Solubilized and cleaved VP7, the outer glycoprotein of rotavirus, induces permeabilization of cell membrane vesicles

A. Charpilienne,1 M. J. Abad,2 F. Michelangeli,2 F. Alvarado,3 M. Vasseur,3 J. Cohen1 and M. C. Ruiz2

1 Laboratoire de Virologie et Immunologie Moléculaires INRA, C.R.J., Domaine de Vilvert, 78350 Jouy-en-Josas, France
2 Laboratorio de Fisiología Gastrointestinal, IVIC. Apartado 21827, Caracas 1020A, Venezuela
3 CIF 94-07 INSERM, Département de Microbiologie et Immunologie, Faculté de Pharmacie, Université de Paris XI, 92296 Chatenay-Malabry, France

It has been previously shown that rotavirus triple-layered particles induce permeabilization of liposomes and membrane vesicles. These effects were mediated by one or both of the solubilized outer-capsid proteins, VP4 and VP7. Permeabilization was dependent on trypsin treatment of the viral particles, suggesting that VP4 was involved. To analyse the respective roles of the outer-capsid proteins in this permeabilization process, we have used membrane vesicles loaded with carboxyfluorescein and virus-like particles derived from insect cells co-expressing various sets of capsid proteins. Virus-like particles containing VP2, VP6 and VP7 (VLP2/6/7) are as efficient in permeabilizing vesicles as triple-layered particles. As with double-layered particles, virus-like particles made of VP2 and VP6 had no effect on vesicle permeabilization. Permeabilization of membrane vesicles required trypsinization of the VP7 solubilized from VLP2/6/7. These results show that solubilized and trypsinized VP7 is able to induce membrane permeabilization, independently of the presence of VP4.

Rotavirus, a member of the family Reoviridae, is a non-enveloped virus which has a triple-layered concentric protein structure containing the genome (11 segments of double-stranded RNA) and two minor proteins, VP1 and VP3 (Yeager et al., 1990; Prasad et al., 1988; Matton et al., 1994). The innermost protein layer is composed of VP2 and the second of VP6. The third, outer layer consists of the glycoprotein VP7 and the protein VP4. Treatment of rotaviral particles by trypsin cleaves VP4 into VP5* and VP8*, and enhances rotavirus infectivity (Clark et al., 1981). Infection of the cell by rotaviruses is thought to be a multi-step process involving attachment to specific membrane receptor(s), entry into the cell and uncoating to trigger virus replication. Rotavirus attachment to the cells seems to involve the binding of VP4 to a membrane receptor (Ludert et al., 1996; Mendez et al., 1996), but the mechanism of rotavirus penetration into the cell remains unknown. Two pathways of entry have been proposed, the direct and the endocytosis pathways (Suzuki et al., 1985). Both of these pathways require the crossing of a cellular membrane, either the plasma membrane in the case of direct entry, or the endosomal membrane following endocytosis.

It has been shown previously that rotavirus has the capacity to induce fusion of plasma membranes and syncytium formation in cultured cells (Falconer et al., 1995). On the other hand, rotavirus can permeabilize liposomes, membrane vesicles and intact cells (Nandi et al., 1992; Ruiz et al., 1994, 1995), but only trypsinized triple-layered particles (TLP) induce this effect. Permeabilization involves interactions between solubilized outer-layer proteins of rotavirus and membrane lipids. It is not known whether one or both of the outer-layer proteins, VP4 and VP7, are necessary to induce membrane permeabilization. The requirement for trypsinization of viral particles suggests the participation of either VP4 or one of its cleavage products, VP5* and VP8*. However, at present there is no direct evidence concerning this point.

