JOURNAL OF VIROLOGY, Dec. 1997, p. 9054–9064 Vol. 71, No. 12
0022-538X/97/$04.00+0

Inhibition of Endoplasmic Reticulum-to-Golgi Traffic by Poliovirus Protein 3A: Genetic and Ultrastructural Analysis

JOHN R. DOEDENS,[1]† THOMAS H. GIDDINGS, JR.,[1] AND KARLA KIRKEGAARD[2]*

Department of Molecular, Cellular and Developmental Biology and Howard Hughes Medical Institute, University of Colorado, Boulder, Colorado 80309,[1] and Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305[2]

Received 16 May 1997/Accepted 28 August 1997

Poliovirus protein 3A, only 87 amino acids in length, is a potent inhibitor of protein secretion in mammalian cells, blocking anterograde protein traffic from the endoplasmic reticulum (ER) to the Golgi complex. The function of viral protein 3A in blocking protein secretion is extremely sensitive to mutations near the N terminus of the protein. Deletion of the first 10 amino acids or insertion of a single amino acid between amino acids 15 and 16, a mutation that causes a cold-sensitive defect in poliovirus RNA replication, abrogates the inhibition of protein secretion although wild-type amounts of the mutant proteins are expressed. Immunofluorescence light microscopy and immunoelectron microscopy demonstrate that 3A protein, expressed in the absence of other viral proteins, colocalizes with membranes derived from the ER. The precise topology of 3A with respect to ER membranes is not known, but it is likely to be associated with the cytosolic surface of the ER. Although the glycosylation of 3A in translation extracts has been reported, we show that tunicamycin, under conditions in which glycosylation of cellular proteins is inhibited, has no effect on poliovirus growth. Therefore, glycosylation of 3A plays no functional role in the viral replicative cycle. Electron microscopy reveals that the ER dilates dramatically in the presence of 3A protein. The absence of accumulated vesicles and the swelling of the ER-derived membranes argues that ER-to-Golgi traffic is inhibited at the step of vesicle formation or budding from the ER.

Poliovirus is a nonenveloped positive-sense RNA virus that interacts extensively with its primate host cells during its lytic infectious cycle. The viral genome contains a 742-nucleotide (nt) 5′ noncoding region, a single 6,627-nt open reading frame encoding the viral polyprotein, 70 nt of 3′ noncoding sequence, and, on average, 65 nt of poly(A) (35, 52). After the virus enters the cell, the viral RNA genome is uncoated and translated to produce a 247-kDa polyprotein which is cleaved by viral proteases to yield individual viral proteins. The first third of the open reading frame encodes the structural proteins that constitute the viral capsid. The remainder of the coding region contains genes for the viral nonstructural proteins which are responsible for proteolytic processing of the viral polyprotein, replication of the viral genome, and the inhibition and possible subversion of normal cellular functions in the infected cell.

The inhibition of host gene expression in poliovirus-infected cells has been studied extensively. Viral protein 2A, either directly (40) or in conjunction with cellular proteins (64), induces the cleavage of p220, a component of the cellular cap-binding complex, resulting in the inhibition of cap-dependent initiation of translation in poliovirus-infected cells (21). Other, as yet undefined alterations in the infected cell may also contribute to the inhibition of cellular translation (11, 33, 49). Translation of poliovirus RNA, one of the few RNAs that can be translated in the infected cell (reviewed in reference 46), is initiated by the internal binding of ribosomes to RNA sequences and structures in the viral 5′ noncoding region (34, 48). Inhibiting host cell translation presumably increases the concentrations of free ribosomes available to translate the poliovirus genome. In addition, the C-terminal cleavage product of p220, in complex with canonical translation factors, stimulates internal binding of ribosomes to the viral 5′ noncoding region directly (39, 67). The virus presumably inhibits cellular translation both to increase the translation rate of the viral genome and, possibly, to thwart any host defense that requires new protein synthesis.

Poliovirus infection also inhibits cellular RNA synthesis (30, 68); transcription factors, including the TATA-binding protein, that are required for transcription by RNA polymerase I, II, and III are inactivated during infection (13, 14, 54). The viral function of this inhibition could be to block host defenses that require new RNA synthesis, to increase the intracellular concentration of ribonucleotides, or to disassemble nuclear complexes.

Protein transport through the host secretory pathway is inhibited during poliovirus infection (19). The function of this inhibition in the viral life cycle is unknown. The inhibition of protein secretion is independent of the shutoff of cellular gene expression, with viral proteins 2B, 2BC, and 3A each being sufficient to inhibit protein secretion in the absence of other viral gene products. Of these three proteins, 3A is apparently the most potent and specific inhibitor, blocking exit of secreted proteins from the endoplasmic reticulum (ER). The work presented here extends our understanding of the inhibition of protein secretion by 3A.

To date, the only activity demonstrated for poliovirus 3A protein in eukaryotic cells is the inhibition of protein secretion (19). When expressed in *Escherichia coli*, 3A increases the permeability of the plasma membrane (37). Expression of 3A does not increase plasma membrane permeability in mammalian cells (19), although it is possible that the permeability of intracellular membranes is affected. Several activities have been demonstrated in vitro for 3A's proteolytic precursor, 3AB. 3AB differs from 3A by the presence of the 22-amino-acid peptide 3B (VPg) at its C terminus. RNA-dependent

* Corresponding author. Phone: (650) 498-7075. Fax: (650) 498-7147. E-mail: karlak@leland.stanford.edu.
† Present address: Immunex, Inc., Seattle, WA 98101.

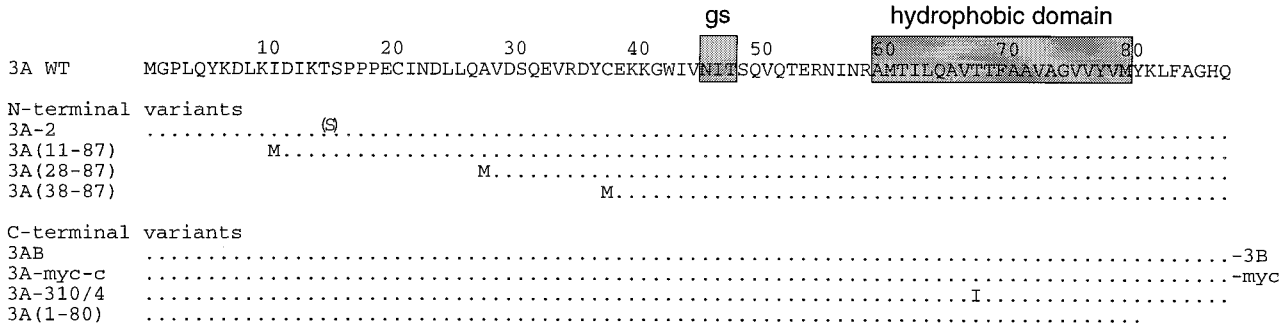

FIG. 1. Sequences of 3A proteins used in this study. The wild-type (3A WT) sequence shown differs from that of the 3A protein expressed in poliovirus-infected cells only by the presence of an initiator methionine residue in the first position. The consensus N-glycosylation sequence (gs) and hydrophobic domain are indicated. Periods indicate unchanged residues, and parentheses indicate amino acid insertions rather than substitutions.

RNA polymerase activity of 3D and proteolytic activity of 3CD are both stimulated by addition of detergent-solubilized 3AB, but not 3A, in vitro (38, 44, 51). Also, detergent-solubilized 3AB, but not 3A, shows both nonspecific RNA-binding activity and specific RNA binding to a cloverleaf structure at the 5′ end of the positive-sense viral genome (29, 47, 65). Membrane-associated 3AB can serve as a substrate for proteolysis by 3C and 3CD in vitro and binds directly to purified 3D polymerase (31). These in vitro properties are consistent with a function for 3AB in the replication of the viral genome, and interpretations of viral defects stemming from mutations in 3A have frequently presumed that the mutations were disrupting 3AB function.

Mutations in the 3A coding region give rise to viruses defective in RNA synthesis (5, 24, 31, 65); however, a specific function for 3A protein in the viral replicative cycle has not been demonstrated. An understanding of the phenotypes of viruses containing mutations in 3A is further complicated by the observation of both recessive (complementable [6]) and *cis*-dominant (noncomplementable [23]) mutations in the 3A coding region. It is likely that alleles with such different genetic properties are defective in different functions of 3A-containing proteins in the viral replicative cycle.

Both 3A and 3AB are membrane associated in infected cells (59); this association is thought to be mediated by the hydrophobic domain near the C terminus of 3A (18, 59). 3A and 3AB expressed by using vaccinia virus vectors showed staining suggestive of ER or Golgi localization; however, no cellular markers were used to identify these intracellular compartments (18). The membrane topology of 3A and 3AB also remains an open question. The need for proteolytic processing at both the N and C termini by cytosolic viral proteases predicts that both termini reside on the cytoplasmic side of the membrane, at least at some time during the synthesis and maturation of the protein. Further, the viral protein 3B (VPg) is a direct participant in the replication of the viral genome and is therefore expected to be cytoplasmic, arguing that the C termini of 3AB and 3A are cytoplasmic. Protease sensitivity experiments on membrane extracts of infected cells suggested that the N terminus of 3AB, and thus probably 3A, was also cytoplasmic (60). Although the 22-amino-acid hydrophobic domain of wild-type 3A is longer than the 20-amino-acid length required to span a lipid bilayer as an α-helix, mutant proteins in which the hydrophobic domain was shortened to only 13 amino acids still retained their association with membranes in vitro (62). The single argument that 3A might span the lipid bilayer, with its N-terminal domain in the lumen of the ER or Golgi, has been the presence of a consensus N-glycosylation site amino terminal to 3A's hydrophobic domain (Fig. 1) and the observation that a fraction of 3A and 3AB was N-glycosylated when translated in vitro in the presence of microsomal membranes (18). It was suggested that the hydrophobic domain of 3A might span the lipid bilayer and that N-glycosylation of 3A-containing proteins might play a functional role during poliovirus infection. In the present study, however, we report that tunicamycin, an inhibitor of N-glycosylation, has no effect on poliovirus yield, in agreement with previous reports (17, 27), arguing that the glycosylation of 3A and 3AB observed in vitro is not relevant to the biology of the virus.

