Nuclear entry of poliovirus protease-polymerase precursor 3CD: implications for host cell transcription shut-off

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Abstract

Host cell transcription mediated by all three RNA polymerases is rapidly inhibited after infection of mammalian cells with poliovirus (PV). Both genetic and biochemical studies have shown that the virus-encoded protease 3C cleaves the TATA-binding protein and other transcription factors at glutamine–glycine sites and is directly responsible for host cell transcription shut-off. PV replicates in the cytoplasm of infected cells. To shut-off host cell transcription, 3C or a precursor of 3C must enter the nucleus of infected cells. Although the 3C protease itself lacks a nuclear localization signal (NLS), amino acid sequence examination of 3D identified a potential single basic type NLS, KKKRD, spanning amino acids 125–129 within this polypeptide. Thus, a plausible scenario is that 3C enters the nucleus in the form of its precursor, 3CD, which then generates 3C by auto-proteolysis ultimately leading to cleavage of transcription factors in the nucleus. Using transient transfection of enhanced green fluorescent protein (EGFP) fusion polypeptides, we demonstrate here that both 3CD and 3D are capable of entering the nucleus in PV-infected cells. However, both polypeptides remain in the cytoplasm in uninfected HeLa cells. Mutagenesis of the NLS sequence in 3D prevents nuclear entry of 3D and 3CD in PV-infected cells. We also demonstrate that 3CD can be detected in the nuclear fraction from PV-infected HeLa cells as early as 2 h postinfection. Significant amount of 3CD is found associated with the nuclear fraction by 3–4 h of infection. Taken together, these results suggest that both the 3D NLS and PV infection are required for the entry of 3CD into the nucleus and that this may constitute a means by which viral protease 3C is delivered into the nucleus leading to host cell transcription shut-off.

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Introduction

Poliovirus (PV) is the prototype agent of a large group of medically important viruses (picornaviruses), which include those inducing infectious hepatitis (hepatitis A), common cold (rhinoviruses), encephalitis, and myocarditis (coxsackieviruses). The single-stranded, plus polarity RNA genome of PV is translated into one large polyprotein which is co-translationally processed by virus-encoded proteases 2A, 3C, and 3CD to generate the mature viral structural and non-structural proteins (Krausslich and Wimmer, 1988; Lawson and Semler, 1990; Leong et al., 2003). The viral proteases have been extensively studied and found to be very specific in polyprotein cleavage; 3C and 3CD cleave the polyprotein at gln–gly (Q–G) pair while the 2A cleaves only at tyr–gly (Y–G) bond. The proteases do not cleave every potential cleavage site within the polyprotein; other determinants such as accessibility and the context of the cleavage site are also important.

Host cell transcription mediated by all three RNA polymerases is rapidly inhibited after infection of mammalian cells with PV (Dasgupta et al., 2003; Kaariainen and Ranki, 1984). RNA polymerase I (pol I), the enzyme responsible for ribosomal RNA (rRNA) synthesis, is inhibited first after infection of HeLa cells with PV. Cellular mRNA synthesis by RNA polymerase II (pol II) is inhibited next, followed by shut-off of RNA polymerase III (pol III) that catalyzes synthesis of tRNA and 5S rRNA. Previous studies from our laboratory have identified four

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sequence-specific DNA binding pol II transcription factors (TF) that are either cleaved or degraded in PV-infected cells: the TATA-binding protein (TBP), the cyclic AMP-responsive element binding protein (CREB), the Octamer-binding factor (Oct-1), and the transcriptional activator p53 (Clark and Dasgupta, 1990; Clark et al., 1993; Weidman et al., 2001; Yalamanchili et al., 1996, 1997a,b). Similarly, the pol III factor, TFIIIC, which interacts with pol III promoters, as well as the 110-kDa TBP-associated factor (TAF 110), a subunit of the pol I factor SL-1 are also cleaved in PV-infected cells (Clark et al., 1991; Rubinstein and Dasgupta, 1989; Rubinstein et al., 1992; and data not shown). Both genetic and biochemical studies have shown that the virus-encoded protease 3C cleaves the transcription factors at glutamine–glycine sites and is directly responsible for host cell transcription shut-off (Dasgupta et al., 2003). The 3C protease of foot-and-mouth disease virus (FMDV), another member of the Picornavirus family, has been reported to induce proteolytic cleavage of host cell histone H3 (Falk et al., 1990). The significance of histone H3 cleavage by FMDV 3C, however, is not known.

PV replicates in the cytoplasm of infected cells. To shut-off host cell transcription, one or more viral gene products must enter the nucleus of the infected host cell. Because PV-encoded 3C protease has been shown to be directly responsible for transcription shut-off, it is likely that 3C must enter the nucleus of infected cells as it is, or in the form of a precursor to bring about transcription shut-off. Previous studies have reported detection of PV proteins including the protease precursor 3CD in the nuclei of infected cells (Bienz et al., 1982; Fernandez, 1982). The viral protease-polymerase precursor 3CD (Harris et al., 1992) is a protease itself, which efficiently cleaves the capsid precursor P1 to VP0, VP3, and VP1 (Parsley et al., 1999; Ypma-Wong et al., 1988) and is also able to auto-catalyze the formation of 3C and 3D polypeptides (Wimmer et al., 1993). These findings suggested to us that 3CD could interact with transcription factors in the nuclei of infected cells. A computer search did not reveal the presence of a nuclear localization signal (NLS) in 3C, although the relatively small size (~ 20 kDa) of 3C may allow diffusion of 3C into the nucleus at sufficiently high concentrations of this protease. Amino acid sequence examination of 3D, however, identified a potential single basic type NLS, KKKRD (Dingwall and Laskey, 1991), between amino acids 125 and 129 within this polypeptide. This sequence is well conserved among enteroviruses and is present within the finger sub-domain of PV 3D (Hansen et al., 1997). Previous studies have shown that triple alanine substitutions within the KKKRD sequence in the PV infectious cDNA clone were lethal (Diamond and Kirkegaard, 1994). Thus, it is possible to envision a scenario in which 3C enters the nucleus in the form of its precursor, 3CD, which then generates 3C by auto-proteolysis leading to cleavage of transcription factors. Using enhanced green fluorescent protein (EGFP) fusion polypeptides, we demonstrate here that both 3CD and 3D are capable of entering the nucleus in PV-infected cells. However, both polypeptides remain in the cytoplasm in uninfected HeLa cells. Triple alanine substitution of the first three or last three amino acids of the NLS sequence prevents nuclear entry of 3D/3CD in PV-infected cells. Using nuclear and cytoplasmic fractions isolated from PV-infected HeLa cells, we demonstrate that 3CD can be detected in the nuclear fraction as early as 2 h postinfection. These results suggest that (i) both the NLS and PV infection are required for the entry of 3CD into the nucleus, and (ii) this 3C-containing precursor may play an important role in host cell transcription shut-off by delivering 3C protease inside the nucleus.

