Rhinovirus 3C protease precursors 3CD and 3CD’ localize to the nuclei of infected cells

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Human rhinovirus (HRV) 3C protease (3Cpro) plays several important roles in the virus replication cycle. This enzyme cleaves the viral polyprotein at discrete sites to produce mature viral proteins and also inhibits cellular RNA transcription. It is not clear, however, whether the observed transcriptional shutoff activities are due to 3Cpro itself or to 3Cpro-containing precursors, and where 3Cpro exerts its effects within infected cells. To address these questions HeLa cells were infected with HRV-16, stained with polyclonal antibodies directed against 3Cpro and then analysed by laser confocal microscopy. Proteins containing 3Cpro accumulated in nuclei 2–4 h post-infection, and progressively increased in the cytoplasm. Analyses of subcellular extracts demonstrated that 3CD1, a minor component among 3Cpro precursors, gave rise to the earliest 3Cpro nuclear signals. Mature 3Cpro and another 3Cpro precursor, 3CD, were also detected in the nucleus, cytoplasm and perinuclear membrane fractions 4 h post-infection. Transfecting cells with 3Cpro, 3CD precursor and 3CDα371 (with deletion of 371 aa at the carboxyl terminus of 3D) demonstrated that the nucleolar localization signal was near the amino terminus of 3D. In addition, 3Cpro precursors were found to co-localize in nuclei with the transcription factor OCT-1 and the nucleolar chaperone B23. Finally, it was demonstrated that HRV-16 3Cpro, 3CD and 3CDα371 could cleave OCT-1. Collectively, these findings suggest that HRV 3CD’ and/or 3CD are specifically localized to the nucleoli of infected cells during the early stage of infection, and contribute to the inhibition of cellular RNA transcription via a proteolytic mechanism.

INTRODUCTION

Human rhinovirus (HRV) 16, like other members of the Picornaviridae, is a positive-sense, single-stranded RNA virus. The viral genome is translated into a single polyprotein, which is processed into individual proteins by self-cleaving, autocatalytic viral proteases. HRV encodes two such proteases, 2Apro and 3Cpro, which are collectively responsible for most of the processing events within the viral polyprotein and also for cleavage of several cellular proteins during infection. These activities have been presumed to occur within the cytoplasm of infected host cells, at the sites of viral translation, RNA synthesis and virion assembly (Racaniello, 2001).

Among the known cellular substrates for picornaviral proteases are several proteins involved in cellular viability, mRNA translation and transcription. The HRV-2 2Apro, for example, can cleave and inactivate eukaryotic translation initiation factor 4G (eIF4G), a necessary component of the eIF4F complex that mediates the binding of capped (cellular) mRNAs to 40S ribosomal subunits during translation initiation (Haghighat et al., 1996; Gradi et al., 1998; Hunt et al., 1999). Since the majority of eukaryotic mRNAs are capped, this 2Apro activity effectively shuts off cellular mRNA translation during infection. In contrast, the infecting virion mRNAs continue to recruit ribosomal subunits and translate their proteins in the absence of intact eIF4F complexes (Jang et al., 1990), because picornaviral translation is directed by cap-independent internal ribosomal entry sites (IRES) that dominate the 5’ untranslated regions (5’ UTR) of the genomes.

In poliovirus-infected cells, viral proteases can further cripple antiviral host cell defence systems by blocking pol-I, -II and -III dependent cellular RNA synthesis. Recombinant poliovirus 3Cpro has been shown to cleave transcription factors TFIIIC and TFIIID (TATA-binding protein) (Clark et al., 1991, 1993), CREB (Yalamanchili et al., 1997a) and OCT-1 (Yalamanchili et al., 1997b) in cell-free assays and in infected cells. Several forms of 3Cpro are synthesized during virus replication because picornaviral polyproteins are cleaved sequentially through a series of intermediate steps (Schultheiss et al., 1994). Several of the 3Cpro precursor forms are enzymically active and competent for polyprotein cleavage, and probably carry out additional key roles in the virus replication cycle (Ypma-Wong et al., 1988; Harris et al., 1994; Xiang et al., 1995; Davis et al., 1997; Probst et al., 1998; Beneduce et al., 1999; Banerjee et al., 2001). Therefore, it is...
not clear whether 3CPro itself or 3CPro-containing precursors are responsible for cellular transcriptional shutoff, and even the subcellular location of the relevant 3CPro activities has never been accurately documented.