In an attempt to correlate viral phenotype with the ability of 3A to inhibit secretion, we assayed protein secretion in cells that expressed either of two characterized mutant 3A proteins; one of these mutant 3A proteins showed a marked reduction in its ability to inhibit secretion compared to wild-type 3A. Examination of 3A variants with altered N and C termini showed that N-terminal residues are required for 3A to inhibit secretion. 3A proteins with altered C termini, including 3AB, were generally expressed at low or undetectable levels, arguing that alterations in this region affect stability of the protein in COS-1 cells. Finally, we show that 3A is localized to the ER in COS-1 cells and that expression of 3A leads to a pronounced swelling of ER membranes detected by electron microscopy.

MATERIALS AND METHODS

Cells, antibodies, and reagents. COS-1 cells (obtained from Robert Schneider, New York University) were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum and 100 U of penicillin and 100 mg of streptomycin per ml. All cells were grown at 37°C in a 5% CO_2 incubator. Rabbit polyclonal antibody to α1 protease inhibitor (A1PI) was provided by Jerry Brown (University of Colorado Health Sciences Center). Tunicamycin was purchased from Calbiochem and stored as a 10-mg/ml stock in dimethyl sulfoxide (DMSO). Fluorescein- and Texas red-conjugated secondary antibodies were purchased from Jackson Immunoresearch and stored according to the manufacturer's instructions. Lipofectamine transfection reagent was from Gibco/BRL.

Construction of plasmids. To construct dicistronic plasmids that encode the desired 3A variant in the first cistron and A1PI in the second cistron, the desired 3A coding region was amplified by PCR from Mahoney type 1 poliovirus cDNA, using deoxyoligonucleotide primers designed to introduce a *Sal*I site and an AUG start codon immediately upstream and a stop codon and a *Sma*I site immediately downstream of the target 3A sequence. The deoxyoligonucleotide primer used to add an epitope recognized by anti-Myc monoclonal antibody 9E-10 added 12 amino acids, MEQKLISEEDLN, to the C terminus of 3A. The resulting PCR products were digested with *Sma*I and *Sal*I and cloned into pLINKα6, a plasmid containing the poliovirus 5′ noncoding region fused to A1PI coding sequence (19). For all plasmids that contain truncated 3A sequences and for the 3A-Cmyc plasmid, the wild-type poliovirus clone pT7pGEMpolio was used as the template for PCRs. The 3A-2 coding sequence was amplified from a plasmid provided by Peter Sarnow (Stanford University) (5). The 3A-310/4 coding sequence was amplified from pKOΔ-310/4 (23, 24), provided by Bert Semler (University of California, Irvine). DNA sequences of all PCR-amplified regions within the dicistronic plasmids were confirmed by sequencing.

DNA transfections. For the secretion and electron microscopy (EM) experiments, plasmid DNA was introduced into COS-1 cells by using Lipofectamine transfection reagent (Gibco). For the protein secretion experiments, COS-1 cells growing in 12-well dishes were transfected with 600 ng of the desired plasmid DNA, and 4 μl of Lipofectamine (Gibco) was used per well. The DNA and Lipofectamine were each diluted to 50 μl of OptiMEM (Gibco)/well. The DNA and Lipofectamine dilutions were combined, mixed by vortexing, and incubated for 20 min at room temperature before the addition of 400 μl of OptiMEM/well. The cells were rinsed once with OptiMEM; then 500 μl of the transfection mixture was added to each well, and the cells were incubated at 37°C. After 5 h at 37°C, 500 μl of DME containing 20% calf serum was added to each well, and incubation was continued overnight. The next morning, the transfection medium was replaced with DME containing 10% calf serum, and incubation was continued at the temperature specified for each experiment. For transfection of COS-1 cells growing on larger dishes, volumes of reagents and amounts of DNA were increased according to the recommendations of the manufacturer.

For immunofluorescence experiments, COS-1 cells growing on coverslips were transfected by using calcium phosphate (3).

SDS-tricine gels and Western blotting. Lysates of transfected COS-1 cells were electrophoresed on tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gels as described previously (55), using a 4% stacking gel and a 16.5% running gel. After overnight electrophoresis, separated proteins were transferred to Immobilon membranes (Millipore) by using a Genie electrophoretic blotter (Idea Scientific). Efficiency of transfer was verified by using prestained molecular weight markers. The resulting membranes were treated for at least 30 min in a solution of 2% bovine serum albumin (BSA) and 0.1% Tween 20 in phosphate-buffered saline (PBS). After blocking, the blots were incubated in anti-3A hybridoma supernatant for at least 1 h at room temperature. The blots were then washed three times for 5 min each in PBS containing 0.1% Tween 20. The blots were then incubated in anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution in PBS containing 2% BSA and 0.1% Tween 20) for 1 h at room temperature. After three washes as described above, immune complexes with bound secondary antibodies were detected by enhanced chemiluminescence (ECL kit; Amersham) according to the manufacturer's instructions.

Protein secretion assays. COS-1 cells growing in 12-well plates were transfected by using Lipofectamine. Temperature shifts were performed 1 day posttransfection as indicated. At 2 days posttransfection, the cells were washed once with PBS^+ (PBS containing 100 μg of both $CaCl_2$ and $MgCl_2$ per ml) and then incubated in DME (200 μl/well) lacking methionine (Gibco) to which [^{35}S]methionine (10 μCi/well; Expre^{35}S^{35}S labeling mix; New England Nuclear) had been added for 30 min at the appropriate temperature. The labeling medium was then removed; the cells were washed once with PBS^+ and then incubated for 2 h at the indicated temperature in DME that contained a large molar excess of unlabeled methionine. At the end of the chase period, the culture medium was removed and saved. The cells were washed once with ice-cold PBS, and 250 μl of ice-cold TX100 lysis buffer (PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride) was added to each well. The plates were then incubated on ice for 30 min with occasional mixing. The resulting lysates were transferred to microcentrifuge tubes, insoluble material was pelleted, and the supernatants were transferred to fresh tubes for immunoprecipitation of A1PI.

The cell lysates and culture supernatents were then subjected to immunoprecipitation to identify the cell-associated and secreted A1PI fractions, respectively. Affinity-purified rabbit polyclonal antibody directed against A1PI was diluted with PBS that contained 1% Triton X-100, 0.5% sodium deoxycholate, 0.5% SDS, and 1% BSA. An equal volume of antibody dilution was added to each lysate sample to be precipitated, and 200 μl of antibody dilution was added to each sample of culture supernatant. Immunoprecipitations were then incubated for at least 2 h on ice, and antibody-antigen complexes were collected by incubation with fixed *Staphylococcus aureus* cells (Gibco) that had been washed three times with TX100 lysis buffer. The cells and bound immune complexes were washed three times in ice-cold PBS that contained 1% Triton X-100, 0.5% deoxycholate, and 1% SDS. Immunoprecipitated A1PI from cell lysates and culture supernatants was released from the *S. aureus* cells by heating to 95°C in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and displayed by SDS-PAGE on 10% gels. Radiolabeled A1PI was visualized and quantified by using a PhosphorImager and ImageQuaNT software (Molecular Dynamics). The percentage of A1PI secreted was determined from the ratio of A1PI secreted to the total amount of A1PI detected in both fractions. The error in Tables 1 and 2 is given as 1 standard deviation from the mean determined from duplicate transfection experiments.

Immunofluorescence microscopy. COS-1 cells were plated onto coverslips 1 day prior to transfection. At 2 days posttransfection, cells were fixed by treatment with methanol for 10 min at −20°C followed by treatment with acetone for 10 min at −20°C. After three washes with PBS, the coverslips were incubated for 1 h at 37°C with primary antibodies diluted in PBS containing either 5% calf serum or 2% BSA as a blocking agent. After three more washes with PBS, the coverslips were incubated for 1 h at 37°C with appropriate secondary antibodies diluted in PBS that contained either 5% calf serum or 2% BSA. The coverslips were washed and mounted in a Mowiol solution containing 2% [wt/vol] 1,4-diazobicyclo-[2.2.2]-octane as an antifade agent (28). The slides were examined on a Zeiss microscope equipped with a 40× or 63× objective and photographed with Kodak TMAX 400 film.

High-pressure freezing, freeze-substitution, and EM. For cryofixation and EM, COS-1 cells growing on 60-mm-diameter dishes were transfected with a pLINK plasmid that expressed 3A in the first cistron and A1PI in the second cistron (19), using Lipofectamine (BRL) as described above. Control cells were transfected with a plasmid that encoded only A1PI. At 2 days posttransfection, cells were released from the plates with a trypsin-EDTA solution (Gibco/BRL) and suspended in a solution of 0.15 M sucrose in PBS^+. The cells were pelleted for 3 min at 240 × *g* and resuspended in a minimal volume of PBS^+–0.15 M sucrose. Aliquots of the resulting slurry were frozen in a Balzers HPM 10 high-pressure freezing apparatus as described previously (15) and stored in liquid nitrogen. Cells infected with type 1 Mahoney poliovirus were harvested similarly, 5 h after infection at a multiplicity of infection of 50.