Results

Expression of EGFP-fusion polypeptides

To investigate nuclear entry of the viral protease 3C, we prepared the following EGFP fusion constructs using the pEGFP-N1 vector: p3C-EGFP, p3D-EGFP, p3CD-EGFP, and pm3CD-EGFP (Fig. 1A). The m3CD-EGFP construct contained a mutation at the Q–G site of the 3C–3D junction to prevent auto-proteolysis of the 3CD polypeptide into mature 3C and 3D. HeLa cells were transfected with these plasmids and cell-free extracts were prepared from transfected cells after 24 h and examined for synthesis of EGFP-fusion proteins by immunoblotting using an antibody to EGFP. As can be seen in Fig. 1B, EGFP was highly expressed in cells transfected with the pEGFP (lane 2). There was fairly good expression of 3C-EGFP (~ 45 kDa) in cells transfected with p3C-EGFP; however, significant amount of free EGFP was also detected in the immunoblot (lane 3). This could be due to proteolysis of 3C-EGFP. Both the ~ 77-kDa 3D-EGFP (lane 4) and ~ 97-kDa 3CD-EGFP (lane 5) were also expressed and appeared to be stable under the conditions used for expression. The expression of m3CD-EGFP was comparable to that of 3CD-EGFP (lane 6). The Western analysis detected a band having an approximate molecular weight of 55 kDa that migrated between 3D-EGFP and 3C-EGFP (indicated by an arrowhead). Although the precise identity of the 55-kDa polypeptide is not known, we assume it contains part of the 3D sequence linked to EGFP.

PV infection results in nuclear entry of 3D and 3CD

The majority of the PV-encoded proteins are present in the cytoplasm of infected cell. To examine whether PV infection leads to nucleo-cytoplasmic relocalization of the protease precursor 3CD, HeLa cells were first transfected with the recombinant pEGFP-fusion plasmids and then either mock-infected or infected with PV. Initially, a time course of infection revealed nuclear entry of significant amounts of 3CD-EGFP between 3 and 4 h of infection
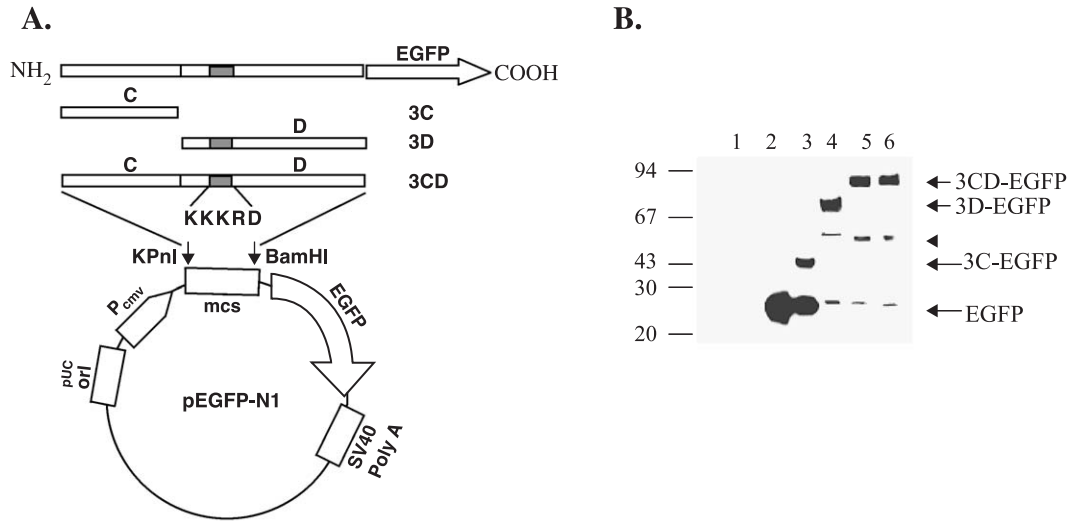
A.

B.

Fig. 1. Expression of EGFP-fusion polypeptides. (A) Schematic representation of the constructs used for expression of p3CD-EGFP, p3D-EGFP, and p3C-EGFP. The shaded area within the 3D sequence shows the location of the NLS (KKKRD, amino acids 125–129). (B) Western blot analysis of cell-free extracts prepared from HeLa cells transfected with no plasmid (lane 1), pEGFP (lane 2), p3C-EGFP (lane 3), p3D-EGFP (lane 4), p3CD-EGFP (lane 5), and pm3CD-EGFP (lane 6). The Q–G pair at the 3C–3D junction in m3CD-EGFP is mutated to an ala–ala pair to prevent processing into 3C and 3D.
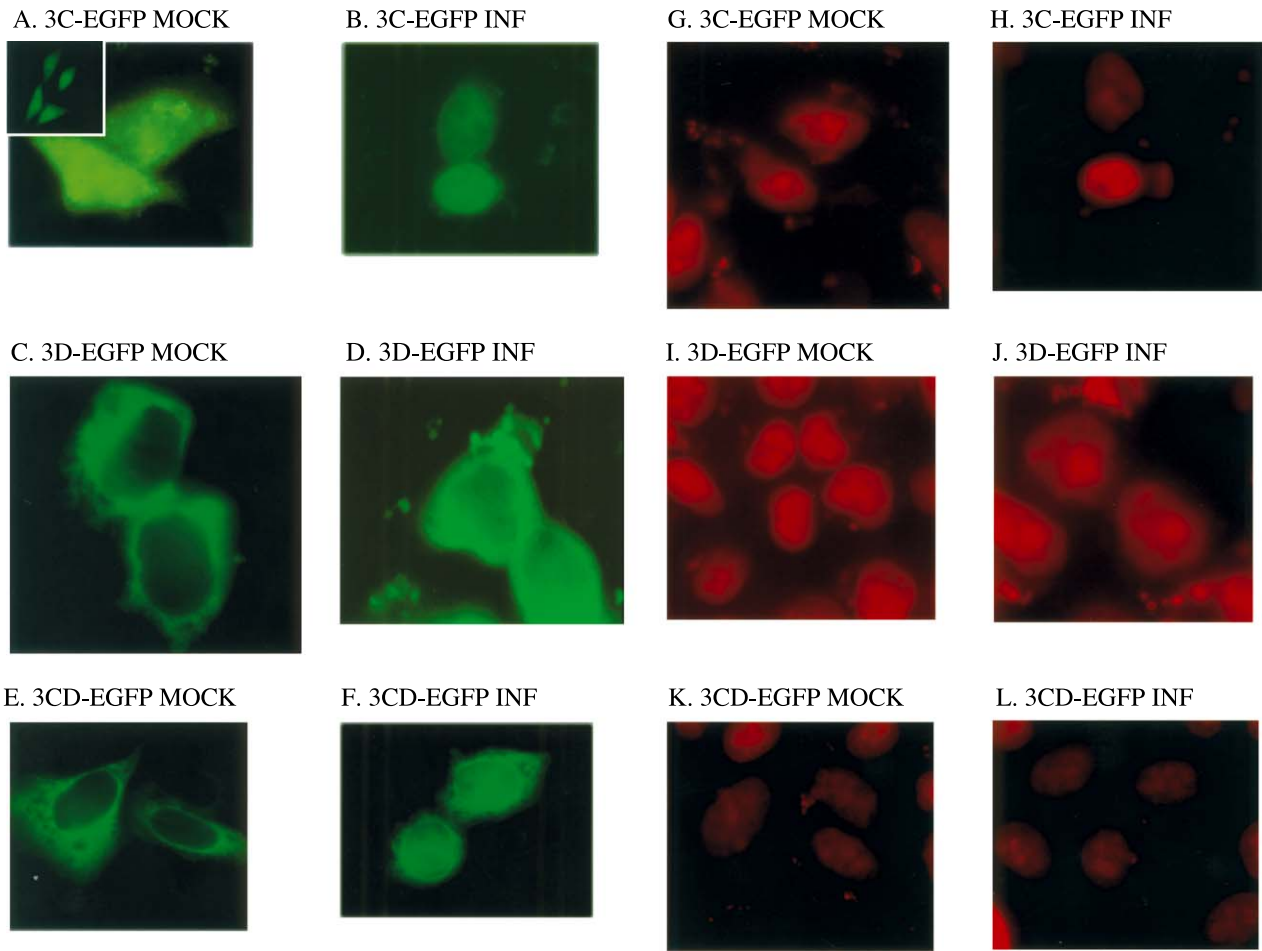

