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Poliovirus-Mediated Entry of Pokeweed Antiviral Protein

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Infection of HeLa cells with poliovirus results in cell permeabilization to pokeweed antiviral protein. Cell permeabilization was dependent on the integrity of virus capsid proteins and directly proportional to the multiplicity of infection. This study demonstrates that virus adsorption is sufficient for the entry of pokeweed antiviral protein into poliovirus-infected cells.

Pokeweed antiviral protein (PAP) is a ribosome-inactivating protein which enzymatically inactivates eucaryotic ribosomes by specifically removing a single adenine residue from a conserved 14-base sequence in 28S rRNA (5). PAP inhibits the multiplication of both DNA- and RNA-containing viruses, including herpes simplex virus (1), influenza virus (17), and poliovirus (18). The antiviral action of PAP directly correlates with the ability of the protein to inactivate ribosomes (10), which indicates that PAP must somehow enter cells during viral infection.

Alpha-sarcin is capable of inhibiting protein synthesis in encephalomyocarditis (EMC) virus-infected cells but not in uninfected cells (7). The entry of alpha-sarcin into infected cells was not prevented by the presence of inhibitors of macromolecular synthesis. Fernandez-Puentes and Carrasco (7) proposed that the permeabilization of HeLa cells by EMC virus takes place very early in virus infection. More recently, Fernandez-Puentes (6) demonstrated that permeability to alpha-sarcin in EMC virus-infected cells is not prevented by the inhibition of either viral uptake or decapsidation. She concluded that membrane permeabilization to alpha-sarcin following infection with EMC virus is induced by the interaction of viral particles with cell membranes. We have recently reported that PAP is slowly cytotoxic to uninfected HeLa cells (2). The fact that PAP enters cells very rapidly during infection but slowly in the absence of virus has prompted us to study the permeabilization of poliovirus-infected cells to the antiviral protein.

HeLa cells (ATCC CCL 2; Flow Laboratories, Inc., McLean, Va.) were grown as monolayer cultures in Eagle modified minimal essential medium as described previously (2). Rabbit antiserum specific for PAP was donated by Jon Robertus (The University of Texas, Austin). The antiserum was prepared in rabbits and the immunoglobulins were concentrated as described by Irvin et al. (11). The final concentration of immunoglobulin was 17.5 mg/ml.

Attenuated poliovirus type I was obtained from B. Sagik (The University of Texas at San Antonio, San Antonio). Virus was propagated in HeLa cells (1) and assayed by an agar cell plaque method (4). UV irradiation of virus was as follows. One-milliliter samples of virus (1.1×10^8 PFU/ml) in 60-mm-diameter dishes at 0°C were exposed at a distance of 12 cm to a UV lamp (The Southern New England Ultraviolet Co., Hamden, Conn.) which emitted a wavelength of 254 nm with an intensity of 1.0×10^5 ergs/s per cm^2 at the surface of the virus suspension. Neutral red-containing virus was prepared by incubating virus-infected cells in a maintenance medium which contained 10 μg of neutral red per ml, as described by Mandel (13).

PAP was extracted from spring leaves of *Phytolacca americana* and purified by ammonium sulfate fractionation followed by ion-exchange chromatography (9). 2-Pyridyldithiopropionyl-PAP (PDP-PAP) was prepared by mixing 0.16 mM PAP with 0.48 mM *N*-succinimidyl 3-(2-pyridyldithio)-propionate as described by Carlsson et al. (3). Iminothiolane (Sigma Chemical Co., St. Louis, Mo.) in Earle's balanced salt solution at final concentrations of 1, 10, and 100 μM was used to induce cross-links between PDP-PAP and poliovirus capsid proteins as follows. Samples of poliovirus which contained 5×10^8 PFU/ml in 0.4-ml volumes were incubated with 0.2 ml of iminothiolane in Earle's balanced salt solution at concentrations of 3, 30, and 300 μM for 30 min at 25°C. PDP-PAP (4 μM) was added to each of the solutions described above at a final concentration of 1 μM and incubated for 60 min at 25°C.

