Membrane Permeabilization by Poliovirus Proteins 2B and 2BC*

(Received for publication, November 13, 1996, and in revised form, March 25, 1996)

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Poliovirus infection leads to drastic alterations in membrane permeability late during infection. Transient expression of each nonstructural protein of poliovirus by means of recombinant vaccinia virus encoding the T7 RNA polymerase indicates that proteins 2B and 2BC strongly enhance membrane permeability to hygromycin B in HeLa cells. Almost no effect on expression of proteins 2C, 3A, 3AB, and 3C was found. Deletions and point mutations in 2B and 2BC have identified sequences in 2B involved in membrane permeabilization. Regions located at both ends of 2B are necessary to bring about these permeability alterations. A deletion of 11 amino acids of 2BC at the junction between 2B and 2C, as well as long deletions in 2C encompassing the GTPase motifs of this protein, do not impair the capacity of 2BC to modify the permeability of the membrane. The release of compounds such as choline or uridine from preloaded cells is also augmented by 2B and 2BC expression.

Infection of cells by cytopathic animal viruses leads to profound alterations in membrane permeability (1, 2). These alterations occur at two well defined moments during virus infection: at early times when virus particles penetrate into cells and late during infection when the majority of viral products are being synthesized (1, 2). Early alterations of the membrane do not require virus gene expression (3, 4). Low molecular weight compounds as well as macromolecules enter cells together with virus particles (3, 5). The exact mechanism of this enhanced permeability at early times of infection is still poorly understood. The suggestion has been put forward that the proton motive force is coupled to the translocation of virus particles and macromolecules into cells during virus entry into cells (6–8).

In addition to this phenomenon, late membrane leakiness requires virus gene expression and involves the diffusion of ions and small molecules but not macromolecules through the plasma membrane (9–12). Picornaviruses have been extensively used as model systems to analyze the mechanism of late membrane leakage in detail (1, 2). As early as 2–3 h postinfection membrane potential drops in picornavirus-infected cells, accompanied by a gradual redistribution of sodium and potassium ions between the culture medium and the cytoplasm (9, 13–15). In addition, increased passive diffusion of compounds such as choline, nucleotides, and low molecular weight antibiotics takes place (16, 17). Phospholipase C is selectively activated in poliovirus-infected cells (18), whereas other lipases, including phospholipase A2, become stimulated in other animal virus-infected cells (19). The exact contribution of lipase activation to membrane leakiness remains to be established, but it seems that a general disorganization of the plasma membrane is generated at late times of poliovirus infection (1, 2).

Not only is the functioning of the plasma membrane altered, but also the vesicular system is profoundly modified. Thus, the Golgi apparatus is not recognized in polio virus-infected cells, and numerous membranous vesicles fill most of the cytoplasm at late times of infection (20, 21). The proliferation of these vesicles is tightly connected to the replication of viral genomes, because inhibitors that interfere with the generation of these membranes block poliovirus RNA synthesis (22–24).

With regard to the picornavirus genes involved in these alterations, it was found recently that 2BC and to a much lower extent 2C induced membrane proliferation when individually expressed in mammalian cells by means of recombinant vaccinia viruses (25, 26). Moreover, our recent results indicate that 2BC induces membrane proliferation and blocks the exocytic pathway in yeast cells (27). Sequences present in both 2B and 2C are required for these alterations to take place. Therefore, 2BC is a protein that interacts with membranes and selectively modifies the vesicular system. Much less is known about the poliovirus proteins responsible for membrane permeabilization. The inducible expression of each poliovirus nonstructural protein in bacteria led to the suggestion that overexpression of 2B or 3A increased permeability of the bacterial membranes (28). Recently, transient expression of 2B or 2BC and to a lower extent 3A enhanced permeability to the hydrophilic antibiotic hygromycin B in COS cells (29). In addition, elegant experiments indicated that both 2B and 3A interfered with glycoprotein trafficking through the vesicular system in mammalian cells (29).

MATERIALS AND METHODS

Cells and Viruses—HeLa, COS, CV2, and 143 TK cells were grown in tissue culture dishes (Nunc) in Dulbecco’s modified Eagle’s medium supplemented with 5% newborn calf serum. The recombinant vaccinia viruses were grown in HeLa cells in Dulbecco’s modified Eagle’s medium supplemented with 2% newborn calf serum. Only the intracellular virus was collected after freezing and thawing the cells three times.