Fig. 2. EGFP fluorescence profiles of cells transfected with various EGFP-fusion constructs. The cells were transfected with pEGFP (A, inset), p3C-EGFP (A, B, G, and H), p3D-EGFP (C, D, I, and J), and p3CD-EGFP (E, F, K, and L). Transfected cells were either mock-infected (MOCK) or PV-infected (INF) for 4 h and EGFP fluorescence visualized using a fluorescence microscope (panels A–F). The cells shown in panels G–L were stained with PI to visualize nuclei.
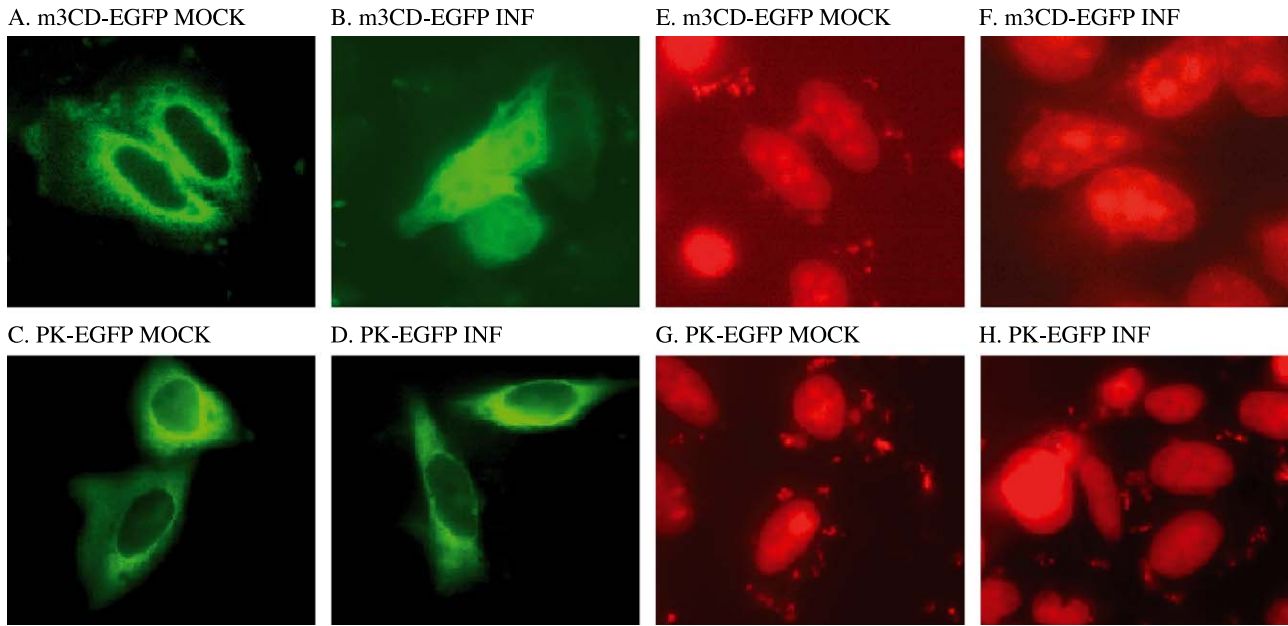
Fig. 3. EGFP fluorescence profiles of cells transfected with EGFP-fusion constructs. The cells transfected with pm3CD-EGFP (A, B, E, and F) and pPK-EGFP (C, D, G, and H) were either mock-infected (MOCK) or PV-infected (INF) and EGFP fluorescence visualized by fluorescence microscopy (panels A–D). The cells shown in panels E–H were stained with PI to visualize nuclei.

(data not shown). We, therefore, chose the 4 h time point to determine nuclear entry of 3CD for all experiments described below. When HeLa cells were transfected with pEGFP, EGFP expression was detected throughout the cell (Fig. 2A, inset). A similar pattern of fluorescence was observed in cells transfected with p3C-EGFP (Fig. 2A). This type of overall distribution of 3C-EGFP could be due to generation of relatively high quantities of free GFP in cells transfected with p3C-EGFP, which was evident from the immunoblot analysis of proteins from p3C-EGFP transfected cells (Fig. 1B, lane 3). Infection of p3C-EGFP transfected cells with PV did not significantly change the EGFP fluorescence profile except that the cells became round in appearance (Fig. 2B). Mock-infected HeLa cells expressing 3D-EGFP showed distinct cytoplasmic/ perinuclear localization (Fig. 2C). In contrast to 3C-EGFP, there was no significant 3D-EGFP fluorescence in the nucleus of mock-infected cells. These results are consistent with a previous report in which PV proteins were expressed following infection of mosquito cells with a recombinant baculovirus (Neufeld et al., 1991). Infection with PV of cells expressing 3D-EGFP resulted in significant increase in fluorescence within the nucleus compared with the mock-infected control (Fig. 2D, also see Fig. 5A). Similar results were observed in 3CD-EGFP-expressing cells; 3CD-EGFP was primarily localized in the cytoplasm in mock-infected cells and infection with PV led to translocation of significant amount of 3CD into the nucleus (Figs. 2E and F). Nuclear staining of these cells by propidium iodide (PI) showed that the nuclei were intact after 4 h of infection (Figs. 2G–L). To rule out the possibility that 3CD-EGFP nuclear relocaliza-