For observation of membrane morphology (Fig. 6), samples were freeze-substituted initially in 0.1% tannic acid plus 0.5% glutaraldehyde in acetone at −80°C. Samples were then rinsed in acetone at −80°C; incubated in 2% osmium tetroxide–0.1% uranyl acetate in acetone at −80°C for 4 h, then at −20°C for 16 h, and at 4°C for 6 h; and embedded in Epon-Araldite resin. For immunolabeling, frozen samples were freeze-substituted in 0.01% osmium tetroxide in acetone at −80°C, warmed to −20°C for 3 h, embedded at −20°C in Lowicryl K4M, and subjected to polymerization under UV light at −35°C. Thin sections were stained with 2% uranyl acetate and lead citrate and then imaged at 80 kV in a JEOL 100C or Philips CM10 electron microscope.

Immunogold labeling of high-pressure frozen cells. Thin sections of high-pressure frozen, freeze-substituted cells embedded in Lowicryl were mounted on Formvar-coated nickel grids and immunolabeled as described previously (57). Mouse monoclonal antibody against poliovirus protein 3A was diluted fivefold in PBS that contained 0.1% Tween 20 and 0.1% BSA. The grids were incubated with antibody for 2 h at room temperature, rinsed in PBS^+, and incubated for 1 h at room temperature in a solution containing a 1:10 dilution of goat anti-mouse secondary antibody conjugated to 15-nm colloidal gold (Ted Pella Inc., Redding, Calif.) in PBS^+ that contained 0.1% BSA.

RESULTS

Tunicamycin does not inhibit poliovirus replication. Poliovirus protein 3A blocks ER-to-Golgi traffic in mammalian cells, causing normally secreted proteins to accumulate in the ER (19). To understand the mechanism by which 3A inhibits protein secretion, it is important to determine the intracellular site of action of 3A. The precise topology within the membranes with which 3A is associated is unknown, although several lines of argument support a peripheral association with the cytoplasmic surface of intracellular membranes in poliovirus-infected cells (see the introduction). However, the presence of a consensus N-glycosylation site in 3A (Fig. 1), the observation that small amounts of poliovirus proteins 3A and 3AB were N-glycosylated when translated in vitro, and the inhibition of poliovirus RNA synthesis by 6-diazo-5-oxo-L-norleucine, an inhibitor of glycoprotein synthesis, suggested a possible requirement for glycosylation of 3A-containing proteins in poliovirus genome replication (18). Such a glycosylation event would argue that these sequences of 3A (Fig. 1) reside, at least transiently, within the lumen of one or more organelles of the secretory pathway. These authors (18) also suggested that the inhibition of poliovirus by brefeldin A (BFA), an inhibitor of transport through the secretory pathway, might stem from the resulting inhibition of Golgi-specific oligosaccharide modifications in BFA-treated cells. Two pieces of data argued against this hypothesis: glycosylated forms of 3A and 3AB were not detected in extracts of poliovirus-infected cells (18), and tunicamycin had been reported to have no effect on poliovirus infection, although little documentation of these experiments was actually published (17, 27). Due to the fragmentary nature of the previous tunicamycin experiments, and the possible significance of the in vitro glycosylation of poliovirus proteins, we tested the effect of tunicamycin on poliovirus growth under conditions in which tunicamycin was shown to be active.

Tunicamycin is a compound that inhibits N-linked glycosylation by blocking the synthesis of the lipid-linked core oligosaccharide which is donated to glycoproteins in the ER (36,

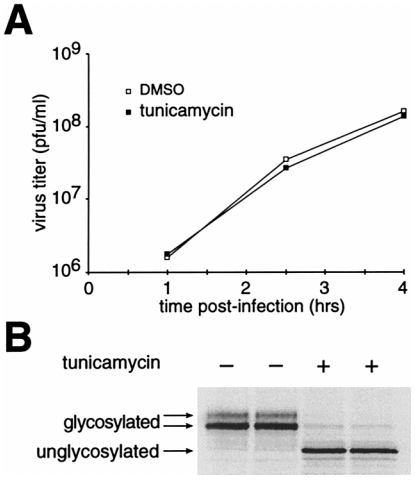

FIG. 2. Effect of tunicamycin on poliovirus production. COS-1 cells were incubated in culture medium with 5 μg of tunicamycin per ml and 0.05% DMSO or in culture medium with 0.05% DMSO for 4 h prior to radiolabeling or infection. (A) Infections of COS-1 cells with poliovirus were performed in the presence of either tunicamycin (5 μg/ml) and 0.05% DMSO or 0.05% DMSO. Plates were harvested at the indicated times postinfection, and virus titers in the resulting cell extracts were determined by plaque assay on HeLa cells. DMSO and tunicamycin were present during virus adsorption and throughout infection. (B) Duplicate plates of COS-1 cells expressing A1PI were labeled with [^{35}S]methionine for 1 h in the presence of tunicamycin or DMSO. A1PI immunoprecipitated from cell lysates was displayed by SDS-PAGE and visualized by using a PhosphorImager; glycosylated and unglycosylated forms of A1PI are indicated.

58). In tunicamycin-treated cells, proteins that are normally N-glycosylated do not receive any of these modifications. Thus, if Golgi-specific oligosaccharide modifications of cellular or viral proteins were required by poliovirus, tunicamycin would be expected to impair virus production.

COS-1 cells were pretreated with either 5 μg of tunicamycin per ml and 0.05% DMSO or 0.05% DMSO alone for 4 h prior to infection. This pretreatment was necessary to observe maximal inhibition of glycosylation by tunicamycin (data not shown). The cells were then infected with poliovirus in the presence of 5 μg of tunicamycin per ml and 0.05% DMSO or 0.05% DMSO only. At various times postinfection, the cells were harvested and intracellular virus production was quantified by plaque assay on HeLa cells (Fig. 2A). No difference in virus yield was seen between the tunicamycin-treated and DMSO-treated cells. In addition, the timing and extent of cytopathic effect, assessed visually as cell rounding and lifting off the substrate, were unaffected by tunicamycin treatment (data not shown).

To confirm that the tunicamycin treatment effectively inhibited glycosylation, COS-1 cells transfected with a plasmid that expresses A1PI, a secreted glycoprotein, were treated with tunicamycin or 0.05% DMSO in parallel with the poliovirus infections. The transfected COS-1 cells were radiolabeled with [^{35}S]methionine for 1 h, and A1PI was immunoprecipitated from lysates of the transfected cells and displayed by SDS-PAGE (Fig. 2B). The lack of glycosylation of A1PI can be visualized as increased mobility of the protein through the gel (42). Quantitation of glycosylated A1PI with a PhosphorImager revealed that greater than 97% of the radiolabeled A1PI from tunicamycin-treated cells was not glycosylated, indicating that tunicamycin had effectively inhibited this modification.

Inhibition of protein secretion by 3A proteins with altered N termini. Several biochemical activities have been demonstrated for poliovirus protein 3AB in vitro; however, the only activities demonstrated for 3A protein in the absence of 3B are (i) the induction of increased membrane permeability when expressed in *E. coli* (37) and (ii) the inhibition of protein secretion when expressed in mammalian cells (19). To investigate the contribution of N-terminal sequences to 3A-induced secretion inhibition, we constructed a series of plasmids that encode N-terminal deletion variants of 3A (Fig. 1). The truncated 3A sequences were cloned into dicistronic plasmids designed to coexpress a single viral protein and A1PI, a secreted glycoprotein. In addition to the N-terminal truncations, the 3A-2 mutation (an insertion of a single serine at position 15) was also introduced into the dicistronic plasmid. The 3A-2 mutation gives rise to mutant viruses that display a cold-sensitive defect in RNA synthesis (5).

To determine whether the 3A truncations could be expressed at intracellular concentrations comparable to those of full-length 3A, extracts of COS-1 cells transfected with the various dicistronic plasmids at 37°C for 2 days were analyzed by Western blotting (Fig. 3A). To analyze the effects of the 3A-2 mutation and wild-type 3A, the effects of incubation at 37°C

