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A Proline-Rich Region in the Coxsackievirus 3A Protein Is Required for the Protein To Inhibit Endoplasmic Reticulum-to-Golgi Transport

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The ability of the 3A protein of coxsackievirus B (CVB) to inhibit protein secretion was investigated for this study. Here we show that the ectopic expression of CVB 3A blocked the transport of both the glycoprotein of vesicular stomatitis virus, a membrane-bound secretory marker, and the alpha-1 protease inhibitor, a luminal secretory protein, at a step between the endoplasmic reticulum (ER) and the Golgi complex. CVB 3A contains a conserved proline-rich region in its N terminus. The importance of this proline-rich region was investigated by introducing Pro-to-Ala substitutions. The mutation of Pro19 completely abolished the ability of 3A to inhibit ER-to-Golgi transport. The mutation of Pro14, Pro17, or Pro20 also impaired this ability, but to a lesser extent. The mutation of Pro18 had no effect. We also investigated the possible importance of this proline-rich region for the function of 3A in viral RNA replication. To this end, we introduced the Pro-to-Ala mutations into an infectious cDNA clone of CVB3. The transfection of cells with in vitro-transcribed RNAs of these clones gave rise to mutant viruses that replicated with wild-type characteristics. We concluded that the proline-rich region in CVB 3A is required for its ability to inhibit ER-to-Golgi transport, but not for its function in viral RNA replication. The functional relevance of the proline-rich region is discussed in light of the proposed structural model of 3A.

Enteroviruses (poliovirus, coxsackievirus, echovirus, and several unnamed viruses) are small viruses that contain a 7.5-kb single-stranded RNA genome with positive polarity. The genomic RNA harbors one large open reading frame that codes for a polypeptide containing several small protein domains (2A pro, 2B, 2C, 3A, 3B, 3C pro, and 3D pol) as well as the replication proteins and the nonstructural replication proteins (2). Replication of the viral RNA (vRNA) takes place in replication complexes in conjunction with secretory pathway-derived membrane vesicles that accumulate in the cytoplasm of the infected cell (2, 24). Enteroviruses are nonenveloped, cytoplasmic viruses that do not rely on an intact secretory pathway to release their virus progeny. Instead, poliovirus (PV) has been shown to induce a general blockage of protein secretion (8). Through the individual expression of the different nonstructural proteins of PV, Doedens and Kirkegaard have shown that proteins 2B and 3A are each sufficient to inhibit transport through the secretory pathway (8). The step blocked by the 2B protein is presently unknown. The expression of the PV 3A protein resulted in the accumulation in the ER of both the G protein of vesicular stomatitis virus (VSVG) and the alpha-1 protease inhibitor (A1PI) (7, 8). Moreover, the inhibition of enterovirus 3A protein is involved in multiple steps in the process of viral RNA replication. The 3A protein is a small hydrophobic protein (87 to 89 amino acids [aa]) that contains a C-terminal hydrophobic anchor which is responsible for its membrane association (29). Several studies have shown that mutations in 3A give rise to defects in viral RNA synthesis (1, 10, 12, 35). The membrane-bound precursor 3AB is most likely the donor of VPg (i.e., 3B), the peptide that serves as the primer for vRNA synthesis, to the membranous replication complex (21). Moreover, 3AB serves as a cofactor for the binding of 3CD to the 5’ and 3’ termini of the RNA genome (11), the polymerase activity of 3D pol (16, 20), and the autocatalytic processing of 3Dpro to 3Cpro and 3Dpol (18).

For this study, we investigated whether the function of the enterovirus 3A proteins in interfering with endoplasmic reticulum (ER)-to-Golgi transport is conserved in the closely related coxsackievirus B (CVB). Expression of the CVB3 3A protein was indeed sufficient to inhibit ER-to-Golgi transport.
All enterovirus 3A proteins contain a proline-rich region in their N termini. The biological significance of this proline-rich region, which may be involved in protein-protein interactions, was investigated by the individual expression of CVB3 3A mutants and the introduction of these mutations into an infectious cDNA clone of CVB3. Our results indicate that the integrity of this proline-rich region is required for the inhibition of ER-to-Golgi transport by the CVB3 3A protein, but not for its functions in mRNA replication.

**MATERIALS AND METHODS**

**Cells and viruses.** Buffalo green monkey (BGM) kidney cells were grown in minimal essential medium (MEM) (Gibco) supplemented with 10% fetal bovine serum. COS-1 cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum. Cells were grown at 37°C in a 5% CO₂ incubator. All viruses used for this study were recombinant CVB3 viruses obtained by the transfection of T7 RNA polymerase-generated run off RNA transcripts from the infectious cDNA clones described below. Virus yields were determined by end-point titration as described previously (30). Virus titers were calculated and expressed as 50% tissue culture infective dose (TCID₅₀) values (23).

