INTRODUCTION

Infection of human cells with poliovirus (PV) leads to rapid inhibition of host cell RNA synthesis catalyzed by RNA polymerases I (Pol I), Pol II, and Pol III (Kaariainen and Ranki, 1984). It has been shown that the inhibition of cellular transcription observed in vivo can be recapitulated in vitro by using extracts prepared from mock- or poliovirus-infected cells (Crawford et al., 1981). For each of the three polymerase systems, in vitro analysis has revealed that the inhibition of transcription by poliovirus infection is a consequence of inactivation of specific transcription factors (Clark and Dasgupta, 1990; Clark et al., 1991, 1993; Kliewer et al., 1988; Rubinstein et al., 1992). We have shown that the TATA-binding protein (TBP), the DNA-binding subunit of transcription factor TFIID, is proteolytically cleaved by the virus-specific protease 3CPro (Clark et al., 1993; Alamanchili et al., 1996; Alamanchili et al., 1997a). This cleavage leads to loss of formation of the TBP–TATA box complex, resulting in inhibition of TATA- and initiator-mediated basal transcription (Alamanchili et al., 1996). Similarly, a Pol III DNA-binding transcription factor, TFIIIC, is cleaved and inactivated by 3CPro (Clark et al., 1991). The α subunit of TFIIIC which contacts the Pol III promoter is the target of the viral protease 3CPro (Shen et al., 1996). An unknown Pol I transcription factor is also cleaved by 3CPro, resulting in inhibition of Pol I transcription in virus-infected cells (Rubinstein and Dasgupta, 1987; Rubinstein et al., 1992).

While the TATA or initiator elements carry out low-level transcription (basal transcription), high-level or activated transcription from many cellular and viral promoters requires interaction of multiple upstream transcription factors. These elements have been found to be required for induction by exogenous substrates such as hormones, phorbol esters, serum, and cyclic AMP (Montminy et al., 1986). One such transcriptional activator is p53. It functions as a typical sequence-specific transcription activator (Farmer et al., 1992; Kern et al., 1991) that binds as a tetramer (Jeffrey et al., 1995) to four tandem, alternatively inverted copies of a 5-bp consensus sequence (Cho et al., 1994; Halazonetis and Kandil, 1993). Considerable evidence suggests that the transcriptional stimulatory activity is necessary for p53 to function as a tumor suppressor (Vogelstein and Kinzler, 1992). It is thought that p53 transcriptionally activates genes that prevent entry into S phase and/or genes required for apoptosis. p53 activation requires that it binds to TFIID (Liu et al., 1993; Thut et al., 1995) through interactions with TATA-box-binding protein (TBP)-associated factors (TAFs) and potentially with TBP (Thut et al., 1995). Recent biochem-

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Poliovirus 3C Protease-Mediated Degradation of Transcriptional Activator p53 Requires a Cellular Activity

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Received May 25, 2001; returned to author July 10, 2001; accepted September 27, 2001
p53 is degraded in poliovirus-infected HeLa cells