tion in infected cells could be due to generation of 3D-EGFP by proteolysis (or auto-proteolysis), a mutant 3CD-EGFP was used to transfect HeLa cells. In this mutant (m3CD-EGFP), the scissile gln–gly bond at the 3C–3D junction was mutated to an ala–ala pair to prevent proteolysis. In vitro protease cleavage assays showed that m3CD-EGFP was completely resistant to cleavage by exogenously added 3C protease (data not shown). As can be seen in Figs. 3A and B, the m3CD-EGFP protein behaved in a similar
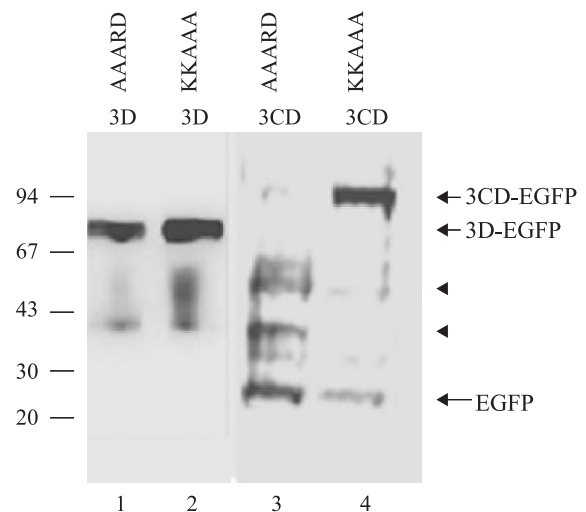

Fig. 4. Expression of 3D-EGFP and 3CD-EGFP NLS mutants. Cell-free extracts recovered from cells transfected with p3D$_{AAARD}$-EGFP (lane 1), p3D$_{KKAAA}$-EGFP (lane 2), p3CD$_{AAARD}$-EGFP (lane 3), and p3CD$_{KKAAA}$-EGFP (lane 4) were analyzed by Western blot using anti-EGFP.

A. **3D** WT (KKKRD)

INF

F. **3CD** WT (KKKRD)

INF

B. **3D** (AAARD)

MOCK

C. **3D** (AAARD)

INF

G. **3CD** (KKAAA)

MOCK

H. **3CD** (KKAAA)

INF

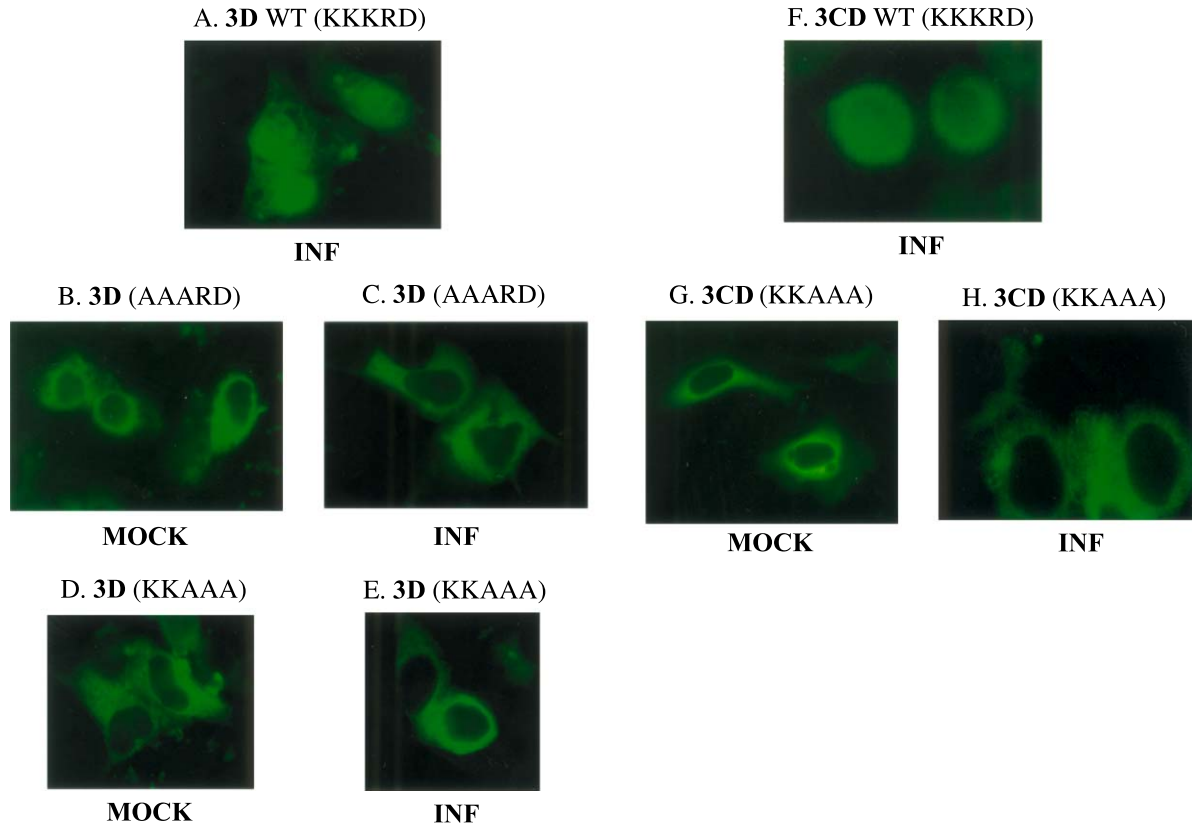D. **3D** (KKAAA)

MOCK

E. **3D** (KKAAA)

INF

Fig. 5. EGFP fluorescence profile of cells transfected with p3D-EGFP and p3CD-EGFP NLS mutants. HeLa cells transfected with wt p3D$_{KKKRD}$-EGFP (A), wt p3CD$_{KKKRD}$-EGFP (F), and NLS mutants p3D$_{AAARD}$-EGFP (B and C), p3D$_{KKAAA}$-EGFP (D and E), and p3CD$_{KKAAA}$-EGFP (G and H) were analyzed by fluorescence microscopy. Mutant 3D-EGFP and 3CD-EGFP transfected cells were either mock-infected (MOCK) or infected with PV (INF). Only infected cells are shown for wt 3D-EGFP and 3CD-EGFP transfected cells.

manner to that observed with the wt 3CD-EGFP (Figs. 2E and F). Majority of the m3CD-EGFP was present in the cytoplasm of uninfected cells and PV infection resulted in nuclear entry of significant amount of this protein. As a control, HeLa cells were transfected with a commercially available soluble protein kinase (PK), which lacks a NLS. Transfection of cells with pPK-EGFP resulted in cytoplasmic expression of this protein as expected (Fig. 3C). Infection of cells expressing pPK EGFP with PV for 4 h did not result in significant nuclear fluorescence of this soluble protein compared with the mock-infected cells (Fig. 3D). Panels E−H (Fig. 3) show staining of nuclei by PI. The results presented in Figs. 2 and 3 suggest that a significant portion of total 3CD and 3D enter the nucleus in cells infected with PV.