**Plasmids.** (i) p5CB3/T7. The CVB3 infectious cDNA clone used for this study was p5CB3/T7, which contains a full-length cDNA of CVB3 (strain Nancy) behind a T7 RNA polymerase promoter. This cDNA clone was constructed by removing the nonviral nucleotides between the T7 promoter and the 5' end of the CVB3 genome from plasmid pCB3/T7 (14). Furthermore, an MluI site was introduced downstream of the 3' end of the CVB3 genome to ensure that in vitro-transcribed RNAs did not contain nonviral nucleotides at their 3' ends, resulting in increased infectivity of the mRNA transcripts (data not shown).

(ii) p5CB3-LUC. Plasmid p5CB3-LUC, which contains the firefly luciferase gene in place of the P1 capsid coding region, was derived from pCB3-LUC (30) and contains the same deletion of nonviral nucleotides at the 5' and 3' termini as p5CB3/T7.

(iii) pSVSG-GFP. The plasmid pSVSG-GFP (28), which encodes the 0.045 VSVG protein fused to enhanced green fluorescent protein (EGFP) at its C terminus, was kindly provided by P. Keller and K. Simons (Max Planck Institute of Molecular Biology and Genetics, Dresden, Germany).

(iv) p3A. For construction of the p3A plasmid, the 3A coding sequence was amplified by use of a forward primer that introduced a SalI restriction site and a start codon preceded by a Kozak sequence (p120-4, 5'-GGG GGG TGC ACC ATG GCA CCA GTA TAC AGA-3' [the SalI site is underlined]) and a reverse primer that introduced a stop codon followed by a BamHI restriction site (p203-5, 5'-GGG TGC ACC TCC CTA TGG AAT GGC GAA GAG GAG-3' [the BamHI site is underlined]). The PCR product was cloned into pEGFP-C3 (Clontech) from which the EGFP coding region had been deleted.

(v) pc-A1PI-EYFP-S-EGFP-3A. The plasmid pCMS-EGFP (Clontech) was used to express A1PI and 3A from a single plasmid. The pCMS-EGFP plasmid contains two promoters, namely, a cytomegalovirus (CMV) promoter followed by a multiple cloning site and a simian virus 40 (SV40) promoter followed by the EGFP coding sequence. The secretory marker A1PI, with enhanced yellow fluorescent protein (EYFP) at its C terminus, was cloned into the multiple cloning site behind the CMV promoter by the use of EcoRI and NotI. The EGFP coding sequence behind the SV40 promoter was replaced with 3A (wild type or mutant) N-terminally fused to EGFP by the use of AgeI and BclI. The fusion of EGFP to the N terminus of 3A did not affect its protein secretion inhibition function (data not shown).

(vi) p5CB3/T7-A1PI-EYFP. For construction of the p5CB3/T7-A1PI-EYFP plasmid, the 5' noncoding region of PV fusid to the coding sequence for the secretory marker A1PI was removed from pLink-2B (8) by the use of SmaI and EcoRI and then cloned into pEGFP-C1 (Clontech) by the use of EcolIII and EcoRI. The A1PI coding sequence was replaced with the coding sequence for A1PI-EYFP by removing A1PI by the use of EcoRV and SmaI and then cloning in the A1PI-EYFP fragment cut with EcoRV and HpaI.

**Mammalian two-hybrid plasmids.** The pACT, pBIND, and pG5lac plasmids were obtained from the mammalian two-hybrid system (Promega). For the construction of pACT-3A and pBIND-3A, the 3A coding sequence was cloned into the BamHI and EcoRV sites of the pACT and pBIND plasmids. To obtain pACT and pBIND-3A mutant plasmids, we replaced the wild-type 3A sequence with mutant 3A sequences by using the enzymes BstI107I and BamHI.

**DNA transfections.** BGM cell monolayers were grown in 6-well plates (for A1PI secretion assays or Western blot analysis) or on coverslips in 24-well plates (for microscopic purposes) to subconfluence and then transfected with 2 µg of plasmid DNA per well of a 6-well plate or with 0.5 µg of plasmid DNA per well of a 24-well plate. COS cell monolayers were grown in 24-well plates to subconfluence and then transfected with 0.75 µg of plasmid DNA (see below). Transfections were performed by use of the FuGENE 6 reagent (Roche) according to the manufacturer’s instructions. Cells were grown at 37°C until further analysis, unless otherwise stated.