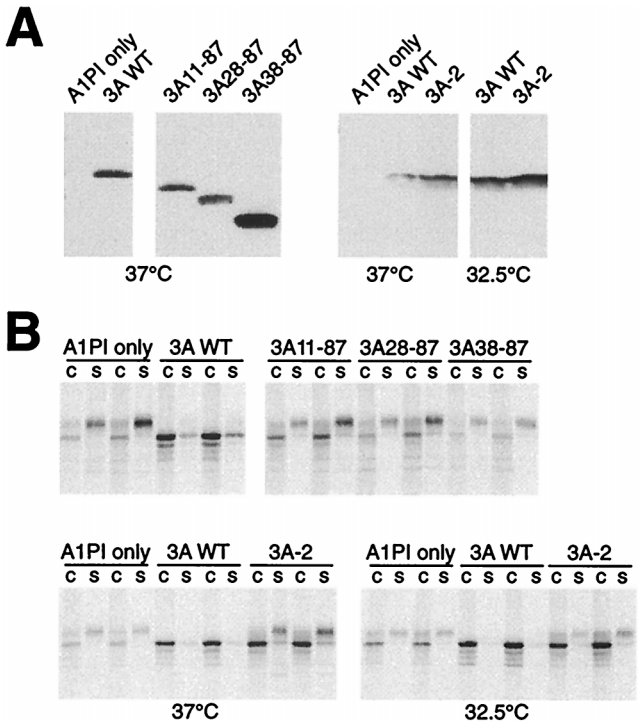

FIG. 3. Effects of 3A proteins with altered N-terminal sequences on A1PI secretion. (A) Expression of wild-type 3A (3A WT) and N-terminal variants. COS-1 cells were transfected with dicistronic plasmids encoding the indicated 3A protein (see Fig. 1) and A1PI. At 1 day posttransfection, the transfected cells were transferred to the indicated temperature. Cell lysates were prepared at 2 days posttransfection, and equivalent amounts of the resulting lysates were separated on SDS-Tricine gels. 3A proteins on Western blots were detected by enhanced chemiluminescence using an anti-3A monoclonal antibody followed by an anti-mouse horseradish peroxidase-conjugated secondary antibody. (B) Effect of 3A mutations on the inhibition of A1PI secretion. COS-1 cells were transfected in duplicate with the indicated dicistronic plasmids and were then incubated as in panel A. At 2 days posttransfection, the cells were labeled with [^{35}S] methionine for 30 min and chased in the presence of unlabeled methionine for 120 min. At the end of the chase period, cell lysates and culture supernatants were collected. Immunoprecipitated A1PI was displayed by SDS-PAGE and was visualized and quantitated with a PhosphorImager. Pairs of lanes show radiolabeled A1PI from a single transfected sample that was either cell associated (c) or secreted (s) after the chase period. The altered mobility of secreted A1PI results from glycosylation and modification by the secretory apparatus.

TABLE 1. Inhibition of A1PI secretion by N-terminal 3A variants

3A variant transfected	% Maximal A1PI secretion (mean ± SD)
37°C	
A1PI only	100 ± 5
3A WT[a]	21 ± 5
3A-2	72 ± 11
3A11-87	72 ± 9
3A28-87	80 ± 2
3A38-87	89 ± 15
32.5°C	
A1PI only	100 ± 3
3A WT	18 ± 3
3A-2	61 ± 3

[a] 3A WT, wild-type 3A.

and of a shift after 1 day of incubation to 32.5°C, the nonpermissive temperature for the 3A-2 virus, were investigated. Proteins present in cellular extracts were separated on SDS-Tricine gels (55) and analyzed by immunoblotting. 3A proteins were detected by using an anti-3A monoclonal antibody that recognizes an epitope located between amino acids 38 and 59 (data not shown).

Deletion of N-terminal sequences did not impair the synthesis or accumulation of 3A, since amounts of 3A11-87, 3A28-87, and 3A38-87 comparable or larger than that of full-length 3A were detected (Fig. 3A). Similarly, amounts of 3A-2 comparable to those of wild-type 3A, if not greater, were detected at both temperatures tested.

The effect of expression of wild-type 3A protein and of N-terminal 3A variants on A1PI secretion was assayed in pulse-chase experiments (Fig. 3B; Table 1). A1PI secretion was monitored by its movement from cells into the medium after a 30-min labeling period, followed by a 2-h chase in the presence of unlabeled methionine (Fig. 3B). The reduction of labeled protein in the cell pellet and the corresponding increase in the medium (S) during the chase period can be quantified as the percent maximal A1PI secretion (Tables 1 and 2). Transit through the secretory pathway is also shown by the change in mobility of A1PI protein, corresponding to glycosylation and modification, as it moves from the cell to the medium (Fig. 3B). Those cases in which A1PI in the medium displays the same electrophoretic mobility as cell-associated A1PI are probably due to breakage of cells during sample preparation.

TABLE 2. Inhibition of A1PI secretion by C-terminal 3A variants

3A variant transfected	% Maximal A1PI secretion (mean ± SD)
37°C	
A1PI only	100 ± 5
3A WT[a]	21 ± 5
3A-310/4	24 ± 4
3A1-80	43 ± 6
3A-MycC	33 ± 2
3AB	87 ± 6
39.5°C	
A1PI only	100 ± 5
3A WT	18 ± 1
3A-310/4	14 ± 2

[a] 3A WT, wild-type 3A.

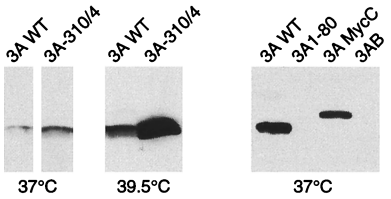

FIG. 4. Expression of 3A proteins with altered C termini. COS-1 cells were transfected with dicistronic plasmids encoding the indicated 3A variants and transferred to the indicated temperatures at 1 day posttransfection. At 2 days posttransfection, lysates were prepared and separated on SDS-Tricine gels. 3A proteins were detected on Western blots as in Fig. 3A. 3A WT, wild-type 3A.

Thus, the values for total protein secreted shown in Tables 1 and 2 are likely to be slightly overestimated.

Despite being expressed at levels comparable to that of the wild-type 3A, all three N-terminal deletions showed significantly reduced ability to inhibit A1PI secretion. Similarly, the 3A-2 protein showed reduced ability to inhibit A1PI secretion at both 32.5 and 37°C. Therefore, the inhibition of protein secretion by poliovirus protein 3A, but not its synthesis or stability, is extremely sensitive to mutation at the N terminus of the protein.

Analysis of 3A variants with altered C termini. A similar set of experiments was performed with 3A proteins bearing altered C termini. Two C-terminal extensions of 3A were tested for the ability to be expressed and to inhibit secretion: 3AB, the naturally occurring fusion protein of 3A and 3B; and 3A-MycC, wild-type 3A with a 12-amino-acid extension containing the Myc epitope tag on its C terminus. In addition, 3A1-80, a seven-amino-acid deletion that retains the hydrophobic domain but not the hydrophilic residues immediately C terminal of the hydrophobic region (Fig. 1), was tested for its effect on cellular protein secretion. Finally, 3A-310/4, a mutation in the hydrophobic domain of 3A that gives rise to temperature-sensitive virus defective in RNA synthesis, was also tested. Transfection with any of these constructs led to efficient A1PI expression (Fig. 3), and therefore the dicistronic RNA that encodes both A1PI and 3A protein variants was likely to have been expressed, and transfection efficiencies were comparable between experiments.

In contrast to the N-terminal deletions, alterations of the C terminus of 3A had dramatic effects on expression levels (Fig. 4). Most notably, expression of the wild-type fusion protein 3AB was not detectable. This observation was surprising since 3AB protein is readily detectable in extracts of poliovirus-infected cells or when expressed by using vaccinia virus vectors (18). Due to the undetectable expression level of 3AB, the observation that it fails to inhibit protein secretion (19) (Table 2) is not interpretable. C-terminal deletion variant 3A1-80 was also expressed at considerably reduced levels compared to full-length 3A. However, extended exposures of Western blots showed the presence of a small amount of 3A1-80, whereas 3AB was not detected even at the longest exposure times (data not shown). Interestingly, even at these reduced expression levels, 3A1-80 showed reduced but significant inhibition of A1PI secretion (Table 2). Therefore, the C-terminal amino acids of 3A, while somewhat dispensable for 3A's activity in inhibiting protein secretion, may modulate the translation or, more likely, the stability of 3A-containing proteins expressed in the absence of other viral proteins.

Two other C-terminal variants of 3A, 3A-Myc and 3A-310/4 (Fig. 4), showed expression levels comparable to that of wild-type 3A at 37°C. Interestingly, 3A-310/4, which gives rise to a temperature-sensitive growth defect in poliovirus, accumulated

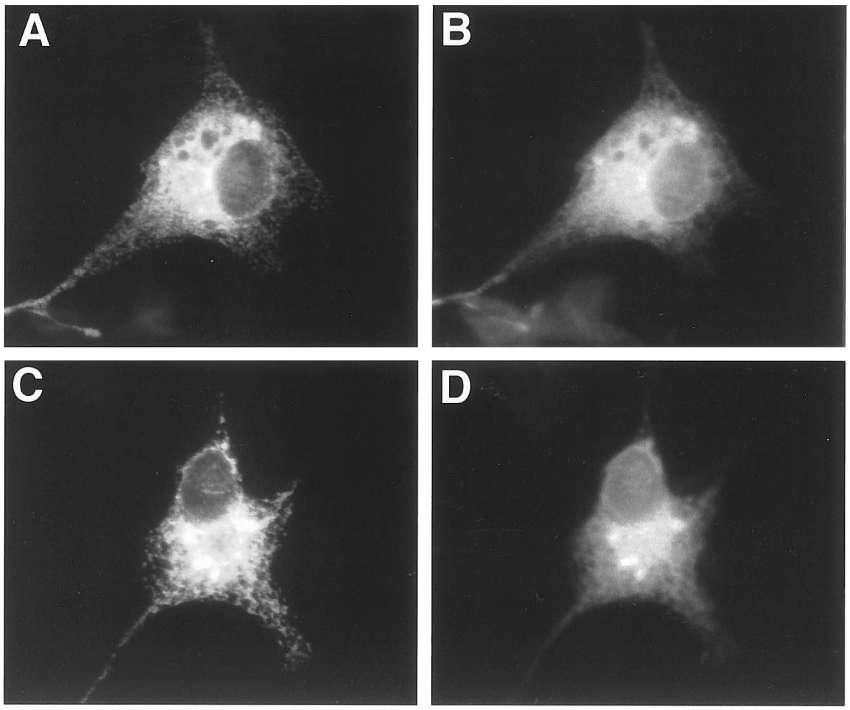

FIG. 5. Localization of 3A and A1PI in transfected COS-1 cells. Double-immunofluorescence experiments display the localization of A1PI, a normally secreted protein coexpressed in transfected cells with poliovirus protein 3A (A), and PDI, a marker for the ER present in all cells in this field (B). A second field shows the localization of A1PI (C) and 3A protein (D), both present only in cells transfected with a plasmid that encodes both proteins from a dicistronic transcript.

to elevated levels in samples incubated at 39.5°C. As shown in Table 2, wild-type 3A, 3A-MycC, and 3A-310/4 at both temperatures tested all inhibited A1PI secretion with comparable efficiencies. Therefore, the temperature-sensitive defect conferred by the 3A-310/4 mutation does not correlate with a failure to inhibit protein secretion. The ability of cells to accumulate 3A-Myc but not 3AB under these expression conditions argues that not all C-terminal additions to the 3A sequence reduce its translatability or stability.