We have cloned, expressed and purified recombinant HRV-16 3CPro from bacteria, and developed antibody reagents that react specifically with this protease and its precursors in infected cells. We now report that cellular fractions from HRV-infected cells indicated that the precursor 3CD, a product of both 3CPro and 2APro-dependent polyprotein cleavages, localized exclusively to nuclei by 2 h post-infection (p.i.). Shortly thereafter a second precursor, 3CD was also found in nuclei. Mature 3CPro, along with a number of its precursors, accumulated in the cytoplasm at 5–8 h after infection. We have determined further that the N terminus of 3D carries a sequence responsible for nucleolar localization of linked 3C precursors. As has been reported with polio, our recombinant HRV 3CPro could cleave the OCT-1 transcription factor in cell-free assays, and moreover, this protein co-localized with 3CD and 3CD during infection. These findings suggest that specific 3CPro precursors contribute to the shutoff of cellular RNA transcription by a process involving nuclear transport, followed by cleavage of vital transcription factors in the nucleus.

METHODS

**Recombinant 3CPro.** 3CPro coding region from plasmid pHRV-16.11 (Lee et al., 1995), which contains a genome length cDNA from HRV-16, was amplified by PCR. One primer contained a synthetic NdeI site, followed by 19 nt derived from the 5'-end of the 3CPro HRV-16 coding region. The second primer contained nucleotides complementary to the 3'-end of the HRV-16 3CPro coding region, a stop codon and a BamHI site. The amplicon was subcloned into pET-41 (Novagen), transformed into E. coli [strain BL21(DE3)PlySs; Novagen] and the bacteria were amplified in 2× YT broth (37°C), supplemented with kanamycin (15 mg ml⁻¹) and chloramphenicol (30 mg ml⁻¹). After induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 5 h, the cells were pelleted, resuspended in buffer A (1/10 volume, 50 mM Tris pH 8.5, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% deoxycholate), lysed by freeze-thaw cycles and subjected to centrifugation (27 000 g, 15 min). The cellular pellet fraction was washed twice (buffer A), and the inclusion bodies were collected by centrifugation. Recombinant 3CPro (r3CPro) was purified according to the method of Cordingly and colleagues (Cordingly et al., 1989) with minor modifications. Briefly, a soluble (supernatant) sample of r3CPro (10 ml) was applied to a High-Q site within the 3' UTR of pHRV-16.11), the plasmid was used to programme [³⁵S]methionine incorporation in a TnT coupled transcription-translation system (Promega). This cDNA directed the synthesis of a nearly full-length HRV-16 polyprotein (ΔA1Δas) under control of an EMCV IRES element (from the pCITE vector). Plasmid pr16-subΔ3C was similar, except that it contained an additional deletion (Small to BstI107) in the viral 3C region (Δ3Cα1,133), which inactivated endogened 3CPro activity within the expressed polyprotein.

**Antibody reagents.** Recombinant 3CPro for antibody induction was isolated from bacterial inclusion bodies by preparative SDSPAGE (12%). The protein bands were visualized after soaking the gel in 0.25 M KCl (10 min, 4°C), excised, then electro eluted (4°C) into buffer (25 mM Tris pH 8.5, 200 mM glycine). The r3CPro samples were concentrated, suspended in complete Freund’s adjuvant (Sigma) then used to immunize female BALB/c mice intraperitoneally (40 μg). A second injection (40 μg in incomplete Freund’s adjuvant) was administered 28 days p.i., followed by a third dose (40 μg without adjuvant) on 42 days p.i. Serumconversion to 3CPro was confirmed by ELISA, using serum samples collected after the final injection (mouse polyconal sera, Ab-r3C). On day 45, the animals were sacrificed and the isolated spleen cells were fused to myeloma cells (SP 2.0) using a polyethylene glycol technique (PEG 1450; Acros), then screened by HAT selection. Cultures positive for r3CPro antibodies (mouse monoclonal, mAb-r3C) were identified by ELISA then cloned twice by limiting dilution (Harlow & Lane, 1998). Rabbit polyclonal serum raised against HRV-16 3CPro (Ab-r3C) was kindly provided by Agouron. Monoclonal antibodies to EMCV 3CPro (mAb-r3C) and to HRV-1B structural protein (also recognizing 1AB precursor, mAb-1AB) were described (Aminev et al., 2003; Mosser et al., 2002). Wheat germ agglutinin conjugated with Texas red (WGA; Molecular Probes) was used to identify Golgi and nuclear membrane. Rabbit polyclonal antibodies to OCT-1 (Ab-OCT-1), B23 protein (Ab-B23), mouse monoclonal antibodies to actin (Ab-actin), anti-mouse or anti-rabbit polyclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or Texas red (TXR) were purchased from Santa Cruz Biotechnology. Anti-mouse or anti-rabbit polyclonal antibodies conjugated with horseradish peroxidase were ordered from Amersham Biosciences.