**VSVG trafficking.** The subcellular localization of VSVG-GFP was determined as described previously (5). Briefly, BGM cells expressing either VSVG-GFP alone or VSVG-GFP together with 3A were incubated at 40°C. After a temperature shift to 32°C, the cells were fixed, and analyzed by confocal laser scanning microscopy (CLSM) under a TCS NT microscope (Leica Lasertechnik GmbH, Heidelberg, Germany). Rabbit polyclonal anti-calreticulin was obtained from Sigma-Aldrich. Mouse monoclonal anti-GM130 was obtained from BD. Alexa fluor 594 goat anti-rabbit immunoglobulin G and Alexa fluor 594 goat anti-mouse immunoglobulin G were obtained from Molecular Probes. Primary antibodies were diluted 1:200, and conjugates were diluted 1:1,500.

**A1PI secretion assay.** BGM cells grown in six-well plates were transfected with plasmid DNAs, and 20 h after transfection, were washed with phosphate-buffered saline (PBS) and incubated in MEM lacking methionine (Sigma) for 30 min at 37°C. Proteins were pulse labeled with [³⁵S]methionine (50 µCi/well) for 30 min at 37°C and then washed twice with PBS, after which 800 µl of fresh serum-free MEM was added. After a 2-h chase, the medium was collected and adjusted to 1% isy buffer by the addition of 200 µl of 5% isy buffer (250 mM Tris [pH 7.4], 750 mM NaCl, 5 mM EDTA, 5% Nonidet P-40, 0.25% sodium deoxycholate) (SDS) (10). The cells were washed twice with PBS and then homogenized in 1 ml of isy buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.05% SDS). An anti-EGFP rabbit polyclonal antiserum (1:1,000) was added to the cell lysate and culture medium, and the mixtures were incubated at 4°C for 16 h. Antibody-protein complexes were collected by the use of protein A-Sepharose (Amersham Biosciences) for 1.5 h, washed twice with dilution buffer (0.01 M Tris [pH 8.0], 0.14 M NaCl, 0.1% bovine serum albumin, 0.1% Triton X-100), once with Tris (0.01 M Tris, 0.14 M NaCl, 0.1% Triton X-100) and once with 0.05 M Tris (pH 6.8), and then precipitated. The samples were resuspended in 25 µl of Laemmli sample buffer, boiled for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The amounts of radiolabeled A1PI-EYFP in the cell fraction and the medium fraction were quantified by the phosphorimaging device (Bio-Rad Multi-Analyzer, version 1.0.1).
transfection, the cells were lysed in 1
means (SEM). Differences were tested for significance by analysis of variance
fection, the cells were lysed and luciferase activities was assayed as described
among the different samples.
activities, encoded by the pBIND plasmid and allowing monitoring of the trans-
globulins (diluted 1:5,000) and visualized with the Lumi-Lightplus Western
SDS–12.5% polyacrylamide gel and transferred to a nitrocellulose membrane
NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.05% SDS). Samples were run in an
previously (30). The RNA transcripts were checked by agarose gel electrophore-
sis. BGM monolayer cells grown to subconfluence in 25-cm2 flasks were trans-
fected with 2.5 μg of RNA transcripts by the DEAE-dextran method as described
previously (30). After transfection, the cells were grown at 37°C. When virus
growth was observed, the cultures were incubated until the cytopathic effect
(CPE) was complete. In cases in which no CPE was observed after 5 days, the
cultures were subjected to three successive cycles of freezing and thawing, and
200 μl was passaged onto fresh BGM monolayer cells, which were grown at 37°C
for another 5 days.
Sequence analysis of viral RNA. RNAs were isolated from virus suspensions
and reverse transcribed as described previously (30). The 3A coding region was
amplified by PCRs with SuperTag DNA polymerase (HT Biotechnology), and
the sequences were analyzed.
Single-cycle growth analysis. Confluent BGM monolayer cells were infected with
virus at a multiplicity of infection (MOI) of 1 TCID50 for 30 min at room
temperature. The cells were washed three times with PBS, supplied with MEM,
and grown at 37°C. At the indicated times, the cells were disrupted by three
cycles of freezing and thawing, and the virus titers were determined by end-point
titration.
Analysis of viral RNA synthesis. BGM cell monolayers were transfected with 1 μg of T7 RNA polymerase-generated RNA transcripts from MluI-linearized
p53CB1/T7-LUC plasmids as described above. At the indicated times posttransfe-
ction, the cells were lysed and luciferase activities was assayed as described
previously (30).
Calculations. Data are presented as mean values ± standard errors of the
means (SEM). Differences were tested for significance by analysis of variance
(least significant difference [LSD]).
Western blot analysis. BGM cells grown in six-well plates were transfected with
pC-A1PI-EYFP-S-EGFP-3A plasmids (wild type or mutant). At 24 h post-
transfection, the cells were lysed in 1× lysis buffer (50 mM Tris [pH 7.4], 150 mM
NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.05% SDS). Samples were run in an
SDS–12.5% polyacrylamide gel and transferred to a nitrocellulose membrane
(Bio-Rad). EGFP fusion proteins were stained with an anti-EGFP polyclonal
antiserum (diluted 1:10,000) and peroxidase-conjugated goat anti-rabbit immu-
noglobulins (diluted 1:5,000) and visualized with the Lumi-Lightplus Western
blotting substrate (Roche Molecular Biochemicals) according to the manufac-