3A protein colocalizes with ER-retained A1PI in transfected COS-1 cells. Previous experiments using immunofluorescence and light microscopy showed that in COS-1 cells that expressed poliovirus 3A protein, ER morphology was grossly altered and that the ER marker protein disulfide isomerase (PDI) colocalized with the normally secreted cellular protein A1PI (19). In most 3A-transfected cells, Golgi staining was not altered, and the Golgi marker wheat germ agglutinin did not colocalize with A1PI. On the other hand, in cells that did not express 3A, intracellular A1PI was predominantly Golgi associated (19). We concluded that 3A expression alters the morphology of the ER and causes normally secreted proteins to be retained in structures derived from these altered ER membranes (19). However, due to the lack of available antibodies specific to poliovirus 3A protein, the site of action of 3A was not tested.

To examine the localization of 3A itself with respect to A1PI during the inhibition of ER-to-Golgi traffic, we performed double-immunofluorescence experiments that used an anti-3A monoclonal antibody and an antibody against the lumenal ER protein PDI. Figures 5A and B show labeling for A1PI and PDI in the same field of COS-1 cells transfected with the dicistronic plasmid that encodes 3A and A1PI. As previously reported, in 3A-expressing cells, A1PI and PDI staining colocalized. Thus, the normally secreted protein A1PI accumulates in the ER of 3A-expressing cells, presumably in the ER lumen (19). Staining a field of 3A-transfected cells for both 3A and A1PI (Fig. 5C and D) showed that 3A itself showed localization similar to that of A1PI by the resolution of light microscopy. Although it is likely that 3A is not in the lumen of intracellular organelles but rather is associated with the cytoplasmic surface of their membranes, this experiment makes it clear that the membranes with which 3A is associated are the same as those in which A1PI accumulates, namely, the ER. Double-immunofluorescence experiments with 3A and PDI were not performed, as secondary antibodies could not readily distinguish between the mouse monoclonal antibodies that recognized these two proteins.

Expression of 3A protein causes pronounced swelling of ER membranes. To examine the ER-derived membranes within 3A-transfected cells in more detail, we examined untransfected and 3A-transfected COS-1 cells by EM, using high-pressure freezing and freeze-substitution methods (reviewed in references 15, 25, and 45). Unlike untransfected cells (Fig. 6A), cells that expressed viral protein 3A accumulated dilated membranes with both tubular and whorled configurations throughout the cell (Fig. 6B). Higher magnification (Fig. 6D) showed that the dilated membranes were contiguous with the nuclear membrane of 3A-transfected cells. Thus, the morphology of these membrane-bound cisternae is consistent with immunofluorescence staining for an ER marker enzyme (Fig. 5), arguing that the 3A-induced membranes are of ER origin. The ER-derived membranes appear to be more prevalent in transfected cells, in part due to their extreme dilation. Whether there is a quantitative increase in ER membrane content remains to be determined.

Expression of 3A protein in these experiments was accomplished by transient transfection of plasmid DNA from which the 3A-encoding RNA was transcribed from the simian virus 40 (SV40) promoter, constitutively expressed in COS-1 cells.

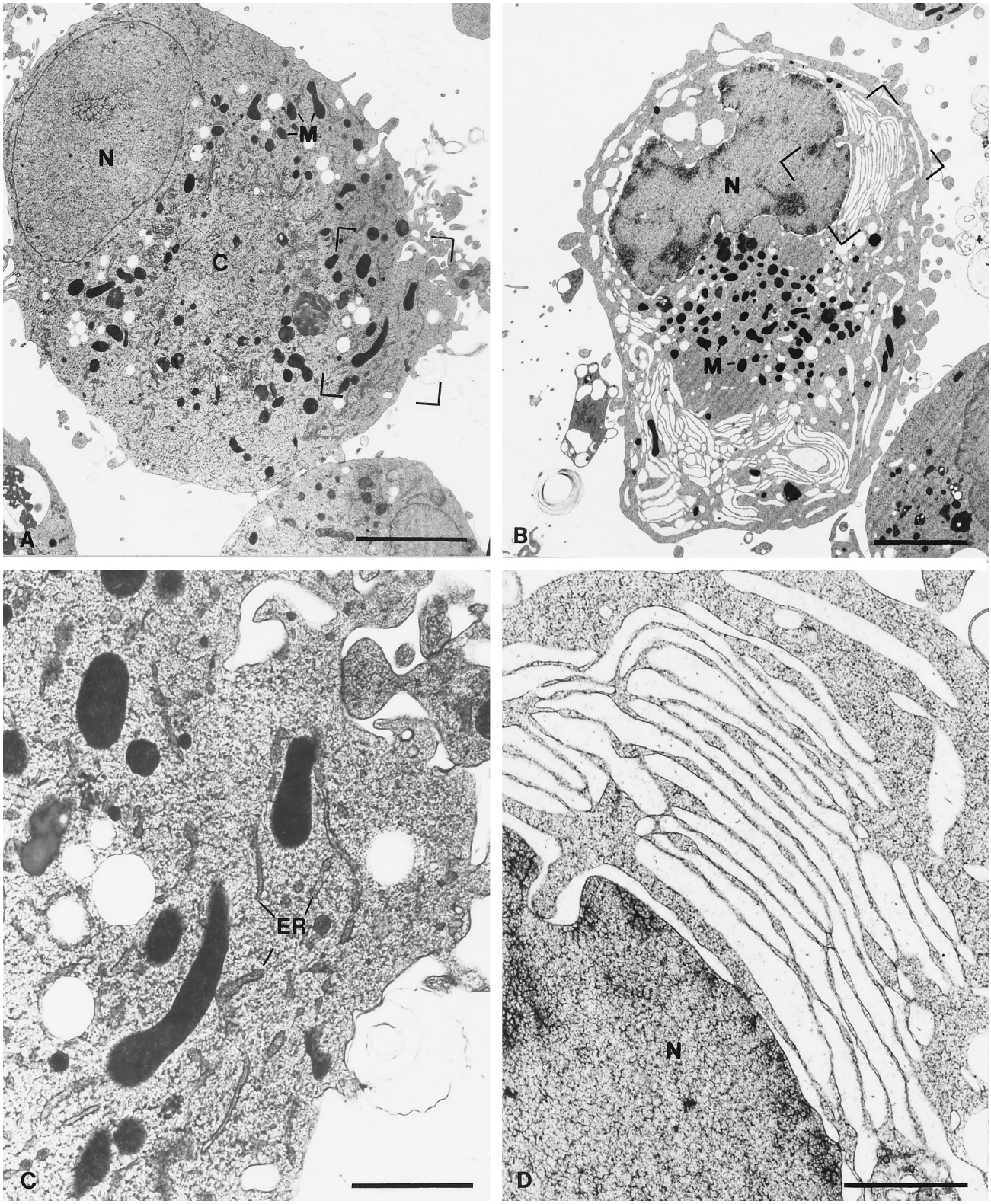

FIG. 6. EM analysis of the effect of 3A expression on COS-1 cells. (A) Control cell from a sample transfected with a plasmid that does not express 3A protein. Membranes of the ER and Golgi complex are prevalent in the cell center (C); the nucleus (N) is also identified. Mitochondria (M) stain very darkly following freeze-substitution fixation in tannic acid and osmium (Materials and Methods). Bar = 4 μm. (B) Cells transfected with plasmid expressing poliovirus protein 3A. Bar = 4 μm. (C) Area within brackets in panel A, shown at higher magnification. ER membranes are marked. Bar = 1 μm. (D) Area within brackets in panel B, shown at higher magnification. Bar = 1 μm.

Under the transfection conditions used (Materials and Methods), 20 to 30% of the cells were routinely transfected, as determined by immunofluorescence (data not shown). By EM, 20 to 30% of the cells displayed morphologies such as that shown in Fig. 5B and C; it seems certain that these are the 3A-expressing cells. However, to demonstrate that 3A expression causes the dilated membrane morphology and to test whether 3A protein is directly associated with the swollen ER-derived membranes, immunostaining of thin sections prepared for EM was performed.

Figure 7 shows the anti-3A staining of both the stacked (Fig. 7A) and whorled (Fig. 7B) membrane morphologies seen following transfection with 3A-expressing plasmids. The gold particles that identify the sites of anti-3A reactivity were found almost exclusively associated with the swollen membranous structures. It is likely that the site of action of 3A protein in inhibiting ER-to-Golgi traffic is similarly localized to ER membranes.