To investigate whether the transcriptional activator p53 is cleaved during infection of HeLa cells with poliovirus, cell-free extracts were prepared from mock- and poliovirus-infected cells and examined by Western blot analysis using a mouse monoclonal antibody against an epitope corresponding to amino acids 11–25 of p53. The antibody recognized the p53 polypeptide from mock-infected cells (Fig. 1A, lane 1). In cells infected with poliovirus for 4 h, p53 was almost totally absent (Fig. 1A, lane 2). Although the intensity of the p53 band was drastically reduced, we were unable to detect any cleaved products of p53 in infected cell extracts. The precise reason for this is not known. The proteolyzed fragments may be unstable in HeLa cell extracts. Alternatively, the monoclonal antibody may somehow have failed to detect the proteolyzed fragments containing the epitope. As a positive control, a similar immunoblot was developed with anti-Oct-1 antibodies. We have previously shown that Oct-1 is cleaved in poliovirus-infected cells. As can be seen in Fig. 1B, the proteolytic cleaved product of Oct-1 was clearly detected in the infected cell extract (lanes 1 and 2). A number of host cell transcription factors including TBP, Oct-1, TFIIIC, and CREB are cleaved by the poliovirus-encoded protease 3Cpro both in vitro and in vivo (Clark et al., 1991, 1993; Yalamanchili et al., 1996, 1997b,c). We, therefore, examined whether 3Cpro was responsible for p53 degradation. A mutant poliovirus (Se1 3C-02; Dewalt and Semler, 1987) which has a valine to alanine substitution at amino acid 54 of 3Cpro was used to determine whether it was defective in p53 degradation. Upon infection of HeLa cells, this virus produces very little active 3Cpro and consequently has a small plaque phenotype. It still grows to nearly wild-type (wt) titers because poliovirus overproduces 3Cpro and other viral proteins (Dewalt and Semler, 1987). It is important to note that this mutant protease is not totally defective but is much less active than the wild-type protease. Earlier studies from our laboratory demonstrated that this mutant virus was defective in shutting off cellular RNA Pol II and III transcription (Clark et al., 1991; Yalamanchili et al., 1996). HeLa cells were either mock-infected or infected with wt poliovirus or the Se1 3C-02 mutant and cells were harvested at various times postinfection. Cell-free extracts were then prepared and subjected to Western blot analysis following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Two p53 antibodies which recognized a 53-kDa protein from mock-, PV-, and Se1 3C-02-infected cells were used in this experiment (Fig. 2). The p53 antibody used in Fig. 2A was a mouse monoclonal antibody which recognized an epitope corresponding to amino acids 11–25. The p53 antibody used in Fig. 2B was a goat polyclonal antibody raised against full-length p53. A mouse monoclonal antibody to β-tubulin (Fig. 2A) or a mouse monoclonal antibody to actin (Fig. 2B) was also used in the Western blot analysis as a loading control. While the intensity of the β-tubulin band remained relatively constant among mock- and Se1 3C-02-infected extracts (Fig. 2A, lanes 1–5 and 11–15), a significant increase in the intensity of the β-tubulin band was observed in PV-infected cell extracts particularly at 4 and 5 h postinfection (lanes 6–10). Actin seemed to be a better loading control as its
levels remained constant throughout the experiment. The intensity of the p53 band did not change significantly up to 2 h of infection. At 3 and 4 h postinfection, the intensity of the p53 band was reduced to 70 and 18% of the zero time control. At 5 h postinfection p53 was no longer detectable (Fig. 2A, lane 10). In comparison, the levels of p53 in mock- and Se1 3C-02-infected cells remained relatively constant throughout infection. These results suggest that the viral protease 3C was responsible for degradation of p53 in poliovirus-infected HeLa cells.

3C\textsuperscript{pro}-mediated degradation of p53 \textit{in vitro}

To determine whether 3C\textsuperscript{pro} is indeed capable of degrading p53, bacterially expressed, purified His-tagged 3C\textsuperscript{pro} was incubated with the uninfected HeLa cell extract and the resulting reactions were analyzed by Western blotting using the p53 antibody. Incubation of mock-infected extract with 0.8 \(\mu\)g of 3C\textsuperscript{pro} resulted in significant degradation (~75%) of p53 compared to the control (Fig. 3A, lanes 1 and 2). At a higher concentration of 3C\textsuperscript{pro}, no p53 was detectable (Fig. 3A, lane 3).

To confirm the above results, p53 was translated \textit{in vitro} in the presence of \(^{35}\)S methionine in rabbit reticulocyte lysate, and the labeled protein was incubated with various concentrations of purified 3C\textsuperscript{pro}. As can be seen in Fig. 3B, at a very low concentration of 3C\textsuperscript{pro} (80 ng), p53 degradation was only 8% compared to the control (compare lanes 1 and 2). However, p53 was almost totally degraded (3 and 6%, respectively) at 0.8 and 8 \(\mu\)g of 3C\textsuperscript{pro} (Fig. 3B, lanes 3 and 4). To rule out the possibility that a contaminating \textit{Escherichia coli} protease was degrading p53, a mutant 3C\textsuperscript{pro} having a single amino acid substitution (3C C147S) was expressed and purified from \textit{E. coli} (Hammerle et al., 1991). As expected, 8 \(\mu\)g of the mutant protease was totally inactive in degrading \textit{in vitro} translated p53 (Fig. 3B, lane 5). Thus, both genetic and biochemical results suggest that degradation of p53 in poliovirus-infected HeLa cells is mediated by the viral protease 3C\textsuperscript{pro}.

