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Proteolytic Cleavage of the Catalytic Subunit of DNA-Dependent Protein Kinase during Poliovirus Infection

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DNA-dependent protein kinase (DNA-PK) is a serine/threonine kinase that has critical roles in DNA double-strand break repair, as well as B- and T-cell antigen receptor rearrangement. The DNA-PK enzyme consists of the Ku regulatory subunit and a 450-kDa catalytic subunit termed DNA-PK$_{CS}$. Both of these subunits are autoantigens associated with connective tissue diseases such as systemic lupus erythematosus (SLE) and scleroderma. In this report, we show that DNA-PK$_{CS}$ is cleaved during poliovirus infection of HeLa cells. Cleavage was visible as early as 1.5 h postinfection (hpi) and resulted in an approximately 40% reduction in the levels of native protein by 5.5 hpi. Consistent with this observation, the activity of the DNA-PK$_{CS}$ enzyme was also reduced during viral infection, as determined by immunoprecipitation kinase assays. Although it has previously been shown that DNA-PK$_{CS}$ is a substrate of caspase-3 in vitro, the protein was still cleaved during poliovirus infection of the caspase-3-deficient MCF-7 cell line. Cleavage was not prevented by infection in the presence of a soluble caspase inhibitor, suggesting that cleavage in vivo was independent of host caspase activation. DNA-PK$_{CS}$ is directly cleaved by a picornaviral 2A protease in vitro, producing a fragment similar in size to the cleavage product observed in vivo. Taken together, our results indicate that DNA-PK$_{CS}$ is cleaved by the 2A protease during poliovirus infection. Proteolytic cleavage of DNA-PK$_{CS}$ during poliovirus infection may contribute to inhibition of host immune responses. Furthermore, cleavage of autoantigens by viral proteases may target these proteins for the autoimmune response by generating novel, or "immunocryptic," protein fragments.

There is considerable literature describing a role for posttranslational modifications of autoantigens in the bypass of immunological tolerance. For example, it has been shown that many autoantigens undergo a variety of modifications, including phosphorylation, dephosphorylation, and citrullination (53). Such modifications can have obvious effects on protein function, and these alterations also have both diagnostic and prognostic significance for patients with autoimmune disease. It has been proposed that posttranslational modifications might generate antigens that appear novel or foreign to the immune system, providing one possible explanation of how immunological tolerance to "self" is broken in the periphery of mammalian organisms (12–14, 21, 55).

Rosen and colleagues recently suggested a possible role for another type of posttranslational modification in the development of autoimmunity, proteolytic antigen cleavage mediated by caspases and granzyme B (grB). They and others have reported that a number of autoantigens are in fact caspase substrates and are cleaved during apoptosis (10, 13, 14, 56). Furthermore, many of these autoantigens are translocated to apoptotic bodies, or apoptotic blebs, suggesting a mechanism whereby they could be presented to effector cells in the immune system (12). In this way, autoantigen proteolysis in a cell undergoing apoptosis may lead to the generation of novel protein fragments (or "neo-epitopes"), which may in turn induce an autoimmune response.

It has been hypothesized that modifications of autoantigens that occur during programmed cell death may play a particularly important role in the initiation of autoimmunity. However, the ubiquitous nature of apoptosis seems inconsistent with the relatively low incidence of autoimmune disease in the general population, implicating a role for other mechanisms. It is widely held that autoimmunity results from a complex interplay of genetic and environmental factors. Genetic components of autoimmunity have been examined in great detail. Examples include major histocompatibility complex genotypes (51), as well as enzyme expression levels, such as DNase activity in systemic lupus erythematosus (SLE) (41, 61).

The association between various environmental factors and autoimmune disease has also been widely studied. Putative environmental triggers of autoimmunity include infectious agents, such as viruses and bacteria. The data suggesting a link between certain infectious agents and autoimmunity are compelling; however, the underlying mechanisms describing how viruses or bacteria might activate autoreactive lymphocytes remain poorly understood. In an extension of the hypothesis put forth by Rosen and others, we set out to examine whether viral proteases can directly recognize autoantigens as substrates. To test this hypothesis, we utilized a model system in which we examined autoantigen cleavage in poliovirus-infected

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HeLa cells. Cell extracts were screened by Western blot analysis, using well-characterized sera derived from patients with autoimmune diseases, as well as antibodies that recognize known autoantigens. Autoimmune sera have proven very useful as molecular probes in the identification of novel autoantigens, as well as in the study of important biochemical processes such as mRNA splicing, endoplasmic reticulum transport, and apoptosis signaling (27, 54).

In this report, we demonstrate the specific cleavage of a lupus- and scleroderma-associated autoantigen, the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{CS}), during poliovirus infection. DNA-PK_{CS} has critical roles in DNA repair, lymphocyte repertoire formation, and the induction of various proinflammatory cytokines involved in innate immune responses. We demonstrate that cleavage of this protein during infection modulates its enzymatic activity and is mediated by a picornaviral 2A protease in vitro. Our findings implicate direct viral protease cleavage of autoantigens as a potential and plausible mechanistic explanation for virus-induced autoimmunity. The results also suggest yet another intriguing mechanism by which a virus can modulate host innate and adaptive antiviral responses.

MATERIALS AND METHODS

Cells and viruses. HeLa and MCF-7 cells were grown as monolayers in Dulbecco's modified Eagle's medium (Gibco, Grand Island, N.Y.) supplemented with 10% calf serum (BioWhittaker, Walkersville, Md.). Mahoney type 1 poliovirus stocks were prepared at 8.4×10^9 PFU/ml, and infections were performed as previously described (57). All infections were carried out at a multiplicity of infection of 50.

Antibodies. SW5 anti-La monoclonal antibodies (kindly provided by W. J. van Venrooij, University of Nijmegen, Nijmegen, The Netherlands) were used at a dilution of 1:10. DNA-PK_{CS}-specific rabbit antiserum (Serotec, Oxford, England) was used at a dilution of 1:2,000. Anti-poly(ADP-ribose) polymerase (PARP) monoclonal antibodies (BD Transduction Labs, San Diego, Calif.) were used at 1:1,000. Rabbit antiserum directed against the N terminus of eukaryotic initiation factor 4G (eIF-4G) was generated as previously described (38) and used at 1:1,000. Antibodies specific for the cleaved form of caspase-3 (Cell Signaling Technology, Beverly, Mass.) were used at 1:1,000.