The effect of inhibition of viral and cellular protein synthesis by cycloheximide on the permeabilization of virus-infected cells to PAP was determined. HeLa cells were infected with poliovirus at a multiplicity of infection (MOI) of 20 PFU per cell in the presence of 0.4 μg of cycloheximide per ml and 3 μM PAP. The rate of incorporation of [^{14}C]leucine into trichloroacetic acid-precipitable materials was determined as described previously (2). Inhibition of protein synthesis was observed in cycloheximide-treated, infected cells in the presence (87% inhibition at 9 h postinfection [p.i.]) but not in the absence of PAP. Therefore, the inhibition of protein synthesis by PAP in virus-infected cells is dependent on viral infection, and permeabilization of virus-infected cells to PAP is not dependent on either viral or cellular protein synthesis. This agrees with the report by Fernandez-Puentes and Carrasco (7), who showed that macromolecular synthesis is not required for the entry of the toxin alpha-sarcin into EMC virus-infected cells.

The failure of cycloheximide to block PAP entry prompted us to determine whether PAP would also be able to enter cells infected with UV-inactivated virus. A short period of viral protein synthesis following the removal of cycloheximide could lead to cell permeabilization, whereas viral protein synthesis could not occur in cells infected with virus whose genomes were inactivated by UV irradiation. If the entry of PAP is not dependent on either viral protein synthesis or the inhibition of cellular protein synthesis, one would expect to observe an inhibition of protein synthesis in

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TABLE 1. Effect of PAP on protein synthesis in cells infected with UV-irradiated poliovirus

UV irradiation time	% Protein synthesis[a]	
	With PAP	Without PAP
20 min	87 (76)	97 (90)
30 s	16 (51)	100 (93)
0 s (unirradiated)	5 (40)	18 (63)

[a] Percent protein synthesis of cells infected at MOIs of 10 and 1 PFU per cell (values in parentheses). MOI was determined from the number of PFU prior to UV irradiation. Results are expressed as percent incorporation of [^{14}C]leucine into PAP-treated, infected cells compared with incorporation into untreated, uninfected cells (100% equals 30,282 cpm).

PAP-treated cells infected with UV-inactivated virus. Cell monolayers were infected with either irradiated or unirradiated poliovirus in either the presence or absence of 3 μM PAP. At 9 h p.i., protein synthesis was determined following a 1-h exposure to [^{14}C]leucine. PAP has an inhibitory effect on protein synthesis in cells infected with short-term-irradiated virus (16% protein synthesis) but not long-term-irradiated virus (87% protein synthesis) (Table 1). High-dose UV irradiation (254 nm, 32 J/m^2 per s, >2 min) is reported to cause structural alterations in poliovirus capsid proteins, whereas virus exposed to low-dose UV irradiation (254 nm, 32 J/m^2 per s, <2 min) does not possess conformational changes in viral capsid proteins (19, 20). Exposure of poliovirus to low-dose UV irradiation results in rapid inactivation of the virus, and the virion is converted to dense particles that are structurally and antigenically related to standard virus (20). Long-term UV irradiation may have caused significant damage to the capsid proteins which, in turn, resulted in the failure of the virus to attach to or penetrate the cells. The data suggest that the entry of PAP into infected cells is dependent upon the integrity of virus capsid proteins, which supports the finding that viral genome expression is not required for PAP entry.

The effect of MOI on permeabilization was determined. HeLa cells were infected with poliovirus at MOIs of 1, 5, 10, 50, and 100 PFU per cell in the presence of 0.1, 3, and 10 μM PAP. Infected and uninfected cells were incubated at 34°C, and protein synthesis was determined at 1 h p.i. Increased inhibition of protein synthesis was observed with increasing MOI from 1 to 100 PFU at both 3 and 10 μM PAP (Fig. 1). The correlation of a decrease in protein synthesis with an increase in the amount of virus inoculum suggests either that PAP is entering the infected cell together with the virus during penetration or that adsorbed virus causes local changes in membrane permeability to the antiviral protein. The observation that the activity of PAP was directly proportional to the MOI is consistent with a virus-dependent transport mechanism as suggested by Ussery et al. (18).