NLS mutation interferes with nuclear entry of 3D and 3CD in PV-infected cells

To determine whether the NLS present within the 3D sequence plays a role in nuclear entry of 3D and 3CD, the wt NLS sequence (KKKRD) was mutated to either AAARD or KKAAA by triple alanine substitution. These mutations, when introduced into the infectious PV cDNA clone, were found to be lethal (Diamond and Kirkegaard,

A. m3CD-EGFP

INF

B. m3CD (KKAAA)-EGFP

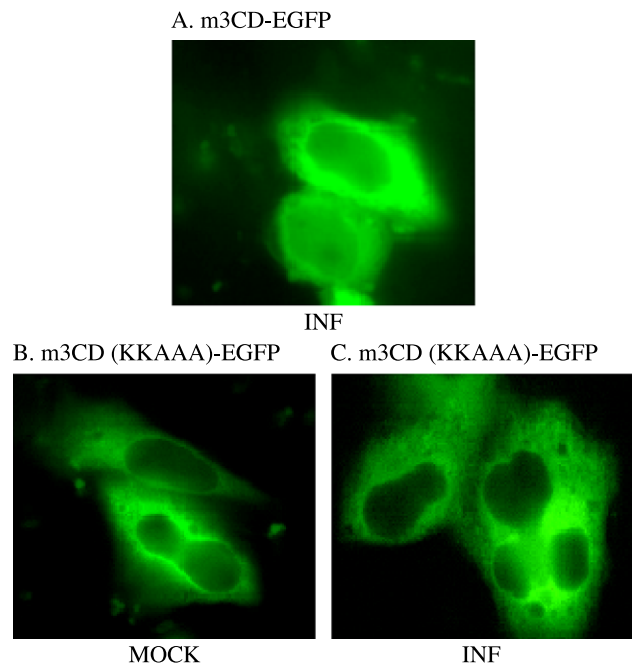MOCK

C. m3CD (KKAAA)-EGFP

INF

Fig. 6. EGFP fluorescence profile of cells transfected with pm3CD-EGFP NLS mutants. The pm3CD-EGFP fusion plasmid with the wt NLS (A), and pm3CD-EGFP containing the mutated NLS sequence (KKKRD to KKAAA) (B, C) were transfected into HeLa cells. Transfected cells were either mock-infected (B) or PV-infected (A and C) for 4 h.

1994). Both mutants were expressed in HeLa cells as EGFP-fusion proteins. Western analyses revealed that both the 3D(AAARD)-EGFP and 3D(KKAAA)-EGFP polypeptides were produced in transfected cells and the level of expression was similar to that of the wt 3D-EGFP (Fig. 4, lanes 1 and 2), and data not shown). Similarly, the recombinant 3CD-EGFP harboring the KKAAA NLS mutation was expressed in significant amounts and was stable under the conditions used for expression (Fig. 4, lane 4). Surprisingly, despite repeated attempts, we were unable to express the full-length, recombinant 3CD-EGFP harboring the AAARD mutation in reasonable quantities. Western analysis revealed that the majority of expressed protein was degraded to a number of lower molecular weight polypeptides that included significant amount of EGFP and at least two other products having molecular masses of approximately 55 and 43 kDa (indicated by arrowheads, Fig. 4, lane 3). The 55-kDa band detected here could be the same polypeptide as seen previously in Fig. 1B (lanes 5 and 6). We do not know the precise reasons for the instability of 3CD(AAARD)-EGFP. It is possible that the mutation

(KKKRD to AAARD) introduced into the protein has somehow made this polypeptide unstable. It should be noted, however, that that the same mutation had no significant effect on the stability of 3D-EGFP (Fig. 4, lane 1). We, therefore, were unable to use the 3CD(AAARD)-EGFP plasmid in the following experiment to delineate the effects of the NLS mutations on nuclear-cytoplasmic distribution of 3CD. The nuclear-cytoplasmic distribution of the mutant polypeptides 3D(AAARD)-EGFP, 3D(KKAAA)-EGFP, and 3CD(KKAAA)-EGFP was examined in PV- or mock-infected cells by fluorescence microscopy. In this particular experiment, the localization of wt 3D-EGFP in PV-infected cells was distinctly nuclear, although some fluorescence was detected throughout the cell cytoplasm (Fig. 5A). Infected cells expressing wt 3CD-EGFP showed both cytoplasmic and nuclear fluorescence (Fig. 5F). Although not shown here, expression patterns of both wt 3D-EGFP and 3CD-EGFP in mock-infected cells were almost identical to that seen previously (Figs. 2C and E). In the absence of PV infection, both the 3CD-EGFP and 3D-EGFP mutants were predominantly localized in the cell cytoplasm (perinuclear,
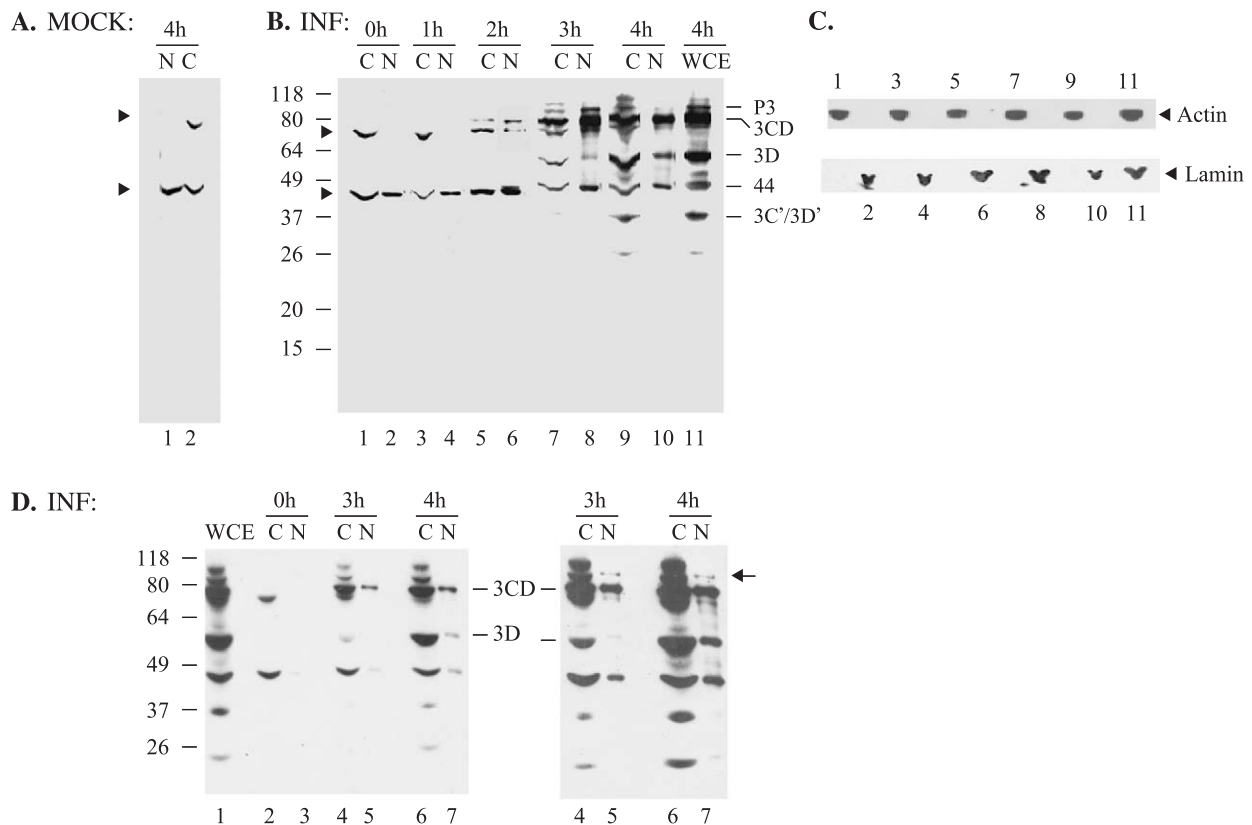