Western blot analysis. Cells were washed in phosphate-buffered saline (PBS), followed by resuspension in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris, pH 7.8, and 1 mM EDTA) supplemented with a protease inhibitor cocktail, which was prepared as previously described (28). Briefly, 100× cocktail was prepared by dissolving 10 mg of chymostatin, 1.5 mg of leupeptin, 7 mg of pepstatin A, 850 mg of phenylmethylsulfonyl fluoride, 500 mg of benzamidine, and 5 mg of aprotinin in 50 ml of ethanol by stirring overnight. Protease inhibitor cocktail was filtered and stored at room temperature. After incubation in lysis buffer at 4°C for 30 min, the cells were centrifuged at $13,000 \times g$ at 4°C for 15 min. The supernatants were heated at 100°C for 3 min in sodium dodecyl sulfate (SDS) loading buffer with 9% 2-mercaptoethanol and separated by SDS-polyacrylamide gel electrophoresis (PAGE) as described (32). Proteins were then transferred to nitrocellulose (Schleicher & Schuell, Keene, N.H.) and blocked with 5% Blotto (Bio-Rad Laboratories, Hercules, Calif.) in PBS-T (PBS with 0.1% Tween 20) overnight at 4°C. Membranes were incubated with antibodies at the dilutions indicated above for 1 h, followed by species-specific antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, Pa.) at 1:5,000, and developed by chemiluminescence performed according to the manufacturer's instructions (Pierce Biotechnology, Rockford, Ill.).

Preparation of apoptotic cell extracts. For UV irradiation, HeLa cells were plated in 100- by 15-mm polystyrene petri dishes (Nalge Nunc International, Rochester, N.Y.) at a concentration of 2×10^6 cells/ml and irradiated in a Stratalinker 2400 (Stratagene, San Diego, Calif.) at a distance of 9 cm for 30s. After irradiation, cells were incubated at 37°C for 5.5 h prior to harvesting. For anisomycin treatment, cells were plated as described above in cell growth medium containing anisomycin (Sigma, St. Louis, Mo.) at 10 μg/ml. The cells were then harvested after incubation at 37°C for 5.5 h. For caspase inhibition experiments, cells were treated with 100 μM benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone (ZVAD-fmk; Calbiochem, La Jolla, Calif.) for 30 min prior to UV irradiation, anisomycin treatment, or infection with virus. A total of 100 μM ZVAD-fmk was also included in the cell growth medium for the entire incubation period. Actinomycin D (ActD; Sigma) and cyclohemixide (Cx; Sigma) were used at concentrations of 5 μg/ml and 100 μg/ml, respectively.

IP kinase assay. DNA-PK_{CS} immunoprecipitation (IP) kinase assays were performed as previously described (27). Briefly, HeLa cells were infected with poliovirus or treated with the combination ActD/Cx as described above. After incubation for 5.5 h, cells were lysed as described above, and the lysates were precleared once with 40 μl of a 50% solution of protein A-Sepharose (Amersham Pharmicia Biotech, Piscataway, N.J.) in NP-40 detergent lysis buffer for 1 h, rotating at 4°C. Two microliters of anti-DNA-PK_{CS} antibody was used for IP experiments. IPs were performed with 0.7 mg of cytosolic protein. NP-40 buffer was added to a total volume of 500 μl, and IPs were rotated at 4°C for 1 h. Precipitates were washed three times in NP-40 lysis buffer, followed by one wash with wash buffer (50 mM Tris, pH 7.4, 5 mM $MnCl_2$). Precipitates were then resuspended in 20 μl of reaction buffer (50 mM Tris, pH 7.4, 5 mM $MnCl_2$, 5 mM dithiothreitol, 5 mM NaF, 40 μM $MgCl_2$, 1 mM sodium orthovanadate, 4 μM ATP, 50-μg/ml salmon sperm DNA) containing 10 μCi of [γ-^{32}P]ATP and 20 μg of α-casein, and then incubated at 30°C for 30 min. Reactions were terminated by boiling in an equal volume of 2× SDS loading buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. The extent of substrate phosphorylation was detected by autoradiography and then quantified with Molecular Imager FX (Bio-Rad Laboratories). Phosphorylation was determined by subtracting background counts (cpm per square millimeter) from total counts (cpm per square millimeter).

Production of viral proteases. CVB3 $2A^{pro}$ was subcloned from the vector pCVB3-20 (15), produced in *Escherichia coli*, and purified as described previously (26, 37). PV $3C^{pro}$ was expressed in *E. coli* and purified as previously described (26). PV $3CD^{pro}$ was expressed in *E. coli* and purified as previously described (45).

Cleavage assays. In vitro cleavage assays using purified recombinant viral proteases were performed as previously described (31). Briefly, 100 ng of $2A^{pro}$ or $3C^{pro}$ or 300 ng of $3CD^{pro}$ was incubated with 100 μg of HeLa cell S10 extract in cleavage buffer (100 mM NaCl, 5 mM $MgCl_2$, 10 mM HEPES-KOH, pH 7.4) for 30 min at 37°C. Reactions were terminated by the addition of loading buffer, and proteins were separated by SDS-PAGE. Proteins were then transferred to nitrocellulose and subjected to immunoblotting with either anti-eIF-4G or anti-DNA-PK_{CS} antibodies, followed by the appropriate secondary antibody. For assessment of protease activity against the 2C3AB substrate, [^{35}S]methionine-labeled 2C3AB protein was generated by coupled in vitro transcription/translation of plasmid pTM1-2C3AB (kindly provided by B. Semler, University of California, Irvine) as previously described (44). One microliter of 2C3AB translation reaction mixture was then incubated with 100 μg of HeLa cell S10 extract in the absence or presence of 100 ng of $2A^{pro}$ or $3C^{pro}$ or 300 ng of $3CD^{pro}$ for 1 h at 37°C. Reactions were terminated by the addition of loading buffer, and proteins were separated by SDS-PAGE. Proteins were then transferred to nitrocellulose and visualized by autoradiography.

RESULTS

The DNA-PK_{CS} autoantigen undergoes proteolysis during poliovirus infection. To determine whether direct cleavage of an antigen by a viral protease during infection of the host could potentially generate novel protein fragments, we used poliovirus infection of the HeLa human cell line as a model system. HeLa cells were infected with poliovirus under permissive conditions, and lysates from the virus-infected cells were screened for autoantigen cleavage by Western blotting. Consistent with previous reports (49), we observed that the La autoantigen was cleaved during poliovirus infection (Fig. 1A, compare lanes 1 and 3). However, despite screening nearly 30 other prominent autoantigens (Table 1), we identified DNA-PK_{CS} as the only other autoantigen that was cleaved during poliovirus infection. A cleavage product of approximately 160 kDa was visible as early as 1.5 h postinfection (hpi). The intensity of this product increased over time, with an approximately 40% reduction in full-length protein by 5.5 hpi (Fig. 1B).

