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Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A

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Poliovirus RNA replication occurs on the surface of membranous vesicles that proliferate throughout the cytoplasm of the infected cell. Since at least some of these vesicles are thought to originate within the secretory pathway of the host cell, we examined the effect of poliovirus infection on protein transport through the secretory pathway. We found that transport of both plasma membrane and secretory proteins was inhibited by poliovirus infection early in the infectious cycle. Transport inhibition did not require viral RNA replication or the inhibition of host cell translation by poliovirus. The viral proteins 2B and 3A were each sufficient to inhibit transport in the absence of viral infection. The intracellular localization of a secreted protein in the presence of 3A with the endoplasmic reticulum suggested that 3A directly blocks transport from the endoplasmic reticulum to the Golgi apparatus.

Key words: endoplasmic reticulum/poliovirus/secretory pathway/virus–host interactions

Introduction

Poliovirus is a non-enveloped positive-sense RNA virus that interacts extensively with its primate host cells during its lytic infectious cycle. After the virus enters the cell, the 7500 nt 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 the viral non-structural proteins, including the proteases involved in polyprotein processing, the RNA-dependent RNA polymerase and several other proteins required for the replication of the viral RNA genome (Wimmer *et al.*, 1993).

As poliovirus infection progresses, the host cell undergoes dramatic metabolic and morphological changes. Poliovirus protein 2A induces the cleavage of p220, a component of the cellular cap binding complex, resulting in an inhibition of cap-dependent initiation of translation in poliovirus-infected cells (Etchison *et al.*, 1982). Translation of poliovirus RNA, one of the few RNAs that can be translated in the infected cell (OH and Sarnow, 1993), is initiated by the internal binding of ribosomes to RNA sequences and structures in the 5′ non-coding region (Pelletier and Sonenberg, 1988; Jang *et al.*, 1989). Presumably the virus inhibits cellular translation to free cellular ribosomes to translate the viral genome and possibly to thwart any host defense that requires new translation. Poliovirus infection also inhibits cellular RNA synthesis (Holland, 1962; Zimmerman *et al.*, 1963); transcription factors required for transcription by RNA polymerases I–III are inactivated during infection (Clark *et al.*, 1991, 1993; Rubinstein *et al.*, 1992). In contrast, lipid synthesis is stimulated by poliovirus infection; much of the newly synthesized lipid is found in membranous vesicles that accumulate in the cytoplasm of infected cells (Mosser *et al.*, 1972). In addition, the plasma membrane of poliovirus-infected cells shows increased permeability to monovalent cations and translation inhibitors that are normally non-permeant (Munoz and Carrasco, 1983; Lopez-Rivas *et al.*, 1987). Other virus-induced changes in cellular morphology include rearrangement of the cellular intermediate filament system (Lenk and Penman, 1979) and rounding and detachment of infected cells from the substrate. Collectively, these biochemical and morphological changes are known as the cytopathic effect.

Although the functions of many poliovirus-induced changes in cellular structure and metabolism remain unknown, several experiments have indicated that the association of viral proteins with cellular membranes and the accumulation of membranous vesicles are required for viral RNA synthesis. Electron microscopy has shown that both newly synthesized viral RNA and viral proteins known to be required for RNA replication are associated with the cytoplasmic surface of the virus-induced vesicles (Bienz *et al.*, 1987, 1990; Troxler *et al.*, 1992). Membrane-containing extracts from infected cells catalyze the linkage of VPg, a 22 amino acid viral protein, to newly synthesized RNA via a covalent tyrosine–phosphate bond identical to that seen in both the positive and negative strands of viral RNA during infection. The production of VPg linked to newly synthesized viral RNA is sensitive to the addition of non-ionic detergents and has not been mimicked in soluble *in vitro* extracts (Takegami *et al.*, 1983; Takeda *et al.*, 1986).

Electron microscopy and inhibitor studies have suggested that the virus-induced vesicles with which viral RNA synthesis is associated are derived from the host cell secretory pathway. Immunoelectron microscopy observations showed a physical association between viral protein 2C and the surface of vesicles which appeared to bud from the endoplasmic reticulum (ER) (Bienz *et al.*, 1987), suggesting that at least some of the virus-induced vesicles are derived from the ER. In addition, poliovirus RNA replication is strongly inhibited by the fungal metabolite brefeldin A (BFA) (Irurzun *et al.*, 1992;

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A

B

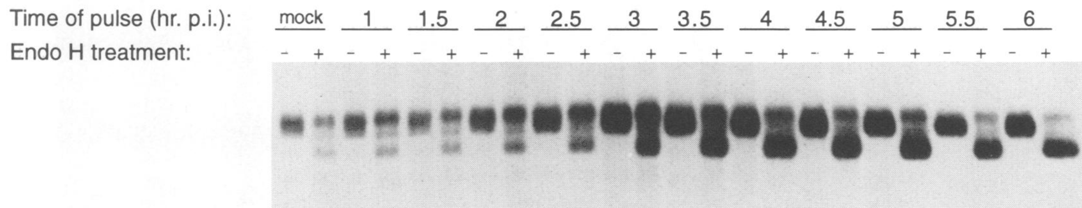


Fig. 1. (**A**) Diagram of the predicted mRNA from pLNCG, a plasmid used for transient expression of VSV-G in poliovirus-infected COS cells. The poliovirus 5′ non-coding region is fused at the start codon to VSV-G cDNA, allowing the mRNA to be translated by a cap-independent mechanism. (**B**) Modification of pulse-labeled VSV-G at various times during poliovirus infection. COS cells transfected with pLNCG were either infected or mock-infected with poliovirus type 1 Mahoney at a MOI of 20 p.f.u./cell. At various times post-infection, the cells were starved of methionine for 30 min, labeled with [^{35}S]methionine for 15 min and chased with unlabeled methionine for 30 min. VSV-G was immunoprecipitated from cell lysates and its glycosylation state was analyzed by digestion with endo H. Each set of lanes is labeled with the time post-infection at which the [^{35}S]methionine pulse was initiated.

Maynell *et al.*, 1992), an inhibitor of secretory pathway membrane traffic in many cell types (Takatsuki and Tamura, 1985; Misumi *et al.*, 1986). In cells possessing a BFA-resistant secretory pathway, poliovirus replication was unaffected by BFA. Thus, a host protein or process required by poliovirus, and not any of the viral products, is sensitive to BFA (Doedens *et al.*, 1994). One explanation for the inhibition of poliovirus RNA synthesis by BFA is that the formation of poliovirus-induced vesicles, like the formation of normal transport vesicles from the ER, is inhibited by BFA. Alternatively, a host protein whose conformation or subcellular localization is affected by BFA treatment might be required for poliovirus RNA synthesis (Maynell *et al.*, 1992).

We predicted that if poliovirus infection sequesters membranes, host proteins or both from the normal protein secretory apparatus, poliovirus infection might inhibit protein transport through the secretory pathway. By constructing plasmids that encode marker proteins whose mRNAs can be translated in poliovirus-infected cells, we have shown that poliovirus infection does lead to an inhibition of transport, apparently at a pre-Golgi step in the secretory pathway. This inhibition does not require viral RNA replication or virus-induced inhibition of host protein synthesis. We have identified two viral proteins, 2B and 3A, that can individually inhibit transport in the absence of poliovirus infection. Immunofluorescence microscopy showed that, in the presence of protein 3A, secreted proteins co-localized with ER markers whereas, in the presence or absence of viral protein 2B, the majority of the secreted protein was found associated with the Golgi apparatus. Thus, the two poliovirus proteins inhibit the protein secretory apparatus by different mechanisms.

Results

Vesicular stomatitis virus envelope glycoprotein expressed during poliovirus infection persists in an ER form

To monitor secretory pathway function during poliovirus infection, the envelope glycoprotein of vesicular stomatitis virus (VSV-G) was used as a marker protein. VSV-G acquires *N*-linked glycosylation at two asparagine residues as it is synthesized and translocated across the ER membrane (Rothman and Lodish, 1977). After the protein reaches the medial Golgi, the high-mannose oligosaccharides of the ER form are modified by the Golgi enzymes *N*-acetylglucosaminyltransferase and mannosidase II (Kornfeld and Kornfeld, 1985). The ER and Golgi forms of VSV-G can be distinguished by treatment with endoglycosidase H (endo H), which specifically cleaves the high-mannose oligosaccharides characteristic of the ER form of glycoproteins while leaving the more complex structures generated in the medial Golgi intact.

Poliovirus infection leads to the inhibition of translation of most non-polioviral mRNAs; therefore, it was necessary to construct a plasmid that would allow expression of VSV-G in infected cells. To accomplish this, the 5′ non-coding region (5′NC) of the poliovirus genome was fused to the VSV-G coding region. The plasmid, pLNCG, also contains an SV40 origin of replication to allow its amplification in COS-1 cells (Gluzman, 1981) and an SV40 late promoter to drive transcription of the poliovirus 5′NC-VSV-G mRNA (Figure 1A). Transfection of COS-1 cells with this plasmid allows VSV-G protein to be synthesized in both infected and uninfected cells.