Fig. 7. Detection of 3D and 3CD in nuclear and cytoplasmic fractions recovered from PV-infected cells. HeLa cells (3×10^6) were mock-infected for 4 h (A) or infected with PV (B) at an moi of 25 for 0 (lanes 1 and 2), 1 (lanes 3 and 4), 2 (lanes 5 and 6), 3 (lanes 7 and 8), and 4 (lanes 9 and 10) h. Nuclear (N) and cytoplasmic (C) fractions prepared from these cells were examined by Western blot analysis using a polyclonal antibody to 3D. In lane 11, a whole cell extract (WCE) from 4 h infected cells was examined by Western analysis using anti-3D antibody. (C) The upper and lower panels show Western analyses of actin and lamin for cytoplasmic and nuclear fractions, respectively, as loading controls. (D) Cytoplasmic (C) and nucleoplasmic (N) fractions recovered from cells infected for 0 (lanes 2 and 3), 3 (lanes 4 and 5), and 4 (lanes 6 and 7) h of infection were used for Western analysis using 3D antibody. Lane 1 shows Western analysis of a whole cell extract recovered from 4 h infected cells. The panel on the right shows an overexposed blot for lanes 4–7 from the same experiment.

Figs. 5B, D and G). Unlike the wt proteins (Figs. 5A and F), infection with PV did not result in significant redistribution of the mutant proteins into the nucleus (Figs. 5C, E and H). These results suggest that triple alanine substitution of the NLS prevent nuclear entry of 3D and 3CD in PV-infected HeLa cells. We also examined the localization pattern of m3CD-EGFP with the KKAAA NLS mutation. The cells transfected with pm3CD-EGFP containing the wt NLS (KKKRD) sequence showed GFP fluorescence both in the cytoplasm and nucleus (Fig. 6A). However, the majority of m3CD(KKAAA)-EGFP was found to localize to the cytoplasm irrespective of whether the cells were mock-infected or infected with PV (Figs. 6B and C). These results suggest that both the NLS and PV infection are required for entry of 3D and 3CD into the nucleus.

Detection of 3CD in nuclear fractions from PV-infected HeLa cells

To confirm results of transient transfection experiments using the pEGFP-fusion plasmids, we examined distribution of viral 3CD/3D polypeptides in nuclear and cytoplasmic fractions of HeLa cells infected with PV. HeLa monolayer cells were infected with PV at a multiplicity of infection (moi) of 25 and cells were harvested at 0, 1, 2, 3, and 4 h postinfection. Cell-free lysates were prepared following cell lysis and removal of membrane and cell debris by centrifugation. Nuclei were separated from the cytoplasmic fraction by centrifugation followed by repeated washing of the nuclei with buffer containing non-ionic detergent NP-40. The washing step was repeated until 3CD could no longer be detected in the wash. Nuclear extracts were then prepared and 20 μg of cytoplasmic and nuclear extracts were examined by Western blot analysis using a polyclonal antibody to 3D. As can be seen in Fig. 7B, no viral protein could be detected in cytoplasmic or nuclear fractions harvested at 0 and 1 h of infection (lanes 1–4). The two background polypeptides migrating at approximately 44 and 69 kDa in the cytoplasmic fraction and one polypeptide (~44 kDa) in the nuclear fraction (indicated by arrowheads, lanes 1–4, Fig. 7B) were also detected in nuclear and cytoplasmic fractions prepared from mock-infected cells (Fig. 7A). A 72-kDa polypeptide that comigrates with 3CD was detected as early as 2 h postinfection in both cytoplasmic and nuclear fractions (Fig. 7B, lanes 5 and 6). At 3 h postinfection, considerable amount of 3CD was detected in the nuclear fraction (lane 8). In contrast to 3CD, very little 3D polymerase was detected in the nuclear fraction at 3 h postinfection (lane 8). Considerable amounts of both 3CD and 3D polypeptides were detected in the nuclear fraction at 4 h postinfection (lane 10). We also detected another virus-encoded protein in the nuclear fraction at 3 h postinfection, which migrated slower than 3CD (lanes 8). The estimated molecular weight (~86 kDa) and migration pattern of this polypeptide was consistent with that of the viral precursor P3. Fig. 7C shows Western analyses of actin and lamin from

the cytoplasmic and nuclear fractions, respectively from infected cells as loading controls. We also examined the possibility that a portion of the 3CD in the nuclear fraction could be associated with the nuclear membrane rather than being nucleoplasmic. Therefore, the nucleoplasmic fraction, after removal of nuclear membranes, was examined by Western blot analysis. As can be seen in Fig. 7D, significant amount of 3CD was still present in the nucleoplasmic fraction at 3 and 4 h postinfection (lanes 5 and 7). Overexposure of the same immunoblot showed considerable amounts of both 3CD and 3D at 4 h as well as a small amount of the precursor predicted to be P3 (indicated by an arrow). These results suggest that 3CD and possibly some other 3D precursors are capable of translocating into the nucleus in PV-infected cells.

Discussion

We have shown that the PV-encoded protease precursor 3CD is capable of translocating into the nucleus in PV-infected cells. Both the transiently expressed 3CD-EGFP-fusion protein and the native 3CD polypeptide synthesized in PV-infected cells were found to translocate into the nucleus within 2–4 h of infection. This ability to enter nucleus resides within the 3D portion of 3CD, which contains a single basic type NLS, KKKRD, between amino acids 125 and 129. Mutation of the NLS sequence prevents entry of 3CD/3D into the nucleus. The results presented here also suggest that the presence of NLS alone is not sufficient for nuclear entry of 3D/3CD; additional alterations in the nuclear membrane brought about by PV infection is also required for successful nuclear translocation of 3CD/3D.