**Construction of plasmids that contain 3C, 3CD and 3CD sequences.** Plasmids that contain coding regions of HRV-16 3C and 3CD under the control of an EMCV IRES were constructed and generously supplied by H. Owen (Institute For Molecular Virology, University of Wisconsin-Madison, Madison, WI, USA). To construct plasmid pIRE3-3CD371, the 3CD protein coding region was amplified by PCR from pHRV-16.11. A primer corresponding to 5'-end of the 3C-coding region was linked to a synthetic NdeI restriction site. A second primer encoding nucleotides complementary to the 3'-end of 3CD was designed to contain the 3CD coding region, a stop codon and a SalI restriction site. The amplicon was subcloned into pCITE-4a plasmid via NdeI and SalI restriction sites within the vector. The r3CD371 (3CD 89–460) with truncated C terminus of 3D (up to putative NLS) was derived from the plasmid by restriction with BstII102 I (unique site within 3D region). Resulting sequence included 267 nt coding 89 aa of the N terminus of 3D protein. Plasmids were transcribed by T7 RNA polymerase in RiboMAX large-scale RNA production system-T7 (Promega) and transcripts were used for the transfection of Hela cells or to programme rabbit reticulocyte extracts containing [³⁵S]methionine to analyse stability of synthesized proteins.

**Western assays.** Protein samples were fractionated by SDS-PAGE then blotted onto polyvinylidene fluoride (PVDF) membranes as described (Mosser et al., 2002). Bands with positive-antibody
reactions against HRV-16 r3Cpro (Abu-r3C), OCT-1 (Ab-OCT-1) or actin (Ab-actin) were visualized by chemiluminescence (ECL kit; Amersham Biosciences).

**Cellular techniques.** Subconfluent HeLa monolayers were infected with HRV-16 (10–25 p.f.u. per cell) as described (Lee et al., 1995). At appropriate times p.i., the cells were collected then fractionated into nuclear, cytoplasmic and membrane components as described (Hu et al., 1998). Briefly, HeLa cells (5 x 10^6) were washed three times with cold PBS, scraped and pelleted (400 g. 1 min, 4°C). The cells were resuspended in buffer C (500 µl, 10 mM Tris pH 7.8, 5 mM MgCl₂, 10 mM KCl, 0.3 mM EGTA, 0.5 mM DTT, 0.3 M sucrose, 10 mM b-glycerolphosphate, 2 mM ZnCl₂) then incubated on ice for 15 min. NP40 detergent was added to 0.5%. The samples were vortexed, cellular membrane fraction and nuclei were harvested after a centrifugation step (7200 g for 20 s, 4°C). The cytoplasmic (supernatant) fractions were removed, and the pellets (cellular membranes and intact nuclei) were washed three times with buffer C, then resuspended in buffer D (125 µl; 20 mM Tris pH 7-8, 5 mM MgCl₂, 320 mM KCl, 0.2 mM EGTA, 0.5 mM DTT, 2 mM ZnCl₂). After incubation on ice (15 min) and sonication (10 s), nuclear extracts and membrane fractions were separated by centrifugation (13500 g, 15 min, 4°C). The membrane fractions (pellets) were washed three times with buffer D then resuspended (125 µl) in the same buffer. The protein concentration in each cellular subfraction was determined using a Coomassie plus protein assay reagent kit (Pierce). The fraction efficiency was assessed in Western blot using antibodies for specific proteins in each fraction, such as actin for the cytoplasmic fraction, nucleolar-specific protein C23 for the nuclear extract.

**Transfection of HeLa cells with RNA.** Transfection of HeLa cells with RNA derived from plasmids pRES-3C, -3CD, -3CDAS71 and -HRV-16 was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. After 24 h of incubation, cells were washed with cold PBS and fixed for the confocal microscopy. To analyse samples in Western blot assay after the washing procedure, cells were collected in sample buffer (Bio-Rad Laboratories).

**Immunofluorescence microscopy.** HeLa cell monolayers were grown on coverslips to 50–70% confluence then infected with HRV-16 (10–50 p.f.u. per cell). At appropriate intervals p.i., the cells were rinsed three times (with cold PBS, fixed with paraformaldehyde (4% for 20 min) then rinsed again. After cell permeabilization (0.3% triton X-100) and incubation with blocking solution (30 min, 10% FBS in PBS) the samples were incubated (1 h, 20°C) with the desired primary antibody in blocking solution, then washed, and reincubated with the corresponding secondary antibody (1 h, 37°C), before a final wash (three times in PBS). The slips were mounted (Vectashield mounting medium; Vectorlab) and labelled location was visualized by laser scanning confocal microscopy. Image capture used MCR 1024 LaserSharp software (Bio-Rad). WGA was used in a similar manner, to highlight and identify Golgi and nuclear membrane locations. Newly synthesized RNA was labelled by incorporation of fluorescein-12-UTP (Sigma) into permeabilized cells before the formaldehyde fixation step as described (Ko et al., 2000).

