a single cleavage fragment of around 150 kDa is consistent with cleavage in the hinge 3 region. Upon immunoprobing with a rabbit antiserum recognizing the dystrophin N terminus, a large fragment of ~280 kDa and similar in size to the rod domain fragment was detectable but not a second fragment of ~65 kDa.

These results demonstrate that Coxsackievirus protease 2A cleaves endogenous rat, mouse, and human dystrophin, yielding fragments consistent with cleavage in the hinge 3 region. In mouse dystrophin, a second rod domain fragment indicated potential cleavage in the spectrin-like repeat 3, although this was not substantiated by the detection of the corresponding N-terminal fragment.

Coxsackieviral Protease 2A Directly Cleaves Dystrophin in the Hinge 3 Region—We sought to map the predominant Coxsackieviral protease 2A cleavage site using recombinant dys-
The mutant construct fully retains the FRET during infection. During viral infection, the emission from YFP. This FRET is partially lost during viral infection, indicating cleavage in the hinge 3 region and dissociation of YFP from CFP.

To test directly whether the hinge 3 region was also cleaved within Coxsackievirus B3-infected cells, green fluorescent protein variants were used as “carrier” molecules, and the human and mouse dystrophin hinge 3 regions were fused in frame between cyan fluorescent (CFP) and yellow fluorescent protein (YFP) (Fig. 3A). This strategy allows quantitation of cleavage using FRET. FRET can occur between CFP and YFP when they are in close proximity, and the emission wavelength from CFP is transferred to YFP, resulting in a shifted emission spectrum with a peak at 526 nm because of the emission from YFP. This FRET is partially lost during viral infection, indicating cleavage in the hinge 3 region and dissociation of YFP from CFP.

Therefore, the cleavage during viral infection, even though protease ZA was present in these samples at equal levels (Fig. 3B). Cleavage can also be demonstrated by analysis of the emission spectrum of these FRET constructs in cell extracts. Transfection of CFP only results in a peak emission at 474 nm upon excitation at 400 nm. Transfected YFP only is barely excitable at 400 nm in the tandem-fused constructs, the emission from the CFP is transferred to YFP, which resulted in a shifted emission spectrum with a peak at 526 nm because of the emission from YFP. This FRET is partially lost during viral infection, indicating cleavage in the hinge 3 region and dissociation of YFP from CFP. The mutant construct fully retains the FRET during infection.

trophin. Because full-length dystrophin is difficult to purify given its large size (data not shown), we elected to use miniproteins. The miniproteins contained the hinge 3 region in the context of adjacent complete spectrin-like repeats, the minimal folding unit of dystrophin (28) (Fig. 2A).

To assess whether Coxsackieviral protease 2A can cleave dystrophin directly, a bacterially expressed and affinity-purified mouse and human dystrophin miniprotein was incubated with Coxsackieviral protease 2A. As shown in Fig. 2B, purified protease 2A is sufficient to cleave both the purified mouse and human dystrophin miniprotein yielding fragments compatible with cleavage in the hinge 3 region. The smaller cleavage product (arrow) was verified as the C-terminal fragment by immunoblotting for the C-terminal Myc epitope tag of the dystrophin miniproteins (data not shown). Purified protease 2A migrates at approximately 16 kDa when present. The free N terminus of the smaller cleavage fragment was sequenced by Edman degradation demonstrating NH2-GASPTQ and NH2-GASASQ for human and mouse dystrophin, respectively.

Therefore, the cleavage in vitro occurs exactly at the computer-predicted residue in the hinge 3 region and is conserved between mouse (amino acid 2427) and human (amino acid 2434) dystrophin.

To examine whether the cleavage activity in Coxsackievirus B3-infected cells is identical to that of purified protease 2A, we cloned and expressed similar miniproteins by in vitro transcription and translation. The radiolabeled mouse wild type construct is cleaved both by purified protease 2A and extracts from virally infected HeLa cells with the same fragments (Fig. 2C). We next mutated the glycine residue at the C-terminal side of the mapped scissile bond to glutamate. Glycine at this position is essential for enteroviral protease 2A cleavage (18). Replacing the glycine with glutamate inhibits the susceptibility of eIF4GI to protease 2A cleavage (29). When introduced into the mouse (G2428E) dystrophin hinge 3 region, this mutation completely abolishes cleavage by both purified protease 2A as well as extracts from virally infected cells (Fig. 2C).