turer’s instructions.
Mammalian two-hybrid analysis. COS cells grown in 24-well plates were transfected with a total of 0.75 μg of plasmid DNA (1:1:1 mix of the pACT,
pBIND, and pG5lac plasmids). At 48 h posttransfection, the cells were lysed, and
both the firefly luciferase and Renilla luciferase enzyme activities were measured
from the same cell lysate by use of a dual-luciferase reporter assay system
(Permea) as described previously (4). An analysis of the Renilla luciferase
activities, encoded by the pBIND plasmid and allowing monitoring of the trans-
fec tion efficiency, revealed no gross differences in efficiencies of transfection
among the different samples.
RESULTS
Inhibition of ER-to-Golgi transport by CVB3 and 3A expres-
sion. To monitor secretory pathway trafficking, we made use of a
GFP fusion of the temperature-sensitive ts045 mutant of
VSVG, a well-known membrane-bound secretory marker
(VSVG-GFP). Figure 1A shows that at the nonpermissive tem-
perature (40°C), VSVG-GFP was improperly folded and, as
a consequence, retained in the ER (as indicated by its colocal-
ization with calreticulin, an ER protein). Upon shifting the
temperature to the permissive temperature (32°C), the protein
was correctly folded and transported out of the ER. After 45
min, the majority of the protein was found at the Golgi com-
plex (as indicated by its colocalization with GM130, a cis-Golgi
protein) (Fig. 1B). After 120 min, the protein was localized
mostly at the plasma membrane (Fig. 1C).
To investigate whether CVB3 inhibits vesicular trafficking,
we transfected BGM cells with the VSVG-GFP expression
plasmid, incubated them at 40°C, and then infected them with
CVB3 at an MOI of 50. The infected cells were further incu-
bated at 40°C and then shifted to 32°C (for 120 min) at 1-h
time intervals between 0 and 5 h postinfection (p.i.). The cells
were fixed and stained, and VSVG-GFP localization was ana-
lyzed by CLSM. In infected cells that were kept at 40°C,
VSVG-GFP was retained in the ER (Fig. 1D). In infected cells
that were shifted to 32°C at 0, 1, 2, or 3 h p.i., VSVG-GFP was
mainly localized at the plasma membrane (similar to the case
shown in Fig. 1C). In cells that were shifted to 32°C at 4 or 5 h
p.i., VSVG-GFP was found to accumulate in both the ER (Fig.
1E) and a dispersed post-ER compartment that also contained
the cis-Golgi marker (Fig. 1F). Together, these results suggest
that in CVB3-infected cells, protein transport is blocked from
about 4 h p.i. at a step between the ER and the Golgi.
Next, we tested whether expression of the CVB3 3A protein
alone was sufficient to inhibit ER-to-Golgi transport. BGM
cells were cotransfected with VSVG-GFP and a 3A expression
plasmid (at a 1:3 ratio to optimize the cotransfection effi-
ciency). The cells were incubated at 40°C for 20 h (Fig. 1G)
and then shifted to 32°C for 120 min. Figures 1H and I show that
in cells cotransfected with VSVG-GFP and 3A, VSVG was
mainly retained in the ER (Fig. 1H) and in a dispersed
post-ER compartment that also contained the cis-Golgi
marker (Fig. 1I), which was more or less similar to the case for
infected cells.
In order to quantify the inhibitory effects of CVB3 infection
and 3A expression on protein secretion, we made use of the
reporter protein A1PI, a secreted soluble glycoprotein.
In pulse-chase experiments, the level of A1PI secretion
can be determined by measuring the percentages of reporter protein
in the cell and medium fractions. For this study, a fusion protein of A1PI and EYFP was used because the A1PI protein
was found to migrate to the same position as a nonspecific
protein band in BGM cell lysates (data not shown). Figure 2A
shows that the A1PI-EYFP fusion protein, which was labeled
for a 30-min pulse period and immunoprecipitated with anti-
EYFP, was efficiently secreted into the medium during a 2-h
chase period.
The inhibitory effect of CVB3 infection on protein secretion
was determined at various times p.i. Because enterovirus in-
fec tion shuts off cap-dependent translation (9), a plasmid
was used that contained the A1PI coding sequence behind the PV
5′ noncoding region. Cells transfected with this construct were
infected with CVB3 (MOI = 50), pulse labeled for 30 min with
[35S]methionine at 2, 4, or 6 h p.i., and subsequently chased for
2 h in the presence of unlabeled methionine. In cells labeled at
2 h p.i. (i.e., chased between 2.5 and 4.5 h p.i.), the majority
of A1PI was secreted into the medium (Fig. 2A). In cells labeled
at 4 or 6 h p.i. (i.e., chased between 4.5 and 6.5 h p.i. and
between 6.5 and 8.5 h p.i., respectively), however, only a small
amount of A1PI was observed in the medium fraction, whereas
a large amount of A1PI was retained in the cell. The amount
of A1PI in the medium fraction was determined as the per-
centage of the total amount of A1PI and compared to that of
control cells (for which the value was normalized to 100% A1PI
secretion). The average results of three independent
experiments are shown in Fig. 2B. The results demonstrate
that CVB3 infection inhibited reporter protein secretion to
approximately 30% compared to control cells from about 4 h
p.i.
To quantify the inhibitory effect of 3A on protein secretion,
we coexpressed the 3A protein (behind an SV40 promoter)
Control cells