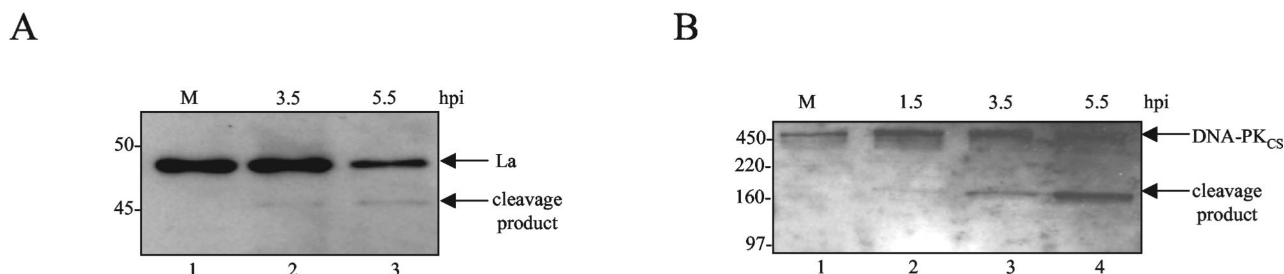

FIG. 1. Western blot analysis of La and DNA-PK_{CS} proteins from extracts prepared from poliovirus-infected cells. HeLa cells were incubated for the indicated times after poliovirus infection. The cells were then lysed in NP-40 detergent lysis buffer and the extracts were analyzed by immunoblotting with SW5 anti-La monoclonal antibody (A) or anti-DNA-PK_{CS} rabbit polyclonal sera (B) as described in Materials and Methods. Extracts from mock-infected cells are indicated with an M. Molecular mass markers are shown on the left (in kilodaltons) for Fig. 1 to 5. The positions of full-length protein and cleavage products are indicated by the arrows on the right.

Caspase-3 is not activated in HeLa cells during poliovirus infection. DNA-PK_{CS} is a well-studied substrate for caspase-3. Therefore, a possible explanation for our findings is that viral infection induces apoptosis, leading to activation of caspase-3 and cleavage of DNA-PK_{CS}. Several experiments were performed to exclude this possibility. First, our screen included autoantigens such as PARP and toposiomerase 1, both of which are known caspase-3 substrates. Neither of these molecules was cleaved during poliovirus infection, despite being cleaved in cells treated with apoptotic stimuli (Fig. 2A and 3C) (data not shown). Second, we were unable to detect activated caspase-3 in lysates prepared from virus-infected cells by Western blotting (Fig. 2B). These observations are consistent with those made by Agol and colleagues, who have previously reported that viral infection under permissive conditions does not involve caspase activation (1, 2, 52). Taken together, these data suggest that the proteolysis of DNA-PK_{CS} during poliovirus infection is not mediated by caspase-3. This is an important point, as caspase-3 is a major effector caspase in the apoptotic cell death pathway, and cleavage of well-defined caspase-3 substrates is a hallmark of apoptosis. Furthermore, several other caspase substrates were included in our initial screen (e.g., SRP72, vimentin, and SRPK1; Table 1). However, none of these was cleaved during poliovirus infection, providing strong evidence that caspase-mediated apoptosis does not occur in response to poliovirus infection under the conditions we utilized.

DNA-PK_{CS} is cleaved during poliovirus infection of a caspase-3-deficient cell line. In order to obtain additional evidence that the DNA-PK_{CS} fragment generated during poliovirus infection in HeLa cells was not due to low-level caspase-3 activation, we infected the MCF-7 human breast cancer cell line, which is deficient in caspase-3 protein and activity (24, 25, 36). As shown in Fig. 2C, DNA-PK_{CS} was still cleaved in this cell line, consistent with the notion that caspase-3 was not responsible for generating the 160-kDa fragment seen in lysates derived from poliovirus-infected HeLa cells. The cleaved fragment observed in poliovirus-infected MCF-7 cells comigrated with the one seen in infected HeLa cells (Fig. 2C, compare lanes 2 and 4). These data definitively demonstrate that DNA-PK_{CS} proteolysis is not mediated by caspase-3 during poliovirus infection.

TABLE 1. Autoantigens screened for cleavage during poliovirus infection

Autoantigen	Cleaved during poliovirus infection	Cleaved by caspases during apoptosis[a]	Disease association[b]
DNA-PK	Yes	3	SLE, SS
La	Yes[c]	Not 1, 2, 3, 8 or 9	SLE, Sjögren's
SRP-72	No	3	DM, PM
U1A/U2B″	No		SLE, MCTD
U1A	No		SLE, MCTD
PCNA	No		SLE
Ro60	No		SLE, Sjögren's
TIA-1	No		SLE
TIA-R	No		SLE
Sm	No		SLE
Jo-1	No		Myositis
Vimentin	No	1, 2, 3, 8, and 12	SLE, RA, Sjögren's
Th/To	No		SS
RNA pol I/III	No		SS
Phospho-SR proteins	No		SLE
All SR proteins	No		SLE
SRPK1	No	8	NONE
SC35	No		SLE
NOR90	No		SLE, SS, Sjögren's
Hsp70	No		SLE
Hsp90	No		SLE
SRP54	No		Myositis
PARP	No	1, 2, 3, and 6	SLE
Ku70	No		SLE
Ku80	No		SLE
MitoW	No		PBC
Topo-1 (Scl-70)	No	3	SS

[a] Reviewed in reference 53. Caspases are listed by number.
[b] DM, dermatomyositis; MCTD, mixed connective tissue disease; PBC, primary biliary cirrhosis; PM, polymyositis; RA, rheumatoid arthritis; SS, systemic sclerosis (scleroderma).
[c] See reference 49.