In the present study, we have analysed the role of the outer-capsid protein VP7 in membrane permeabilization by using membrane vesicles loaded with a fluorophore, and virus-like particles (VLP) derived from insect cells co-expressing capsid proteins VP2, VP6 and VP7 (VLP2/6/7), but not VP4. VLP were produced by co-infecting Sf9 cells with recombinant baculoviruses at an m.o.i. of 5 p.f.u. per cell. The recombinant baculoviruses BacRF2, BacRF6 and BacRF9 containing genes 2, 6 and 9 of the bovine RF strain, respectively, were prepared as previously described (Labbé et al., 1991; Tosser et al., 1992; Ruiz et al., 1996). VLP were purified by banding twice in CsCl gradients in 20 mM PIPES pH 6.6.
containing 10 mM CaCl$_2$ (18 h at 35 000 r.p.m. in an SW50.2 rotor). The TLP used as controls were grown in MA104 cells in the presence of trypsin (0.044 mg/ml, Sigma type IX) and purified after Freon 113 extraction by banding twice in CsCl gradients.

Membrane permeabilization assays were performed by using brush border membrane vesicles loaded with the self-quenching fluorophore 6-carboxyfluorescein (CF) as previously described (Ruiz et al., 1994). Vesicles were prepared from pig jejunum by using the magnesium–EGTA precipitation method of Hauser et al. (1980). Vesicles loaded with the fluorophore were resuspended in a standard assay medium (SAM) consisting of 100 mM sorbitol and 200 mM Tris–HCl in a pH 7.4 buffer (20 mM HEPES/10 mM Tris). CF release was monitored at 37 °C as a fluorescence increase due to dilution dequenching in a spectrofluorometer (Photon Technology International) equipped with stirrer and temperature control. Excitation and emission wavelengths were 496 and 520 nm, respectively. The results are expressed as percentages of the total fluorescence, determined after the addition of digitonin (0.13 mg/ml final concentration) at the end of each experiment.

Changes in the diameters of VLP2/6/7 and VLP2/6 were monitored by 90° light scattering. An 8 µl volume of purified VLP at a concentration of 1.5 mg/ml was added to the cuvette of the fluorometer (37 °C) which contained 1 ml of SAM supplemented with 1 mM CaCl$_2$. Slits were adjusted to 0.5–1 nm and both monochromators were set at a wavelength of 300 nm.

Protein modifications resulting from trypsin treatment of the different types of particles were analysed by SDS–PAGE
on 12% polyacrylamide gels. Gels were stained with Coomassie blue.

Addition of VLP2/6/7 to vesicles resuspended in SAM containing 1 mM EGTA had no effect, but CF release was observed as soon as trypsin was added to the cuvette (Fig. 1A). This effect was not obtained with trypsinized or untreated VLP2/6, or when trypsin alone was added. The addition of VLP2/6/7 into SAM supplemented with 1 mM CaCl2 and trypsin did not induce vesicle permeabilization (Fig. 1B). However, CF release took place immediately after Ca2+ chelation by EGTA. Comparison of the kinetics of CF release induced by VLP2/6/7 (Fig. 1B) and by TLP (Fig. 1A), revealed a similar pattern.

Since it has been shown that low Ca2+ concentration induces VP7 solubilization from VLP2/6/7 (Ruiz et al., 1996), we studied the kinetics of this process and compared it with the time-course of the permeabilizing effect. We observed that the addition of 10 mM EGTA to VLP2/6/7 induced an immediate decrease in the scattering signal corresponding to a reduction in the diameter of the particles (Fig. 1C). The time necessary for total VP7 solubilization (at 37 °C and zero Ca2+) was much shorter than that preceding the onset of permeabilization. No change in scattering signal was observed when VLP2/6 were treated with EGTA. Addition of trypsin before or after EGTA did not modify the apparent diameter of the particles (not shown). These results show that the permeabilizing effect of VLP2/6/7 required not only treatment of VP7 with trypsin, but also solubilization of this viral protein. Results are similar to those previously described for TLP, where both solubilization and trypsinization of the outer-capsid proteins were required for permeabilization (Ruiz et al., 1994). For TLP, it has been shown that permeabilization was due to the solubilized outer-capsid proteins (Ruiz et al., 1994).