To analyze protein transport in poliovirus-infected cells, we monitored the glycosylation state of VSV-G synthesized at various times after infection with poliovirus. COS-1 cells were transfected with pLNCG and then, 48 h

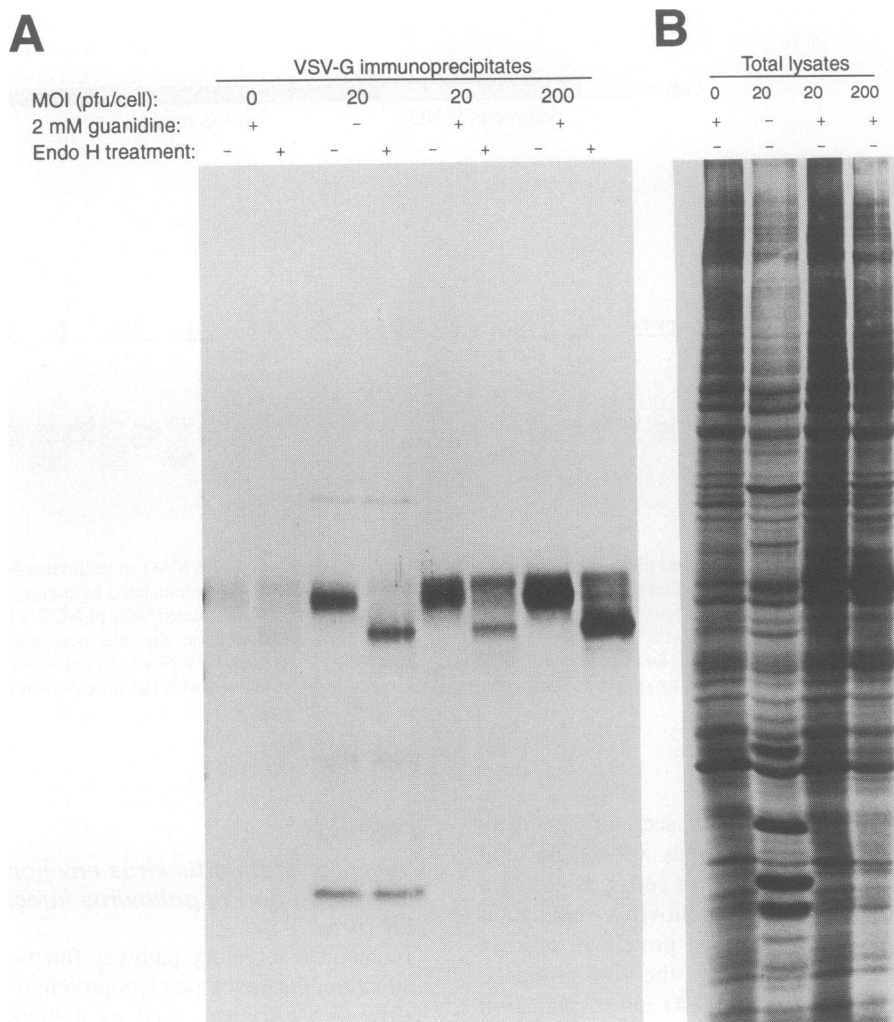


Fig. 2. Effect of inhibition of poliovirus RNA synthesis on VSV-G modification in poliovirus-infected cells. COS cells transfected with pLNCG were infected or mock-infected with poliovirus at a MOI of 20 or 200 p.f.u./cell in the presence or absence of 2 mM guanidine to inhibit viral RNA synthesis. At 3.5 h post-infection, cells were pulse labeled with [^{35}S]methionine for 15 min, chased for 30 min and cell lysates were prepared. (**A**) Aliquots of each lysate were immunoprecipitated for VSV-G and the glycosylation state of immunoprecipitated VSV-G was analyzed by digestion with endo H followed by SDS–PAGE and autoradiography. (**B**) Samples of each lysate prior to immunoprecipitation were electrophoresed in parallel with the immunoprecipitated samples; a shorter exposure of the non-precipitated samples is shown. The additional bands present in the MOI 20 without guanidine lanes in (A) are poliovirus-specific proteins which are precipitated with low efficiency under these conditions.

later, either infected or mock-infected with poliovirus. At various times post-infection, cells were incubated with [^{35}S]methionine for 15 min to label newly synthesized proteins, followed by incubation with higher concentrations of unlabeled methionine for 30 min. VSV-G was immunoprecipitated from each cell lysate and half of each immunoprecipitate was digested with endo H. Digested and undigested samples were then analyzed by SDS–PAGE and autoradiography (Figure 1B). At early times post-infection, VSV-G protein from infected cells and mock-infected cells contained similar modifications, with the majority of the labeled protein having acquired endo H resistance in the 30 min chase period. At 3 h post-infection, however, a reduction in the proportion of endo H-resistant VSV-G was seen and at 4 h post-infection nearly all the newly expressed VSV-G protein accumulated in an endo H-sensitive form, indicating that it had not reached the medial Golgi.

Although the cell lysates from each time point contained identical amounts of total protein, the amount of labeled VSV-G increased dramatically during poliovirus infection. The increased translation of mRNAs containing the 5′ non-coding region of poliovirus has been observed previously and may result from both transactivation of translation by poliovirus protein 2A (Hambidge and Sarnow, 1992) and the increased availability of translation machinery due to the inhibition of cap-dependent translation by poliovirus (Percy *et al.*, 1992).

Inhibition of protein secretion by poliovirus does not require viral RNA synthesis or inhibition of host translation

To determine whether viral RNA replication was required for the observed transport inhibition, we examined the transport of VSV-G in cells infected with poliovirus in the presence of 2 mM guanidine HCl. Low concentrations of guanidine specifically inhibit polioviral RNA synthesis (Caliguiri and Tamm, 1968), while having no toxic effect

on cells. Mutations that render poliovirus resistant to guanidine map to viral protein 2C (Pincus and Wimmer, 1986), known to be required for viral RNA synthesis (Li and Baltimore, 1988).

We examined VSV-G modification in cells infected at both low and high multiplicities of infection (MOI) in the presence and absence of guanidine. The lower MOI, 20 plaque forming units (p.f.u.)/cell, was the same as that used in the infections shown in Figures 1 and 2 in the absence of guanidine. The higher MOI, 200 p.f.u./cell, was used to increase the level of viral proteins in the infected cells in the absence of RNA replication; for example, sufficient amounts of poliovirus protein 2A are made at MOI 100 in the presence of guanidine to inhibit cap-dependent translation in HeLa cells (Bonneau and Sonenberg, 1987). COS cells transfected with pLNCG were labeled with [^{35}S]methionine at 4 h post-infection with poliovirus and chased with unlabeled methionine for 30 min. The resulting lysates were immunoprecipitated for VSV-G and aliquots were digested with endo H (Figure 2A). Samples of untreated lysates were electrophoresed in parallel with the immunoprecipitates to evaluate the amount of host translational inhibition and viral protein synthesis in each sample (Figure 2B).

At a MOI of 20 p.f.u./cell in the presence of guanidine, there was only a slight effect on VSV-G modification compared with uninfected cells treated with guanidine (Figure 2A). However, at a MOI of 200 in the presence of guanidine, Golgi-specific VSV-G modification was inhibited to a similar degree as in poliovirus-infected cells without guanidine present. Thus, either the proteins translated from the virion RNAs or the poliovirions themselves are sufficient to inhibit Golgi-specific modification of VSV-G. Figure 2B reveals that although VSV-G modification was dramatically inhibited at the time point shown, translation was not yet inhibited in the cells infected with poliovirus at a MOI of 200 in the presence of guanidine. Thus, inhibition of protein transport in poliovirus-infected cells is not a consequence of the inhibition of host cell translation by the virus.

Viral proteins 2B and 3A are each sufficient to inhibit Golgi-specific modification of VSV-G

To test whether any individual viral protein could inhibit VSV-G transport, each of the viral proteins in the non-structural region of the viral coding region was tested independently for its effect on VSV-G modification. We focused on the non-structural proteins because poliovirus genomes with the capsid-encoding region deleted can replicate (Kaplan and Racaniello, 1988) and it is likely that the inhibition of protein secretion by poliovirus is related to formation of the virus-induced membranous vesicles thought to be required for RNA replication. The structure of the poliovirus genome and the products of translation and polyprotein processing are represented diagramatically in Figure 3A. A series of plasmids which should each express both a polioviral non-structural protein and VSV-G from a single dicistronic RNA molecule (Figure 3B) was constructed. The first cistron, encoding a poliovirus protein, should be translated by a cap-dependent mechanism like any cellular mRNA, while translation of the second cistron should be initiated by internal ribosome binding to the poliovirus 5′ non-coding region inserted between the two cistrons (Pelletier and Sonenberg, 1988). Translation initiation and termination codons were introduced during cloning of the viral coding regions; the resulting proteins should differ from the authentic proteins expressed during poliovirus infection only by an initiator methionine introduced at the first position.

COS cells were transfected with plasmids that encoded each of the individual viral non-structural proteins 2B, 2C, 3A and 3B in dicistronic mRNAs that also encoded VSV-G. Plasmids encoding 2BC and 3AB, which have been proposed to function as fusion proteins during the poliovirus replicative cycle (Semler *et al.*, 1982; Bienz *et al.*, 1983), were also tested. Transport of VSV-G translated from each of the dicistronic RNAs was monitored by endo H digestion of immunoprecipitates following labeling with [^{35}S]methionine and a chase for 30 min with unlabeled methionine. Expression of VSV-G from the second cistron of the dicistronic mRNAs was used to ensure that every cell that expressed VSV-G also expressed the poliovirus protein encoded in the first cistron.