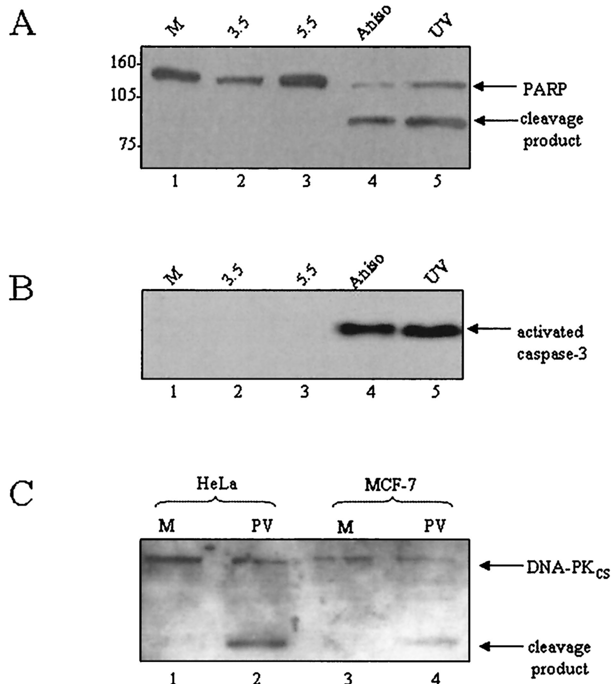

FIG. 2. (A) PARP, a well-characterized caspase-3 substrate, is not cleaved during poliovirus infection. Lysates from mock (M)- and poliovirus-infected cells were subjected to blotting with anti-PARP antibody, which recognizes both the full-length form of the PARP protein (116 kDa) and the 85-kDa cleavage product generated during apoptosis. Extracts from mock- and poliovirus-infected cells (lanes 1 to 3) are labeled as in Fig. 1. Lysates from anisomycin (Aniso)- and UV-treated HeLa cells are shown for comparison (lanes 4 and 5). (B) Caspase-3 is not activated during poliovirus infection of HeLa cells. HeLa cells were treated as previously described and subjected to blotting with an antibody that recognizes only the activated form of the caspase-3 enzyme, which migrates at approximately 19 kDa. Lysates from anisomycin- and UV-treated HeLa cells are shown as positive controls (lanes 4 and 5). (C) Poliovirus infection of a caspase-3-deficient cell line. HeLa and MCF-7 human breast carcinoma cells were infected with poliovirus and harvested in detergent lysis buffer at 5.5 hpi (lanes 2 and 4). Lanes 1 and 3 show lysates prepared from mock-infected HeLa and MCF-7 cells, respectively. The extracts were analyzed by blotting with anti-DNA-PK_{CS} antibody as described above.

Cleavage of DNA-PKcs during poliovirus infection is not prevented by caspase inhibitors. It has previously been reported by several groups that the DNA-PK_{CS} autoantigen is cleaved during apoptosis (10, 13, 35, 40, 50), and it has also been shown that DNA-PK_{CS} is a substrate for caspase-3 in vitro (10, 50). The results presented above strongly suggest that caspase-3 is not involved in the proteolysis of DNA-PK_{CS} during poliovirus infection. However, the DNA-PK_{CS} cleavage product seen during viral infection is approximately the same size as the fragment generated during apoptosis (see Fig. 5C, lanes 2 and 3), suggesting that cleavage of DNA-PK_{CS} could simply be due to viral activation of an endogenous host caspase with caspase-3 like activity. To test this possibility, we pretreated HeLa cells with the broad-spectrum caspase inhibitor ZVAD-fmk and then infected the cells with poliovirus. Caspase inhibitor was included in the cell growth medium at a concentration sufficient to inhibit endogenous caspases throughout the course of infection. The cells were then lysed, and the lysates were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blotting with anti-DNA-PK_{CS} antibody. As shown in Fig. 3A (lanes 3 and 4), the DNA-PK_{CS} protein was cleaved during viral infection in the presence of 100 μM ZVAD-fmk. Importantly, the caspase treatment regimen we utilized prevented apoptosis induced by the drugs anisomycin (an inhibitor of protein synthesis) and staurosporine (a protein kinase inhibitor), as well as by UV irradiation (Fig. 3B and C) (data not shown). These results strongly suggest that cleavage of DNA-PK_{CS} during poliovirus infection is not mediated by endogenous host caspases.

DNA-PKcs is a substrate for picornavirus protease 2A. The poliovirus genome encodes three proteases, termed $2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$, which are involved in protein processing during viral replication. The proteolytic activities of these proteases are essential for inhibition of host cell cap-dependent protein synthesis and viral propagation. We tested the hypothesis that DNA-PK_{CS} was an in vitro substrate for enterovirus proteases 2A, 3C, and 3CD. We used purified, recombinant coxsackievirus $2A^{pro}$, which has the same substrate specificity as poliovirus $2A^{pro}$ (23), and recombinant poliovirus proteases $3C^{pro}$ and $3CD^{pro}$ in our assays. As shown in Fig. 4A, the control substrate eIF-4G is specifically cleaved by $2A^{pro}$ but not by $3C^{pro}$ or $3CD^{pro}$ (lanes 2 to 4). Similarly, $3C^{pro}$ and $3CD^{pro}$ activities were verified by using the control substrate 2C3AB, a poliovirus nonstructural polyprotein precursor (Fig. 4B, see lanes 3 and 4). The recombinant $3CD^{pro}$ utilized in these studies does not support *cis*- or *trans*-cleavage into 3C and 3D. Furthermore, the aliquots of $3C^{pro}$ and $3CD^{pro}$ used correspond to equimolar amounts and possess equal protease activity at the P2-P3 junction of the poliovirus polyprotein (44).

We next tested the ability of these recombinant proteases to cleave DNA-PK_{CS} derived from HeLa cell cytoplasmic extract. As shown in Fig. 4C, DNA-PK_{CS} was a substrate for $2A^{pro}$ in an in vitro cleavage assay (lane 2). Conversely, $3C^{pro}$ and $3CD^{pro}$ did not cleave DNA-PK_{CS} (Fig. 4C, lanes 3 and 4). We cannot completely rule out a possible role for proteolytic cleavage of DNA-PK_{CS} by $3C^{pro}$ or $3CD^{pro}$ during poliovirus infection. However, the in vitro DNA-PK_{CS} cleavage product generated by incubation with $2A^{pro}$ was approximately the same size as the fragment generated in vivo during poliovirus infection of HeLa cells. Taken together, these results demonstrate that the DNA-PK_{CS} protein is an in vitro substrate for $2A^{pro}$ and strongly suggest that $2A^{pro}$ cleaves DNA-PK_{CS} during poliovirus infection.

