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This article has been published in final form at:
http://dx.doi.org/10.1128/JVI.01748-08
RHINOVIRUS 3C PROTEASE CAN LOCALISE IN THE NUCLEUS AND ALTER ACTIVE AND PASSIVE NUCLEOCYTOPLASMIC TRANSPORT

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Word Count: 1578

Keywords: Rhinovirus, 3C protease, Nuclear cytoplasmic trafficking
Abstract

The degradation of nuclear pore components and disruption of nucleocytoplasmic trafficking during rhinovirus infection has been attributed to viral 2A protease. Here we show for the first time that rhinovirus 3C protease may also have a role. Specifically, we show that 3C and its precursor 3CD can target GFP to the nucleus of living cells leading to degradation of nuclear pore components, and that incubation with recombinant 3C disrupts active and passive nucleocytoplasmic transport in a semi-intact cell nuclear transport system dependent on 3C protease activity. 3C may thus contribute to host cell shutoff in infected cells by localizing in the nucleus and facilitating nuclear pore breakdown.
Human rhinoviruses (HRV) are positive-strand RNA viruses belonging to the *Picornaviridae* family that includes poliovirus. Although picornavirus replication is completed within the cytoplasm, many nuclear factors have been implicated in the life cycle of both HRV and polioviruses. Gustin and Sarnow (11) previously showed mislocalization of cellular proteins late in infection in HRV-infected cells that was attributed to inhibited nuclear import due to degradation of several nucleoporins (Nup153 and Nup62) that are critical components of the nuclear pore, the only avenue for transport into and out of the nucleus. Degradation of Nup153 and Nup62 has also been observed in poliovirus infection (10). Recent work (17) indicates that another nucleoporin, Nup98, is also degraded in poliovirus-infected cells and that this precedes cleavage of Nup153 and Nup62.

Importantly, results from *in vitro* cleavage experiments using HRV 2A protease were used to implicate picornavirus 2A protease as the mediator of cleavage of Nup98 (17), but 2A protease cleavage of Nup98 alone was not found to be sufficient to induce alterations in nuclear pore permeability. Importantly, a role in this context was not considered for the other major protease of HRV, 3C. Both 3C protease and its precursor form 3CD have been observed in the nucleus of cells infected with HRV or transfected to express 3CD (1), meaning that 3C is an ideal candidate to mediate effects on host cell nuclear transport.

Here we present evidence for the first time that HRV 3C protease has intrinsic nuclear targeting potential, and, dependent on its protease activity, is able to disrupt both active and passive nucleocytoplasmic transport. The results suggest that 3C protease, like 2A (17), is likely to contribute to the disruption of host cell nuclear transport, a key factor in host cell shut down.

To test 3C’s nuclear targeting ability, the coding sequences for HRV16 3CD and 3C were both cloned into the pEPI-DESTC Gateway™ vector (9) and expressed as C-terminal GFP fusion derivatives in transfected Vero cells with GFP alone (pEPI-GFP) as a control. Localization of GFP fluorescence was followed by live cell confocal laser scanning microscopy (CLSM) at 18 h post
transfection and the ImageJ1.62 shareware used to analyze the digital images to determine the relative intensity of fluorescence in the nucleus compared to that in the cytoplasm (Fn/c, the ratio of nuclear (Fn) to cytoplasmic (Fc) fluorescence, after the subtraction of fluorescence due to background/autofluorescence; Figure 1 A/B). GFP alone was present throughout the transfected cell (Figure 1A, upper image), while GFP-3C (centre image) and GFP-3CD (lower image) showed increased accumulation in the nucleus. The ratio of fluorescence in the nucleus to that in the cytoplasm (Fn/c) increased significantly from about 1.5 for GFP to 1.9 and 1.8 for GFP-3C and GFP-3CD respectively (Figure 1B). Clearly, both 3C and 3CD are able to target a heterologous protein (GFP) to the nucleus and hence have intrinsic nuclear localizing ability. The fact that 3C/3CD is nuclear in infected cells, and as shown by Amineva et al. (1), untagged 3C is nuclear in transfected cells, implies that the results are unlikely to stem from sequence anomalies in the GFP fusion constructs.

Cell lysates were prepared (15) from COS-7 cells transfected to express GFP, GFP-3C or GFP-3CD and subjected to Western analysis using monoclonal antibody Mab414 that specifically recognizes common motifs within nucleoporins such as Nup358, Nup214, Nup153 and Nup62, and has been used successfully to document degradation of nucleoporins in HRV infected cells (11). Expression of GFP-3C or GFP-3CD led to degradation of three Mab414-recognised proteins corresponding to Nup153, Nup214 and Nup358 (Fig. 1C; see also (11)). There was no evidence for degradation of the band corresponding to Nup62. Clearly, 3C or 3CD alone are sufficient to effect degradation of specific nuclear pore components, independent of other HRV-encoded gene products.