**RESULTS**

**Recombinant 3Cpro**

The 3Cpro gene from HRV-16 was cloned into cDNA, expressed in E. coli, and the soluble, recombinant protein was purified to about 90% homogeneity (Fig. 1a). A typical yield was about 3 mg of protease per 100 ml of bacterial culture. The activity of 3Cpro was assayed in cell-free reactions containing radiolabelled HRV-16 polyprotein substrates harbouring deletions in the endogenous 3Cpro (Fig. 1b). Samples showed the progressive accumulation of viral protein cleavage products (1AB, 1C, 1D, 2C, 3CD), and the sizes of these cleavage products, as estimated by SDS-PAGE, were consistent with 3Cpro-mediated processing at multiple P1, P2 and P3 polyprotein sites, including 1C/1D, 2B/2C and 2C/3A. These locations are the preferred trans cleavage targets for other picornaviral 3Cpro enzymes (Racaniello, 2001), and we conclude that our r3Cpro was active and able to react with its natural HRV-16 substrate in a manner analogous to that of the endogenous enzyme.

Several protein bands could not be identified on the basis of molecular mass. Two precursors (approximately 80 and 90 kDa) were present in samples of translated pRV16 sub3C. The smaller protein was not detected after incubation with r3Cpro, while the larger protein was not affected by the protease. Although the precise nature of these proteins is unknown, they may represent the stable products of 2Apro processing of viral polyprotein. It is known that 3D protein has at least one site for 2A cleavage (Lee & Wimmer, 1988), and at least two other potential sites on the surface of this protein can be identified by computer analysis [NetPicoRNA V1.0, The Center for Biological Sequence Analysis at the Technical University of Denmark (Blom et al., 1996)].

**Protein localization**

The subcellular distributions of 3C-containing proteins and 1AB capsid sequences in HRV-16-infected HeLa cells were monitored after staining with specific antibodies, and visualization by confocal microscopy (Fig. 2). Viral translation, replication and assembly processes take place on membranous fractions associated with the Golgi and endoplasmic reticulum membranes (Egger et al., 2000), and indeed this localization was observed when cells were co-stained with a mAB specific for a structural protein (mAB-1AB) and WGA (Fig. 2b), a Golgi and nuclear membrane marker (Silver, 1991; Dingwall & Laskey, 1992). In contrast, the 3C signals were distributed quite differently. Beginning at 2 h p.i., there was strong, punctate 3C labelling throughout the nuclei as well as in the cytoplasm. The nuclear localization of 3C-containing proteins overlapped with SYTOX (Fig. 2a), a nucleic acid marker (Molecular Probes) and was particularly strong near the perinuclear membrane and in the regions that probably mark transcriptionally active sites including nucleoli. Pulse-labelling experiments with FITC-UTP confirmed that 3C-containing proteins accumulated in nuclei at the sites of ongoing RNA synthesis (Fig. 2d). Moreover, the sites of heaviest 3C stain overlapped exactly with those of an antibody against nucleophosmin B23 (Fig. 2c), a nucleolar molecular chaperone protein that contributes to the assembly of ribosomal subunits (Sipos & Olson, 1991; Szébeni et al., 1995, 1997; Scheer & Hock, 1999; Szébeni & Olson, 1999). Similar experiments conducted with antibodies
against nucleolin (C23), another nucleolar marker, also
demonstrated a very strong 3C signal in nucleoli (not
shown).

Although 3C-containing proteins were not uniquely local-
lized to nucleoli, a dense stain in this locale was evident even
at the earliest times p.i. (Fig. 2d and 2e), and especially if the
cells were infected at high multiplicity (i.e. 50 p.f.u. per cell,
Fig. 2d). Discernable nucleolar 3C stain persisted until cell
death (about 8–10 h p.i.), but as is typical for Rhinovirus
and Enterovirus infections, the nuclei and nucleoli began to
swell noticeably at about 3 h p.i. The nuclear membranes
became progressively indistinct as the replication cycle
progressed, and by 5–6 h p.i. the membranes were so
fragmented in appearance that it was sometimes difficult to
discern the nuclear edges by microscopy (Fig. 2b, d).
Throughout this process, nuclear transcription, as assessed
by FITC-UTP, diminished progressively, to be replaced by
cytoplasmic UTP-labelling that presumably marked the sites
of virion RNA synthesis (Fig. 2d). In parallel, the 3C stain
intensified throughout the cytoplasm as new protein was
synthesized or as the previously synthesized protein leaked
from the nuclei and was recruited to the replication com-
plexes. By 8 h p.i., the 3C signal was very intense and
ubiquitous throughout the cells, and it became difficult to
determine any particular localization of 3C-containing pro-
teins (data not shown).