To test directly whether the hinge 3 region was also cleaved within Coxsackievirus B3-infected cells, green fluorescent protein variants were used as “carrier” molecules, and the human and mouse dystrophin hinge 3 regions were fused in frame between cyan fluorescent (CFP) and yellow fluorescent protein (YFP) (Fig. 3A). This strategy allows quantitation of cleavage using FRET. FRET can occur between CFP and YFP when they are in close proximity, and the emission wavelength from CFP is transferred to YFP, resulting in a shifted emission spectrum (30). Upon cleavage in the hinge 3 “linker,” YFP dissociates from CFP, and the FRET is lost (31, 32). Using this design, the dystrophin hinge 3 regions can be expressed in HeLa cells, and the constructs are cleaved near the middle following infection with Coxsackievirus B3 (Fig. 3B). The mutant mouse (G2428E) and human (G2435E) dystrophin hinge 3 regions are completely cleavage-resistant in vivo during viral infection, even though protease ZA was present in these samples at equal levels (Fig. 3B). Cleavage can also be demonstrated by analysis of the emission spectrum of these FRET constructs in cell extracts. Transfection of CFP only results in a peak emission at 474 nm upon excitation at 400 nm. Transfected YFP only is barely excitable at 400 nm (Fig. 3C). Fusing the mouse wild type hinge 3 region in frame between CFP and YFP results in strong energy transfer between the two and a shifted emission spectrum because of YFP emission at 526 nm. This FRET is partially lost during infection. In contrast, the cleavage-resistant mouse G2428E hinge 3 region completely retains the FRET during viral infection (Fig. 3C).

These data demonstrate that the Coxsackieviral protease 2A
Materials and Methods. Cleavage was assessed by SDS-PAGE and autoradiography. Incubated either with purified Coxsackieviral protease 2A or extracts from HeLa cells infected with Coxsackievirus B3 as described under "Materials and Methods." Cleavage was assessed by SDS-PAGE and autoradiography. aa, amino acids.

directly cleaves dystrophin in the hinge 3 region and that the dystrophin cleavage activity in Coxsackievirus B3-infected cells is identical to that of the purified protease 2A because the cleavage occurs at exactly the same residue. This suggests that protease 2A cleaves dystrophin directly during Coxsackievirus B3 infection.

The Hinge 3 Region Is the Primary Protease 2A Cleavage Site in Dystrophin—To investigate whether any other site in mouse dystrophin could be cleaved, a set of overlapping miniproteins spanning from the N terminus to the end of the rod domain was constructed and expressed using in vitro transcription and translation. In contrast with the miniprotein containing the hinge 3 region, neither the miniprotein containing the third spectrin-like repeat nor any other miniprotein was cleaved by the addition of purified Coxsackieviral protease 2A or extracts from virally infected cells (Fig. 4). Additionally, FRET constructs with the complete human or mouse third spectrin-like repeat were generated that contained the putative cleavage site at amino acid 588 of the human or 590 of the mouse dystrophin sequence. Following transfection into HeLa cells and infection with Coxsackievirus B3, both constructs remained intact without evidence of proteolytic degradation during Coxsackievirus B3 infection (data not shown). These results demonstrate that the hinge 3 region appears to be the primary site in dystrophin that is cleaved by Coxsackieviral protease 2A or by extracts from virally infected cells.