Figure 3C shows the result of co-expressing VSV-G with several different proteins encoded by the P2 region of the viral genome. Expression of 2B or 2BC caused VSV-G to remain predominantly in an ER-specific form, whereas expression of 2C did not alter the VSV-G modification pattern from that of cells that expressed VSV-G alone. Because 2BC is a fusion protein of 2B and 2C, and since 2C did not affect VSV-G in this assay, 2B was likely to be the minimal active domain in the 2BC experiment. The effects of expression of proteins from the P3 region of the viral genome are shown in Figure 3D. Expression of 3A, but not 3AB or 3B (VPg), caused VSV-G to remain predominately in an ER form. Thus, the addition of the 22 amino acid protein 3B to the C-terminus of 3A abolished the effect of 3A in this assay. By comparing Figure 3 with Figure 1, it can be seen that the individual effects of 2B and 3A on VSV-G modification were comparable in magnitude with the effect of poliovirus infection.

The effects of poliovirus proteins 2A, 3C, 3D and 3CD on VSV-G transport were also tested, but cells transfected with plasmids encoding these poliovirus proteins in dicistronic mRNAs displayed virtually no VSV-G expression. Expression of these proteins may have been sufficiently toxic or deleterious to remove the transfected cells from the population by selection or these proteins or the sequences encoding them may have interfered with VSV-G expression. Toxic effects of 2A- and 3C-encoding plasmids would not be surprising. Protein 2A is a protease that, in conjunction with cellular proteins, induces cleavage of p220 of eIF4F (Wyckoff *et al.*, 1990). Protein 3C is also a protease, whose cellular targets include transcription factors required for transcription by RNA polymerases II and III (Clark *et al.*, 1991, 1993). Although we would expect that inhibition of protein secretion by 2B and 3A would eventually be cytotoxic, these effects were not apparent by 48 h post-transfection, as measured by VSV-G expression.

Endocytosis is not inhibited in 2B- or 3A-expressing cells

To examine whether the effects of poliovirus proteins 2B and 3A on VSV-G modification were specific to the

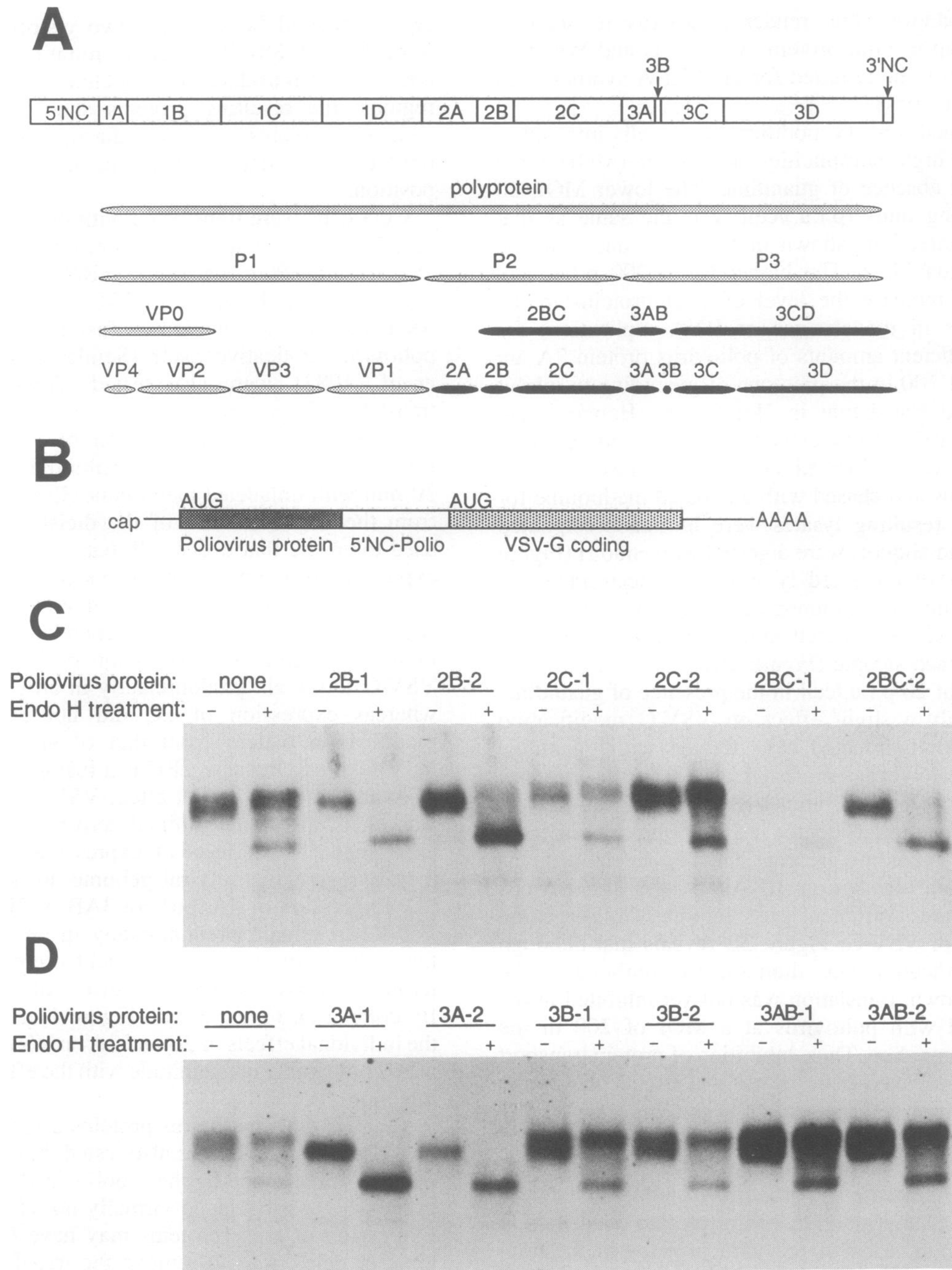
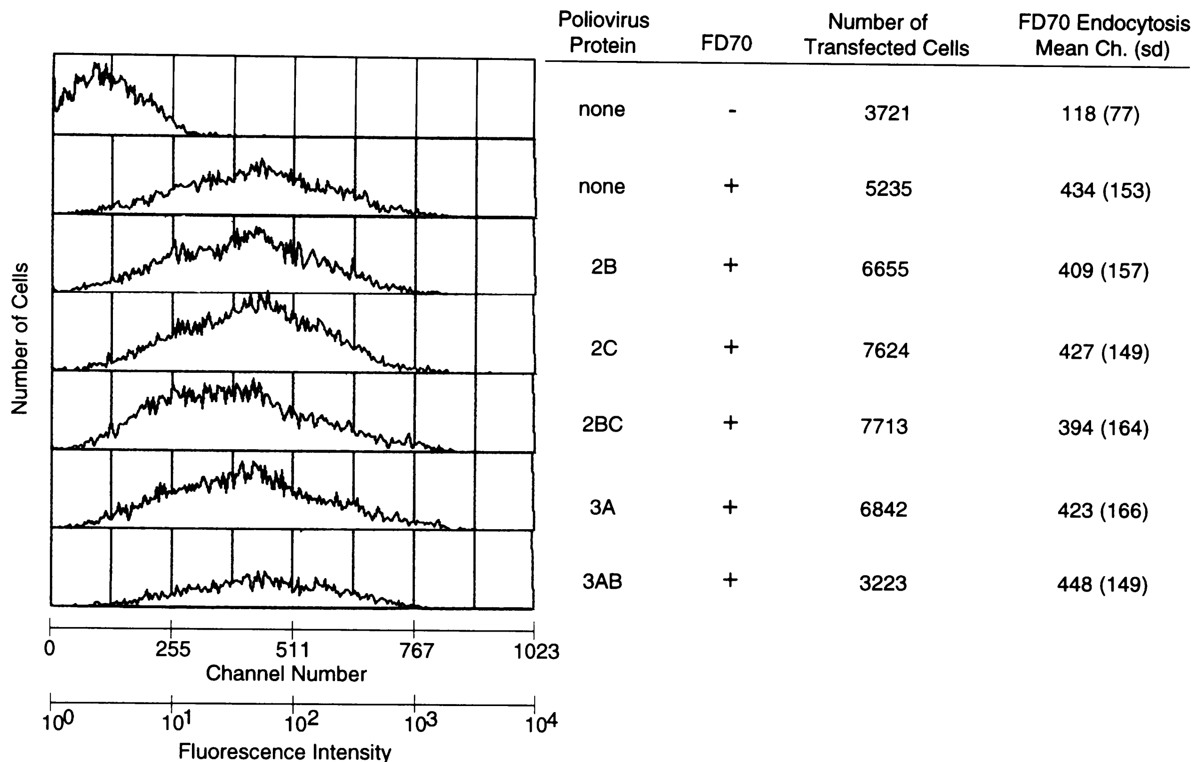


Fig. 3. (**A**) Structure of the poliovirus genome and products of polyprotein processing. Coding regions for the darkly shaded, non-structural viral proteins were amplified by PCR to introduce start and stop codons and convenient restriction sites. They were then cloned upstream of the poliovirus 5′NC–VSV-G fusion to produce a series of plasmids encoding dicistronic mRNAs generically diagramed in (**B**). (**C** and **D**) COS cells were transfected with dicistronic plasmids containing coding regions for individual poliovirus proteins and VSV-G. At 2 days post-transfection, the cells were pulse labeled with [^{35}S]methionine for 15 min. At the end of a 30 min chase period, the cells were harvested and lysates were prepared. Immunoprecipitated VSV-G was digested with no enzyme or endo H as indicated and the resulting products were analyzed by SDS–PAGE and autoradiography. (C) VSV-G glycosylation in cells expressing proteins 2B, 2C and 2BC from the P2 region of the viral genome. (D) VSV-G glycosylation in cells expressing proteins 3A, 3B and 3AB from the P3 region of the viral genome. Duplicate transfections that used independent dicistronic plasmids constructed by PCR amplification of the poliovirus-derived sequences are indicated.

secretory pathway or whether other membrane traffic events were also inhibited by these proteins, we assayed fluid phase endocytosis in cells expressing poliovirus proteins. Fluorescently labeled dextrans have been used frequently as markers for endocytosis in flow cytometry assays (Wilson and Morphy, 1989); we adapted this approach for analysis of endocytosis in COS cells that transiently expressed poliovirus proteins.