Cell fractionation

The confocal microscopy experiments demonstrated that
3Cpro protein(s) could be detected immunogenically quite
early after infection and a substantial portion of this material
localized to the nuclei by 2 h p.i. However, in situ antibody

signals could not distinguish between the mature form of
3Cpro and any of several other immunoreactive polyprotein
precursors. Accordingly, HRV-16-infected cells were col-
lected at 2, 4, 6, 8 and 24 h p.i., and then fractionated into
lysates enriched for nuclear, cytoplasmic and membrane
proteins. After SDS-PAGE and transfer to membranes, the
samples were evaluated relative to standard, HRV-16 pro-
teins (Fig. 3). At the earliest time points (2 h p.i.), the pre-
cursor 3CD (74 kDa) dominated the cytoplasmic fractions.
In repeated experiments (duplicates not shown), there was
no evidence of larger precursors (e.g. 3ABCD, 86 kDa) or
mature forms of the enzyme (20 kDa) in the early cyto-
plasmic samples. Presumably, this indicates that cleavage
between 3AB and 3CD is the preferred processing pathway
for the earliest polyproteins.

Surprisingly, a lower molecular mass precursor, 3CD’,
appeared in the nuclear fractions 2–6 h p.i. (Fig. 3). A
previously published report has identified similar protein
(3C’) in poliovirus-infected cells, and demonstrated it
originates from a 2A-mediated cleavage within 3CD at a
tyrosine-glycine site (Pallansch et al., 1984). As the infection
progressed, the nuclear 3CD’ band intensified (Fig. 3, 4–
6 h p.i.), and additional bands representing 3CD as well as
mature 3Cpro became evident. It was not until the onset of
RNA synthesis (4–6 h p.i.) (Wang et al., 1998) that the
cellular membrane fractions showed evidence of other
3Cpro-containing sequences, starting with 3CD (4 h p.i.), and
then accumulating 3CD’, 3ABC and/or 3C’ (3C and a
part of 3D, another product of 3CD cleavage catalysed by
2Apro), and 3Cpro itself. Yet another precursor, slightly larger
than 3CD, which might represent 3BCD (76 kDa) or
3ABCD (86 kDa), also became evident in the membrane
fractions at 6–8 h p.i. In samples collected at 24 h p.i., the

\[ \text{Fig. 1. Purification and activity of r3C}^{\text{pro}}. \]
(a) r3Cpro was isolated from IPTG-induced E. coli lysate (fraction 0) and was applied to a
High-Q cartridge, eluted with the linear gradient of NaCl then dialysed. After con-
centration the protein fractions were ana-
lysed by SDS-electrophoresis, and the bands were visualized by Coomassie blue.
(b) A radiolabelled HRV-16 polyprotein sub-
strate, lacking endogenous 3Cpro activity, was synthesized in reticulocyte extracts as
indicated in Methods. Samples (1 µl) were
supplemented with r3Cpro then incubated at
30 °C for 12 h before fractionation by SDS-
PAGE (12.5%). The bands were visualized
by autoradiography of the dried gel. The locations of molecular mass markers (left)
and known viral proteins (right) are indicated.
(c) Schematic representation of the
rhinoviral polyprotein. The HRV 3Cpro clea-
vage sites are indicated by open arrow-
heads, the 2Apro sites by filled arrowheads.
Detailed is the 3CD region with marked site
for 2Apro cleavage at the position Y325.
Fig. 2. Cellular localization of 3C-containing proteins. HeLa monolayers grown on coverslips were infected with HRV-16 (10 p.f.u. per cell). At the indicated times (h p.i.), the cells were fixed, stained and examined by confocal microscopy as described in Methods. Photography used a 560 nm filter for detection FITC and a 680 nm filter for Texas Red detection or SYTOX. Both filter sets were used for merged images. (a) Green: primary antibody was Ab1-r3C (murine polyclonal against r3C), followed by anti-mouse FITC-conjugated antibodies. Orange: SYTOX stain, localizing dsDNA. (b) Green: primary antibody was mAb-1A (murine monoclonal against 1A), followed by anti-mouse FITC-conjugated antibodies. Red: Texas Red-conjugated wheat germ agglutinin (WGA), localizing Golgi and nuclear membranes. (c) Green: primary antibody was mAb-r3C (murine monoclonal against HRV r3C pro) followed by anti-mouse FITC-conjugated antibodies. Red: primary antibody was rabbit anti-B23 (polyclonal), followed by Texas Red-conjugated anti-rabbit antibodies. (d) Infection used 50 p.f.u. per cell. Green: 30 min before fixation, cells were treated with Triton X-100 and FITC-conjugated UTP to visualize nascent RNA. Red: primary antibody was Ab1-r3C (murine polyclonal against r3C), followed by anti-mouse FITC-conjugated antibodies. (e) Green: primary antibody was mAb-r3C or mAb-r3C EMCV (murine monoclonal against HRV or EMCV r3C), followed by anti-mouse FITC-conjugated antibodies. Red: primary antibody was rabbit anti-OCT-1 (polyclonal), followed by Texas Red-conjugated anti-rabbit antibodies.
Fig. 3. Western analysis of cellular fractions. HeLa cells (1 × 10⁵) were infected with HRV-16 (5–10 p.f.u. per cell), and harvested at the indicated times p.i. Cytoplasmic (C), nuclear (N) and cellular membrane (M) fractions were separated as described in Methods. The extracts were fractionated by SDS-PAGE (12.5%), transferred onto PVF membranes then probed with Ab-2-r3C (rabbit polyclonal against r3C). The bands were visualized after reaction with HRP-conjugated anti-rabbit antibodies.