Infection of cells with poliovirus leads to profound rearrangements of intracellular membranes, causing the appearance of cytoplasmic vesicles (7, 8, 16), many of which we have recently shown to be bounded by double lipid bilayers (57). The formation of vesicles with morphologies similar to those found in poliovirus-infected cells can be observed in cells that express only viral protein 2BC (1, 12). Immunolabeling of infected cells and isolated vesicles has revealed that antigenic determinants from poliovirus proteins 2C (7, 9), 2B (20), and 3D (57) as well as capsid precursors (50) are found on the cytoplasmic surface of these vesicles. Purification of membrane fractions from poliovirus-infected cells has revealed the presence of specific viral proteins and precursors 2B, 2BC, 3A, 3AB, 3CD, and 3D (20, 59–61), all thought to be required for viral RNA synthesis (reviewed in reference 63). The immunostaining of infected cells with the anti-3A monoclonal antibody (Fig. 7C) shows that antigenic determinants from viral protein 3A are associated with the poliovirus-induced vesicles in infected cells. Comparison of the morphologies of the poliovirus-induced vesicles with the membrane alterations seen following expression of 3A protein alone reveals that these morphologies are quite distinct.

DISCUSSION

Tunicamycin does not inhibit poliovirus growth. The finding that a subpopulation of poliovirus proteins 3A and 3AB were N-glycosylated when translated in vitro in the presence of microsomal membranes suggested a possible requirement for glycosylation during poliovirus infection (18). If poliovirus required glycosylation of viral or cellular proteins in its replicative cycle, then the inhibition of poliovirus by BFA (32, 41) might be explained as a defect in protein glycosylation (18). However, neither intracellular virus production nor cytopathic effect visible by light microscopy was affected by tunicamycin. Any effect of tunicamycin late in poliovirus infection, on cell lysis or virus release, for example, was not tested. However, it is clear that N-glycosylation is not required for viral translation, RNA replication, or RNA packaging during poliovirus infection.

These findings, combined with the failure to detect glycosylated forms of 3A or 3AB in extracts of poliovirus-infected cells (reference 18 and data not shown), suggest that the observation of glycosylation of proteins 3A and 3AB in vitro may not be relevant to their functions in infected cells. Instead, the existing data (see the introduction) argue that both 3A and 3AB are associated peripherally with the cytoplasmic surface of intracellular membranes, although critical experiments to determine the membrane topology of 3A or 3AB have not yet been performed.

Analysis of characterized 3A mutations: relationship between secretion inhibition and viral phenotype. The two previously characterized 3A mutants tested for the ability to inhibit protein secretion gave strikingly different results. 3A-310/4, which contains a mutation in the hydrophobic domain near the C terminus, inhibited secretion comparably to wild-type 3A. The phenotype of 3A-310/4 virus therefore cannot be ascribed to a defect in this activity. This finding was not surprising since the defect in the 3A-310/4 virus is *cis* dominant, not being complemented by wild-type 3A (23). Efficient rescue by the presence of wild-type 3A protein would be predicted for any viral phenotype that arose solely from the inability of mutant 3A to inhibit protein secretion.

In contrast, the 3A-2 mutant protein, an N-terminal variant, reduced the ability of 3A to inhibit secretion at both 37 and 32.5°C. This defect in inhibiting protein secretion was therefore not conditional with respect to temperature. The RNA synthesis defect displayed by the 3A-2 virus was more pronounced at 32.5°C, although the amounts of RNA synthesis observed at 39.5°C were certainly not comparable to those of wild-type virus (5). The lack of perfect correlation between the biochemical defect in protein secretion inhibition and the RNA synthesis defect in infected cells could argue that the defect in 3A-2 virus is not due solely to the reduced ability of the mutant protein to inhibit secretion. Additional functions for 3AB are implied by in vitro studies (29, 38, 44), and these activities are sensitive to mutations within the 3A coding region (65). Alternatively, it is possible that the ability of the 3A protein to inhibit protein secretion is less rate limiting to virus growth at 39.5°C than at 32.5°C. The recessive character of the 3A-2 mutation is consistent with a defect in the inhibition of protein secretion by the mutant 3A-2 protein, since the inhibition of protein secretion by wild-type 3A protein should be provided effectively in *trans*.

Analysis of 3A truncations. Analysis of mutant 3A proteins with altered C termini suggested that integrity of the C terminus is required for accumulation of the protein to detectable levels in COS-1 cells. No 3A protein was detected in COS-1 cells transfected with a plasmid encoding 3A1-59 or 3AB, and only trace amounts of 3A1-80 could be detected (Fig. 4 and data not shown). The presence of significant levels of A1PI protein in extracts of cells transfected with these plasmids argued that the defect in 3A accumulation seen for these constructs was not due to problems with transfection of the DNA, amplification of the plasmids in COS-1 cells, or transcription of dicistronic mRNAs encoding the 3A variants and A1PI. Impaired translation and decreased stability of altered 3A proteins remain as possible explanations for the apparent lack of expression of these 3A variants. Interestingly, 3AB and a truncation of 3A removing 18 amino acids from its C terminus have been successfully expressed in HeLa cells by using a recombinant vaccinia virus system (18). Thus, the lack of expression of 3A molecules with altered C termini observed in this work may be specific to COS-1 cells or the particular expression plasmid used in these experiments.

Truncation of the N terminus of 3A severely impaired the ability of 3A to inhibit secretion despite the fact that the protein still accumulated to significant levels (Fig. 3). Removal of the N-terminal 10 amino acids reduced activity substantially, arguing that this portion of the molecule is required either for interactions involved in secretion inhibition or for proper folding of the entire 3A molecule. The importance of this region of 3A is consistent with the defect seen for the 3A-2 mutation, which inserts a single amino acid at position 15 and results in

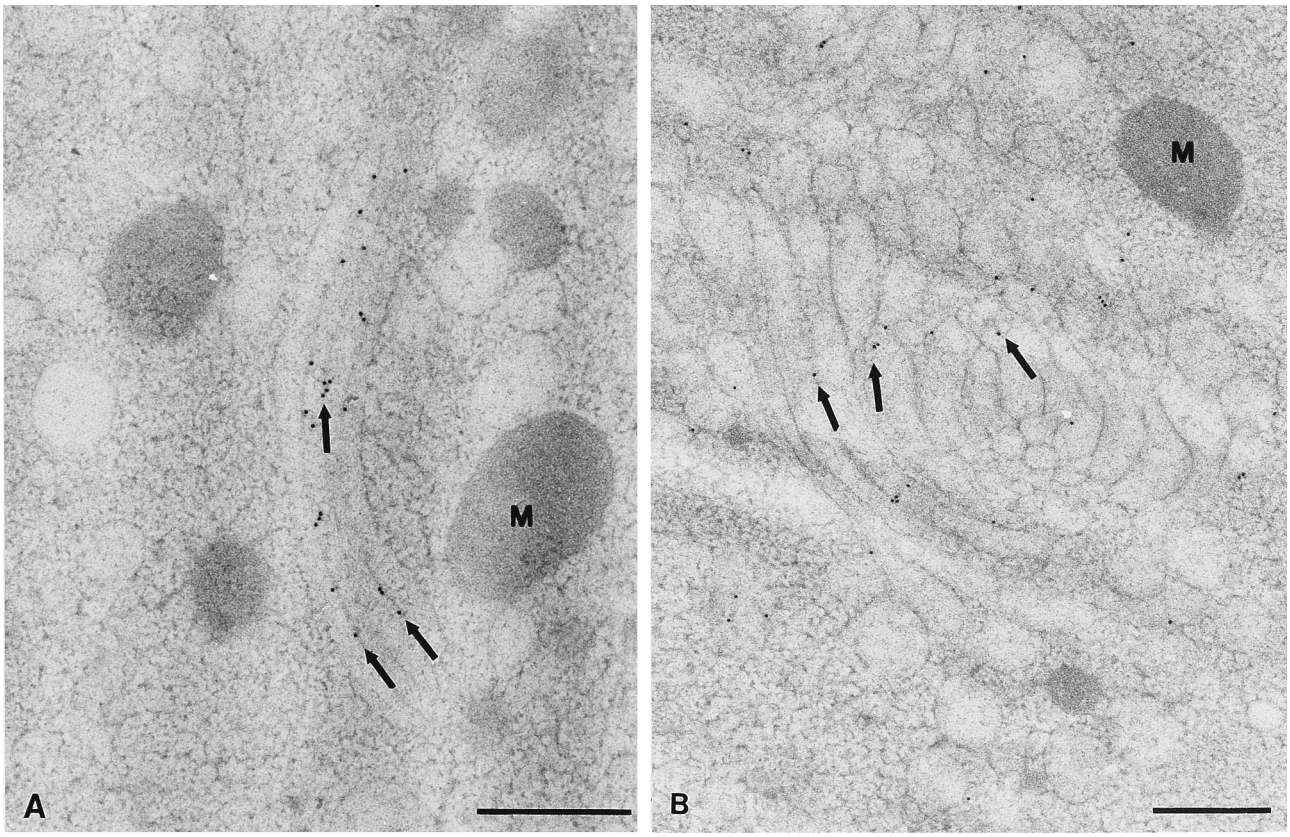

FIG. 7. Immunolocalization of poliovirus protein 3A in transfected and infected COS-1 cells. (A and B) Immunolabeling of cells transfected with plasmid that encodes 3A protein. Cells with dilated membranes were found to stain with anti-3A antibody; 15-nm gold particles were observed along the membranes limiting the cisternae (arrows). M, mitochondria. (C) Poliovirus-infected COS-1 cells immunolabeled with anti-3A antibody and 15-nm gold particles (arrows). The light labeling was specific for the double-membraned vesicles characteristic of poliovirus-infected cells (57). Bars = 0.5 μm.

a diminished ability to inhibit protein secretion. On the other hand, removal of the C-terminal seven amino acids of 3A, while reducing its level of accumulation, did not destroy its ability to inhibit protein secretion.