Initial characterization of p53 degradation in poliovirus-infected cells

In order to determine whether proteolytic degradation products of p53 can be detected early during the reaction, a time kinetics of degradation was performed. \textit{In vitro} translated, \(^{35}\)S methionine-labeled p53 in rabbit reticulocyte lysate was incubated with various concentrations of purified 3C\textsuperscript{pro}. The degradation was monitored by Western blotting using a monoclonal anti-p53 antibody. As can be seen in Fig. 2A, lane 10, p53 degradation was significant (~75%) at a low concentration of 3C\textsuperscript{pro} (80 ng). At a higher concentration, 3C\textsuperscript{pro} was almost totally degraded (3 and 6%, respectively) at 0.8 and 8 \(\mu\)g of 3C\textsuperscript{pro} (Fig. 2B, lanes 3 and 4). To rule out the possibility that a contaminating \textit{Escherichia coli} protease was degrading p53, a mutant 3C\textsuperscript{pro} having a single amino acid substitution (3C C147S) was expressed and purified from \textit{E. coli} (Hammerle et al., 1991). As expected, 8 \(\mu\)g of the mutant protease was totally inactive in degrading \textit{in vitro} translated p53 (Fig. 2B, lane 5). Thus, both genetic and biochemical results suggest that degradation of p53 in poliovirus-infected HeLa cells is mediated by the viral protease 3C\textsuperscript{pro}.
ticulocyte lysate was mixed with 3C\textsuperscript{pro} at 30°C and aliquots of the reactions were withdrawn at 0 min, 30 min, 1 h, 1.5 h, and 2 h (Fig. 4A, lanes 1–5). Only 10% reduction in the intensity of the labeled p53 band was observed at 30 min postincubation compared to the zero time control (Fig. 4A, compare lanes 1 and 2). However, 1 h after the reaction close to half (~47%) of the total p53 was degraded (Fig. 4A, lane 3). Yet no cleaved products of p53 were detectable at this time point. Continued incubation through 2 h resulted in further disappearance of p53 without concomitant generation of proteolyzed products (Fig. 4A, lanes 4 and 5).

FIG. 3. Poliovirus-encoded 3C\textsuperscript{pro} degrades p53 in vitro. (A) Two hundred micrograms of mock-infected HeLa extract was incubated in the absence (lane 1) and in the presence of 0.8 µg (lane 2) and 8 µg (lane 3) of the wild-type 3C\textsuperscript{pro}. Reactions were analyzed by Western blotting using anti-p53 antibody. (B) In vitro translated, \[^{35}S\]methionine-labeled p53 in reticulocyte lysate was incubated in the absence (lane 1) and in the presence of 0.08 (lane 2), 0.8 (lane 3), and 8 µg (lane 4) of 3C\textsuperscript{pro} or 8 µg of the 3C\textsuperscript{pro} mutant, 3C C147S (lane 5). Labeled proteins were analyzed by SDS–PAGE as described under Materials and Methods. Migration of marker proteins is shown on the left.

FIG. 4. Characterization of p53 degradation. (A) Kinetics of 3C\textsuperscript{pro}-induced degradation of p53. \[^{35}S\]Methionine-labeled p53 translated in rabbit reticulocyte lysates was incubated with 1 µg of 3C\textsuperscript{pro} at 30°C for 0 min (lane 1), 30 min (lane 2), 1 h (lane 3), 1.5 h (lane 4), and 2 h (lane 5). Labeled proteins were analyzed by SDS–PAGE. The \(^{14}C\)-labeled molecular weight markers are shown on the left (in kilodaltons). The position of full-length p53 is indicated on the right. (B) 3C\textsuperscript{pro}-catalyzed degradation of p53 at various temperatures. \[^{35}S\]Methionine-labeled p53 translated in reticulocyte lysates was incubated without (lanes 1, 3, 5, 7, and 9) or with 0.5 µg 3C\textsuperscript{pro} (lanes 2, 4, 6, 8, and 10) at 4°C (lanes 1 and 2), 24°C (lanes 3 and 4), 30°C (lanes 5 and 6), 37°C (lanes 7 and 8), and 65°C (lanes 9 and 10). Labeled proteins were analyzed by SDS–PAGE. The migration of marker proteins is shown on the left.
Varying the temperature during incubation of labeled p53 with 3CPro showed very little degradation at 4°C (Fig. 4B, lanes 1 and 2), and approximately 50% degradation was observed at 24°C (lanes 3 and 4). Increasing the incubation temperature to 30 and 37°C resulted in close to 70% and more than 94% degradation, respectively (Fig. 4B, lanes 5–8). Increasing the temperature to 65°C almost completely blocked cleavage (Fig. 4B, lanes 9 and 10). Again, no proteolytic fragments of p53 were detectable during the reaction at various temperatures.