A

40°C
left: VSV-G
middle: ER marker

B

40°C-32°C (45 min)
left: VSV-G
middle: Golgi marker

C

40°C-32°C (120 min)
left: VSV-G

CVB3 infected cells

D

40°C
left: VSV-G
middle: ER marker

E

40°C-32°C (120 min)
left: VSV-G
middle: ER marker

F

40°C-32°C (120 min)
left: VSV-G
middle: Golgi marker

3A-expressing cells

G

40°C
left: VSV-G
middle: ER marker

H

40°C-32°C (120 min)
left: VSV-G
middle: ER marker

I

40°C-32°C (120 min)
left: VSV-G
middle: Golgi marker
and the A1PI secretion marker (behind a CMV promoter) from a single plasmid. The 3A protein was expressed as an EGFP-3A fusion protein (the fusion of EGFP to the N terminus of 3A did not affect its protein secretion inhibition function [data not shown]). As a control, a plasmid was constructed that contained the EGFP sequence behind the SV40 promoter. Western blot analysis showed that both proteins were efficiently expressed (Fig. 2C). Figure 2D shows that the expression of the 3A protein resulted in a severely reduced amount of labeled reporter protein in the medium fraction. A quantitative analysis of A1PI secretion in three independent experiments showed that 3A expression inhibited reporter protein secretion to approximately 25% of that of control cells (Fig. 2E) (the percentage of A1PI secretion was calculated as described above).

**Importance of proline-rich region in 3A for inhibition of ER-to-Golgi transport.** The enterovirus and rhinovirus 3A proteins are characterized by the presence of a proline-rich region in the N terminus (Fig. 3). This proline-rich region is located at aa 17 to 19 (numbering refers to the CVB3 3A protein). Pro17 and Pro19 are present in all 63 enterovirus and rhinovirus 3A proteins that have been sequenced to date (except for enterovirus [EV] type 71). Pro18 is present in nearly all enteroviruses and rhinoviruses (except for coxsackievirus A16 [CVA16], CVA24, EV70, and EV71). All human enterovirus group B (HEV-B) members (which include all CVB types, all echoviruses, and all EV71) have Pro18. Pro17 and Pro18 are present in all 63 enterovirus and rhinovirus 3A proteins that have been sequenced to date (except for EV71). Pro18 is present in nearly all enteroviruses and rhinoviruses (except for CVA16, CVA24, EV70, and EV71). All human enterovirus group B (HEV-B) members (which include all CVB types, all echoviruses, and all EV71) have Pro18.
ruses, some CVA types, and several unassigned EVs) contain additional Pro residues at positions 14 and 20.

Proline-rich regions are often involved in protein-protein interactions (reviewed in reference 13). To investigate the functional importance of the proline-rich region in the CVB3 3A protein, we constructed mutant 3A expression plasmids. Since Pro17, Pro18, and Pro19 are the most conserved proline residues, we first replaced these three residues simultaneously with Ala residues (P17A/P18A/P19A). Figures 4A and B show that this 3A mutant was no longer able to inhibit protein secretion. This was not due to reduced expression of the mutant protein, as it was expressed at a similar level as wild-type 3A (Fig. 4E). Moreover, in cells that coexpressed this mutant 3A protein and VSVG-GFP, we observed that VSVG accumulated at the plasma membrane (data not shown). Thus, the proline-rich region is required for the ability of 3A to inhibit protein secretion.