COS cells transfected with dicistronic plasmids that encode the poliovirus protein of interest in the first cistron and VSV-G in the second cistron were incubated in the presence of a 70 kDa fluoresceinated dextran (FD70) for 4 h. After being released from the plates, the cells were fixed, permeabilized, incubated with a rabbit polyclonal antiserum directed against VSV-G and stained with phycoerythrin-conjugated anti-rabbit secondary antibodies. The



Poliovirus Protein	FD70	Number of Transfected Cells	FD70 Endocytosis Mean Ch. (sd)
none	-	3721	118 (77)
none	+	5235	434 (153)
2B	+	6655	409 (157)
2C	+	7624	427 (149)
2BC	+	7713	394 (164)
3A	+	6842	423 (166)
3AB	+	3223	448 (149)

Fig. 4. Effect of poliovirus proteins on fluid phase endocytosis. Fluorescence histograms showing the effect of individual poliovirus proteins on endocytosis of FD70. COS cells transfected with plasmids encoding the indicated poliovirus proteins and VSV-G were incubated in the absence or presence of FD70 as indicated and then processed for flow cytometry as described in Materials and methods. VSV-G staining served as a marker for transfection; the number of VSV-G-positive cells in each sample is indicated. Transfection efficiencies were typically 5–10% of the total number of cells. The logarithmic scale on the x axis shows the relative fluorescence intensities of the samples, while the linear scale indicates the channel numbers on which the data were collected. The amount of FD70 uptake for each sample is indicated by the mean channel number of fluorescein fluorescence for the transfected population. Since the channel numbers are proportional to the log of the actual fluorescence intensities, the values for experimental samples (+FD70) reflect actual fluorescence intensities 12- to 19-fold above background due to autofluorescence (–FD70). Standard deviations are given in parentheses.

samples were then analyzed by flow cytometry. Phycoerythrin fluorescence was used to identify the subpopulation of transfected cells within each sample, while fluorescein fluorescence was used to measure endocytosis within this transfected subpopulation.

In control experiments, the fluorescence signal obtained was proportional to the time of incubation with FD70 and the concentration of FD70 used (not shown). In addition, cells incubated with FD70 at 4°C showed no fluorescence above that observed with cells that were not exposed to FD70 (not shown), arguing that the signal we detected was due to endocytosis of the FD70 and not non-specific binding to the cell surface.

In transfected COS cells, uptake of FD70 was not significantly affected by any of the poliovirus proteins tested (Figure 4), indicating that not all membrane traffic events were inhibited by expression of 2B or 3A and suggesting that these viral proteins might act specifically on the secretory pathway.

Transport of a secreted protein is also inhibited by 2B and 3A

The experiments performed with VSV-G relied on glycosylation state as an indicator of transit through the secretory pathway. It was possible that all the effects observed in the above experiments were due to virus-induced alterations in Golgi-specific modification activities, rather than to inhibition of the secretory pathway itself. It was also possible that the observed inhibition of VSV-G modification by proteins 2B and 3A was due to a direct interaction with VSV-G, rather than the inhibition of host cell protein secretion. To test these possibilities, the effects of co-expression of 2B and 3A on the transport of another reporter protein, one that was secreted from the cell, were monitored.

The second reporter protein used was human α_1 proteinase inhibitor (A1PI), a secreted glycoprotein expressed primarily in liver cells (Laurell and Jeppsson, 1975). The A1PI coding region was substituted for the VSV-G sequences in the 2B-, 2C-, 3A- and 3AB-expressing dicistronic plasmids described previously. COS cells transfected with these plasmids were labeled with [^{35}S]methionine and chased with unlabeled methionine. To control for varying transfection efficiencies between plates of cells, complete time courses were performed on individual plates. After 30 and 60 min of chase, the culture medium was removed from each plate and replaced with fresh medium. After 120 min of chase, the culture medium was again removed from each plate and the cells were harvested. A1PI protein was collected from the 0–30, 30–60 and 60–120 min aliquots of culture medium, as well as the cell lysates, by immunoprecipitation and the labeled proteins were displayed by SDS–PAGE. The amount of A1PI in each sample was determined by PhosphorImager analysis.

In 3A-expressing cells, secreted A1PI was barely detect-

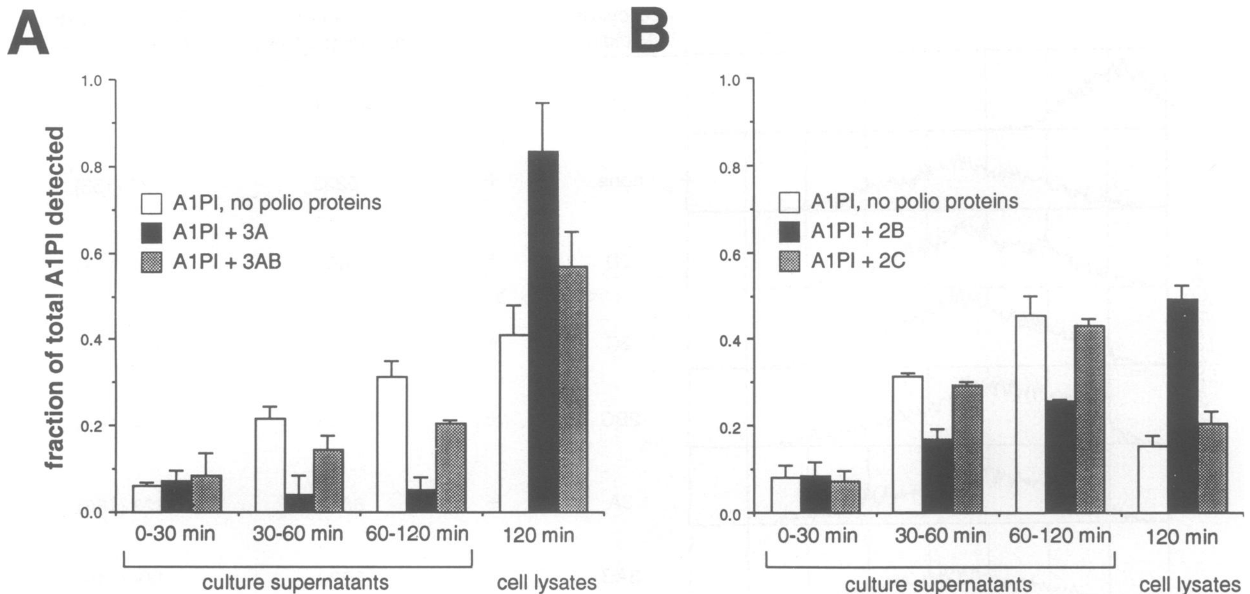


Fig. 5. Effect of individual poliovirus proteins on extracellular secretion of A1PI. COS-1 cells transfected with the indicated dicistronic plasmids containing poliovirus proteins and A1PI as the first and second cistrons respectively were labeled with [^{35}S]methionine for 15 min and chased with unlabeled methionine for various periods of time. Duplicate plates were analyzed for each plasmid. At 30 and 60 min of chase, the culture supernatants were removed and fresh medium was added. After 120 min of chase, both the medium and the cells were harvested. The 0–30, 30–60 and 60–120 min supernatants, as well as the cell lysates, were immunoprecipitated with antibodies to A1PI and displayed by SDS–PAGE; A1PI in each sample was quantitated by PhophorImager analysis. (**A**) Effect of poliovirus proteins 3A and 3AB on A1PI secretion. (**B**) Effect of 2B and 2C on A1PI secretion in an independent experiment. Error bars indicate the standard deviations of measurements from duplicate plates of cells.

able in any of the culture supernatants. After 120 min of chase, 83% of the labeled A1PI still remained within the cells (Figure 5A), compared with 42% in cells lacking 3A. Further, compared with the control cells, the cell-associated A1PI in 3A-expressing cells was incompletely glycosylated (not shown), consistent with our findings with VSV-G. Co-expression of 2B blocked A1PI secretion to a lesser extent, with amounts of A1PI significantly above background, yet below that observed in cells not expressing 2B, clearly detected in the culture supernatants. Still, by the end of the experiment, there was a 3-fold greater amount of intracellular A1PI in the 2B-expressing cells (Figure 5B), indicating that 2B also inhibited protein secretion in this assay.

By quantifying the amount of intracellular and extracellular A1PI, we have measured secretory transport directly, confirming that the impaired glycosylation observed with VSV-G corresponded to actual inhibition of secretory transport in cells expressing polioviral proteins. Because the effect on protein transport was seen with both VSV-G and A1PI, the observed transport inhibition is unlikely to be due to interactions between viral and reporter proteins.

Expression of 2B, but not 3A, causes an increase in cell membrane permeability to hygromycin B

The plasma membrane of poliovirus-infected cells is known to become more permeable to monovalent cations and small molecules, such as the translation inhibitor hygromycin B (Munoz and Carrasco, 1983; Lopez-Rivas *et al.*, 1987); it is not known which protein or proteins are responsible for this effect. However, expression of poliovirus protein 2B, 3A or 3AB in *Escherichia coli* causes the bacterial cell membrane to become more permeable to several small molecules (Lama and Carrasco, 1992). These observations suggested to us that 2B, 2BC and 3A might inhibit secretion by inducing non-specific membrane leakiness.