The ubiquitin pathway of protein degradation is ATP-dependent and operates in rabbit reticulocytes, HeLa cells, and many other eukaryotic cells (Finley and Chau, 1991; Ciechanover et al., 1991). Previous studies have shown that many oncogenic viral proteins (such as the papilloma virus E6 transforming protein) induce degradation of p53 via the ubiquitin pathway (Scheffner et al., 1991). p53 is one of the many proteins in the cell which is marked for protein degradation. Such proteins are polyubiquitinated and then rapidly degraded by proteasomes. In order to determine whether ubiquitination played a role in the p53 degradation, we examined the effect of a proteosome inhibitor on HeLa cells during poliovirus infection. HeLa cell spinner cultures were incubated with (Fig. 5A, lanes 4–6) and without (lanes 1–3) the proteosome inhibitor MG132 (10 μM). The cells were incubated in the presence of the inhibitor for 15 h at 37°C. The cells were then mock-infected or poliovirus-infected (lanes 3 and 6) for 5 h or incubated with TNF-α for 6 h at 37°C. HeLa whole-cell extracts (150 μg) were loaded onto a 5–14% SDS–PAGE gel, transferred to nitrocellulose, and probed with a p53 polyclonal antibody, an Igβ-α polyclonal antibody, and an actin monoclonal antibody. (B) γ-thio ATP does not inhibit 3CPro-mediated degradation of p53. In vitro translated [35S]methionine-labeled p53 in reticulocyte lysate was incubated in the absence (lane 1) and in the presence of 1 μg 3CPro (lanes 2–4) and with 5 mM γ-thio ATP (lane 4) and 10 mM γ-thio ATP (lane 3) or without γ-thio ATP (lane 2). Lanes 5 and 6 show cleavage of [35S]methionine-labeled TBP in the absence (lane 6) and in the presence of 1 μg 3CPro (lane 6). Molecular weight markers are shown on the left.

Degradation of p53 by 3CPro in HeLa cells is inhibited by vaccinia virus infection

To examine whether 3CPro directly cleaves p53 it was necessary to purify p53 from HeLa cells. Because p53 is not an abundant protein in HeLa cells, a recombinant vaccinia virus harboring an epitope-tagged p53 was used to infect HeLa cells and extracts were prepared from these cells. Western analysis using the p53 antibody showed that a significant amount of p53 was made in these cells (Fig. 6A, lane 1). No endogenous p53 was detectable when 10 or 20 μg of uninfected HeLa cell extracts was used in this assay (data not shown). interestingly, when purified 3CPro was incubated with the
vaccinia-infected p53-containing extract, degradation of p53 was not observed (Fig. 6A, compare lane 2 with lanes 3–5). In fact, even very high concentrations of purified 3C<sup>pro</sup> (8 μg) were unable to mediate degradation of p53 (Fig. 6A, compare lane 2 with lane 5). Under similar conditions, however, much smaller amounts of 3C<sup>pro</sup> were sufficient to cleave transcription factor TBP in the same extract (Fig. 6B), indicating that the 3C<sup>pro</sup> activity was not blocked in extracts prepared from vaccinia-infected cells. It is important to note that degradation of endogenous HeLa p53 by 3C<sup>pro</sup> was detected in our previous experiment with as little as 0.8 μg of 3C<sup>pro</sup> (Fig. 3A). To investigate the effect of vaccinia infection on endogenous p53 levels, HeLa cells were infected with vaccinia virus for 20 h. Extracts were prepared from these cells and analyzed by Western blot with p53 antibody (Fig. 6C). Increasing amounts of purified 3C<sup>pro</sup> were incubated with the cellular extracts. As little as 0.5 μg of 3C<sup>pro</sup> in mock-infected extracts decreased the levels of endogenous p53 by 54% (Fig. 6C, lanes 1 and 2). When 1 or 2 μg of 3C<sup>pro</sup> was added (Fig. 6C, lanes 3 and 4), the level of p53 present decreased by 89 and 95.5%, respectively, whereas in vaccinia virus-infected extracts, the level of p53 did not decrease even when 2 μg of 3C<sup>pro</sup> was added (lane 8). It appears, therefore, that vaccinia virus infection somehow interferes with 3C<sup>pro</sup>-mediated cleavage of p53.

Vaccinia virus is known to use a novel strategy to prevent IL-1 action (anti-inflammatory response) through the inactivation of IL-1β-converting enzyme (ICE). Two vaccinia virus-encoded proteins, SPI-2 and CrmA, inhibit ICE (Caspase 1), preventing the intracellular conversion of the inactive precursor IL-1β to the active secreted form (Ray et al., 1992). In this way CrmA functions as a viral anti-inflammatory molecule. To investigate whether the ICE inhibition by vaccinia-encoded proteins results in the inhibition of the cellular activity necessary for 3C<sup>pro</sup>-mediated degradation of p53, we utilized the ICE inhibitor, Z-VAD-fmk (Barco et al., 2000). HeLa cells were preincubated for 16 h with caspase inhibitor (Z-VAD-fmk) or caspase inhibitor control (F-A-fmk). Cells were then mock- or PV-infected [multiplicity of infection (m.o.i.) of 25] for 5 h. Whole-cell extracts were then subjected to Western blot analysis using anti-p53 and anti-β-tubulin antibodies. Extracts from cells which were mock-infected in the absence (Fig. 7, lane 1) or in the presence of either 20 μM caspase I inhibitor (lane 3) or 20 μM caspase inhibitor control (lane 4) all showed relatively similar levels of p53. In all extracts infected with PV (Fig. 7, lanes 2 and 5–10), p53 was completely degraded regardless of whether caspase I inhibitor was present (lanes 5–7) or not (lanes 2 and 8–10). Western blot analysis of the same extracts with anti-PARP antibody was performed to demonstrate that the caspase inhibitor and caspase inhibitor control were working under the same assay conditions (data not shown). An additional control was used in which Western blot analysis of the extracts was performed with anti-CREB antibody to demonstrate that the caspase inhibitor and caspase inhibitor control had no significant effect on 3C<sup>pro</sup> catalytic activity (data not shown). These results indicate that the IL-1β converting enzyme is not the cellular activity inhibited in vaccinia virus-infected extracts that is necessary for 3C-mediated degradation of p53.