DNA-PK_{CS} kinase activity is reduced in poliovirus-infected cells. We next wanted to ascertain whether viral infection had any effect on the function of the DNA-PK_{CS} enzyme. We harvested poliovirus-infected HeLa cells at 5.5 hpi and then immunoprecipitated the DNA-PK_{CS} protein by using a rabbit polyclonal antibody. We then tested the ability of the immunoprecipitated protein to phosphorylate α-casein, a known substrate of DNA-PK (9). As shown in Fig. 5A, kinase activity was reduced by approximately 30% in virus-infected cells, when compared with mock-infected controls (compare lanes 1 and 2; phosphorimager results quantitated in panel B, as described in Materials and Methods). This decrease in kinase activity was accompanied by a roughly 40% reduction in the amount of full-length DNA-PK_{CS} protein during poliovirus

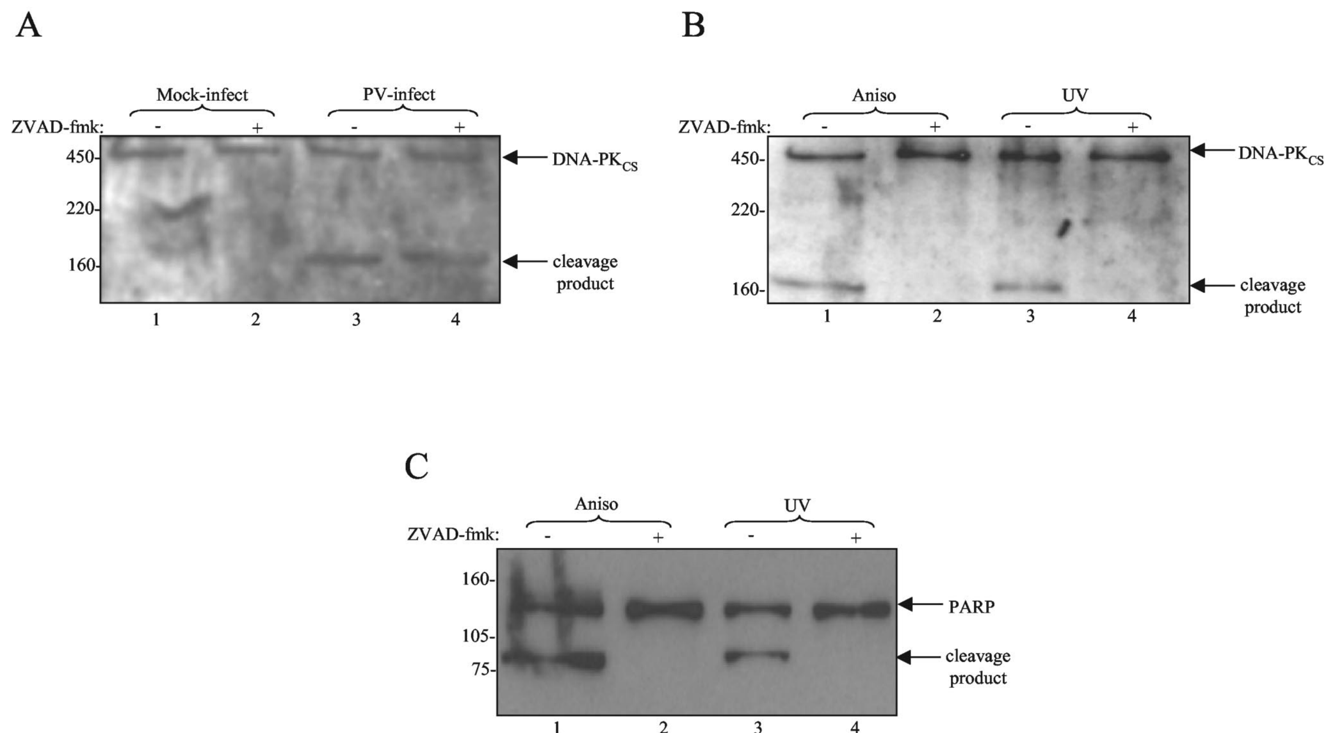

FIG. 3. Poliovirus infection of HeLa cells in the presence of a caspase inhibitor. (A) HeLa cells were pretreated with 100 μM ZVAD-fmk for 30 min prior to infection with poliovirus (PV). A total of 100 μM ZVAD-fmk was present in the cell growth medium throughout the course of infection. Cells were harvested in detergent lysis buffer at 5.5 hpi, and the extracts were analyzed by immunoblotting with anti-DNA-PK_{CS} antibody. Lanes 1 and 2 show lysates from mock-infected cells that were incubated in the presence of ZVAD or vehicle alone. (B) Treatment of HeLa cells with apoptotic stimuli in the presence of a caspase inhibitor. HeLa cells were pretreated with 100 μM ZVAD-fmk for 30 min prior to treatment with anisomycin (Aniso) or exposure to UV irradiation (lanes 2 and 4). Caspase inhibitor was present in the cell growth medium throughout the 5.5-h incubation period. At 5.5 h after anisomycin treatment or UV irradiation, cell extracts were analyzed by immunoblotting with anti-DNA-PK_{CS}. Lanes 1 and 3 show lysates from anisomycin- and UV-treated HeLa cells incubated in the absence of ZVAD, respectively. (C) HeLa cells were treated as described above and subjected to blotting with anti-PARP monoclonal antibody.

infection (as determined by Western blotting; Fig. 5C, compare intensities of 460-kDa bands in lanes 1 and 2).

Caspase-3-mediated cleavage of DNA-PK_{CS} in vitro leads to a significant decrease in the protein's kinase activity (10, 50), and a reduction in DNA-PK_{CS} activity is considered to be a hallmark of apoptosis (35, 40, 50). Thus, as an additional control, we immunoprecipitated the DNA-PK protein from HeLa cells that had been cotreated with ActD and Cx, drugs that are potent inducers of apoptosis when used in combination. As predicted, ActD/Cx treatment led to caspase-mediated DNA-PK_{CS} cleavage (Fig. 5C, lane 3). DNA-PK_{CS}-mediated phosphorylation of the α-casein substrate was reduced by nearly 70%, serving as confirmation of the reliability of our kinase assay (Fig. 5B, far-right panel). Taken together, the results suggest that $2A^{pro}$-mediated cleavage of DNA-PK_{CS} during poliovirus infection causes a significant reduction in enzymatic activity. Notably, the DNA-PK_{CS} fragment generated during poliovirus infection comigrates with the product observed during apoptosis (Fig. 5C, compare lanes 2 and 3), suggesting that the $2A^{pro}$ and caspase-3 recognition sites are in close proximity to one another. This is reminiscent of the La autoantigen; the $3C^{pro}$ and caspase cleavage products of La both migrate at approximately 45 kDa by SDS-PAGE (47).