To investigate the importance of the Pro residues individually, we constructed mutant 3A proteins in which single Pro residues at positions 14, 17, 18, 19, and 20 were replaced with Ala residues (mutations P14A, P17A, P18A, P19A, and P20A, respectively). The P19A mutation completely abolished the ability of 3A to inhibit A1PI secretion (to a similar extent as the P17A/P18A/P19A mutant) (Fig. 4C and D). The P14A, P17A, and P20A mutations also impaired the ability of 3A to inhibit protein secretion, although to a lesser extent. The P18A mutation had no effect on the secretion inhibition activity of 3A. Western blot analysis showed that all mutant 3A proteins were efficiently expressed (Fig. 4E). Thus, the Pro residues at positions 14, 17, 19, and 20, but not that at position 18, are important for the ability of 3A to inhibit protein secretion.

**Importance of proline-rich region in 3A for its function in viral replication.** To study the effect of 3A mutations on viral RNA replication and virus growth, we introduced mutations into an infectious cDNA clone of CVB3. For each mutation, two p53CB3/T7 clones were tested, derived from two independently generated site-directed mutagenesis clones. The effects of the P17A/P18A/P19A, P14A, P17A, P18A, P19A, and P20A mutations on virus viability were studied by the transfection of BGM cells with RNA transcripts. The results obtained for the P17A/P18A/P19A mutant are described below. Cells transfected with RNA transcripts carrying single Pro mutations exhibited complete CPE in all transfections (Fig. 5A). vRNAs were isolated from these cell cultures, and the 3A coding region was amplified by reverse transcription-PCR and then sequenced. In all cases, the original mutations were retained in the vRNAs, and no secondary amino acid replacements had occurred. The mutant viruses were further characterized by single-cycle growth analysis (Fig. 5B). Viruses carrying mutations exhibited wild-type growth characteristics. This indicated that the mutant 3A proteins were correctly folded. In conclusion, mutation of the individual Pro residues had no effect on viral replication.

**Identification of a second-site suppressor mutation.** Cells transfected with RNA transcripts containing the P17A/P18A/P19A mutations exhibited no CPE up to 5 days posttransfection. After passage to fresh BGM cells, CPE was observed in only one of eight transfected cell cultures (Fig. 6A). A sequence analysis of the 3A coding region showed that the introduced mutations were retained in the obtained virus but that a second-site suppressor mutation, A→T, had occurred at nt 5119. This nucleotide mutation resulted in a Ser-to-Cys

![Graphical representation of the alignment of enterovirus and rhinovirus 3A proteins](image-url)
substitution at aa 31 of the 3A protein. To demonstrate that this amino acid change in 3A was indeed responsible for the growth of this mutant virus, we introduced the P17A/P18A/P19A/S31C mutations into the infectious cDNA clone. BGM cells transfected with RNA transcripts of this mutant showed CPE on all occasions. A sequence analysis of the 3A coding regions of the resulting viruses showed that the introduced mutations were retained in the vRNAs. Thus, a S31C mutation can indeed rescue the defect in virus growth caused by the P17A/P18A/P19A mutations. Single-cycle growth analysis showed that the mutant virus exhibited a severe delay in virus growth (Fig. 6B).

To investigate whether the quasi-infectious phenotype [a definition that indicates that the mutation disrupts vRNA replication to such an extent that (pseudo)reversion mutations or second-site suppressor mutations can arise but that no virus progeny can be observed harboring the original mutation] of the P17A/P18A/P19A mutant was due to a defect in polyprotein processing, we performed in vitro translation reactions with RNA transcripts generated from the wild type and the mutant cDNA clone. An analysis of the [35S]methionine-labeled translation products by SDS-PAGE showed no differences between the wild type and the mutant, arguing that it is unlikely that the growth defect of the mutant was due to aberrant processing of the viral polyprotein (data not shown). To investigate whether the primary defect occurred at the level of vRNA replication, we introduced the P17A/P18A/P19A mutations into p53CB3/T7-LUC, a subgenomic replicon that contains the luciferase gene in place of the P1 coding region. BGM cells were transfected with RNA transcripts of this clone, and at 10 h posttransfection, the cells were lysed and luciferase expression was analyzed. Transfected cells were grown in the absence or presence of guanidine hydrochloride (GuHCl), an inhibitor of enterovirus replication that is used to determine luciferase levels in the absence of replication (under this condition, luciferase production only reflects translation of the transfected replicon RNA). Figure 6C shows that luciferase production by replicons carrying the P17A/P18A/P19A mutant was the same in the absence or presence of GuHCl and also the same as that observed for wild-type replicons when replication was inhibited by GuHCl. However, replicons carrying the P17A/P18A/P19A/S31C mutant showed an increase in luciferase production. These findings provide evidence that the quasi-infectious phenotype of the P17A/P18A/P19A mutant was due to a primary defect in vRNA replication.