FIG. 6. Vaccinia virus inhibits 3C<sup>pro</sup>-mediated degradation of p53. (A) HeLa cells were infected with vaccinia virus as described under Materials and Methods. Cell-free extracts were prepared and analyzed by Western blotting using the anti-p53 monoclonal antibody. Ten or 20 μg of total VV-ep53-infected cell extract was incubated in the absence of purified 3C<sup>pro</sup> (lanes 1 and 2, respectively) and 20 μg of extract was incubated in the presence of 0.08 (lane 3), 0.8 (lane 4), and 8 (lane 5) μg of purified 3C<sup>pro</sup>. (B) The same extracts were also used in Western blot analysis using anti-TBP antibody following incubation in the absence (lane 1) and in the presence of 0.08 (lane 2) and 0.8 (lane 3) μg of purified 3C<sup>pro</sup>. (C) HeLa cells were mock-infected or vaccinia virus-infected with recombinant vaccinia virus which expresses T7 at an m.o.i. of 10. Cell-free extracts were prepared and analyzed by Western blotting using the anti-p53 monoclonal antibody. Two hundred micrograms of total mock- or VV-T7-infected cell extracts was analyzed by Western blotting using the anti-p53 monoclonal antibody. Ten or 20 μg of extract was incubated in the absence (lanes 1 and 5, respectively) and in the presence of 0.5 μg (lanes 2 and 6, respectively), 1 μg (lanes 3 and 7, respectively), and 2 μg (lanes 4 and 8, respectively) μg of purified 3C<sup>pro</sup>.
Requirement of a cellular factor(s) for 3CPro-mediated p53 degradation

To address the possibility that one or more HeLa cell proteins (or activities) might be involved in 3CPro-mediated p53 degradation, epitope-tagged p53 was purified from the recombinant vaccinia virus-infected cells by affinity chromatography and its degradation by 3CPro was examined in the presence and in the absence of uninfected HeLa cell extract by Western blot analysis. Addition of 0.8 mg of purified 3CPro to the affinity-purified epitope-tagged p53 decreased the intensity of the p53 band by 25% of the control (compare lanes 1 and 2 in Fig. 8). At the lowest concentration of HeLa extract (0.5 μg), degradation of p53 by 3CPro was not significant (Fig. 8, lane 3). However, at 1 and 2 μg of HeLa cell extract, 75–85% of 3CPro-mediated degradation of p53 was observed compared to the control (Fig. 8, lanes 4 and 5). At these concentrations of HeLa cell extracts (1 and 2 μg), the endogenous p53 was beyond detection by Western analysis (data not shown). Two micrograms of HeLa extract in the absence of 3CPro had no significant effect (Fig. 8, lane 9). Heating the HeLa extract at 85°C for 10 min almost totally abolished its p53 degradation activity in the presence of 3CPro (Fig. 8, lane 7). 3CPro-mediated p53 degradation in the presence of 2 μg of rabbit reticulocyte lysates was more complete than that observed with 2 μg of HeLa extract (Fig. 8, compare lane 8 with lane 5). Incubation of purified p53 with reticulocyte lysate alone had no significant effect on p53 degradation (Fig. 8, lane 11). Approximately 40% degradation of p53 was observed in the presence of 2 μg wheat germ extract (in the presence of 3CPro) compared to the control (lane 6). These results suggest that both reticulocyte lysate and HeLa cell extracts contain activities that are required for 3CPro-mediated degradation of purified p53 and that this activity in HeLa cell extract is heat labile.