Table I. Sensitivity of A1PI translation to hygromycin B in cells expressing poliovirus proteins

Poliovirus protein[a]	Poliovirus infection	Ratio of A1PI synthesis ± hygromycin B[b]
None	–	0.89
None	+	0.30
2B	–	0.20
2BC	–	0.14
2C	–	1.03
3A	–	0.77
3AB	–	0.85

[a]COS-1 cells were transfected with dicistronic plasmids containing the indicated coding region as the first cistron and A1PI as the second cistron.
[b]Ratio of specific activities (A1PI PhophorImager counts/µg total protein) of [^{35}S]methionine-labeled A1PI synthesized in the presence and absence of hygromycin B.

To assess the leakiness of COS cells that expressed individual poliovirus proteins, we assayed the effect of hygromycin B on translation in pulse labeling experiments. Hygromycin B has been reported to have little or no effect on total protein synthesis in uninfected cells, while translation is reduced several fold in poliovirus-infected cells (Munoz and Carrasco, 1983). The increased sensitivity of translation to hygromycin B in infected cells is thought to be due to a virus-induced increase in permeability of the plasma membrane. To assay translation specifically in the transfected subpopulation of COS cells in our transfection experiments, we measured incorporation of [^{35}S]methionine into one of the reporter proteins, A1PI. COS cells transfected with dicistronic plasmids that

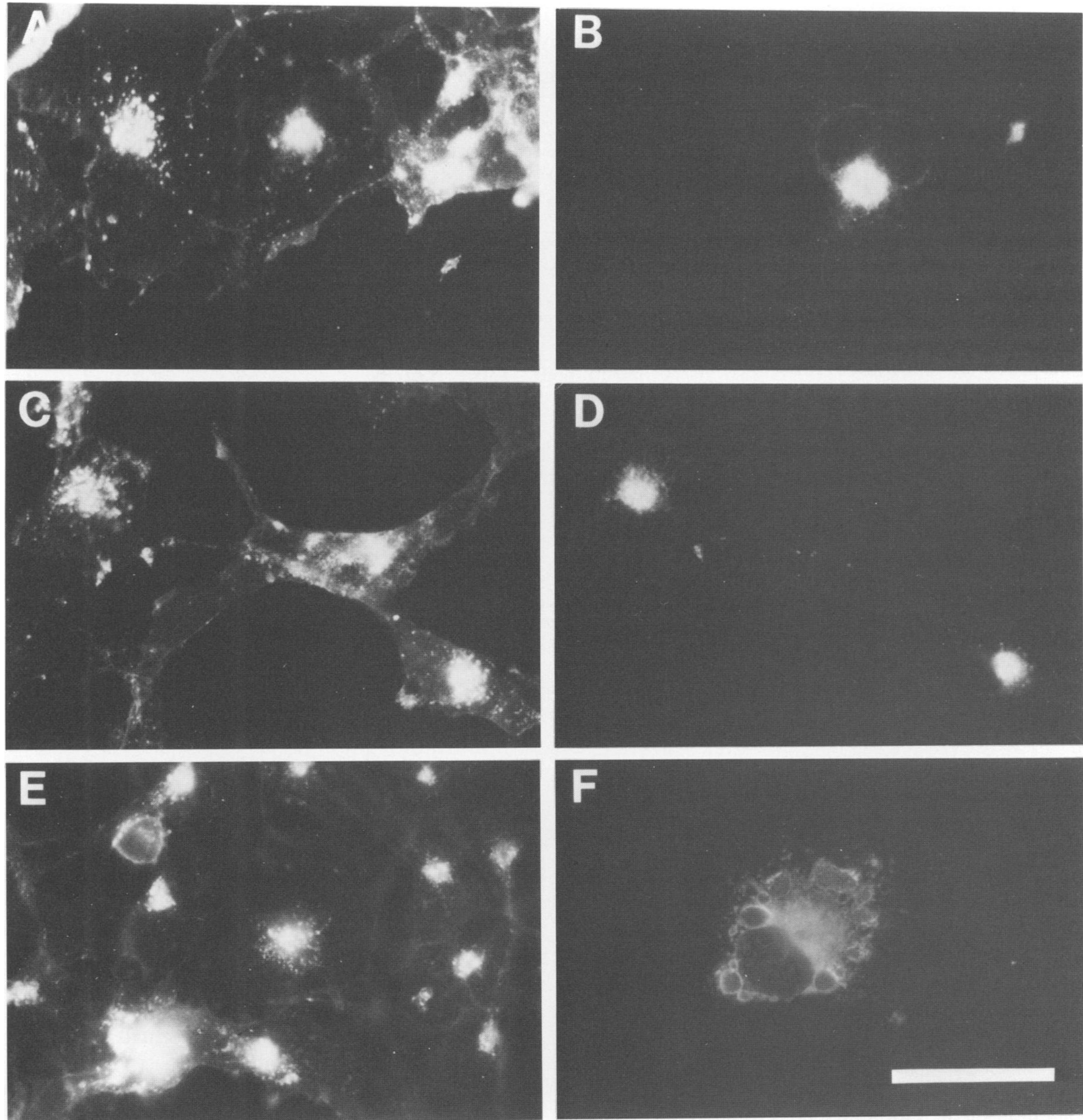


Fig. 6. Intracellular localization of A1PI and the Golgi marker WGA in the presence of individual poliovirus proteins 2B and 3A. COS-1 cells were transfected with the appropriate dicistronic plasmids and processed for indirect immunofluorescence 2 days post-transfection. A1PI was detected with a rabbit anti-A1PI polyclonal serum followed by donkey anti-rabbit–Texas red secondary antibody. Fluorescein-conjugated WGA was included in the secondary antibody incubation. Pairs of panels show the same fields stained with WGA (A, C and E) and anti-A1PI (B, D and F). (**A** and **B**) Cells expressing A1PI and no poliovirus proteins. (**C** and **D**) Cells expressing A1PI and poliovirus protein 2B. (**E** and **F**) Cells expressing A1PI and poliovirus protein 3A. Bar, 5 μm.

each encoded an individual poliovirus protein and A1PI were pulse labeled in the presence and absence of hygromycin B. The effect of hygromycin B on A1PI synthesis was determined for each of the viral proteins assayed.

Table I shows that hygromycin B reduced translation of A1PI roughly 3-fold in poliovirus-infected cells, consistent with previous reports (Munoz and Carrasco, 1983). Expression of either 2B or 2BC dramatically increased the sensitivity of translation to hygromycin, indicating that these proteins did indeed alter membrane permeability. Protein 2C did not increase membrane permeability to hygromycin, indicating that the 2B domain was most likely responsible for the effect seen in the 2BC-expressing cells. This finding parallels the effects of 2B, 2C and 2BC on both VSV-G modification (Figure 3) and A1PI secretion (Figure 5). In contrast, hygromycin had little effect on translation in 3A- and 3AB-expressing cells; it is not clear whether the values obtained for 3A and 3AB reflect meaningful increases in permeability to hygromycin, since transfected cells expressing no poliovirus proteins also showed a slight reduction in translation in the presence of hygromycin. However, any increase in membrane permeability detected in the 3A and 3AB samples is not likely to be relevant to the poliovirus-induced inhibition of secretion, since 3A strongly inhibits protein secretion, whereas 3AB does not (Figures 3 and 5).

Effect of 2B and 3A on subcellular localization of a secreted protein

The glycosylation and secretion results with VSV-G and A1PI showed that the viral proteins 2B and 3A could each inhibit secretory transport. To determine where proteins transiting the secretory apparatus accumulated when secretory transport was inhibited by viral proteins, we examined the subcellular localization of A1PI in the presence and absence of co-expressed poliovirus proteins by fluorescence microscopy.

To test the location of A1PI with respect to the Golgi apparatus, COS-1 cells transfected with plasmids expressing A1PI alone, 2B and A1PI or 3A and A1PI were fixed and processed for immunofluorescence microscopy. A1PI was detected using a rabbit polyclonal antiserum and the Golgi apparatus was identified using fluorescein-conjugated wheat germ agglutinin (WGA), which has been shown to stain medial and *trans*-Golgi cisternae (Tartakoff and Vassalli, 1983). In each case, A1PI staining could only be observed in the subset of cells that were transfected. In cells transfected with plasmids encoding only A1PI, the most pronounced A1PI-specific staining (Figure 6B) co-localized with Golgi staining (Figure 6A), as shown previously (McCracken *et al.*, 1989). The A1PI staining in the presence of 2B (Figure 6D) remained co-localized with the Golgi apparatus and WGA staining of the Golgi appeared normal as well (Figure 6C).

In contrast, the pattern of A1PI staining was drastically affected by 3A expression. The most obvious change, seen in all of the 3A-expressing cells, was the disappearance of A1PI staining (Figure 6F) from the Golgi structures identified by WGA staining (Figure 6E). The A1PI staining identified distinct structures within the cytoplasm, however, the identity of these structures was not obvious from these experiments. The pattern of WGA staining was also affected to various extents in the 3A-expressing cells. In some of the cells, the Golgi staining was indistinguishable from that in cells expressing no viral proteins. In others, the WGA staining appeared to be dispersed somewhat from its concentrated juxtanuclear location (Figure 6E). In the cells most strongly affected by 3A expression, WGA staining of the Golgi appeared to have disappeared (not shown), suggesting that the Golgi had either been dispersed throughout the cytoplasm or had disappeared entirely.