cell-associated proteins could no longer be separated into cytoplasmic or nuclear fractions because the nuclei were largely destroyed or ruptured in these cells. Among the remaining viral proteins, however, 3ABC and/or 3C' and 3Cpro were the majority species of immunogenic bands. The appearance of 3ABC during the replication process, and its accumulation as a majority 3Cpro species at late times of infection, may mark a switch in polyprotein cleavage pathway preferences, perhaps denoting a difference in intramolecular (early) versus intermolecular (late) 3Cpro activities.

Protein mapping

The above experiments demonstrated that the large 3C-containing precursors can enter the nuclei of infected cells. The next step was to map the segments that are required for active nuclear translocation. The primary sequence of the 3C-3D region of the polyprotein shows at least one region, near the N terminus of 3D (Fig. 4) that is similar to the nuclear localization sequence (NLS) of yeast ribosomal proteins (Stugel et al., 2000), and is conserved among rhino-, polio- and enteroviruses. To assess whether this region of 3D was responsible for the nuclear signals by 3C and its precursors, plasmids encoding 3C, 3CD or 3CD lacking 371 aa from the C terminus of 3D (3CD₃₇₁), but still containing the putative NLS were constructed. The size and stability of these proteins was confirmed by in vitro translation reaction (data not shown). The consequent RNAs were transfected into HeLa cells, and 24 h later, the cells were fixed and then stained with antibodies and WGA (Fig. 5). Cells transfected with pIRE-3C RNA showed light diffuse staining throughout the nucleus and mostly in the cytoplasm (Fig. 5a). Cells transfected with pIRE-3CD or pIRE-3CD₃₇₁ had a different staining pattern (Fig. 5b, c), in that most of the 3C-signal localized to the nucleus, and in most of the cells the nucleoli were clearly visible.

**OCT-1 cleavage by 3Cpro**

It is well established that during poliovirus infections, pol-I, II and -III dependent host transcription is rapidly inhibited (Rubinstein et al., 1992). The culpable agent in most experiments is believed to be 3Cpro or its precursors, reacting with vulnerable transcription factors such as TATA-binding protein (Clark et al., 1993; Yalamanchili et al., 1996), OCT-1 (Yalamanchili et al., 1997b), CRE-binding protein and TFIIC (Clark et al., 1991). Few studies, however, have evaluated the site(s) of transcription factor cleavage within infected cells. Perhaps this is due to a general assumption that nuclear and cytoplasmic proteins might easily have co-mingled when the nuclei swell and become porous during an infection. Since transcriptional shutoff (2–3 h p.i.) clearly occurs before nuclear swelling (about 6 h p.i.), it was opportune to revisit this question with the HRV-16 r3Cpro and its antibodies.