A Dystrophin-based Fluoromethyl Ketone Inhibits Coxsackieviral Protease 2A in Vitro—The enteroviral protease 2A possesses a catalytic cysteine residue, and its substrate recognition is dependent on the 4–6 amino acids upstream of the scissile bond (33). Because direct cleavage of the mouse dystrophin hinge 3 region was very efficient (Fig. 2B), we first utilized an octapeptide (RAPGLSTT) based on the mouse dystrophin recognition sequence that was found to directly interact with the Coxsackieviral protease 2A. However, this unmodified peptide did not inhibit the Coxsackieviral protease 2A (data not shown). Therefore, we synthesized a modified tetrapeptide containing a fluoromethyl ketone group at the C terminus (z-LSTT-fmk). The fluoromethyl ketone can form a covalent bond with the catalytic cysteine leading to protease inactivation. Peptide-fmk inhibitors, such as z-VAD-fmk, have been previously described for caspases (34). A second substrate analogue (z-LSTT-fmk) based on a binding peptide of the related poliovirus protease 2A was also tested (35). The resulting components z-LSTT-fmk and z-LSTL-fmk inhibited the Coxsackieviral protease 2A (1 μM) in vitro in a dose-dependent manner with half-maximal inhibition at 550 nM and 1050 nM, respectively (Fig. 5).

DISCUSSION

The main finding of this manuscript is that the computer-predicted cleavage site in the hinge 3 region of human and mouse dystrophin directly interacts with and is cleaved by the Coxsackieviral protease 2A. The same residue is cleaved within cells infected with Coxsackievirus B3, indicating that the protease 2A also directly cleaves dystrophin in vivo. Based on the protease 2A cleavage sequence found in dystrophin, a potent substrate analogue protease inhibitor was generated and tested in vitro.

Our results are the first prospective validation of a cleavage prediction made by the neural network (NetPicoRNA V1.0) (18) that was originally used to identify dystrophin as a potential cellular substrate of the enteroviral protease 2A (19). Mapping of the dystrophin cleavage site to the hinge 3 region explains why the cleavage functionally impairs dystrophin with dissociation of the rod domain from the sarcolemmal fraction. Cleavage in the hinge 3 region disconnects the N-terminal two-thirds of dystrophin with the actin-binding sites (4, 36) from the distally localized β-dystroglycan anchoring site (37) that links dystrophin to the transmembrane glycoprotein complex (4).

The high proline content of the hinge 3 region was suggested by others to lead to an open flexible conformation (26). Accessibility of a substrate to a given protease is important for cleavage to occur (38) and thus explains why dystrophin can be cleaved in the hinge 3 region by the protease 2A.

The results from epitope mapping of native dystrophin digested with Coxsackieviral protease 2A as well as the data obtained with recombinant dystrophin miniproteins suggest that the hinge 3 region is the primary site in mouse and human dystrophin that is directly cleaved by the viral protease 2A. Other than the dystrophin segment that spanned the hinge 3 region, miniproteins that spanned the N-terminal and rod do-

![Fig. 4. The hinge 3 region is the predominant cleavage site in mouse dystrophin.](http://www.jbc.org/Downloaded_from)
The importance of the dystrophin cleavage can be elegantly tested in a mouse model that carries a cleavage-resistant dystrophin mutant.

The enteroviral protease 2A is, in analogy to the HIV protease, an attractive target for antiviral drug development because both proteases are essential for viral life cycles (17, 38). Previously described inhibitors of the picornaviral protease 2A were either originally designed against host enzymes such as neutrophil elastase (elastatinal, MPCMk) (40) or originally identified as inhibitors of rhinoviral protease 3C (homophthalimidines) (41). In contrast, the potent z-LSTT-fmk was specifically designed for Coxsackievirus protease 2A. A closely related compound, z-LSTL-fmk, inhibited protease 2A less efficiently and demonstrates a degree of specificity for the tetrapeptide recognition sequence. The fluorescence resonance energy transfer-based protein substrates of the enteroviral protease 2A will allow rapid kinetic assays ideal for further inhibitor screening.

In summary, the enteroviral protease 2A cleavage site in human and mouse dystrophin was molecularly mapped to the hinge 3 region in vitro and in vivo. The identified cleavage motif was used to generate a potent peptidomimetic protease 2A inhibitor potentially useful for anti-enteroviral therapy.

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