To test the possibility that A1PI localized with the ER when co-expressed with poliovirus protein 3A, transfected cells were subjected to double immunofluorescence for A1PI and protein disulfide isomerase (PDI), a lumenal ER protein (Figure 7). In cells that expressed A1PI alone, the most intense A1PI staining was once again seen in a juxtanuclear region that was shown to co-localize with the Golgi apparatus in Figure 6. In this experiment, fainter staining of ER structures that co-localized with PDI staining could also be seen (Figure 7A and B). A1PI staining was again unaffected by 2B expression (Figure 7D) and ER structures were also undisturbed (Figure 7C). In 3A-expressing cells, however, the altered A1PI staining first observed in Figure 6F was found to co-localize with PDI (Figure 7E and F); staining of both A1PI and PDI was distributed throughout the cytoplasm and the pattern of PDI staining was clearly different from that seen in cells not expressing 3A.

In no case was the juxtanuclear staining for A1PI characteristic of Golgi localization seen in the 3A-expressing cells; instead, co-localization of A1PI and PDI was consistently observed. Most of the 3A-expressing cells showed a morphology similar to that shown in Figures 6F and 7F, although some displayed even more severely disturbed patterns of both A1PI and PDI staining. Thus, the inhibition of protein secretion by protein 3A, which blocked the modifications attendant on successful ER to Golgi transport, causes A1PI to accumulate in the ER and also results in a striking alteration in ER organization.

Discussion

Inhibition of protein secretion by 2B and 3A

We have shown that poliovirus infection leads to a potent inhibition of protein secretion and that the poliovirus proteins 2B and 3A are each sufficient to inhibit secretory traffic in the absence of other viral gene products. These two proteins therefore represent novel inhibitors of secretory pathway function. Although dominant mutations in cellular proteins ARF1 (Dascher and Balch, 1994; Teal *et al.*, 1994), rab1 and rab2 (Tisdale *et al.*, 1992), as well as over-expression of ERD-2 and ELP-1, receptors for ER proteins being recycled from the Golgi (Hsu *et al.*, 1992), lead to inhibition of mammalian protein secretion *in vivo*, the two proteins identified here represent the first virally encoded inhibitors of mammalian protein secretion. Furthermore, unlike the other proteins mentioned, the natural function of these two viral proteins may be to inhibit protein secretion in the host cell. In mammalian cells, an unknown protein target of the $cdc^{2/28}$ kinase is thought to be the inhibitor of membrane fusion activity that causes vesiculation of the Golgi during mitosis (Tuomikoski *et al.*, 1989). An understanding of the mechanisms of inhibition of protein secretion by 2B and 3A will be of great interest in understanding both the poliovirus infectious cycle and normal functioning of the mammalian secretory pathway.

Examination of the 2B and 3A protein sequences reveals little about how they inhibit secretion. They are both relatively small proteins and neither shows significant sequence similarity to known cellular proteins. Protein 3A, 87 amino acids in length, contains a 22 amino acid hydrophobic domain near its C-terminus that is believed to mediate its association with cellular membranes. Both 3A and 3AB have been shown to associate with intracellular membranes when expressed in human cells using vaccinia vectors (Datta and Dasgupta, 1994). Protein 2B is also relatively hydrophobic, containing 31 hydrophobic residues out of 97 amino acids, but contains no continuous region of hydrophobic amino acids as long as that seen in 3A.

When expressed in *E.coli*, both 2B and 3A lead to an increase in the permeability of the bacterial cell membrane to small molecules such as antibiotics and nucleotides; any effect on permeability to larger molecules was not tested (Lama and Carrasco, 1992). In our experiments, expression of either 2B or 2BC led to an increase in the permeability of the plasma membrane to hygromycin B, raising the possibility that 2B and 2BC inhibit secretion by inducing non-specific leakiness of organelle membranes.

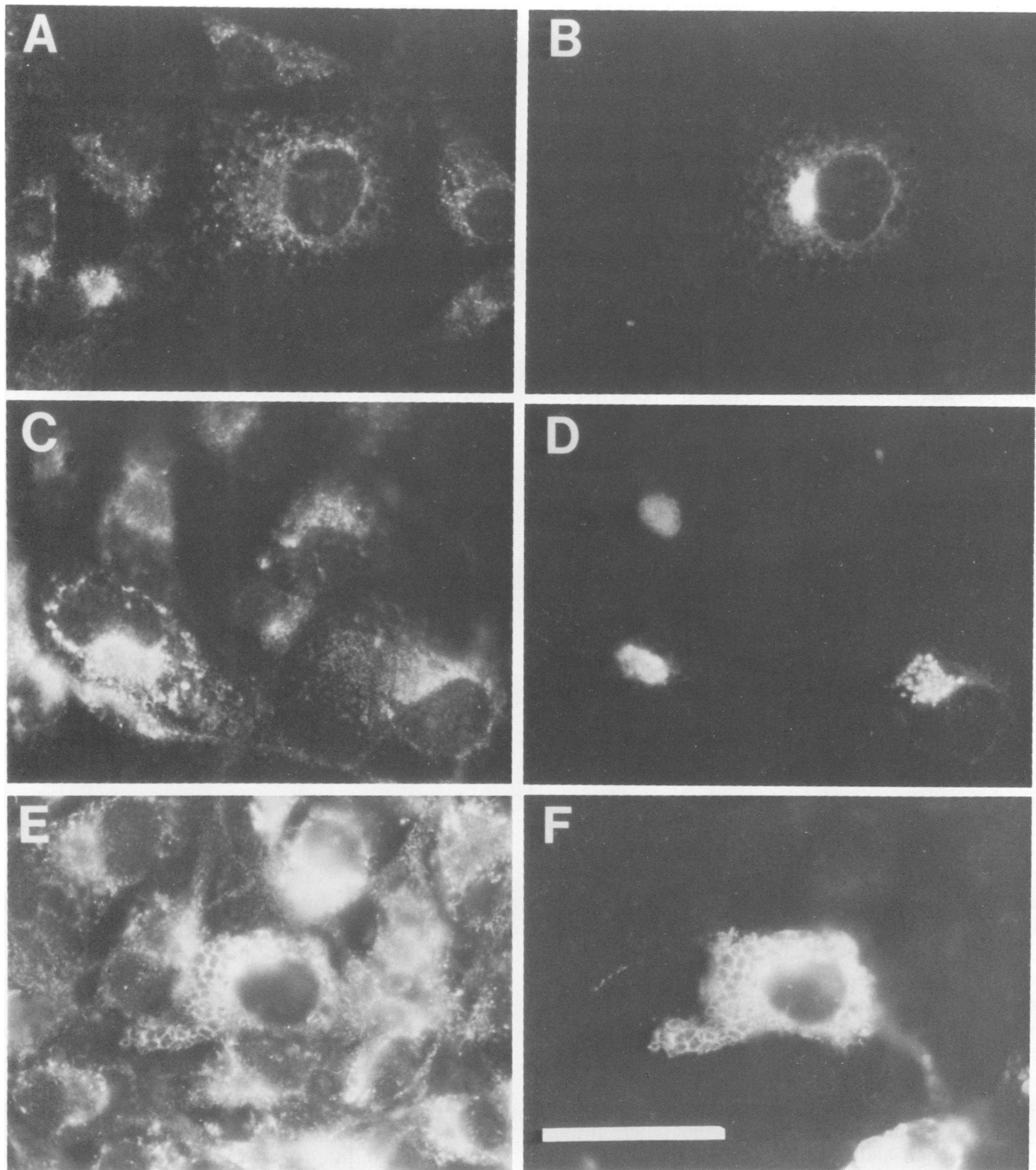


Fig. 7. Intracellular localization of A1PI and PDI, a lumenal ER protein, in the presence of individual poliovirus proteins 2B and 3A. COS cells transfected with the appropriate plasmid were processed for indirect immunofluorescence at 2 days post-transfection. Cells were stained with rabbit anti-A1PI and mouse anti-PDI primary antibodies and donkey anti-rabbit–Texas red and donkey anti-mouse–DTAF secondary antibodies. Pairs of panels show the same fields stained for PDI (A, C, E) and A1PI (B, D, F). (**A** and **B**) Cells expressing A1PI and no poliovirus proteins. (**C** and **D**) Cells expressing A1PI and poliovirus protein 2B. (**E** and **F**) Cells expressing A1PI and poliovirus protein 3A. Bar, 5 μm.

Such a permeabilization of ER or Golgi membranes might inhibit transport by altering lumenal concentrations of ions or small molecules required for proper modification and transport of protein through the secretory pathway.

Both 2B and 3A inhibit protein secretion and cause VSV-G to persist in an ER-specific glycosylation state in pulse–chase experiments. Thus, the first step at which protein secretion is inhibited by both proteins lies between the ER and medial Golgi compartments. However, 2B and 3A must inhibit protein secretion by mechanisms that differ in detail, based on the subcellular localization of the secreted protein A1PI when co-expressed with the two different poliovirus proteins. When 3A and A1PI were co-expressed, a majority of the A1PI was found to co-localize with ER-specific elements dispersed throughout the cytoplasm of the transfected cells. This pattern is consistent with the specific inhibition of ER to Golgi traffic by 3A. Depending on the step or steps of ER to

Golgi transport inhibited by 3A, this inhibition could possibly cause or contribute to an accumulation of transport vesicles en route from the ER to the *cis*-Golgi.