Plasmid Construction—The expression plasmids pTM1–2B, pTM1–2BC, and pTM1–2C were constructed using polymerase chain reaction techniques as described previously (28). For the construction of the plasmids pTM1–3A, pTM1–3AB, and pTM1–3C, the amplified products obtained using the primers 5′ 3A.EY (GGCCGGGATCCATGGGACCTTACTAGTGAAG) and 3′ 3A.E1A (GGCCGCCAGTCATCAGGCTTACTAGTGAAG) for the amplification of 3A, the primers 5′ 3A.EY and 3′ 3B.E1A (GGGCCCAGCTCATCAGGCTTACTAGTGAAG) for the amplification of 3AB, and the primers 5′ 3C.EY (GGCCGGATCCATGGGACCTTACTAGTGAAG) and 3′ 3C.E1A (GGCCGCCAGTCATCAGGCTTACTAGTGAAG) for the amplification of 3C, were digested with NcoI and SacI and cloned in pTM1 digested with the same endonucleases.
The 2Bc variant designated as 2bc(ΔSphI) was generated by digesting the cDNA of pTM1–2BC with SphI, eliminating a 650-bp fragment of 72 nucleotides, and self-ligating the vector. The mutant 2Bc(1–258) and 2b(70–30N) (which encode 7 amino acids not present in the original sequence) were generated from pEMBLyex–2BC (27) by digestion with SpeI and SmaI. The other 2Bc variants were obtained from the corresponding construct in pEMBLyex (27) and cloned into the vector pTM1–2Bc previously digested with NcoI and StuI. The plasmids pTM1–2Bc(I) and pTM1–2BC(D) were constructed after digestion of the corresponding pEMBLyex construct with AfiI and PstI and sub-cloned in pTM1–2Bc digested in the same way. pTM1–2Bc(D) was generated after digestion of pEMBLyex 2BC(D) with BstEII and SphI. The DNA fragment obtained was subcloned in pTM1–2Bc digested with the same enzymes. The construct pTM1–2Bc(Δ30) was obtained by digestion of pEMBLyex 2BC(Δ30N) with NcoI, and the fragment was subcloned in pTM1–2Bc previously digested with NcoI, eliminating 231 nucleotides. The pTM1–2BC(Δ60N) plasmid was generated by digesting with BspHI and SacI pTM1 and the polymerase chain reaction product obtained with primers 5′2B.60 and 3′2C.E1A, after digestion pTM1 and the polymerase chain reaction product were ligated. The mutant 2b(ΔSphI) was obtained digesting the construction pTM1–2Bc with SpeI, blunt-ended with Klenow enzyme, and self-ligated.

Transfection of DNAs with the VT7 Expression System—For transfection experiments, cells were plated in 24-well dishes (Nunc) 24 h before infection with vaccinia virus bearing the T7 RNA polymerase (VT7) (multiplicity of infection, 5) (kindly given by Dr. B. Moss, National Institutes of Health, Bethesda). After 45 min of virus adsorption, a mixture of DNA (0.5 g/well) and Lipofectin (2 g/well) was added to cells in Dulbecco’s modified Eagle’s medium as described by the manufacturer (Life Technologies, Inc.). Cells were harvested at the times indicated in this figure legend.

Protein Analysis by SDS-Polyacrylamide Gel Electrophoresis: Immunoblot Assays—To estimate protein synthesis cells were labeled with 25 μCi/ml [35S]methionine (1.45 Ci/mmol, Amersham) in methionine-free medium. To examine the radiolabeled proteins, cell monolayers were dissolved in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 0.1 M dithiothreitol, 17% glycerol, and 0.024% bromophenol blue as indicator), loaded onto 15% SDS-PAGE gels and electrophoresed at 80 V for 16 h. Immunoblot analysis of the proteins was as described (30).

Immunoprecipitation—Transfected cells were radiolabeled for 1 h at 7 h.p.i. with 50 μCi/ml [35S]Translabel (Amersham Corp.) in cytoine- and methionine-free Dulbecco’s modified Eagle’s medium. After labeling, the medium was removed and the cells were washed three times with phosphate-buffered saline before adding the lysis buffer (20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% Nonidet P-40, 10 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin). After incubating the cells for 30 min at 4°C, the lysates were recovered and cleared by centrifugation (15 min, 14,000 rpm, 4°C). The supernatants were incubated with protein A-Sepharose beads and preimmune serum overnight at 4°C. After 5 h of incubation the immunoprecipitates were washed five times with the lysis buffer and analyzed by 15% SDS-PAGE.