DISCUSSION

Poliovirus is a positive-stranded RNA virus whose replicative cycle is carried out entirely in the cytoplasm of the host cell. Poliovirus polypeptides are generated by cotranslational and posttranslational cleavage of a 220-kDa polyprotein precursor encoded by a unique long open reading frame within the virus RNA (46). Three viral proteases—$2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$ (which is a precursor of $3C^{pro}$)—are involved in viral protein processing during replication of the virus (33, 42), and these proteases also cleave several host proteins directly. The proteolytic degradation of host proteins serves an important function in the viral life cycle, ensuring that host protein synthesis is halted while viral protein synthesis is preferentially activated. For example, $3C^{pro}$ functions to inhibit transcription through proteolysis of transcription factors such as TATA-binding protein (17, 18) and CREB (60). $2A^{pro}$ induces the shutoff of cap-dependent translation initiation by cleaving eIF-4G (29). It has been shown previously that the La autoantigen is cleaved during poliovirus infection by $3C^{pro}$, and this may contribute to accumulation of La in the cytoplasm of infected cells (49). Relocalization of La to the cytoplasm favors survival of the virus, since La enhances translation from the viral internal ribosomal entry site. However, there have been

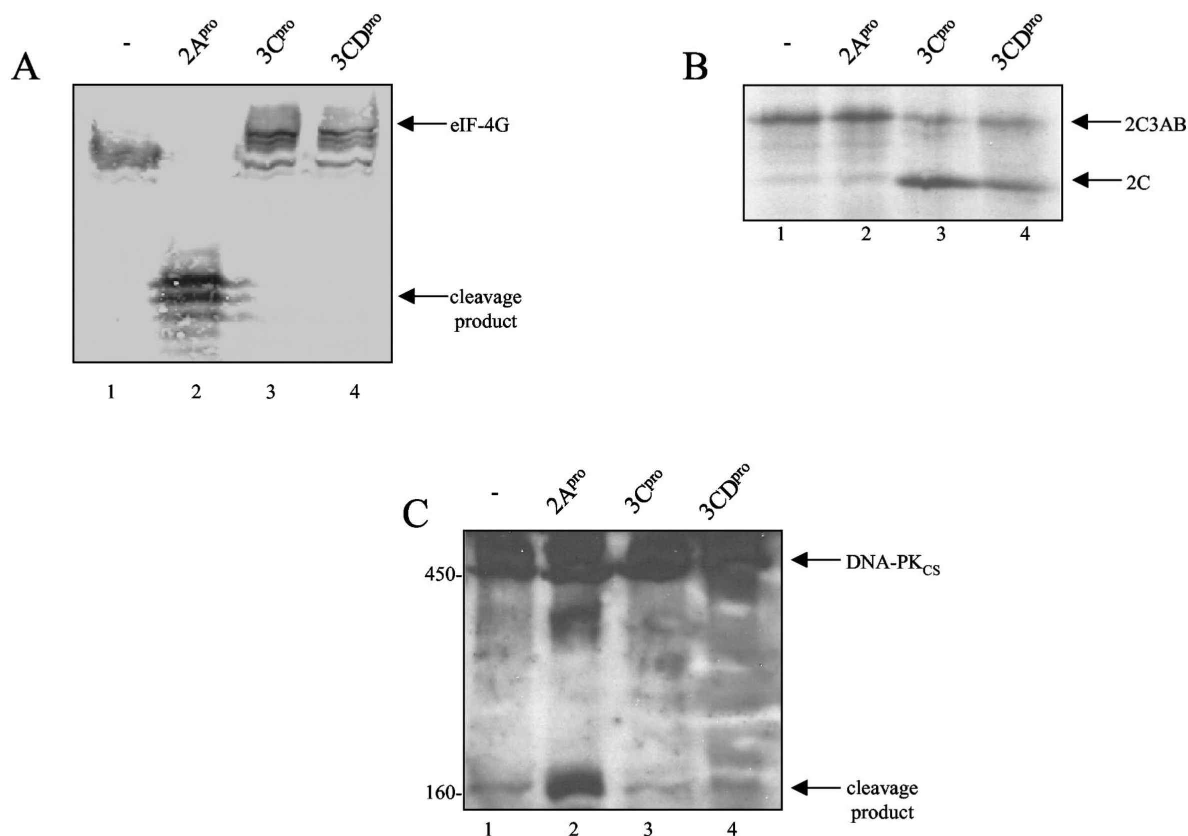

FIG. 4. Cleavage of DNA-PK_{CS} by 2A protease. (A) One hundred micrograms of HeLa cytoplasmic extract was incubated in the presence or absence (−) of coxsackievirus $2A^{pro}$, poliovirus $3C^{pro}$, or poliovirus $3CD^{pro}$. Reactions were carried out for 30 min at 37°C in cleavage buffer (see Materials and Methods), and eIF-4G was analyzed by Western blotting. The position of full-length eIF-4G and the cleavage fragment are shown on the right. (B) $2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$ were incubated with [^{35}S]methionine-labeled 2C3AB produced in vitro. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and the reactions were analyzed by autoradiography. The positions of the 2C3AB polyprotein and the 2C cleavage product are indicated on the right. (C) $2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$ were incubated with HeLa cytoplasmic extract as in panel A, and DNA-PK_{CS} was analyzed by Western blotting.

no previous reports describing the specific cleavage of other autoantigens by a poliovirus protease.

Picornavirus proteases are highly specific, as evidenced by the fact that a limited number of poliovirus $2A^{pro}$ substrates have been identified in mammalian cells. Poly(A)-binding protein (26), eIF-4G (29), and TATA-binding protein (59) represent known substrates that are directly recognized by $2A^{pro}$. More recently, it has been shown that cytoskeletal proteins are also specifically targeted by the $2A^{pro}$ of rhino- and coxsackieviruses. For example, cytokeratin 8 is a substrate for rhinovirus and coxsackievirus B4 $2A^{pro}$ (48), and coxsackievirus B3 (CVB3) $2A^{pro}$ cleaves dystrophin in myocytes, suggesting a possible mechanism for the pathogenesis of acquired cardiomyopathy (6). It has been suggested that disruption of the cytoskeletal network may also facilitate virus release from the host cell (48). However, in contrast to substrates directly involved in host cell transcription and translation, cytoskeletal proteins appear to be targeted relatively late in the infection cycle.