In the presence of viral protein 2B, on the other hand, prominent staining for A1PI remained co-localized with the Golgi complex. Thus, although VSV-G co-expressed with 2B accumulated in an ER-specific form, acquiring medial Golgi-specific modifications only very slowly in pulse–chase experiments, transport of A1PI from the ER to the Golgi was not absolutely blocked. Several mechanisms for the action of viral protein 2B are consistent with these observations. First, 2B could block transport specifically between the *cis*- and *medial* Golgi, leaving ER to *cis*-Golgi transport intact. Second, 2B could inhibit, but not absolutely block, many different steps of the secretory pathway, which could give rise to a steady-state immunofluorescence pattern indistinguishable from that seen in the absence of 2B. However, in a pulse–chase experiment, inhibition of modification would be seen at the first inhibited step after co-translational insertion into the ER. Finally, if 2B increases the permeability of membranes in the secretory pathway as it does the plasma membrane, molecules crucial for modification or transport could escape. Thus, A1PI could be transported as far as the medial Golgi and still not receive the appropriate modifications. Determination of the precise mechanism by which poliovirus proteins 2B and 3A inhibit secretory traffic awaits analysis of their function in cell-free extracts and the identification of intracellular targets.

Role of 2B and 3A in poliovirus infection

The functions of 2B and 3A in the poliovirus replicative cycle are incompletely characterized. Viruses with mutations in either of these coding regions display primary defects in RNA synthesis (Giachetti and Semler, 1991; Johnson and Sarnow, 1991; Giachetti *et al.*, 1992). Protein 3AB has been proposed as donating 3B (VPg) as a primer during viral RNA replication (Takegami *et al.*, 1983), however, this activity has not been demonstrated. Addition of purified 3AB can stimulate the RNA-dependent RNA polymerase activity of poliovirus protein 3D *in vitro*, demonstrating a direct interaction between 3AB and 3D and suggesting a direct role for 3AB in RNA replication (Lama *et al.*, 1994).

Prior to this study, no specific functions during poliovirus infection had been identified for either 2B or for 3A in the absence of 3B. We do not yet know the significance to the poliovirus infectious cycle of inhibiting protein secretion. That 2B and 2BC increased plasma membrane permeability when expressed individually suggests that these proteins are responsible for the plasma membrane alterations seen in poliovirus-infected cells; altering membrane permeability is therefore a second identified function for these viral proteins.

Is the inhibition of protein secretion by 2B or 3A a direct result of their causing the formation of membranous vesicles upon which the poliovirus replication complexes assemble? If transport were blocked at a step subsequent to vesicle budding, then the block might function to cause both the accumulation of cytoplasmic vesicles and the inhibition of protein secretion. This hypothesis would coincide well with the electron microscopic observation of vesicles with polioviral proteins on their surface apparently budding from the ER (Bienz *et al.*, 1987). Electron microscopic examination of poliovirus-infected cells in which complete processing of the viral polyprotein was inhibited showed a correlation between the accumulation of viral-induced vesicles and the presence of detectable levels of 2BC (Bienz *et al.*, 1983). Expression studies of proteins encoded by cowpea mosaic virus (CPMV), a plant comovirus, showed that a 60 kDa protein could specifically induce the formation of small membranous vesicles in insect cells (van Bokhoven *et al.*, 1992). The similarity in genetic organization of RNA viruses suggests that the 60 kDa protein from CPMV corresponds to the poliovirus fusion protein 2BC3AB and may therefore include functional homologs of both poliovirus proteins we have found to inhibit protein secretion. However, the only region of significant amino acid similarity between the 60 kDa protein of CPMV and poliovirus proteins corresponds to sequences in poliovirus protein 2C and not to 2B or 3A (Argos *et al.*, 1984; Franssen *et al.*, 1984).

Recently, the expression of poliovirus protein 2C from vaccinia vectors was shown to result in the accumulation of small membranous vesicles in human cells; 2BC expression also caused vesicle accumulation (Cho *et al.*, 1994). One hypothesis is that the viral protein or proteins that cause the accumulation of membranous vesicles in poliovirus-infected cells also directly cause inhibition of protein secretion. However, this idea is not supported by the observations that 2C alone can cause vesicle formation and 2B and 3A, but not 2C, can inhibit protein secretion.

It is possible that a host protein involved in membrane traffic functions as a direct participant in poliovirus RNA replication and that vesicle accumulation, the inhibition of secretion or both are secondary consequences of the interaction between viral proteins and this putative host protein or proteins. The dramatic inhibition of poliovirus RNA replication by BFA (Irurzun *et al.*, 1992; Maynell *et al.*, 1992) suggests a handful of candidates for such a host factor. BFA blocks transport through the secretory pathway by inhibiting the binding of ARF protein and, as a result, of coatomer proteins to ER and Golgi membranes (Donaldson *et al.*, 1992a). The specific reaction inhibited by BFA appears to be the exchange of GTP onto ARF, displacing bound GDP (Donaldson *et al.*, 1992b; Helms and Rothman, 1992). ARF-GDP is found in soluble form within the cytoplasm, whereas ARF-GTP is membrane bound (Walker *et al.*, 1992). By inhibiting this nucleotide exchange reaction, BFA treatment is believed to drive the cellular pool of ARF into the soluble, GDP-bound form and thus prevent the formation of coatomer-coated vesicles. If poliovirus replication complexes used coatomer proteins, ARF or the membrane-bound ARF nucleotide exchange factor as either an active constituent or as a membrane attachment site, then the inhibition of secretory pathway function by poliovirus could be easily explained by titration by viral proteins of a cellular factor required for secretory transport. Such an interaction might lead to the budding of vesicles that were incompetent to be properly targeted or fused to their normal acceptor membrane, perhaps resulting in the accumulation of vesicles seen during poliovirus infection.

Although poliovirus proteins 2B and 3A do not show similarity to any known cellular proteins, they both show extensive sequence similarity to the presumably homo-

logous proteins encoded by closely related picornaviruses, such as rhinoviruses and coxsackieviruses. Inhibiting or modifying the protein secretory apparatus could provide a direct advantage to viruses, especially non-enveloped viruses such as poliovirus and other picornaviruses, which do not need the protein secretory apparatus to modify their own proteins. In tissue culture cells, poliovirus inhibits protein secretion even earlier in the infectious cycle than it inhibits host cell translation (Figure 2). Crucial responses of mammalian host cells to viral infection, such as interferon secretion and antigen presentation in the context of MHC class I molecules, may be inhibited concomitantly with the inhibition of protein secretion. Advantages to the spread of non-enveloped viruses within infected organisms could therefore accrue directly from inhibiting the protein secretory pathway.

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 (DMEM) supplemented with 10% calf serum and 100 units/ml penicillin and 100 mg/ml streptomycin at 37°C in a 5% CO_2 incubator.

Mouse monoclonal antibody against VSV-G (Lefrancois and Lyles, 1982) was provided by Paul Melançon (University of Colorado). Rabbit polyclonal anti-VSV-G was provided by Kathryn Howell (University of Colorado Health Sciences Center). Affinity-purified rabbit polyclonal anti-A1PI was provided by Jerry Brown (University of Colorado Health Sciences Center). Mouse monoclonal 1D3 against protein disulfide isomerase was provided by Steven Fuller (EMBL, Heidelburg). Fluorescein dichlorotriazine (DTAF)-conjugated donkey anti-mouse IgG, Texas red-conjugated donkey anti-rabbit IgG and *r*-phycoerythrin-conjugated donkey anti-rabbit IgG were purchased from Jackson ImmunoResearch (West Grove, PA).

Guanidine HCl was purchased from Sigma (St Louis, MO). Hygromycin B was from Boehringer Mannheim. Fluorescein-conjugated WGA was purchased from Vector (Burlingame, CA). Fixable fluorescein-conjugated dextran (70 kDa) was purchased from Molecular Probes (Eugene, OR) and reconstituted as a 10 mg/ml solution in phosphate-buffered saline (PBS).

Construction of plasmids

The poliovirus 5′ non-coding region was fused to the VSV-G coding region by ligating a polymerase chain reaction (PCR) product containing the first 137 nucleotides of the VSV-G coding sequence (digested with *Bsp*HI and *Dra*I) with both a *Dra*I–*Eco*RI fragment containing the remainder of VSV-G and a fragment from p5NC-polio-LUC (Hambidge and Sarnow, 1992) digested with *Eco*RI and partially digested with *Nco*I. pSVGL (Rose and Bergmann, 1982), provided by Jack Rose (Yale University), served as both the template for PCR reactions and the source of the *Dra*I–*Eco*RI fragment containing the remainder of the VSV-G coding sequence. No predicted amino acid changes were introduced into the VSV-G coding region by this cloning scheme. The resulting plasmid, p5NC-G, was sequenced throughout the region that was amplified by PCR. pLNCG was constructed from p5NC-G in two steps. First, a *Pvu*II–*Stu*I fragment containing the 5′NC-VSV-G fusion from p5NC-G was inserted into a filled-in *Sal*I site of an intermediate plasmid to introduce *Sal*I sites at both ends of the fragment. The *Sal*I fragment was then inserted into *Xho*I-digested pSVGL to generate pLNCG.

Dicistronic plasmids to express both individual poliovirus proteins and VSV-G were constructed in two steps. First, pLINK was constructed by inserting a deoxyoligonucleotide linker containing *Sal*I and *Sma*I sites into a *Bam*HI site of pLNCG upstream of the poliovirus 5′NC. Individual viral protein coding regions were amplified with deoxyoligonucleotide primers designed to introduce a *Sal*I site and an AUG codon at the 5′ end and a stop codon followed by a *Sma*I site at the 3′ end of each coding region. *Sal*I- and *Sma*I-digested PCR products were inserted into pLINK in a three piece ligation using the *Sal*I and *Sma*I sites in the linker and a *Bst*XI site in the SV40 early region of the plasmid. To minimize the likelihood of mutations introduced by PCR affecting the results, the PCR reactions were limited to five cycles and two independent clones of each dicistronic plasmid were isolated and tested. In all cases, the duplicate clones behaved identically. The 2B inserts were sequenced and found to be wild-type; both a wild-type 3A insert and one containing a mutation that resulted in the conversion of amino acid 85 from glycine to alanine showed the effect on protein secretion reported here.