Cells infected with PV undergo numerous changes that include shut-off of host cell translation and transcription (Dasgupta et al., 2003; Etchison et al., 1982; Gradi et al., 1998), inhibition of host protein secretion (Doedens and Kirkegaard, 1995), both induction and inhibition of apoptosis (Agol et al., 2000; Tolskaya et al., 1995), and the conversion of ER membranes to viral replication complexes (Bienz et al., 1983; Suhy et al., 2000). Whether one or more of these processes play any role in 3CD/3D nuclear translocation is not known. However, recent studies have shown that PV infection can cause inhibition of nuclear-cytoplasmic trafficking leading to accumulation of nuclear proteins in the cytoplasm (Belov et al., 2000; Gustin and Sarnow, 2001, 2002). Nuclear proteins such as the La autoantigen, Sam 68, and nucleolin accumulate in the cytoplasm of infected cells and interact with either viral RNA or virus-encoded proteins (McBride et al., 1996; Meerovitch et al., 1993; Waggoner and Sarnow, 1998). The cytoplasmic retention of La in PV-infected cells may, at least in part, be due to truncation of La by the 3C protease resulting in the loss of NLS (Shiroki et al., 1999). However, many proteins that relocate to the cytoplasm retain their NLS (Belov et al.,

2000; Gustin and Sarnow, 2001, 2002). Accumulation of the latter class of proteins in the cytoplasm could result from alteration of the nuclear pore complex as well as inhibition of active nuclear import. The TATA-binding protein (TBP), which is the major target for PV protease 3C, was found not to relocalize to the cytpoplasm of infected cells (Gustin and Sarnow, 2001). The inability of TBP to be translocated to the cytoplasm of infected cells necessitates entry of 3C and/ or 3CD or some other 3C-containing precursor into the nucleus and subsequent cleavage of TBP leading to shut-off of host cell transcription. It is interesting to note that despite accumulation of cellular proteins in the infected cell cytoplasm, virus-encoded proteins (3CD/3D) are capable of moving in the opposite direction in a NLS-dependent fashion. Therefore, nuclear import pathways must be operational in spite of general inhibition of nuclear import in infected cells. Indeed, a previous study found that not all nuclear transport/import systems are affected in PV-infected cells (Gustin et al., 2001).

The results presented in this paper do not rule out the possibility that 3C protease itself could enter the nucleus, leading to cleavage of TBP or other transcription factors. In fact, overexpression of 3C alone in mammalian cells leads to shut-off of pol III transcription (Clark et al., 1991). At sufficiently high concentrations, such as overexpression or late times postinfection, 3C could conceivably diffuse into the nucleus and inhibit transcription. However, this may not be the case early during infection when the concentration of 3C is not high enough to successfully defuse into the nucleus. This is particularly relevant for RNA pol I transcription shut-off, which occurs very early (1.5–2 h) postinfection accompanied by multiple cleavage of the TBP-associated factor (TAF) 110, a pol I factor, at gln–gly sites (Dasgupta et al., 2003; Banerjee and Dasgupta, unpublished results). We could only detect 3CD but no free 3C in the infected cells between 1.5 and 2 h postinfection. Thus, it is conceivable that 3CD might translocate into the nucleus as early as 1.5–2 h postinfection to shut-off RNA pol I catalyzed synthesis of rRNA. Although purified 3CD does not directly cleave purified TBP in vitro (Dasgupta et al., 2003), we cannot rule out the possibility that it could cleave transcription factors in vivo. Efficient processing of the viral capsid precursor P1 by 3CD appears to be dependent on a cellular polypeptide (Blair et al., 1993). It is, therefore, possible that 3CD in association with cellular proteins could cleave TBP or other transcription factors in infected cells.

The lack of distinct localization of 3C-EGFP in p3C-EGFP transfected cells did not allow us to make any valid conclusions regarding nuclear entry of 3C. This was due, at least in part, to generation of relatively high amounts of free EGFP in these cells, which made it difficult to determine intracellular localization of 3C-EGFP. The precise reason for the generation of high amounts of EGFP in p3C-EGFP transfected cells is not known. The cloning strategy used to make the 3C-EGFP-fusion polypeptide did not generate

potential 3C cleavage sites at or near the junction of 3C and EGFP. We were also surprised with the finding that almost no full-length protein was found in cells transfected with the p3CD-EGFP containing the AAARD NLS mutation (Fig. 4, lane 3). The same mutation in 3D-EGFP, however, did not result in the lack of synthesis/breakdown of the full-length protein (Fig. 4, lane 1). It is possible that the NLS mutation (KKKRD to AAARD) introduced into the protein might have destabilized the polypeptide leading to its cleavage and/or degradation. We also do not know whether viral (3C) or cellular proteases contribute to the instability of this polypeptide in vivo. Further studies will be required to answer these questions.

The results presented here suggest that cleavage between 3C and 3D is not necessary for nuclear entry of 3CD-EGFP. First, a significant fraction of m3CD-EGFP in which the Q–G bond between 3C and 3D is not cleaved in vitro (data not shown), translocates into the nucleus following PV infection (Figs. 3A and B). Secondly and more importantly, when the KKAAA NLS mutation is introduced into m3CD-EGFP, the protein is found almost exclusively in the cytoplasm of PV-infected cells (Fig. 6C). This is in contrast to the m3CD-EGFP with the wt NLS, a significant amount of which migrates to the nucleus following PV infection (Figs. 3A and B). Thus, both the wt 3CD and m3CD behave similarly with respect to cellular localization in PV-infected HeLa cells.

Why does 3D-EGFP (or 3CD-EGFP) not enter nucleus in transfected cells (in the absence of virus infection) despite the presence of a fairly well conserved NLS sequence? It is possible that the NLS sequence may not be exposed in the absence of other viral proteins. Virus infection may be required for interaction of 3CD/3D with one or more viral or cellular proteins before entry into the nucleus. It is interesting to note that bipartite nuclear signal sequences, called NLS I and II, were required for efficient entry of potyviral NIa, the VPg-protease precursor, into the nucleus of infected cells. When used individually, NLS I and II could facilitate very low level of translocation of the precursor protein (Carrington et al., 1991). The potyviral NIb, the RNA-dependent RNA polymerase, also contains an NLS and mutations in this sequence interfere with nuclear localization of NIb (Li and Carrington, 1995; Li et al., 1997; Schaad et al., 1996).

The results of transient transfections using pEGFP-fusion plasmids are consistent with the observation that both virus-encoded 3CD and 3D are detected in the nuclear fraction in PV-infected HeLa cells (Fig. 7). In fact, considerable amount of 3CD is detected in the nuclear fraction by 3 h postinfection. Also, significant amounts of 3CD and 3D could be detected in the nucleoplasm of infected cells (Fig. 7D). A 3D-containing viral precursor protein with an approximate molecular mass of 86 kDa was also detected in the nuclear fraction (Figs. 7B and D). The estimated molecular weight and migration pattern of this polypeptide are consistent with that of the precursor P3. Although this observation raises

the possibility that 3CD and 3D may be generated in the nucleus by processing of P3, the transient transfection data clearly show that both 3CD and 3D are capable of entering the nucleus on their own.