To see whether trypsin cleavage of VP7 could take place in either the solubilized or the native state, controlled trypsinization was performed both before and after VP7 solubilization by EGTA treatment (Fig. 1D). Addition of a suspension of VLP2/6/7, in which VP7 was first solubilized by EGTA pre-treatment outside the cuvette, to vesicles incubated in SAM containing 2 mM CaCl2 and trypsin, induced CF release (Fig. 1D, curve a). This result indicates that solubilized VP7 can be modified by the action of trypsin and thereby induce permeabilization even in the presence of 1-3 mM free Ca2+. This shows that Ca2+ does not inhibit either vesicle permeabilization or the action of trypsin on VP7 when this protein is first solubilized. When intact VLP2/6/7 (in the presence of 1 mM Ca2+) were treated with trypsin and added to the vesicle suspension in the presence of 1 mM Ca2+, no CF release was observed (Fig. 1D, curve b). Further addition of EGTA to solubilize VP7 had only a small effect, most probably due to the low concentration of trypsin in the cuvette derived from the VLP sample. Full restoration of permeabilization was attained when more trypsin was added to the cuvette. This result indicates that trypsin had no effect whatsoever when VP7 was attached to the intact VLP2/6/7 particle, but was effective on the solubilized protein.

The rate of vesicle permeabilization was a function of trypsin concentration (Fig. 2A). The trypsin concentrations used were in the same range as those needed either to enhance rotavirus infectivity (Clark et al., 1981) or to activate untrypsinized TLP in the CF release assay (Ruiz et al., 1994). The effect of trypsin does not seem to take place directly over
the membrane. Vesicles treated with trypsin, and then washed, did not permeabilize by themselves or upon the addition of VLP2/6/7 in the presence of EGTA (not shown). The action of trypsin was specific since chymotrypsin did not have the same effect (Fig. 2B) and was inhibited by TLCK (Nα-p-tosyl-L-lysine chloromethyl ketone), a specific inhibitor of trypsin (Fig. 2C).

To detect the protein modifications induced by trypsin, we analysed the mobility of viral proteins from TLP and VLP2/6/7 by SDS–PAGE after various treatments (Fig. 3). The gels indicate that trypsin digestion of VLP2/6/7 or TLP treated with EGTA to solubilize the outer-capsid proteins resulted in the disappearance of the VP7 band and the appearance of new bands of lower molecular mass. On the other hand, the mobility of VP7 was not affected when the protein was bound to the particles (VPL2/6/7 or TLP) during trypsin treatment. Solubilization of the outer-capsid proteins by EGTA did not affect the migration of the TLP and VLP2/6/7 proteins (data not shown).

It is well known that trypsin induces the cleavage of VP4 but, to our knowledge, there is no report of any effect of trypsin on VP7. Our experiments indicate that the sites of trypsin cleavage on VP7 are inaccessible in the intact particle. Solubilization appears to expose trypsin sites. One or several of the resulting peptides would be able to destabilize the vesicle membrane and induce the leakage of CF.

In light of these and other results, we suggest that VP4 also has permeabilizing capacity. Trypsinized TLP, in which we now know that VP4 is cleaved but not VP7, are able to elicit permeabilization upon solubilization of the outer capsid by EGTA (Ruiz et al., 1994; Nandi et al., 1992). Furthermore, the trypsinized supernatant of a lysate of Sf9 cells expressing VP4 induced permeabilization (Alaoui et al., 1997). Therefore, it appears that both cleaved VP4 and VP7 are each able to induce permeabilization independently. It seems clear that both VP7 and VP4 need to be solubilized and trypsinized to induce destabilization of the membrane, although the exact role of each one of the proteins in this process and, moreover, in the replication of rotavirus, is not known. Interestingly, this permeabilizing property is also shared by the nonstructural rotavirus protein NSP4 (Tian et al., 1996). Interaction of hydrophobic regions of viral proteins with membrane lipids may play an important role in the different stages of the virus replication cycle. The specific interactions of VP4 and VP7 with the cellular membrane remain to be elucidated in order to understand the penetration mechanism of infectious rotavirus particles into the cell.

This work was supported in part by grants S1-95000520 from CONICIT, Venezuela, from the French Embassy at Caracas, from the Fondation pour la Recherche Médicale, Paris, France, and from the INCO programme from the European Community (grant ERB3514FL950019). We thank Dr Ferdinando Liprandi for constructive criticisms and Aleida Sanchez for her technical assistance.

References


Membrane permeabilization by rotavirus VP7


Received 8 July 1996; Accepted 7 February 1997