DNA-PK is composed of a large catalytic subunit, termed DNA-PK_{CS}, and a regulatory factor, Ku. Ku is comprised of two subunits, termed Ku70 and Ku80. We report here that DNA-PK_{CS} is cleaved during poliovirus infection in a caspase-independent manner and that cleavage in vitro is mediated by the 2A protease. Notably, neither Ku70 nor Ku80 was cleaved during infection in our system (Table 1) (data not shown). This is similar to what other investigators have observed during apoptosis, where DNA-PK_{CS} is cleaved while the Ku subunits remain stable (35, 40, 50). DNA-PK_{CS} degradation occurs relatively early in the course of infection (Fig. 1), correlating closely with cleavage of previously described poliovirus protease substrates.

The specific, early cleavage of DNA-PK_{CS} by poliovirus raises an interesting teleological question: Why would a virus with such a limited genome target a protein not directly involved in initiation of host transcription or translation? The answer may lie in the fact that many viruses have developed a number of strategies aimed at subverting host immune responses. DNA-PK is required for proper B- and T-cell antigen receptor rearrangement, and DNA-$PK_{CS}^{-/-}$ mice develop severe combined immunodeficiency (30). Furthermore, Alt and colleagues have recently described a role for DNA-PK_{CS} in immunoglobulin (Ig) class switching to the murine IgG2a isotype, an antibody subclass traditionally associated with cell-mediated immune responses (39).

Raz and colleagues have also demonstrated a critical role for

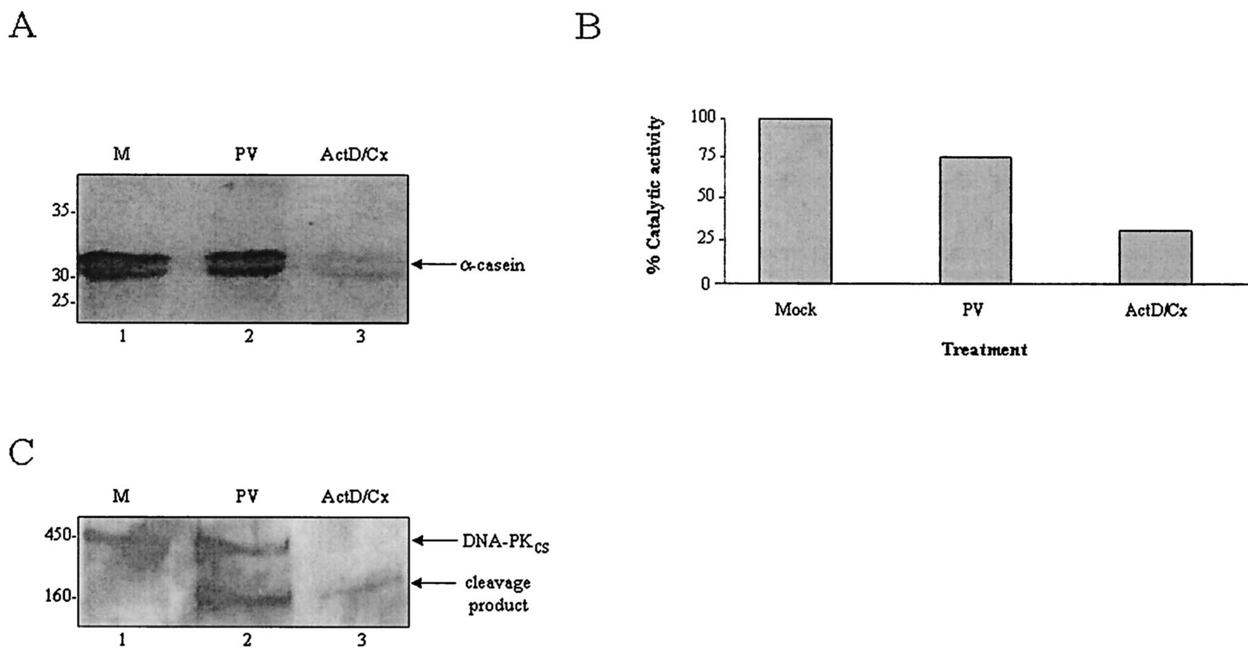

FIG. 5. Kinase activity of DNA-PK$_{CS}$ protein in poliovirus-infected HeLa cells. (A) HeLa cells were infected with poliovirus (PV) and harvested in detergent lysis buffer at 5.5 hpi. DNA-PK$_{CS}$ activity was then assayed by immunoprecipitation, using α-casein as a substrate as described in Materials and Methods. Extracts derived from mock-infected (M) and ActD/Cx-treated cells are shown for comparison (lanes 1 and 3). The position of the α-casein substrate is indicated by the arrow. (B) The extent of phosphorylation was detected by autoradiography and quantified by using Molecular Imager FX (Bio-Rad Laboratories). Results are expressed as a percentage of the activity present in mock-infected cells. (C) The cleavage status of DNA-PK$_{CS}$ was verified by Western blotting. The positions of full-length DNA-PK$_{CS}$ and the cleavage fragment are shown on the right. Results shown are representative of two independent experiments.

DNA-PK$_{CS}$ in the induction of innate immune responses to bacterial DNA and immunostimulatory oligodeoxyribonucleotides (16). The authors showed that DNA-PK$_{CS}$ is activated by immunostimulatory oligodeoxyribonucleotides and contributes to the activation of IKKβ, which in turn phosphorylates the NF-κB transcription factor. This allows NF-κB to translocate into the nucleus, where it subsequently activates transcription of proinflammatory cytokines such as interleukin-6 (IL-6) and IL-12. Kirkegaard and colleagues recently reported that poliovirus protein 3A inhibits host secretion of IL-6 (as well as IL-8 and beta interferon) during viral infection (19). Thus, it is apparent that poliovirus has devised numerous strategies aimed specifically at inhibiting host innate immune responses. Cleavage of DNA-PK$_{CS}$ may represent one of many means to this particular end. It is also possible that proteolytic degradation of DNA-PK$_{CS}$ alters localization of the enzyme, further impairing its ability to interact with cognate substrates and to carry out its normal functions. Experiments are in progress to determine whether cellular redistribution of DNA-PK$_{CS}$ occurs in response to poliovirus infection.