Accordingly, infected cells (10 p.f.u. per cell) were harvested at 2 and 4 h p.i. then co-stained with mAb-r3C and Ab-OCT-1 (Fig. 2e). The merged images showed a high degree of correspondence at both time points, within dense foci in transcriptionally active nuclear and nucleolar regions. In control cells, infected with EMCV (Fig. 2e) and co-stained with mAb-r3C and OCT-1, 3Cpro signal was also detected in the nucleus, but did not co-localize with OCT-1. Cell extracts from similar samples harvested at 4, 6 and 24 h p.i., were fractionated by SDS-PAGE and then probed in Western assays with Ab-OCT-1 or an Ab-actin control (Fig. 6). The OCT-1 bands, but not the actin bands were reduced significantly as the infection progressed (Fig. 6a). On a per cell basis, at least 60% of the OCT-1 signal was already gone by 4 h and 70% by 6 h p.i. Analysis of nuclear fractions of infected cells revealed the accumulation of OCT-1 cleavage products (about 54 kDa) in the nucleus and relocalization (or accumulation) of OCT-1 in the cytoplasm (Fig. 6c).

To confirm the active role of 3Cpro in the OCT-1 cleavage, lysates from uninfected cells were incubated with r3Cpro (Fig. 6b). OCT-1 was readily cleaved in reactions, and this
Fig. 5. 3C-containing precursors were cloned into pCITE plasmids under the control of an EMCV-IRES. HeLa cells were transfected with RNA transcripts from each construct. Plasmid contained the sequence of HRV 3Cpro (a), 3CD protein (b) and 3CD_{371} (c). After 24 h, the cells were fixed, stained with Ab_{2-3C} (green) and Golgi marker (WGA, red), and visualized by confocal microscopy. The position of putative NLS on the 3D protein is highlighted.
cleavage was inhibited by 2 mM ZnCl₂, a known inhibitor of 3Cpro (Koran & Butterworth, 1976).

At the earlier time points p.i., we observed the presence of only large 3C-precursors such as 3CD and 3CD₁ in the nucleus of infected cells. To test the ability of these proteins to cleave OCT-1, lysates of HeLa cells transfected with pIRES-3C, -3CD, -3CD₁₃₇₁, and -HRV-16 RNA were analysed in Western blot assay (Fig. 6d). The results demonstrate a meaningful reduction of the OCT-1 amount in all transfected cells compared with mock control cells.

DISCUSSION

In this set of experiments, the cleavage specificity of 3Cpro was evaluated using a natural polyprotein substrate and the subcellular localization of 3Cpro was determined by confocal microscopy. Our results demonstrate that the mature form of 3Cpro is capable of multiple cleavages in the P1, P2 and P3 regions of homologous polyprotein substrates. In fact, both endogenous 3Cpro (pR16-sub) and r3Cpro produced detectable 1AB and 1D bands, although the 1C bands were consistently weaker in the r3Cpro samples. These results differ from the previous studies of HRV-14 r3CD and r3CD proteins (Davis et al., 1997), in which neither recombinant proteinase cleaved substrates from the capsid region. One explanation for this difference is that we used native polyprotein substrates, instead of synthetic peptides; it is possible that the conformation of the substrate has significant influence on the recognition of the cleavage sites. Capsid region processing by enteroviruses but not cardioviruses is known to require 3CD for efficient cleavage at the 1AB/1C...
site (Ypma-Wong et al., 1988; Jore et al., 1988; Davis et al., 1997). Although our r3C<sup>pro</sup> did produce small amounts of 1AB in the absence of 3CD, the overall results suggest that 3CD or another P3-region precursor is the preferred agent for cleaving the 1AB/1C site in rhinoviruses. These results support the fundamental proposal that different forms of protease may have different activities or specificities throughout the infectious life cycle.

Unexpectedly, 3C<sup>pro</sup> is not confined to the Golgi or cellular membranes that are the sites of viral genome translation and replication. Rather, punctate foci of 3C<sup>pro</sup>, or more specifically the 3CD′ precursor, were detected in nuclei as early as 2 h.p.i. with a distribution that overlapped signals for nucleolar B23 and active RNA transcription (FITC-UTP). The processing of 3CD into 3C<sup>pro</sup> and 3D<sup>pol</sup> occurs at the glutamine-glycine cleavage site, and 3C<sup>pro</sup> (and/or its precursors) are responsible for this cleavage. In contrast, in poliovirus cells the 3CD protein is processed by 2A<sup>pro</sup> at a tyrosine-glycine pair within the 3D region to produce 3C′ and 3D′ (Hanecak et al., 1982; Pallansch et al., 1984; Toyoda et al., 1986). In rhinovirus-infected cells, we clearly observed another product of 3CD cleavage - 3CD′ protein (scheme on Fig. 1). According to the computer analysis of potential cleavage sites for 2A<sup>pro</sup> on HRV 3D<sup>pol</sup> (NetPicorna VI.0) the tyrosine-glycine pair at position 325 is the first preferable cleavage site. Although previously dismissed as minor product of an unimportant cleavage pathway, our fractionated lysates from HRV-16-infected cells clearly indicate that 3CD′ is the first 3C<sup>pro</sup> form to appear in nuclei. At later times p.i., other 3C-containing proteins, including 3CD and then 3C, 3C′ and/or 3ABC are also detectable in the nuclei. Experiments are in progress to determine precisely which forms of the protease have nuclear localization potential. The presence of other 3C-containing precursors in the nucleus might explain observations by Lee and Wimmer (1988) that the elimination of 2A cleavage site on 3D protein did not have detrimental effect on virus replication.