The effect of 3C on active nuclear transport and permeability of the nuclear pore to large molecules was studied at the single cell level using mechanically perforated HTC rat hepatoma cells in conjunction with CLSM as previously (8). This system, also applicable to other adherent cell types, is completely comparable to digitonin permeabilised cells in terms of dependence for transport on
Importins, Ran, etc, has been used successfully to study the nuclear transport of various cargos (4, 5, 7, 9, 12, 13, 18). Nuclear localization signal (NLS)-dependent nuclear protein import can be reconstituted in this system through the exogenous addition of cytosolic extract, an ATP regenerating system (0.125 mg/mL creatine kinase, 30 mM creatine-phosphate, 2 mM ATP), and GTP (2 mM). Active nuclear import of GFP-T-ag(111-135), which accumulates in the nucleus through recognition of its NLS by the importin-α/β heterodimer (2), was followed by CLSM in the presence or absence of HRV 3C (Novagen) (Figure 2). CLSM files were analyzed as above, with the results plotted and curve fitting performed using the SigmaPlot software to determine the kinetics of nuclear transport as previously (9). Typical CLSM images are shown at various times (Figure 2A) with quantitative analysis shown in Figure 2B. In contrast to in the absence of 3C, where GFP-T-ag accumulated actively in the nucleus (Fn/c of 3), treatment with 3C led to a marked decrease in nuclear accumulation in a dose-dependent fashion, with the Fn/c value tending to 1 by 25 min in the presence of 1.4 U 3C, indicative of free diffusion of GFP-T-ag through the nuclear pore.

The commercial HRV 3C used is a recombinant polyhistidine-tagged protein purified from bacteria; to eliminate the possibility that the observed inhibitory effect on nuclear import in Figure 2AB was simply due to the basic polyhistidine tag, 3C was substituted in the assay by hexahistidine-tagged H2B (19), a histidine-tagged, bacterially expressed protein of comparable size to 3C and able to localize in the nucleus. As shown in Figure 2C, H2B had no significant effect on nuclear import of GFP-T-ag, the clear implication being that the effect on transport by recombinant 3C was specific. To examine this further, we tested the effect of 3C in the presence or absence of zinc chloride, which inhibits 3C protease activity (6). Preincubation of 3C with various concentrations of zinc chloride before addition to perforated cells resulted in a reduction of the inhibitory effect of 3C to a significant (p = 0.0011) extent in the case of 200 mM zinc chloride. Zinc chloride alone did not
have a significant effect on nuclear accumulation of GFP-T-ag. Thus, 3C appears to inhibit nuclear import of GFP-T-ag specifically, dependent on its protease activity.

To assess nuclear pore integrity changes effected by 3C activity, 150 kDa FITC-Dextran (FD150) which is too large to diffuse through the nuclear pore and is normally excluded from the nucleus, was used (16). CLSM images taken at various times with and without added 1.4 U HRV 3C (Figure 3A) and quantitative analysis (Figure 3B) indicate that 3C treatment leads to a lack of nuclear exclusion of FD150. Clearly, 3C activity perturbs the passive nuclear transport properties of the nuclear pore.

To test whether 3C can inhibit nuclear import pathways other than that of T-ag, which uses importin α/β (14), we examined the effect of 3C on nuclear import of GFP-TRF1, which is imported actively into the nucleus via binding to Importin-β, and retained in the nucleus by binding to nuclear factors (8). Similar to its effect on T-ag nuclear import, 3C inhibited the nuclear import of GFP-TRF1 (Figure 3C).

To determine if 3C could induce a similar disruption of nuclear transport pathways in intact cells, we expressed GFP-3C in COS-7 cells and examined the localization of endogenously expressed SV40 T-ag in these cells (Figure 4). Nuclear accumulation of T-ag in cells expressing GFP-3C was significantly lower than in cells expressing GFP alone. Additionally, expression of 3C appeared to lead to a loss of fluorescent signal for T-ag; whether this is a direct result of proteolytic activity (see (1)) is unclear.

Together, our data suggest that 3C treatment results in changes to the nuclear pore *in vitro* such that large molecules (FD150) are able to freely diffuse across the nuclear pore, whilst NLS-containing cargoes (GFP-TRF1, GFP-T-ag) are no longer able to accumulate in the nucleus; 3C had no effect
on diffusion of 20kDa-Dextran-Texas Red (FD20) which is small enough to diffuse freely across
the nuclear pore (Figure 3C). The functional changes in nuclear pore permeability induced by 3C
are presumably due to the degradation of specific nucleoporins such as Nup153, Nup214 and
Nup358 as observed in transfected cells (Figure 1C).

Our study shows, for the first time, that passive as well as active transport across the nuclear pore
can be disrupted by 3C, presumably as a direct result of degradation of specific nucleoporins.
Previous studies have shown a general inhibition of active nuclear import of NLS-containing
cargoes in poliovirus and HRV-infected cells due to degradation of key components of the nuclear
pore, with 2A protease being implicated (10, 11, 17). Electron microscopic studies also show that
the structure of the nuclear pore may be changed physically in poliovirus infected cells (3). Our
finding that large, normally excluded molecules, are able to diffuse into the nucleus in the presence
of HRV 3C is consistent with a breakdown of the nuclear pore in HRV-infected or 3C-expressing
transfected cells.