It is interesting to note that the putative NLS (KKKRD) in the 3D coding region is partially contained within a sequence, KKRDI, which is common among all known picornaviral RNA polymerases. This suggests that this sequence might have an important function in the virus life cycle. One distinct possibility why it is so conserved could be that this sequence might act as a true NLS and that nuclear translocation of certain viral proteins could be essential for efficient replication and spread of the virus in the infected host. Further characterization of the role of the putative NLS in nuclear transport of PV non-structural proteins will add to our understanding of how and why cytoplasmic RNA viruses interact with the nuclei. In this regard, it can be said that a portion of life cycle of a cytoplasmic RNA virus does include interaction with the host cell nucleus.

Materials and methods

Cells and viruses

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin. DNA transfections were performed using the lipofectamine plus reagent (Gibco/BRL) following the manufacturer's recommendations. HeLa cells were seeded in six-well plates and allowed to reach 70% confluency. Transfection was performed by addition of 1.5–2.0 μg of DNA, 5 μl lipofectamine and 4 μl plus reagent to HeLa cells for 3 h. Cells were then fed with regular medium and allowed to grow for 24 h. Cells were infected with PV (type 1, Mahoney strain) at an moi of 25 as previously described (Clark et al., 1991).

Subcellular fractionation

HeLa monolayer (3×10^6) cells were infected or mock-infected with PV (type 1, Mahoney strain) at an moi of 25 for 0, 1, 2, 3, and 4 h. The cells were collected at appropriate times postinfection and fractionated into nuclear and cytoplasmic fractions as previously described (Hu et al., 1998). Briefly, the cells were washed 3 times with PBS, scraped into a tube, and peletted by centrifugation at $400 \times g$ for 1–2 min at 4 °C. The cells were resuspended in 500–600 μl buffer C (10 mM Tris, pH 7.8, 5 mM $MgCl_2$, 10 mM KCl, 0.3 mM EGTA, 0.5 mM DTT, 0.3 M sucrose, 10 mM β-glycerol phosphate, and 2 mM $ZnCl_2$). After incubating for 15 min on ice, NP-40 was added to a final concentration of 0.5%. The cells were then vortexed, and nuclei were isolated after centrifugation at $7200 \times g$ for 20–30 s at 4 °C. The nuclei were washed 5 times with

buffer C. In some experiments, the nuclear fraction was resuspended in buffer D (20 mM Tris, pH 7.8, 5 mM $MgCl_2$, 320 mM KCl, 0.2 mM EGTA, 0.5 mM DTT, and 2 mM $ZnCl_2$). After sonication for 10–20 s, nuclear soluble fraction (nucleoplasm) was separated from nuclear membrane fraction (pellets) by centrifugation at $13,500 \times g$ for 15 min at 4 °C.

Plasmids and cloning

The cloning strategy for EGFP-fusion proteins is depicted in Fig. 1A. Briefly, the DNA sequences encoding 3C, 3D, and 3CD were fused with GFP sequence at the N terminus using pEGFP-N1 N-terminal protein fusion vector (Clontech). The multiple cloning site (MCS) in the pEGFP-N1 vector is between the immediate early promoter of CMV and the EGFP coding sequences. Genes cloned into the MCS will be expressed as fusions to the N terminus of EGFP if they are in the same reading frame as EGFP and there are no intervening stop codons. SV-40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3′ end of the EGFP mRNA. The DNA sequences encoding 3C, 3D, 3CD, and m3CD were PCR amplified using specific primers containing the Kpn1 and BamH1 restriction sites. Following amplification and gel purification, the viral protein coding sequences were cloned between the Kpn1 and BamH1 sites of the pEGFP-N1 MCS. All constructs were verified by DNA sequencing as well as immunoblotting with antibodies directed against GFP (Clontech). The following primers were used for PCR amplification:

3C (forward): 5′GACGGTACCTATGGGACCAGG-GTTCGATTACGCA 3′
3C (reverse): 5′GGTGGATCCATTTGACTCTGAGT-GAAGTATGA 3′
3D (forward): 5′GACGGTACCTATGGGTGAAATC-CAGTGGATGAGA 3′
3D (reverse): 5′GGTGGATCCATAAATGAGTCAAGC-CAACGGCG 3′.

For amplification of 3CD, the 3C forward and 3D reverse primers were used.

The following primers were used to mutate the KKKRD NLS sequence to AAARD:

(forward primer): 5′TATGTAGCAATGGGAGC-CGCGGCGAGAGACATCTTFAAC 3′
(reverse primer): 5′GTTCAAGATGTCTCTCGC-CGCGGCTCCCATTGCTACATA 3′.

The following primers were used to alter KKKRD NLS sequence to KKAAA:

(forward primer): 5′GCAATGGGAAAGAAGGCGGC-CGCGATCTTGAACAAACAA 3′,

(reverse primer): 5′TTGTTTGTTCAAGATCGCGGCC-GCCTTCTTTCCCATTGC 3′.

The pm3CD-EGFP construct is same as p3CD-EGFP except that the gln−gly pair at the 3C−3D junction has been mutated to an ala−ala pair.

The following forward and reverse primers were used to mutate the gln−gly pair at the 3C−3D junction:

5′GATCATACTTCACTCAGAGTGCGGCTGAAAT-CCAGTGGATG 3′, and
5′CATCCACTGGATTTCAGCCGCACTCTGAGT-GAAGTATGATC 3′.

Immunoblotting

HeLa cell-free lysates were prepared by washing cells once with PBS, followed by resuspending them in $1\times$ passive lysis buffer (Promega). Cells were lysed by repeated freeze−thawing in dry ice−alcohol bath followed by incubation at 30 °C. The lysate protein concentration was then determined by the Bradford method (Bio-Rad) and proteins separated by 4−15% gradient SDS-polyacrylamide gel electrophoresis. The proteins were then transferred to nitrocellulose membrane, and the membrane probed with a rabbit polyclonal antibody specific to GFP (Clontech and Molecular Probe). Immunoreactive proteins were detected with goat anti-rabbit antibody conjugated to horseradish peroxidase (Roche) using the supersignal chemiluminescence kit (Pierce). For detection of PV proteins in infected cells, 20−40 µg of cell-free extracts were used in Western analysis using a rabbit polyclonal antibody against bacterially expressed 3D polypeptide. The antibody to 3D was generously supplied by Dr. Ellie Ehrenfeld, NIH.

Microscopy

HeLa cells were washed once with PBS 24 h after transfection, fixed for 20 min in chilled absolute methanol at −20 °C, and again washed with PBS-CM-BSA (PBS containing 0.9 mM $CaCl_2$, 0.83 mM $MgCl_2$, and 3 mg/ml BSA) 2 times. Cells were then incubated with 20 µg/ml RNase for 30 min, washed 3 times with PBS-CM-BSA, and incubated with PI for 15 min at 4 °C. The cells were then washed again with PBS-CM-BSA 2 times and mounted on slides (Vectashield). Cells were visualized using a Nikon Diaphot 200 inverted fluorescence microscope attached with a CCD camera.

Acknowledgments

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