To determine whether the inhibitory effect of PAP on virus multiplication could be enhanced by tight association with the virion, HeLa cell monolayers were infected with poliovirus at a MOI of 20 PFU per cell which had been cross-linked to PDP-PAP. Under conditions favoring the cross-linking of PAP to poliovirus capsids by iminothiolane, the virus yield (22%) was slightly lower than the yield obtained in the absence of the cross-linking agent (39%) (Table 2). The results indicate that PAP activity does not depend upon a strong contact with the virus particle during infection. In addition, the activity of PAP was not affected by decapsidation enzymes present in phagolysosomes if indeed PAP does enter simultaneously with virus particles.

To determine whether either viral penetration or uncoating is required for cell permeabilization, PAP-treated cells were infected with photosensitized virus in the presence of either 10 mM sodium azide or 20 mM methylamine. After incubation at 37°C for 30 min, the cells were illuminated, washed twice with EBSS, and then supplied with maintenance medium containing 3 μM PAP. We observed that the inhibitory effect of PAP on protein synthesis in infected cells is not affected by the presence of either sodium azide or methylamine. Inhibition of protein synthesis in both illuminated and unilluminated samples ranged from 51 to 63%. Sodium azide and methylamine inhibit virus penetration and decapsidation, respectively, but they do not inhibit binding of the virus to cell membranes (14). Photoinactivation of the parental genome prior to the removal of sodium azide and methylamine ensured that viral expression would not be a factor in PAP entry. Therefore, virus-induced events which follow penetration and uncoating would not influence the

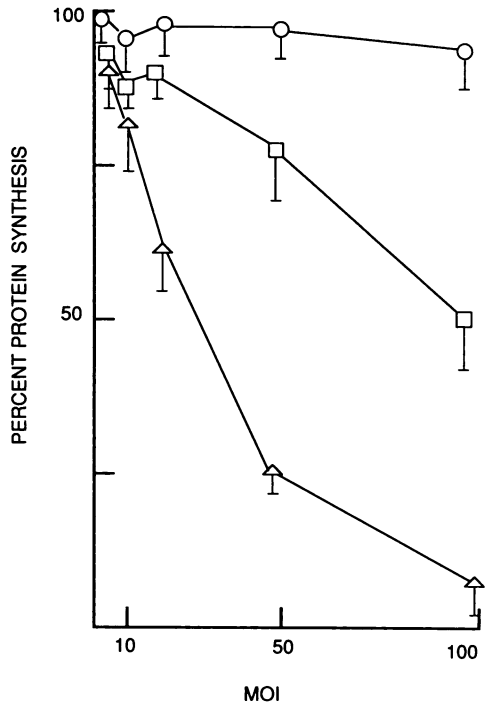

FIG. 1. Effect of MOI. Results are expressed as percent protein synthesis in untreated, uninfected cells. Symbols: ○, 0.1 μM PAP; □, 3 μM PAP; △, 10 μM PAP. Error bars represent standard deviation values.

TABLE 2. Effect of cross-linking PAP to poliovirus capsids on virus multiplication

Virus pretreatment	Virus yield[a]	
	10^7 PFU/ml[b]	%[c]
IT + PDP-PAP[d]	1.4 ± 1.9	22
PDP-PAP (1 μM)	2.5 ± 2.1	39
IT (100 μM)	6.0 ± 1.5	92
None	6.5 ± 2.4	100

[a] Virus yields were determined at 24 h p.i. after three cycles of freezing and thawing.

[b] Mean ± standard deviation of the number of plaques from three separate experiments assayed in triplicate.

[c] Percentage of virus yield from cells infected with untreated virus.

[d] Cell monolayers were infected with poliovirus which had been cross-linked with 1 μM PDP-PAP by 100 μM iminothiolane (IT).