We also investigated whether the second-site suppressor mutation could rescue the effect of the P17A/P18A/P19A mutant on protein secretion. To this end, we introduced the P17A/P18A/P19A and S31C mutations into the 3A protein and tested their effect on A1PI secretion as described above. The P17A/P18A/P19A/S31C mutant caused a similar disruptive effect on the ability of 3A to inhibit A1PI secretion as the

FIG. 4. Proline-rich region is required for secretion inhibition by CVB3 3A. Cells were transfected with plasmids expressing the indicated 3A mutant proteins from an SV40 promoter and the A1PI reporter protein from a cytomegalovirus promoter. Twenty hours after transfection, the cells were labeled with [35S]methionine for 30 min and chased in the presence of unlabeled methionine for 2 h. Cell (C) and medium (M) fractions were collected, immunoprecipitated with an anti-EGFP serum, analyzed by SDS-PAGE (A and C), and quantified by phosphorimaging (B and D). The amount of secreted A1PI in the absence of 3A was normalized to 100%, and the ability of 3A and 3A mutants to secrete A1PI was calculated as a percentage of the A1PI secretion in control cells without 3A. Values represent means ± SEM of three independent experiments. a, significantly different from A1PI secretion in control cells without 3A; b, significantly different from A1PI secretion in the presence of wild-type 3A (calculated by analysis of variance with LSD; *P < 0.05). (E) Western blot of EGFP-3A wild-type and mutant proteins.
P17A/P18A/P19A mutant (Fig. 6D). Thus, the second-site suppressor mutation S31C did not rescue the defect in protein secretion inhibition caused by the P17A/P18A/P19A mutations.

It has been suggested that the enterovirus 3A protein forms a homodimer. Cross-linking and analytical ultracentrifugation studies showed that the first 60 aa of the PV 3A protein form a homodimer (26). Evidence for PV 3A homomultimerization was also obtained by yeast two-hybrid analysis (34). The possibility was considered that the second-site suppressor mutation S31C might restore 3A-3A interactions that were disrupted by the P17A/P18A/P19A mutations. We investigated homomultimerization reactions of CVB3 wild-type 3A and 3A proteins carrying the P17A/P18A/P19A or P17A/P18A/P19A/S31C mutations by use of a mammalian two-hybrid system (4). To this end, we cloned the 3A sequences in the correct reading frame into the expression plasmids pACT (which provides the yeast Gal4 DNA binding domain) and pBIND (which provides the herpes simplex virus type 1 VP16) activation domain of herpes simplex virus type 1 VP16). To this end, we cloned the 3A sequences in the correct reading frame into the expression plasmids pACT (which provides the yeast Gal4 DNA binding domain) and pBIND (which provides the herpes simplex virus type 1 VP16) activation domain of herpes simplex virus type 1 VP16).

FIG. 5. Proline-rich region is not important for CVB3 growth. (A) Effect of the indicated 3A mutations on virus growth. Mutations were introduced into the infectious cDNA clone p53CB3/T7. Copy RNA transcripts were transected into BGM cells and examined for the ability to yield viruses. All mutations resulted in viable viruses except the P17A/P18A/P19A mutations, which caused a quasi-infectious (q.i.) phenotype. (B) Single-cycle growth analysis of mutant viruses. BGM cells were infected at an MOI of 1 and incubated at 37°C. At 2, 4, 6, or 8 h postinfection, viruses were released from the infected cells by three cycles of freezing and thawing. Virus titers were determined by titration on BGM cells and expressed in TCID₅₀ values.

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DISCUSSION

In this study, we showed that CVB3 infection and expression of the 3A protein alone interfere with protein secretion by blocking ER-to-Golgi transport. Moreover, we demonstrated that the proline-rich region in the N terminus of 3A is required for this ability. Pro₁⁷, Pro₁⁸, and Pro₁⁹ are evolutionarily conserved in nearly all enteroviruses and rhinoviruses. Of these residues, Pro₁⁸ was found to be the most important for 3A to inhibit protein secretion. Mutation of this residue almost completely abolished this activity. The mutation of Pro₁⁷, on the other hand, was far less disruptive for the inhibitory activity of 3A. Mutation of this residue was even less disruptive than mutations of the less-conserved Pro₁⁴ and Pro₂⁰ residues, which are conserved in all HEV-B members (i.e., all CVBs and echoviruses, some CVAs, and some unnamed enteroviruses) but not in other enteroviruses and rhinoviruses. Remarkably, the mutation of Pro₁⁸ had no notable effect.

The mechanism by which 3A inhibits protein secretion is still unknown. Therefore, we can only speculate about a role of the proline-rich region in the inhibition of protein secretion. Pro residues can play an important role in protein-protein interactions (reviewed in reference 32). We propose that the 3A protein exerts its activity by tethering a cellular protein through an interaction with its proline-rich region.