Morphology of cells that express poliovirus protein 3A. Expression of 3A drastically alters the intracellular localization of A1PI, a normally secreted protein found predominantly in the Golgi apparatus in the absence of 3A expression (19). When 3A protein was expressed, A1PI was found to colocalize with the ER marker protein PDI (19) and with 3A protein itself (Fig. 5). It therefore appears that transport of proteins out of the ER is inhibited by 3A protein by virtue of its direct association with ER membranes. This idea was further supported by EM analysis of cells that expressed 3A, in which a distinctive accumulation of dilated tubular and whorled membranes was observed (Fig. 6). That these structures are derived from the ER can be argued from our previous studies demonstrating that the membranes altered in the presence of 3A contained ER but not Golgi markers (19), that the normally secreted protein A1PI colocalized with ER (Fig. 5 and reference 19) but not Golgi (19) markers, and that the dilated membranes observed upon 3A expression were directly contiguous with the nuclear membrane (Fig. 6C). The presence of 3A protein on these altered ER membranes was shown directly by immunolabeling of EM sections (Fig. 7A, B).

Inhibition of ER-to-Golgi transport by 3A could result from a specific interaction between 3A and a host protein required for secretory transport. Alternatively, accumulation of overexpressed 3A on ER membranes could nonspecifically disrupt the ability of vesicles to bud from these membranes. Foreign proteins can be expressed to very high levels in COS-1 cells because these cells support the amplification of plasmids that contain the SV40 origin of replication (26). Two lines of evidence argue that concentrations of 3A lower than those expressed in the COS-1 cell system reported here are sufficient to inhibit ER-to-Golgi traffic. Inhibition of A1PI secretion was observed following transfection of the 3A-encoding plasmid into HeLa cells (data not shown), which do not express SV40 T antigen. In addition, secretion inhibition was observed in COS-1 cells that expressed the truncated protein 3A1-80, a 3A variant that was expressed at greatly reduced levels compared to wild-type 3A (Fig. 4).

The observation of swollen ER structures is consistent with the hypothesis that 3A blocks transport out of the ER. Accumulation and swelling of ER membranes might be expected to occur as a result of the synthesis and insertion of new proteins into the ER, the synthesis of new lipids, which occurs in the ER (10), and the retrograde transport of proteins and lipids from the Golgi to the ER. Consistent with this observation, dilated ER membranes have been seen under several conditions of impaired secretory function (22, 43, 53, 66), although not to the extent seen in 3A-expressing cells (Fig. 6). Thus, the observed dilation of the ER-derived membranes could reflect the mechanism by which 3A blocks ER-to-Golgi traffic or simply be a consequence of that block. In either case, since accumulation of transport vesicles was not observed in 3A-expressing cells, it is likely that 3A inhibits ER-to-Golgi traffic by blocking the formation or budding of the COP II-coated vesicles known to mediate anterograde transport from the ER (reviewed in references 2 and 56). This could be accomplished either by interfering with the formation or budding processes themselves or by altering or aggregating the proteins to be secreted such that they do not serve as adequate cargo (4).

A likely purpose of the inhibition of secretory function for a nonenveloped virus such as poliovirus is to block two defenses of organisms to viral infection that require a functional secretory apparatus, namely, interferon secretion and antigen presentation in the context of major histocompatibility complex class I molecules. Alternatively, the alteration of ER membranes by viral protein 3A may serve a function in the assembly of the membrane-associated viral RNA replication complexes. Given the multifunctional nature of many viral proteins, several of these roles for 3A protein may be utilized during the infectious cycle of poliovirus.

ACKNOWLEDGMENTS

We thank Steve Schultz for providing the purified N-terminal domain of 3A for antibody production. We are grateful to Frank Luca and Kurt Christensen at UCHSC for help with anti-3A antibody preparation, to Jerry Brown and Oswald Pfenniger for providing affinity-purified antibodies to A1PI, to Bert Semler and his laboratory for provision of the 3A-310/4 cDNA, and to Anne McBride for help with the figures. We thank Peter Sarnow, Stephen Deitz, Dana Dodd, Verena Gauss-Müller, and David Suhy for comments on the manuscript.

This work was supported by the Howard Hughes Medical Institute and the David and Lucile Packard Foundation. J.R.D. was supported by a predoctoral fellowship from the ARCS Foundation.

REFERENCES

1. **Aldabe, R., and L. Carrasco.** 1995. Induction of membrane proliferation by poliovirus proteins 2C and 2BC. Biochem. Biophys. Res. Commun. **206:**64–76.
2. **Aridor, M., and W. E. Balch.** 1996. Principles of selective transport: coat complexes hold the key. Trends Cell Biol. **6:**315–320.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1990. Current protocols in molecular biology. Greene Publishing Associates, Wiley Interscience, New York, N.Y.
4. **Bannykh, S. I., T. Rowe, and W. E. Balch.** 1996. The organization of endoplasmic reticulum export complexes. J. Cell Biol. **135:**19–35.
5. **Bernstein, H. D., and D. Baltimore.** 1988. Poliovirus mutant that contains a cold-sensitive defect in viral RNA synthesis. J. Virol. **62:**2922–2928.
6. **Bernstein, H. D., P. Sarnow, and D. Baltimore.** 1986. Genetic complementation among poliovirus mutants derived from an infectious cDNA clone. J. Virol. **60:**1040–1049.
7. **Bienz, K., D. Egger, and L. Pasamontes.** 1987. Association of polioviral proteins of the P2 genomic region with the viral replication complex and virus-induced membrane synthesis as visualized by electron microscopic immunocytochemistry and autoradiography. Virology **160:**220–226.
8. **Bienz, K., D. Egger, Y. Rasser, and W. Bossart.** 1983. Intracellular distribution of poliovirus proteins and the induction of virus-specific cytoplasmic structures. Virology **131:**39–48.
9. **Bienz, K., D. Egger, M. Troxler, and L. Pasamontes.** 1990. Structural organization of poliovirus RNA replication is mediated by viral proteins of the P2 genomic region. J. Virol. **64:**1156–1163.
10. **Bishop, W. R., and R. M. Bell.** 1988. Assembly of phospholipids into cellular membranes: biosynthesis, transmembrane movement, and intracellular translocation. Annu. Rev. Cell Biol. **4:**579–610.
11. **Bonneau, A., and N. Sonenberg.** 1987. Proteolysis of the p220 component of the cap-binding protein complex is not sufficient for complete inhibition of host cell protein synthesis after poliovirus infection. J. Virol. **61:**986–991.
12. **Cho, M. W., N. Teterina, D. Egger, K. Bienz, and E. Ehrenfeld.** 1994. Membrane rearrangement and vesicle induction by recombinant poliovirus 2C and 2BC in human cells. Virology **202:**129–145.
13. **Clark, M. E., T. Hammerle, E. Wimmer, and A. Dasgupta.** 1991. Poliovirus proteinase 3C converts an active form of transcription factor IIIC to an inactive form: a mechanism for inhibition of host cell polymerase III transcription by poliovirus. EMBO J. **10:**2941–2947.
14. **Clark, M. E., P. M. Lieberman, A. J. Berk, and A. Dasgupta.** 1993. Direct cleavage of human TATA-binding protein by poliovirus protease 3C in vivo and in vitro. Mol. Cell. Biol. **13:**1232–1237.
15. **Dahl, C. A., R. P. Schall, H. He, and J. S. Cairns.** 1992. Identification of a novel gene expressed in activated natural killer cells and T cells. J. Immunol. **148:**597–603.
16. **Dales, S., H. J. Eggers, I. Tamm, and G. E. Palade.** 1965. Electron microscopic study of the formation of poliovirus. Virology **26:**379–389.
17. **Dargan, D. J., C. B. Galt, and J. H. Subak-Sharpe.** 1992. The effect of cicloxolone sodium on the replication in cultured cells of adenovirus type 5, reovirus type 3, poliovirus type 1, two bunyaviruses and Semliki Forest virus. J. Gen. Virol. **73:**407–411.
18. **Datta, U., and A. Dasgupta.** 1994. Expression and subcellular localization of poliovirus VPg-precursor protein 3AB in eukaryotic cells: evidence for glycosylation in vitro. J. Virol. **68:**4468–4477.
19. **Doedens, J. R., and K. Kirkegaard.** 1995. Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A. EMBO J. **14:**894–907.
20. **Egger, D., L. Pasamontes, R. Bolten, V. Boyko, and K. Bienz.** 1996. Reversible dissociation of the poliovirus replication complex: functions and interactions of its components in viral RNA synthesis. J. Virol. **70:**8675–8683.
21. **Etchison, D., S. C. Milburn, I. Edery, N. Sonenberg, and J. W. B. Hershey.** 1982. Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. J. Biol. Chem. **257:**14806–14810.
22. **Fujiwara, T., K. Oda, S. Yokota, A. Takatsuki, and Y. Ikehara.** 1988. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. J. Biol. Chem. **263:**18545–18552.
23. **Giachetti, C., S. S. Hwang, and B. L. Semler.** 1992. *cis*-acting lesions targeted to the hydrophobic domain of a poliovirus membrane protein involved in RNA replication. J. Virol. **66:**6045–6057.
24. **Giachetti, C., and B. L. Semler.** 1991. Role of a viral membrane polypeptide in strand-specific initiation of poliovirus RNA synthesis. J. Virol. **65:**2647–2654.
25. **Gilkey, J. C., and L. A. Staehelin.** 1986. Advances in ultrarapid freezing for the preservation of cellular ultrastructure. J. Electron Microsc. Tech. **3:**177–210.
26. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23:**175–182.
27. **Goldstein, G., and L. E. Guskey.** 1984. Poliovirus and vesicular stomatitis virus replication in the presence of 6-diazo-5-oxo-L-norleucine or 2-deoxy-D-glucose. J. Med. Virol. **14:**159–167.
28. **Harlow, E., and D. Lane.** 1988. Antibodies, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Harris, K. S., W. Xiang, L. Alexander, W. S. Lane, A. V. Paul, and E. Wimmer.** 1994. Interaction of poliovirus polypeptide 3CDpro with the 5′ and 3′ termini of the poliovirus genome. J. Biol. Chem. **269:**27004–27014.