Fractionation of cellular activity

In order to isolate the cellular activity involved in the 3CPro-mediated degradation of p53, 9 liters of HeLa cells...
The evidence that 3C<sup>P<sub>pro</sub></sup> is required for p53 degradation comes from both genetic and biochemical studies. First, a mutant poliovirus (Se1 3C-02) defective in the 3C-protease function is ineffective in catalyzing p53 degradation in infected cells compared to the wild-type virus (Fig. 2). Second, a purified preparation of bacterially expressed 3C<sup>P<sub>pro</sub></sup> is capable of catalyzing degradation of endogenous p53 in HeLa cell extract or p53 synthesized in reticulocyte lysate by <i>in vitro</i> translation (Fig. 3). Only the wild-type protease and not an inactive mutant protease was able to degrade p53 (Fig. 3). One of the differences between the cleavage of other transcription factors (such as TBP, CREB, Oct-1) and p53 by 3C<sup>P<sub>pro</sub></sup> was that no distinct proteolytically cleaved products of p53 were detectable both <i>in vitro</i> and <i>in vivo</i>. The precise reason for this is not known. It is possible that the p53 antibodies used were unable to react with the cleaved products. Alternatively, cleaved products may have been degraded quickly by cellular proteases. The 3C<sup>P<sub>pro</sub></sup>-catalyzed degradation of p53 may not involve the ubiquitin pathway of protein degradation because p53 degradation in poliovirus-infected cells is not inhibited by the proteasome inhibitor, MG132 (Fig. 5A).

Many DNA tumor viruses appear to inhibit p53 function, which apparently leads to cellular transformation. It is believed that the p53 transcriptional activator function is necessary for p53 to function as a tumor suppressor (Vogelstein and Kinzler, 1992). The large T antigen of SV-40 forms a complex with p53, inhibiting its DNA-binding activity and consequently its transcriptional activity (Bargonetti <i>et al.</i>, 1992; Farmer <i>et al.</i>, 1992). Interestingly, the human papilloma virus type 16 (HPV-16) E6 protein complexes with p53 and, in cooperation with a
cellular protein, promotes polyubiquitination of p53, resulting in its degradation (Scheffner et al., 1990).

The evidence that p53 is not directly cleaved by 3CPro came from the experimental result that infection of cells with a recombinant vaccinia virus expressing the p53 protein resulted in the lack of p53 degradation in these extracts by 3CPro (Fig. 7). Indeed, purified p53 is not degraded by 3CPro and is degraded only when incubated with both 3CPro and HeLa cell extract (Fig. 8). Finally, we were able to partially purify the cellular activity required for 3CPro-mediated degradation of p53. The activity required along with 3CPro to degrade p53 appears to be heat labile, suggesting that it is a protein (Fig. 9). Reticuloocyte lysates appear to contain a considerable amount of this activity.

The fact that vaccinia virus inhibits the cellular activity required for p53 degradation suggests that it may belong to one of those inflammatory response proteins (enzymes) that are inhibited by vaccinia virus (Ray et al., 1992). It is now well established that a vaccinia virus gene product (p38) [cytokine response modifier (Crm A) protein] inhibits IL-1β matrerase (ICE protease), a protease involved in the processing of the IL-1β precursor to mature IL-1β. It is possible that the poliovirus 3CPro activates a similar cellular protease, which in turn degrades p53. In fact, the poliovirus 3CPro has been shown to induce apoptosis in HeLa cells (Barco et al., 2000). The 3CPro-mediated apoptosis is blocked by the caspase inhibitor Z-VAD-fmk, suggesting that caspase 1 (ICE protease) may be responsible for 3CPro-induced apoptosis. The experiments reported here, however, suggest that the cellular activity required for p53 degradation is probably not the ICE protease as degradation is not inhibited by the ICE inhibitor Z-VAD-fmk (Fig. 7). The concept of indirect cleavage of cellular proteins by a viral protease already exists for the poliovirus 2A protease. It is believed that 2APro activates a cellular enzyme which then cleaves the cap-binding protein necessary for cap-dependent translation leading to cellular translation shut-off (Etchison et al., 1982; Lloyd et al., 1986; Krausslich and Wimmer, 1988; Lawson and Semler, 1990). However, some recent reports suggest that the 2APro is capable of cleaving the cap-binding protein directly (Haghighat et al., 1996; Sommergruber et al., 1994). In addition, cleavage of the poliovirus capsid precursor (P1) by the viral protease 3CD is facilitated by a cellular cofactor. This cofactor facilitates recognition of the P1 precursor (Blair et al., 1993). It was previously found that the cellular protein human heat shock protein 70 (HSP70) is associated with the capsid precursor P1 of poliovirus and cowpoxvirus B1 in infected HeLa cells. It was observed that anti-virion serum communoprecipitated HSP70 from virus-infected cell extracts, but not from mock-infected cell extracts (Macejak and Sarnow, 1992).