Two well-known protein interaction domains that interact with Pro residues are Src homology 3 (SH3) and WW domains. SH3 domains recognize proline-rich sequences containing the core PXXP, where “X” denotes any amino acid. WW domains bind ligands containing PPXY or PPLP core motifs, usually flanked by additional Pro residues (13, 17). It seems unlikely, however, that 3A interacts with proteins containing an SH3 or a WW domain. When considering putative interaction domains, one should take into account the fact that the Pro₁⁴ and Pro₂⁰ residues are not conserved among all enteroviruses and rhinoviruses. The 3A proteins of CVB3 and PV (which lacks Pro₁⁴ and Pro₂⁰) most likely use identical mechanisms to inhibit protein secretion. Given the observation that a mutation of Pro₁⁸ had little effect on this ability, these proteins most likely only share Pro₁⁷ and Pro₁⁹ for their secretion inhibition function. Therefore, alternative interaction domains or mechanisms should be considered.

Recently, the structure of the first 60 aa of the PV 3A protein was determined by nuclear magnetic resonance spectroscopy (26). The PV 3A protein was found to form a classic homodimer. Dimerization occurs through ionic interactions, hydrogen bonds, and hydrophobic interactions between two helices formed by aa 23 to 29 and aa 32 to 41. Figure 7 shows the putative structure of the first 60 aa of CVB3, obtained by molecular modeling of the published PV 3A structure. The structure suggests that the proline-rich regions of two interacting 3A proteins are oriented in such a way that they are contiguous and thereby form a Pro platform. We suggest that this Pro platform, rather than specific SH3 or WW binding domains, may be the interaction domain responsible for the binding of a (still unknown) cellular protein.

The CVB3 3A protein inhibited protein secretion to an
extent similar to that previously described for the 3A protein of the closely related PV (7). A PV mutant (mutant 3A-2) containing a Ser insertion between 3A residues 14 and 15 (corresponding to aa 15 and 16 in CVB3) was strongly impaired in inhibiting A1PI secretion (7). The PV carrying this insertion mutation showed a reduced ability to inhibit the presentation of MHC I-antigen complexes (3) and to block the secretion of cytokines and interleukins (6). The introduction of this mutation into CVB 3A (i.e., a Ser insertion between residues 15 and 16) also interfered with the ability of 3A to inhibit A1PI secretion (data not shown). The mutation disrupted this 3A function to a similar extent as the P19A mutation. It is therefore reasonable to assume that CVB3 carrying Pro-to-Ala mutations (in particular P19A) is also impaired in the ability to suppress MHC I-antigen presentation and to block the secretion of cytokines and interleukins.

Enterovirus genome replication takes place at secretory pathway-derived membrane vesicles that accumulate in the cytosol of the infected cell. The 2BC protein has been identified as playing a major role in the accumulation of these vesicles (2, 24, 25). It has been speculated that the 3A-induced inhibition of ER-to-Golgi transport might also contribute to the accumulation of the vesicles with which the viral replication complexes are associated (27). However, in the present study, we showed that viruses carrying mutations in 3A that interfered with its ability to inhibit ER-to-Golgi transport replicated with wild-type growth characteristics, arguing against a possible role of 3A in the accumulation of replication vesicles. It
cannot be excluded that the 3A protein contributes to an accumulation of vesicles, but this activity does not seem to be essential for viral RNA replication, at least not in vitro.

In contrast to the wild-type phenotype of viruses carrying the single Pro-to-Ala mutations, the P17A/P18A/P19A mutant yielded a quasi-infectious phenotype. Upon multiple RNA transfections, only on one occasion was virus obtained. A sequence analysis of the viral RNA showed that a second-site suppressor mutation at position 31 of 3A (S31C) had occurred. This second-site suppressor mutation rescued a defect in 3A in viral RNA replication, but not its ability to inhibit protein secretion. Since the single Pro-to-Ala mutations did not affect viral RNA replication, it can be hypothesized that the P17A/P18A/P19A mutations interfere with the conformation of 3A or its precursor 3AB and thereby with its function in vRNA replication. Indeed, we found that the P17A/P18A/P19A mutation interfered with 3A homomultimerization in a mammalian two-hybrid system. This finding is remarkable because the structural model predicts no important role for the proline-rich region in dimerization. Together, these data are consistent with the idea that the P17A/P18A/P19A mutation causes a general disruption of the 3A structure. How the S31C suppressor mutation, which is present in the loop between the two α-helices implicated in dimerization (Fig. 7), can (partially) rescue the defect in vRNA replication imposed by the P17A/P18A/P19A mutation remains to be established. Further research is needed to establish the requirement of 3A dimerization for its function in vRNA replication and its ability to inhibit ER-to-Golgi trafficking.

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