Estimation of Choline and Uridine Release—To determine changes in membrane permeability, the radioactivity released from [3H]choline- or [3H]uridine-preloaded HeLa cells was measured. HeLa cells were loaded with 2 μCi/ml of [3H]choline chloride (80 Ci/mmol, Amersham Corp.) or [5,6-3H]uridine (35 Ci/mmol, ICN) for 14 h. Then the cells were transfected with the different plasmids, 3 h.p.i. of the medium was removed, and fresh medium was added. 1 h later the process was repeated to eliminate the effects of the Lipofectins. At the indicated times the medium was recovered, centrifuged at 15,000 rpm for 5 min, and 3/4 of each supernatant was mixed with L-929 scintillation mixture (DuPont) to quantitate the radioactivity released to the medium.

RESULTS AND DISCUSSION

Previous results from our laboratory indicated that the poliovirus nonstructural proteins 2B and 3A (or 3AB) enhanced membrane permeability to several compounds in Escherichia coli cells (28, 31). Other recent findings also showed that protein 2B and marginally 3A (but not 3AB) permeabilized mammalian cells to hygromycin B (29). Therefore, we expressed other poliovirus nonstructural proteins, 2B, 2Bc, 2C, 3A, 3AB, and 3C, in the vaccinia system to determine which of them modified membrane permeability. To this end, proteins were labeled in the absence or in the presence of hygromycin B, immunoprecipitated with specific polyclonal antibodies, and analyzed by SDS-PAGE. Clearly, the synthesis of proteins 2B and 2BC totally disappears in the presence of the aminoglycoside antibiotic (Fig. 1A). Note that the anti-2B antibodies immunoprecipitate a protein from control vaccinia virus-infected cells that migrates slightly more slowly than poliovirus 2B. Poliovirus proteins 2C, 3A, and 3AB do not enhance the entry of the inhibitor (Fig. 1, A and B), whereas some permeabilizing capacity occurs with protein 3C (Fig. 1C). However, this modification of the membrane by 3C is less than that observed with 2B or 2BC.

To determine which regions of 2B and 2BC are involved in the enhancement of hygromycin B entry into cells, a number of deletion variants of these proteins was constructed (Fig. 2A). Unfortunately, some of the 2B deletion mutants obtained were not efficiently precipitated by the anti-2B antibodies employed, i.e. 2bΔ30N, 2bΔ3SpeI, and 2b(d). But, in the case of 2B (70a) a clear band was obtained that was observed both in the absence or in the presence of hygromycin B, suggesting that deletion of 20 amino acids at the carboxyl terminus of 2B abolishes its capacity to modify the membrane. An alternative possibility is that this protein is made at lower levels than 2B, which may not be sufficient to alter membrane permeability. We do not favor this possibility because mutant 2bc(ΔSphI) is not deleted at lower levels, does enhance the entry of the antibiotic. The other 2B variants, mainly 2bΔ30N, 2bΔ3SpeI, and 2b(d) do not promote hygromycin B entry (Fig. 2B).

The effects of the 2BC mutant 2Bc(1–258) indicate that large portions of 2C can be deleted without diminishing its perme-
The GKS motif present in protein 2C, which encompasses amino acid residues 281–283 of 2BC, is absent in variant 2BC(1–258). A similar conclusion applies to the deletion affecting the junction between 2B and 2C (mutant 2bc(D_SphI)), whereas the presence of 30 amino acids of 2B located at the amino terminus are crucial for the permeabilization of the membrane induced by 2BC to occur (mutant 2b(Δ30N) and 2bC(Δ60N)). This result is of interest because this deletion does not affect the two hydrophobic regions present in 2B that theoretically could be involved in the interaction of 2B with membranes. To assay the importance of the hydrophobic region present in 2B for the modification of membrane permeability, one or two point mutations were generated in this hydrophobic region of 2B. One 2BC variant had a V52D mutation, whereas another had two substitutions, V52D and I54K. Analysis of the Kyte and Doolittle hydrophobic profiles of these variant 2BC proteins indicated that the hydrophobic characteristics of 2B in this region were greatly diminished.

The 2bC(s) mutant was expressed at low levels, yet it clearly permeabilized the membrane to hygromycin B. The second variant 2bC(D) showed partial reduction of its synthesis in the presence of the antibiotic. These results indicate that the integrity of the most hydrophobic region present in 2B is not fully required by 2BC to increase hygromycin B entry. However, protein 2b(D) does not permeabilize cells, suggesting that 2BC is more active than 2B in this respect.