DNA-PK$_{CS}$ has been shown to play an important role in the life cycle of other viruses. For example, herpes simplex virus (HSV) replicates more efficiently in a cell line deficient in DNA-PK$_{CS}$ than a wild-type control cell line (34). Consistent with this observation, DNA-PK$_{CS}$ appears to be degraded by the proteosome during HSV-1 infection of mammalian cells (43). However, in contrast to the HSV studies, we do not observe complete degradation of the DNA-PK$_{CS}$ protein in response to poliovirus infection. Rather, our data demonstrate site-specific cleavage of DNA-PK$_{CS}$ by a viral protease.

While there is relatively little direct evidence implicating viruses in the initiation of autoimmunity, there is considerable epidemiological data suggesting a causal relationship between certain viral infections and autoimmune disease. For example, Epstein-Barr virus has been linked with SLE and rheumatoid arthritis, and infections with coxsackievirus and hepatitis C virus have been associated with myocarditis and myasthenia gravis, respectively (3). "Molecular mimicry" (defined as cross-recognition of microbial and self-epitopes by autoreactive lymphocytes) and "bystander activation" (which describes the release of normally sequestered proteins as a result of microbe-induced host cell destruction) represent two popular mechanistic explanations for virus-induced autoimmunity (4, 7). However, the fact that DNA-PK$_{CS}$ is an autoantigen associated with SLE and scleroderma (13, 53) and is cleaved by 2A^{pro} in vitro raises the intriguing possibility that direct cleavage of a host protein by a viral protease could generate novel protein fragments (neo-epitopes) and trigger an autoimmune response.

Our system currently does not allow us to examine the immunogenic or autoimmune potential of poliovirus protease cleavage products. However, Rosen and colleagues have recently examined in detail the interaction of several autoantigens with another protease, grB. Their data lend considerable support to this hypothesis. In their studies, grB cleaved several autoantigens (including DNA-PK$_{CS}$) at unique sites both in vivo and in vitro, generating fragments distinct from those produced by caspases (5, 11). Novel protein fragments for most autoantigens were identified based on differential migration of caspase and granzyme protein products, as determined by

Western blot analysis. However, grB and caspase-3 cleave DNA-PK_{CS} at closely spaced, but distinct, sites. grB-mediated cleavage of DNA-PK_{CS} generated a 250-kDa N-terminal fragment that comigrated with the caspase-3 cleavage product. A distinct DNA-PK_{CS} cleavage site for grB was confirmed by performing mutational analysis of in vitro-translated DNA-PK_{CS} fragments, as well as immunoblot analysis with a C-terminal antibody directed against DNA-PK_{CS}. Validation of unique substrate fragments was also aided by the existence of multiple grB cleavages sites within the protein (5). grB is expressed by natural killer cells and $CD8^+$ cytolytic T cells, both of which have central roles in antiviral and antitumor immunity. Thus, antiviral innate and adaptive immune responses could activate host proteases, which might in turn induce immune responses to self-antigens. Our observations and those of other investigators add to increasing evidence directly implicating autoantigen cleavage by various proteases as a potential contributor to the pathogenesis of autoimmune disease.

Cleavage of DNA-PK_{CS} by caspase-3 DNA-PK_{CS} occurs C terminal to the aspartate residue located at position 2712 (10, 40, 50), generating polypeptides of approximately 307 and 161 kDa. While the $2A^{pro}$ cleavage site of DNA-PK_{CS} remains to be determined, we predict that $2A^{pro}$ recognizes a single, distinct site located very close to Asp2712. DNA-PK_{CS} cleavage by a viral protease near Asp2712 would explain the apparent comigration of the caspase-3 and poliovirus cleavage products (Fig. 5C, compare lanes 2 and 3), as the DNA-PK_{CS} antiserum utilized in our studies recognizes a region in the C terminus of the protein. Analysis of the complete amino acid sequence of DNA-PK_{CS} implicates the bond between Val2696 and Gly2697 as a likely $2A^{pro}$cleavage site. Proteolytic cleavage at this site would satisfy $2A^{pro}$ sequence requirements for recognition and cleavage. These requirements include a hydrophobic L or M at the P4 position, a hydroxyl-containing S or T at the P2 position, a critical G at the P1′ position, and a P at the P2′ position (8, 62). The disparate substrate specificity of caspases and viral proteases, combined with the degenerate nature of antigen recognition by lymphocyte receptors (58), may account for the predicted autoantigenic potential of viral protease cleavage products. Proteolysis of DNA-PK_{CS} by $2A^{pro}$, which preferentially cleaves before glycine residues, instead of by caspase-3, which cleaves after aspartic acid residues, could result in the generation of novel protein fragments. In this way, cleavage by $2A^{pro}$ may result in immunocryptic epitopes for presentation to autoreactive lymphocytes.

Poliovirus has never been directly associated with autoimmunity, and we do not intend to suggest a causal relationship between this particular picornavirus and autoimmune disease. However, it is interesting to note that poliovirus is closely related to another picornavirus, coxsackievirus, and the data supporting a link between coxsackievirus and autoimmunity are compelling. Epidemiological studies have shown an increased incidence of viral myocarditis following endemic coxsackievirus B infections (22). It has also been proposed that molecular mimics of coxsackievirus B3 contribute to heart disease in a murine model of myocarditis (20). Coxsackievirus has also been associated with diseases such as cardiomyopathy and diabetes (4). Thus, while the molecular tools for examining autoantigen cleavage by coxsackievirus are readily available, we believe that a poliovirus model system offers an informative and safe alternative. It is worth noting that poliovirus was used during our studies of DNA-PK_{CS} cleavage during viral infection in vivo, while our in vitro cleavage assays utilized recombinant coxsackievirus $2A^{pro}$. However, coxsackievirus $2A^{pro}$ shares cleavage site specificity with its poliovirus counterpart (23), suggesting that poliovirus may indeed be a useful model for mechanistic studies on how viruses potentially contribute to the induction of autoimmunity.

In conclusion, we have identified an autoantigen that is a novel substrate for a viral protease. These data lend credence to the notion that autoantigen cleavage by a viral protease may target a molecule for an autoimmune response by revealing cryptic fragments and further highlight the role of DNA-PK_{CS} in both innate and adaptive antiviral immunity. Future studies of DNA-PK_{CS} proteolysis by $2A^{pro}$ should enhance our understanding of how viruses can modulate the immune system, as well as provide important insights into more basic questions concerning the evolution of virus-host relationships.

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