The mechanism by which both the 3CPro and the cellular activity participate in p53 degradation is not known. A likely scenario is that 3CPro may activate a cellular protease which in turn degrades p53. Alternatively, interaction of one or more cellular proteins with p53 may lead to alteration of p53 secondary structure, resulting in 3CPro-mediated cleavage. Preliminary experiments, however, have shown that preincubation of the cellular activity (0.6 M wash from phosphocellulose column) with 3CPro, but not with purified p53, significantly increases the kinetics of p53 degradation in vitro (data not shown). Thus, an interaction between the cellular factor(s) and 3CPro may result in modification and/or activation of the cellular activity required for p53 degradation.

A number of viruses including poliovirus affect the balance between apoptosis-inducing and apoptosis-suppressing functions that ultimately help viral replication and spread of virus infection (Teodoro and Branton, 1997; Tolskaya et al., 1995; Agol et al., 1998). While regulated expression of 3CPro has been shown to induce apoptosis (Barco et al., 2000), degradation of p53 in poliovirus-infected HeLa cells (this report) could be a mechanism by which apoptosis could be delayed or prevented. Thus, 3CPro-induced degradation of p53 in PV-infected cells could not only be involved in inhibition of transcriptional activation but also result in delay or prevention of apoptosis. The latter could help ensure that progeny viruses are made and released without interference from apoptosis.

Future studies will focus on the purification and identification of the cellular activity required for 3CPro-mediated degradation of p53 from HeLa cells and/or rabbit reticulocytes. Such studies should shed more light onto the mechanisms by which poliovirus manipulates cellular functions for its own benefit.

MATERIALS AND METHODS

Cells and viruses

HeLa cells were grown in spinner culture in minimum essential medium (Gibco BRL) supplemented with 5% calf serum, 1 g glucose per liter, and 10^5 units per liter penicillin or as monolayers in Dulbecco’s modified Eagle’s medium (Gibco BRL) supplemented with 5% newborn calf serum, 5% fetal bovine serum, and 10^6 units per liter of penicillin/streptomycin. Cells were infected with poliovirus type 1 (Mahoney strain) at a multiplicity of infection of 25, as previously described (Dasgupta, 1983). HeLa cells were also infected with a poliovirus mutant SE1 3C-02 at a multiplicity of infection of 25, as previously described (Clark et al., 1991). Vaccinia virus infection of HeLa cells was performed as described previously (Yew et al., 1994).

Preparation of extracts

Nuclear extracts were prepared from mock- and poliovirus-infected HeLa cells as previously described (Dignam et al., 1983).
Expression and purification of p53

pTM.1-ep53, expressing epitope-tagged p53, was constructed by PCR amplification of human p53 from the pcDNA-p53 plasmid (Liu et al., 1993), using primers that introduce an NcoI site and the amino acid sequence MYPYDVPDYA (influenza virus epitope) at the 5' end and a BamHI site at the 3' end. The amplified DNA fragments were cloned between the NcoI and the BamHI sites of the pTM.1 vector (Elroy-Stein et al., 1989). Recombinant vaccinia virus expressing epitope-tagged human p53 was constructed by the procedure previously described by Elroy-Stein et al. (1989). Correct recombinant vaccinia virus was confirmed by Western analysis of cell extracts prepared from infected cells. Recombinant vaccinia virus (VV-ep53) was plaque purified and viral titers were determined by plaque assay on HeLa cells.

Epitope-tagged p53 was purified from vaccinia virus-infected HeLa cell monolayers. Approximately 5.5 × 10⁶ cells were infected with VV-ep53 and V-TF7-3, which expresses the T7 RNA polymerase, at an m.o.i. of 10 and 5, respectively. Infection was stopped at 30 h postinfection and whole-cell extracts were made by freeze/thawing the cells in phosphate-buffered saline (PBS). One milliliter of nuclear extract was incubated with 20 μl of packed beads of protein A–Sepharose covalently linked to monoclonal antibody HA.11 (BabCo) at 4°C for 16 h (Liu et al., 1993). The HA11 affinity beads were washed three times with 0.4 M KCl buffer D [20 mM HEPES (pH 7.9), 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF] and two times with the same buffer containing 0.1 M KCl. Epitope-tagged p53 was eluted with 100 mM PMSF and two times with the same buffer containing 0.4 M KCl buffer D [20 mM HEPES (pH 7.9), 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF]. The purified epitope-tagged p53 was dialyzed three times with 0.4 M KCl buffer D [20 mM HEPES (pH 7.9), 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF] and two times with the same buffer containing 0.1 M KCl. Epitope-tagged p53 was eluted with 100 mM of the influenza virus HA peptide (Sigma/BabCo) (YPYDVPDYA) at 100 mg/ml in the same buffer containing 0.1 M KCl. The purified epitope-tagged p53 was dialyzed against buffer D containing 0.1 M KCl overnight at 4°C in a dialysis membrane with a molecular weight cutoff of 30,000.