30. **Holland, J. J.** 1962. Inhibition of DNA-primed RNA synthesis during poliovirus infection of human cells. Biochem. Biophys. Res. Commun. **9:**556–562.
31. **Hope, D. A., S. E. Diamond, and K. Kirkegaard.** 1997. Genetic dissection of interaction between poliovirus 3D polymerase and viral protein 3AB. J. Virol. **71:**9490–9498.
32. **Irurzun, A., L. Perez, and L. Carrasco.** 1993. Brefeldin A blocks protein glycosylation and RNA replication of vesicular stomatitis virus. FEBS Lett. **336:**496–500.
33. **Irurzun, A., I. N. Sanchez-Palomino, and L. Carrasco.** 1995. Monensin and nigericin prevent the inhibition of host translation by poliovirus, without affecting p220 cleavage. J. Virol. **69:**7453–7460.
34. **Jang, S. K., M. V. Davies, R. J. Kaufman, and E. Wimmer.** 1989. Initiation of protein synthesis by internal entry of ribosomes into the 5′ nontranslated region of encephalomyocarditis virus RNA in vivo. J. Virol. **63:**1651–1660.
35. **Kitamura, N., B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecak, J. J. Lee, S. van der Werf, C. W. Anderson, and E. Wimmer.** 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature **291:**547–553.
36. **Kornfeld, R., and S. Kornfeld.** 1985. Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **54:**631–664.
37. **Lama, J., and L. Carrasco.** 1992. Expression of poliovirus nonstructural proteins in Escherichia coli cells. Modification of membrane permeability induced by 2B and 3A. J. Biol. Chem. **267:**15932–15937.
38. **Lama, J., A. V. Paul, K. S. Harris, and E. Wimmer.** 1994. Properties of purified recombinant poliovirus protein 3AB as substrate for viral proteinases and as co-factor for RNA polymerase 3Dpol. J. Biol. Chem. **269:**66–70.
39. **Lamphear, B. J., R. Kirchweger, T. Skern, and R. E. Rhoads.** 1995. Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. J. Biol. Chem. **270:**21975–21983.
40. **Liebig, H. D., E. Ziegler, R. Yan, K. Hartmuth, H. Klump, H. Kowalsdi, D. Blaas, W. Sommergruber, L. Frasel, and B. Lamphear.** 1993. Purification of two picornaviral 2A proteinases: interaction with eIF-4 gamma and influence on in vitro translation. Biochemistry **32:**7581–7588.
41. **Maynell, L. A., K. Kirkegaard, and M. W. Klymkowsky.** 1992. Inhibition of poliovirus replication by brefeldin A. J. Virol. **66:**1985–1994.
42. **McCracken, A. A., K. B. Kruse, and J. L. Brown.** 1989. Molecular basis for defective secretion of the Z variant of human alpha-1-proteinase inhibitor: secretion of variants having altered potential for salt bridge formation between amino acids 290 and 342. Mol. Cell. Biol. **9:**1406–1414.
43. **Misumi, Y., Y. Misumi, K. Miki, A. Takatsuki, G. Tamura, and Y. Ikehara.** 1986. Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. J. Biol. Chem. **261:**11398–11403.
44. **Molla, A., K. S. Harris, A. V. Paul, S. H. Shin, J. Mugavero, and E. Wimmer.** 1994. Stimulation of poliovirus proteinase $3C^{pro}$-related proteolysis by the genome-linked protein. J. Biol. Chem. **269:**27015–27020.
45. **Moor, H.** 1987. Theory and practice of high pressure freezing, p. 175–191. *In* R. A. Steinbrecht and K. Zierold (ed.), Cryotechniques in biological electron microscopy. Springer-Verlag, Berlin, Germany.
46. **Oh, S. K., and P. Sarnow.** 1993. Gene regulation: translational initiation by internal ribosome binding. Curr. Opin. Genet. Dev. **3:**295–300.
47. **Paul, A. V., X. Cao, K. S. Harris, J. Lama, and E. Wimmer.** 1994. Studies with poliovirus polymerase 3Dpol. J. Biol. Chem. **269:**29173–29181.
48. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature **334:**320–325.
49. **Perez, L., and L. Carrasco.** 1992. Lack of direct correlation between p220 cleavage and the shut-off of host translation after poliovirus infection. Virology **189:**178–186.
50. **Pfister, T., L. Pasamontes, M. Troxler, D. Egger, and K. Bienz.** 1992. Immunocytochemical localization of capsid-related particles in subcellular fractions of poliovirus-infected cells. Virology **188:**676–684.
51. **Plotch, S. J., and O. Palant.** 1995. Poliovirus protein 3AB forms a complex with and stimulates the activity of the viral RNA polymerase, 3Dpol. J. Virol. **69:**7169–7179.
52. **Racaniello, V. R., and D. Baltimore.** 1981. Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc. Natl. Acad. Sci. USA **78:**4887–4891.
53. **Rossi, G., K. Kolstad, S. Stone, F. Palluault, and S. Ferro-Novick.** 1995. BET3 encodes a novel hydrophilic protein that acts in conjunction with yeast SNAREs. Mol. Biol. Cell. **6:**1769–1780.
54. **Rubinstein, S. J., T. Hammerle, E. Wimmer, and A. Dasgupta.** 1992. Infection of HeLa cells with poliovirus results in modification of a complex that binds to the rRNA promoter. J. Virol. **66:**3062–3068.
55. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range of 1 to 100 kDa. Anal. Biochem. **166:**368–379.
56. **Scheckman, R., and L. Orci.** 1996. Coat proteins and vesicle budding. Science **271:**1526–1533.
57. **Schlegel, A., T. H. Giddings, M. S. Ladinsky, and K. Kirkegaard.** 1996. Cellular origin and ultrastructure of membranes induced during poliovirus infection. J. Virol. **70:**6576–6588.
58. **Schwarz, R. T., and R. Datema.** 1982. Inhibition of the dolichol pathway of protein glycosylation. Methods Enzymol. **83:**432–443.
59. **Semler, B. L., C. W. Anderson, R. Hanecak, L. F. Dorner, and E. Wimmer.** 1982. A membrane-associated precursor to poliovirus VPg identified by immunoprecipitation with antibodies directed against a synthetic heptapeptide. Cell **28:**405–412.
60. **Takegami, T., B. L. Semler, C. W. Anderson, and E. Wimmer.** 1983. Membrane fractions active in poliovirus RNA replication contain VPg precursor polypeptides. Virology **128:**33–47.
61. **Tisdale, E. J., J. R. Bourne, R. Khosravi-Far, C. J. Der, and W. E. Balch.** 1992. GTP-Binding mutants of rab1 and rab2 are potent inhibitors of vesicular transport from the endoplasmic reticulum to the Golgi complex. J. Cell Biol. **119:**749–761.
62. **Towner, J. S., T. V. Ho, and B. L. Semler.** 1996. Determinants of membrane association for poliovirus protein 3AB. J. Biol. Chem. **271:**26810–26818.
63. **Wimmer, E., C. U. T. Hellen, and X. Cao.** 1993. Genetics of poliovirus. Annu. Rev. Genet. **27:**353–436.
64. **Wyckoff, E. E., J. W. B. Hershey, and E. Ehrenfeld.** 1990. Eukaryotic initiation factor 3 is required for poliovirus 2A protease-induced cleavage of the p220 component of eukaryotic initiation factor 4F. Proc. Natl. Acad. Sci. USA **87:**9529–9533.
65. **Xiang, W., A. Cuconati, A. V. Paul, X. Cao, and E. Wimmer.** 1995. Molecular dissection of the multifunctional poliovirus RNA-binding protein 3AB. RNA **1:**892–904.
66. **Zhang, C. J., A. G. Rosenwald, M. C. Willingham, S. Skuntz, J. Clark, and R. A. Kahn.** 1994. Expression of a dominant allele of human ARF1 inhibits membrane traffic in vivo. J. Cell Biol. **124:**289–300.
67. **Ziegler, E., A. M. Borman, F. G. Deliat, H. D. Liebig, D. Jugovic, K. M. Kean, T. Skern, and E. Kuechler.** 1995. Picornavirus 2A proteinase-mediated stimulation of internal initiation of translation is dependent on enzymatic activity and the cleavage products of cellular proteins. Virology **213:**549–557.
68. **Zimmerman, E. F., M. Heeter, and J. E. Darnell.** 1963. RNA synthesis in poliovirus-infected cells. Virology **19:**400–408.