In conclusion, HRV 3C has intrinsic nuclear targeting ability as well as the ability, dependent on its
protease activity, to alter the permeability of the nuclear pore. In addition to transcription factor
cleavage (1), these properties, together with those of 2A (17), are likely to contribute integrally to
host cell shutoff in HRV-infected cells.

ACKNOWLEDGEMENTS

The authors thank Belinda Thomas and Hayat Dagher for cell culture of mammalian cells and Alex
Fulcher for assistance with in vitro nuclear transport assays.
REFERENCES


FIGURE LEGENDS

Figure 1.

HRV 3C and 3CD can target GFP to the nucleus and degrade nucleoporins.
A. Vero cells were transfected with equal amounts of pEPI-to express GFP (top image), pEPI-GFP-3C (central image) or pEPI-GFP-3CD (bottom image) and localization of GFP followed by CLSM 18 h later. B. Images such as those shown in A were analyzed using the ImageJ1.62 software to determine the nuclear to cytoplasmic fluorescence ratio (Fn/c), [Fn/c = (Fn - Fb)/(Fc - Fb), where Fn is the nuclear fluorescence, Fc the cytoplasmic fluorescence, and Fb the background fluorescence (autofluorescence)]. Results are the mean ± SEM, n ≥ 50. C. COS-7 cells were transfected with pEPI-GFP, pEPI-GFP-3C or pEPI-GFP-3CD, lysed with Laemmli buffer and subjected to SDS-PAGE (25 µg total protein per lane) on a 10% gel followed by Western transfer to a PVDF membrane. After blocking in 5 % milk, the membrane was probed with Mab414 (Abcam), followed by species-specific secondary antibodies conjugated to horseradish peroxidase and detection by enhanced chemiluminescence (Perkin Elmer). Molecular weight markers are indicated on the left in kDa, with specific nucleoporins indicated on the right.

Figure 2. HRV 3C inhibits nuclear import of GFP-T-ag in vitro.
Active nuclear import across the nuclear membrane in the absence or presence of recombinant HRV 3C was studied in mechanically perforated HTC rat hepatoma cells as described in the text in the presence of cytosolic extract, an ATP regenerating system, GTP (2 mM), and GFP-T-ag(111-135).
A. Nuclear import/entry of GFP-T-ag alone (-HRV 3C, upper panels) or in the presence of 1.4 U HRV 3C (+HRV 3C, lower panels) was followed for 25 min; images of one field at 5 min, 10 min and 25 min are presented. B. Images such as those in A were analyzed by ImageJ, the Fn/c calculated and curve generated with SigmaPlot; data for GFP-T-ag alone (no add) or in presence of 0.8 U or 1.4 U of HRV C (+ 3C) are presented. Each data point represents the mean ± SEM of ≥ 15 cells. C. The nuclear import of GFP-T-ag alone or in the presence of 3C or histone H2B in the
presence or absence of zinc chloride (ZnCl₂) was followed as in B; data are mean ± SEM for 3 separate experiments each of which represents n ≥ 15, where the Fn/c at 25 min is presented as % Fn/c, with 100% being the Fn/c at 25 min for GFP-T-ag in the absence of 3C.

Figure 3. HRV 3C treatment results in a general disruption of nuclear transport in vitro.

Nuclear-cytoplasmic distribution in the absence or presence of recombinant HRV 3C was studied as described in Figure 2. A. Images such as those in Figure 2A are presented for FITC-150 kDa Dextran (FD150) imaged by CLSM at the indicated times B. Images such as those in A were analyzed by ImageJ, the Fn/c calculated and curve generated with SigmaPlot; data for FD150 alone (no add) or in presence of 1.4 U of HRV C (+ 3C) are presented. Each data point represents the mean ± SEM of ≥ 15 cells. C. Extent of nuclear accumulation in the absence or presence of 1.4 U 3C at 5 and 25 min for GFP-T-ag, GFP-TRF1, 20kDa-Dextran (FD20) and FITC-150 kDa-Dextran (FD150). Results are the mean ± SEM for 3 separate experiments, each of which represents n ≥ 15 measurements.

Figure 4. Over expression of GFP-3C can reduce nuclear accumulation of T-ag in COS-7 cells.

COS-7 cells grown on coverslips were transfected to express either GFP alone or GFP-3C, fixed 18 h later with 4 % formaldehyde followed by permeabilisation of membranes with 0.2 % Triton X-100. Cells were probed with rabbit polyclonal antibody to SV40 T-ag (Santa Cruz Biotechnology) followed by detection with Alexafluor 568 conjugated antibodies to rabbit immunoglobulin. Coverslips were mounted on glass slides and CLSM and image analysis performed as described in legend to Figure 1. (A) Single images of cells expressing GFP alone, or GFP-3C as indicated; green channel (GFP, GFP-3C) on the left and red channel (T-ag) on the right. (B) Images such as those shown in A were analyzed as in Figure 1. Results are the mean ± SEM, n ≥ 15.
A

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<tr>
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B

![Graph showing GFP-T-ag levels over time with different conditions.]

C

![Bar graph showing % Fn/c with different conditions.]

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N.S. = Not Significant

P = 0.0011