Accumulation of some viral proteins in the nuclei of picornavirus (polio)-infected cells was described years ago (Bienz et al., 1982; Fernandez-Tomas, 1982). Fernandez-Tomas demonstrated the presence of viral-induced protein with an approximate molecular mass of 75 kDa in nuclei isolated from poliovirus-infected cells at 3 h.p.i., however, the composition was not determined. Another group of investigators described and characterized by molecular mass a different set of polioviral-induced proteins (P1, P2, P3, 2BC and 3CD) in nuclei of infected cells (Bienz et al., 1982). The subcellular distribution of rhinoviral proteins has not been previously described.

How does 3C or its precursors enter nuclei? All picornaviruses including Rhino- and Enterovirus have a highly conserved segment region (PnKTKLnPS) near the N-terminal portion of 3D<sup>pol</sup> (Aminev et al., 2003). The conservation of the sequences implies that the element is functionally important, and indeed, mutations in this region prevent cardiovirus RNA synthesis. It has been suggested that the site binds B23, and serves as a signal for nuclear localization (Aminev et al., 2003). This concept is further supported by demonstrating with confocal microscopy that 3C protein co-localized with B23 protein, and by published reports that B23 interacts with some viral proteins to function as a transporter (Szebeni et al., 1997). Our results demonstrated that transfecting cells with 3C (pIRE-3C) led to a diffuse pattern of staining in the nucleus (Fig. 5a). This may have occurred via passive diffusion since 3C<sup>pro</sup> is small enough to pass through the nuclear membrane. In contrast, fully processed 3C<sup>pro</sup> was not found in the nuclei of cells that were infected; only large molecular mass precursors were visualized in the nuclei and these large precursors likely required the active transport with NLS. Alternately, another region of poliovirus 3D protein (KKKRD) was described as a potential NLS (Weidman et al., 2003). We are currently investigating these potential mechanisms for nuclear localization of rhinovirus 3C<sup>pro</sup>.

What might be the function of 3C<sup>pro</sup> in nuclei? It is well established that poliovirus 3C<sup>pro</sup> cleaves transcription factors TFIIIC (Clark et al., 1991), TBP (Clark et al., 1993), Yalamanchili et al., 1996), p53 (Weidman et al., 2001), OCT-1 (Yalamanchili et al., 1997b), CREB (Yalamanchili et al., 1997a) and the La-autoantigen (also known as SS-B) (Shiroki et al., 1999), thereby leading to the inhibition of pol-I, -II and -III activity in infected cells. Although there is less information about the effects of rhinovirus infection on the same transcriptional factors, our data indicate that OCT-1 is cleaved by 3C<sup>pro</sup> during rhinovirus infection. Furthermore, OCT-1 is cleaved in cells transfected with RNA encoding different forms of 3C<sup>pro</sup> (3C<sup>pro</sup>, 3CD and 3CD<sub>371</sub>). Moreover, OCT-1 and 3C co-localize in infected cells. It is therefore reasonable to speculate that natural cleavage of this factor, and likely other transcriptional factors as well, is catalysed by 3C<sup>pro</sup>, or its precursors 3CD′ and 3CD within nuclei and nucleoli during the first hours of infection.

Collectively, the data suggest that soon after infection, certain 3C precursors (3CD′ in particular), enter the nucleus, where the 3C moiety (in a form of free protease or a precursor) serves to cleave particular host transcriptional factors (e.g. OCT-1) leading to the rapid inhibition of cellular transcription. A consequent loss of nuclear integrity, as indicated by nuclear swelling and perinuclear membrane perforation follows. The fact that both poliovirus and rhinovirus have been shown to cause the alteration of nuclear import, and enhance degradation of nuclear pore complex components suggest this mechanism may be a common feature of infection with these picornaviruses (Belov et al., 2000; Gustin & Sarnow, 2001, 2002). At later times, there is an accumulation of 3C-containing proteins in the cytoplasm, at sites closely associated with viral RNA replication. It is important to understand the various pathways leading to early versus late 3C-precursor processing because this enzyme and its activities control many of the mechanistic elements leading to viral pathogenesis.
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