To fuse the 5′ non-coding region of poliovirus to the A1PI coding region, the A1PI coding region was amplified from plasmid pSV-A1PI-polio-LUC (P.Sarnow, University of Colorado Health Sciences Center) by PCR using deoxyoligonucleotide primers designed to introduce an *Msc*I site at the second codon of A1PI and an *Eco*RI site downstream of the stop codon. The *Msc*I- and *Eco*RI-cut A1PI PCR product was ligated into p5NC-polio-LUC (Hambidge and Sarnow, 1992) that had been partially digested with *Nco*I, filled-in with T4 DNA polymerase and cut with *Eco*RI. The sequence across the *Nco*I–*Msc*I junction was confirmed by sequencing; the resulting plasmid was named p5NC-α6.

To construct dicistronic plasmids encoding various poliovirus proteins and A1PI, an ~1600 bp fragment from the *Esp*I site in the poliovirus 5′NC to the *Eco*RI site downstream of the A1PI coding region in p5NC-α6 was substituted for a corresponding fragment in the dicistronic plasmids encoding proteins 2B, 2C, 3A or 3AB and VSV-G. A1PI expressed from PCR-generated A1PI-expressing plasmids behaved identically in pulse–chase experiments to A1PI expressed from the original A1PI plasmid, pSV-A1PI-polio-LUC, indicating that no mutations affecting A1PI glycosylation, transport, interaction with anti-A1PI antibodies or gel mobility had been introduced during plasmid construction.

Transfections and infections

DNA was transfected by a calcium phosphate method (Ausubel *et al.*, 1990) into COS-1 cells growing on 60 mm dishes, with each plate receiving 10 µg plasmid DNA. At 4 h post-transfection, the medium was aspirated and 1 ml of 10% glycerol in PBS was added to each plate. After 90 s, the cells were washed three times with PBS^+ (PBS containing 0.01% $CaCl_2$ and 0.01% $MgCl_2$), fresh medium was added and incubation was continued.

Virus infections with wild-type poliovirus type I Mahoney were performed as previously described (Maynell *et al.*, 1992) at a MOI of 20 p.f.u./cell unless otherwise indicated. Virus titers were measured by plaque assay on COS-1 cells. For infections performed in the presence of guanidine, guanidine HCl was added from a 50 mM stock solution to the culture medium to a final concentration of 2 mM. For the experiment shown in Figure 2, viral yields at 4.75 h post-infection were reduced 500-fold by guanidine treatment relative to infected cells not treated with guanidine, indicating that the virus was effectively inhibited by guanidine in this experiment.

Labeling of cells and preparation of lysates

To pulse label cells with $[^{35}S]$methionine, transfected COS cells were washed with PBS^+ and incubated in DMEM lacking methionine (Gibco, Boston, MA) for 30 min at 37°C. $[^{35}S]$Methionine (Expre^{35}S^{35}S label; New England Nuclear, Gaithersburg, MD) was then added and the cells were incubated for 15 min, washed once with PBS^+ and placed in fresh culture medium containing 0.23 mM unlabeled methionine. After the desired period of chase, the cells were washed with ice-cold PBS and harvested by scraping into ice-cold PBS. The cells were collected by centrifugation at 300 *g* for 5 min, the supernatant was removed and the cell pellet was lysed by vigorous mixing in RSB-NP40 (10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, 1% NP40) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Nuclei and insoluble debris were pelleted by centrifugation at 2000 *g* for 10 min and the supernatant was saved for further analysis.

For analysis of A1PI secretion, transfected COS cells were labeled as described above. After 30, 60 and 120 min of chase, the entire culture medium was removed and centrifuged for 10 min at 2000 *g* to remove any cellular debris. At 30 and 60 min of chase, fresh culture medium was added to each plate and at 120 min of chase, cell lysates were prepared as described above.

Immunoprecipitations and glycosidase digestions

In the VSV-G experiments, the same amount of total protein, as determined by Bradford assay (BioRad, Hercules, CA), was used in all immunoprecipitations. For experiments examining A1PI secretion, entire culture supernatants and cell lysates were used for immunoprecipitation. Prior to immunoprecipitation, cytoplasmic extracts were pre-cleared by incubating for 1 h on ice with fixed *Staphylococcus* A (Staph A) cells (Gibco) that had been washed three times and resuspended to a concentration of 10% w/v in RSB-NP40. The Staph A cells were

collected from the cytoplasmic extracts by centrifugation for 3 min at 16 000 g in a microcentrifuge, the supernatant was transferred to a new tube and the appropriate antibody was added to the pre-cleared sample. After 1 h on ice, 50 µl of washed Staph A cells was added to each immunoprecipitiation. After 1 h incubation on ice, the cells were pelleted and the supernatant was discarded. The pelleted cells with bound immune complexes were washed once in RSB-NP40, once in RSB-NP40 containing an additional 500 mM NaCl and once more in RSB-NP40.

For analysis of immunoprecipitates by SDS–PAGE, the immune complexes were resuspended in 25 µl of SDS–PAGE loading buffer (Laemmli, 1970), heated to 95°C for 3 min and centrifuged at 16 000 g in a microcentrifuge for 3 min at room temperature. The supernatants were then analyzed by SDS–PAGE. For experiments in which endo H digestions were performed before SDS–PAGE, immune complexes were released by resuspending the Staph A-containing pellet in 0.1 M NaOAc, pH 5.5, 0.5% SDS, heating to 95°C for 3 min and centrifuging for 5 min at 16 000 g. The supernatants were collected, diluted with an equal volume of a solution containing 0.1 M NaOAc, pH 5.5, and 1 mM PMSF and divided into equal aliquots. Each aliquot received either no enzyme or 1 mU endo H. Digested and undigested samples were incubated overnight at 37°C and were then analyzed by SDS–PAGE. Polyacrylamide gels were Coomassie brilliant blue or silver stained, treated with Autofluor (National Diagnostics, Atlanta, GA) and processed for autoradiography. Gels quantitated on the PhosphorImager (Molecular Dynamics, Sunnyvale, CA) were not treated with Autofluor.

Flow cytometry

COS-1 cells were transfected with the appropriate dicistronic plasmids. At 2 days post-transfection, the cells were loaded with FD70 diluted to 1 mg/ml in culture medium or mock-loaded with a 1:10 dilution of PBS in culture medium. After 4 h, the cells were washed extensively with PBS^+ to remove free FD70, harvested by incubating in PBS containing 50 mM EDTA and pipetting up and down, pelleted at 300 g for 5 min and resuspended in 0.5 ml PBS. Formaldehyde (6%, 0.5 ml) in PBS was then added to fix the cells. After 10 min at room temperature, the cells were pelleted and permeabilized in 0.1% Triton X-100 in PBS for 10 min at room temperature. After pelleting and washing twice with PBS, the cells were then stained for VSV-G using a rabbit polyclonal antiserum followed by *r*-phycoerythrin-conjugated donkey anti-rabbit antibodies. The stained cells were analyzed on a Becton Dickinson FACScan flow cytometer with compensation set to eliminate crossover between *r*-phycoerythrin and fluorescein fluorescence. Data were gathered for both transfected and untransfected cells within a sample; subsequent quantitation was performed on transfected cells as identified by VSV-G staining.

Permeability of cells expressing poliovirus proteins to hygromycin B

COS-1 cells were transfected with dicistronic plasmids containing a poliovirus protein as the first cistron and A1PI as the second cistron. At 1 day post-transfection, each transfected plate was split onto two plates to eliminate variations in transfection efficiency. On the second day post-transfection, the duplicate plates of cells were starved of methionine for 15 min in the presence or absence of 500 µg/ml hygromycin B. The cells were then labeled for 30 min with [^{35}S]methionine in the presence or absence of hygromycin B, placed on ice and washed with ice-cold PBS. Lysates were prepared as described above. An aliquot of each was assayed for protein content by a modified Lowry assay (BioRad), while A1PI was immunoprecipitated from another aliquot. Immunoprecipitates were displayed by SDS–PAGE and A1PI-specific radioactivity was quantitated on a PhophorImager. The specific activity of A1PI in each sample was calculated as A1PI radioactivity/µg total protein used for immunoprecipitation. The specific activity of A1PI synthesized in the presence of hygromycin B divided by that of A1PI synthesized in the absence of hygromycin B was used to measure the effect of hygromycin B on translation in cells expressing a single poliovirus protein.

Indirect immunofluorescence

COS-1 cells were plated onto cover slips 1 day prior to transfection. At 2 days post-transfection, 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 cover slips were incubated for 1 h at 37°C with primary antibodies diluted in PBS containing 5% calf serum. After three more washes with PBS, the coverslips were incubated for 1 h at 37°C with appropriate secondary antibodies diluted in PBS containing 5% calf serum. The coverslips were washed and mounted in a Mowiol solution containing 2% w/v 1,4-diazobicyclo-[2.2.2]-octane as an anti-fade agent (Harlow and Lane, 1988). The slides were examined under a Zeiss microscope using a 40× objective and photographed with Kodak TMAX 400 film.

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