Poliovirus infection enhances membrane permeability in both directions across the membrane; not only impermeant antibiotics readily pass from the medium into the cell, but also other compounds such as choline or uridine are released from the infected cells to the culture medium (2). Therefore, we wished to assay the release of these compounds from cells expressing the different poliovirus nonstructural proteins. These experiments posed a number of experimental problems, such as the permeabilizing capacity of vaccinia virus itself to choline and uridine at late times of infection (from 8–10 h.p.i.), which necessitated testing the release of these compounds at earlier times of the expression of poliovirus proteins. Another problem found in this type of experiment is that Lipofectin itself may affect the assay and must be washed off thoroughly after transfection. Despite these problems, when cells are loaded with choline overnight and poliovirus proteins are expressed, there is a clear enhancement of [3H]choline released from cells that express proteins 2B or 2BC (Fig. 3, A and B). Consistently, in the majority of experiments conducted, 2BC has a greater permeabilizing capacity than 2B. This is clearly observed when [3H]uridine release is tested (Fig. 3C); in this assay, only 2BC has a significant effect, whereas the other poliovirus nonstructural proteins, namely 2C, 3A, 3AB, or 3C, did not significantly alter membrane permeability in either of the assays.

Despite the wealth of information on the alterations of cellular membranes induced by animal viruses, very little is known about the specific virus products involved and their exact mode of action (1, 2). Poliovirus nonstructural proteins...
may induce three types of membrane modifications upon expression in cells: 1) morphological changes in cytoplasmic vesicles characterized by a huge proliferation of membranous vacuoles of different sizes (25–27); 2) functional modifications of the vesicular system that involve the inhibition of glycoprotein trafficking (27, 29); and 3) increased membrane permeability that takes place at the plasma membrane level (1, 2). Although the relationship between the three phenomena is unknown, recent findings from several laboratories have shed some light on their effects.

Initial attempts to identify the poliovirus nonstructural proteins implicated in triggering membrane permeabilization involved the cloning and expression in an inducible manner of these proteins in bacteria (28, 31). Poliovirus proteins 2B or 3A were able to enhance membrane permeability in E. coli, and their expression was highly toxic for the bacterial cells (28, 31). The action of 2BC in this system has not yet been tested, although 2BC is the only protein that permeabilizes yeast cells. There is a correlation between the capacity of 2BC to induce vesicle proliferation (27) and permeabilization to hygromycin B in yeast cells. In the case of mammalian cells, both proteins 2B and 2BC enhanced the entry of hygromycin B as measured by the co-expression of the poliovirus protein and α, proteinase inhibitor labeled with [35S]methionine, but a lower permeabilizing effect was found with 3A (29). Our findings with the VT7 system clearly show that 2B and more markedly 2BC permeabilize the plasma membrane to various compounds. Therefore, there is also a parallelism between the induction of cytoplasmic vesicle and membrane permeability in HeLa cells. Perhaps the formation of these membranous vacuoles affects the integrity of the plasma membrane in an indirect way. Alternatively, 2B or 2BC may themselves act directly at the plasma membrane. The possibility that 2B or 2BC directly affect the plasma membrane is not supported by our immunolocalization studies, indicating that these proteins concentrate in the new vesicles formed and there are little if any of these proteins at the plasma membrane level. This is, however, a negative result and should not be considered as definitive proof that some traction of 2B or 2BC associates with the plasma membrane. Both proteins 2BC and (to a lesser extent) 2C induce membrane proliferation in HeLa cells (25, 26), but the morphology and the kind of the membranes induced by 2C are different from those induced by 2BC. This may explain why 2C is totally devoid of permeabilizing capacity. The possibility that the induction of new vesicles affects the permeability barrier of the plasma membrane is very attractive, but our data do not prove it.

Finally, it could be speculated that the induction of glycoprotein traffic enhances plasma membrane permeability non-specifically. We consider this possibility unlikely because of a number of considerations. First, there is no correlation between the activity of poliovirus proteins to block glycoprotein traffic (3A<2BC<2B) and their permeabilizing capacity (2BC<2B<3A). In addition, a compound that interferes with glycoprotein traffic, brefeldin A, has no effect on membrane permeability to hygromycin B.

The action of 2B or 2BC in the poliovirus replication cycle remains poorly understood (32, 33). The finding that these proteins are responsible for the permeabilization induced by poliovirus infection in the infected cells is clear, but the exact molecular mechanism by which modification of the plasma membrane is achieved by 2B and 2BC remains puzzling. Nevertheless, the present studies point to protein 2B and particularly to 2BC as the major determinant of the enhanced permeabilization observed in poliovirus-infected cells. The capacity of 2BC to induce vesicle proliferation, to interfere with protein trafficking, and to enhance membrane permeability is intriguing. Further studies in this direction will be aimed at elucidating the exact mode of action of 2BC and variant proteins on membranes.

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J. Biol. Chem. 1996, 271:23134-23137.
doi: 10.1074/jbc.271.38.23134

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