Protease reactions

The poliovirus protease 3CPro was PCR amplified from poliovirus infectious cDNA pT7PV1 and cloned into pQE30 (Qiagen) in the BamHI and HindIII sites as described previously (Yalamanchili et al., 1996). 3CPro DNA was used for transforming E. coli expression cells M15pRep4, and the plasmid 3CM15pRep4 at an optical density of 0.7 was induced with 1 mM isopropyl thiogalactoside for 4 h. The histidine-tagged 3CPro was purified from induced cells by nondenaturing nickel affinity chromatography as previously described (Hammerle et al., 1991; Nicklin et al., 1988). The indicated amounts of 3CPro were incubated with mock-infected extracts, affinity-purified p53 or in vitro translated p53 at 30°C for 4 h as previously described (Yalamanchili et al., 1996). Reactions were performed in the presence of 100–200 μg of HeLa cell extract or rabbit reticulocyte lysate (Promega) unless otherwise indicated.

Antibodies

The p53 antibodies consisted of either a mouse monoclonal antibody (DO-1, Santa Cruz) raised against an epitope corresponding to amino acids 11–25 or a goat polyclonal antibody raised against full-length p53 (FL-393, Santa Cruz Biotechnologies). The Oct-1 antibody (C-21, Santa Cruz Biotechnologies) was a rabbit polyclonal antibody which recognizes the carboxy terminus of Oct-1 of human origin. The β-tubulin antibody (KMX-1, Boehringer Mannheim) was a mouse monoclonal antibody from mouse–mouse hybrid cells. The actin antibody (ILA20, Oncogene Research Products) was a mouse monoclonal antibody raised against chicken cytoskeletal proteins and recognized α-, β-, and γ-actin. The PARP antibody (Boehringer Mannheim) was a rabbit polyclonal antibody. The TBP antibody (SI-1, Santa Cruz Biotechnologies) was a rabbit polyclonal antibody prepared by immunization with full-length E. coli-produced TBP of human origin. The 1κβ-α antibody (C-21, Santa Cruz Biotechnologies) was a goat polyclonal antibody raised against the carboxy terminus of 1κβ-α.

Western blot analysis

Proteins were separated by SDS–14% PAGE. The separated proteins were electrophoretically blotted onto nitrocellulose. The nitrocellulose was blocked for 1 h with 1% blocking reagent (Boehringer Mannheim). The blot was probed with the specific primary antibody for 1 h. All corresponding secondary antibodies were horseradish peroxidase conjugates obtained from Boehringer Mannheim. The immunoblot was developed using the chemiluminescence detection system (Pierce) as described by the manufacturer.

Proteasome inhibitor studies

HeLa cell spinner cultures (7.2 × 10⁷ cells) were preincubated with the proteasome inhibitor MG132 (Sigma) at a concentration of 10 μM for 15 h at 37°C. The cells were then mock-infected, poliovirus-infected, or treated with TNF-α (20 ng/ml, Sigma) for 5–6 h at 37°C. The cells were pelleted and washed twice with ice-cold PBS, and whole-cell extracts were made and assayed by Western blot analysis.

Caspase inhibitor studies

HeLa cell monolayers (2.5 × 10⁶ cells) were preincubated with the caspase inhibitor Z-VAD-fmk or with the caspase inhibitor control F-A-fmk (Enzyme Systems Products), in the indicated amounts. The preincubation lasted approximately 16 h at 37°C. The cells were then mock- or poliovirus-infected and incubated for 5 h at
37°C. The cells were scraped in PBS, and whole-cell extracts were made and assayed by Western blot analysis.

Partial purification of the cellular activity from HeLa cells

Nuclear extract was prepared from 9 liters of HeLa cell spinner culture as previously described (Digman et al., 1983). Nuclear extracts were precipitated with finely ground ammonium sulfate at 40, 80, and 100% saturation. The pellets were resuspended in Buffer D and dialyzed against this buffer overnight at 4°C. The dialyzed ammonium sulfate fractions were assayed for p53 degradative activity as described for the protease reaction above. The reminder of the 40–80% ammonium sulfate saturated fraction was run over a phosphocellulose column following the protocol provided by the manufacturer (Sigma). The column was equilibrated with Buffer D containing 0.1 M KCl and eluted with Buffer D containing 0.3, 0.6, and 1 M KCl. These fractions were dialyzed against Buffer D overnight at 4°C. Aliquots of each elution were stored at −70°C for future use.

ACKNOWLEDGMENTS

This work was supported by NIH Grant AI 27451 to A.D. PY, was supported by Public Health Service Awards AI-07323 and GM-07104 from the National Institutes of Health. We are grateful to E. Berlin for her excellent secretarial assistance.

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