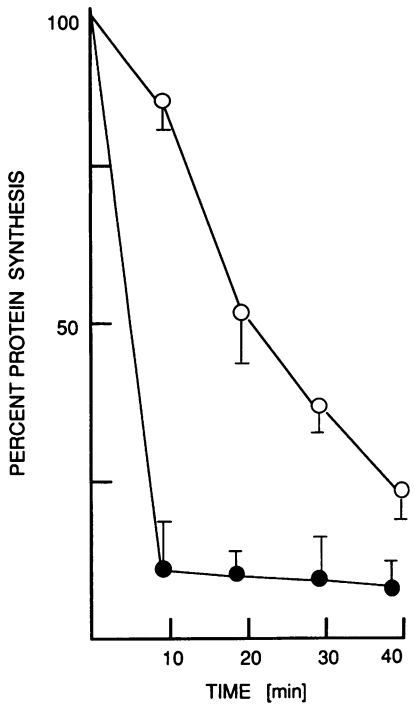

FIG. 2. Kinetics of permeabilization. Sodium azide was present during exposure of the cells to PAP antiserum and adsorption of virus. Protein synthesis was determined at 7 h p.i. Symbols: ●, PAP plus poliovirus followed by PAP antiserum; ○, PAP followed by PAP antiserum and poliovirus. Error bars represent standard deviation values.

entry of PAP. The results indicate that virus penetration or decapsidation is not required for permeabilization of cells to PAP. This suggests that the entry of PAP is induced solely by the adsorption of virus to the cell.

The rate of cell permeabilization to PAP induced by virus infection was determined. HeLa cell monolayers were preincubated with 10 mM sodium azide for 15 min at 37°C. PAP (3 μM) and sodium azide (10 mM) were added to HeLa cell monolayers, and then a mixture of 10 mM sodium azide and antiserum was added at 10-min intervals. In one series of experiments, UV-irradiated poliovirus was added simultaneously with PAP, and antisera were added at 10 min intervals. In duplicate monolayers, PAP was added to cell monolayers in the absence of virus, and antisera were added at the indicated times. Virus was added to all of the monolayers 10 min after the last addition of antisera. When antiserum was added as early as 10 min after the addition of PAP, protein synthesis was inhibited by 90% when cells were exposed to PAP and virus simultaneously, whereas protein synthesis was reduced by only 10% when PAP was added before virus (Fig. 2). A 50% inhibition of protein synthesis was observed when PAP was allowed to associate with the cells for 20 min prior to the addition of virus and antiserum. The inability to neutralize 50% of the activity of PAP suggests that membrane association of PAP occurs in the absence of virus. This association may play a role in the reported cytotoxicity of cultured cells following long-term exposure to PAP (2). However, viral infection caused the protection from antibody and permeabilization of the cell membrane to PAP. This uptake is similar to that of dimeric toxins containing B chains which induce permeabilization to the ribosome-inactivating chain (15). The data indicate that PAP enters infected cells both rapidly and early following exposure to poliovirus and that viral penetration is not required. A rapid membrane association appears to be the first stage of PAP entry, which is slower in the absence of virus. After membrane association, virus is capable of mediating PAP transport to the cytosol even if PAP has associated with the membrane prior to viral adsorption (Fig. 2).

We have previously shown that PAP slowly enters cells presumably via fluid-phase endocytosis (2), but such a slow internalization cannot account for the more-rapid effects observed during viral infection. A number of factors could account for capsid-induced permeabilization of membranes. It is possible that virion capsid proteins could bind to cell membrane receptors and induce changes in membrane permeability to PAP. Paramyxovirus infection has been shown to cause changes in membrane integrity including leakage of ions (16), modification of the resting membrane potential (8), and increased fluidity of the cell membrane (12). Thus, changes in membrane integrity could allow PAP to enter virus-infected cells.

In conclusion, the results presented in this study support the view that the entry of PAP into poliovirus-infected cells is induced by viral adsorption and that subsequent steps in the viral infection cycle such as penetration, uncoating, viral macromolecular synthesis, and inhibition of host protein synthesis are not essential. The results of this study indicate that the antiviral activity of PAP does not depend upon a strong association of PAP with the virus during entry and is most likely due to virus-induced permeabilization of the membrane.

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