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MODIFICATION OF MEMBRANE PERMEABILITY BY ANIMAL VIRUSES

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I. Introduction

Animal viruses permeabilize cells at two well-defined moments during infection: (1) early, when the virus gains access to the cytoplasm, and (2) during the expression of the virus genome. The molecular mechanisms underlying both events are clearly different; early membrane permeability is induced by isolated virus particles, whereas late membrane leakiness is produced by newly synthesized virus protein(s) that possess activities resembling ionophores or membrane-active toxins.

Detailed knowledge of the mechanisms by which animal viruses permeabilize cells adds to our understanding of the steps involved in virus replication. Studies on early membrane permeabilization give clues about processes underlying entry of animal viruses into cells; understanding gained on the modification by viral proteins of membrane permeability during virus replication indicates that membrane leakiness is required for efficient virus release from infected cells (or virus budding, in the case of enveloped viruses). In addition, the activity of these membrane-active virus proteins may be related to virus interference with host cell metabolism and with the cytopathic effect that develops after virus infection.

Apart from the interest in studying membrane permeabilization from a basic viewpoint, these studies can also provide novel, useful techniques. For instance, the finding that animal viruses increased membrane permeability to a number of compounds during the late phase of infection provided a new approach to block translation specifically in the infected cells. This selective inhibition of animal virus-infected cells was achieved by means of hydrophilic inhibitors of protein synthesis that do not permeate the membranes of normal cells, but readily penetrate into virus-infected cells (Carrasco, 1978). The use of nonpermeant translation inhibitors now constitutes a simple assay for changes in the permeability of the plasma membrane (virus induced or not) in both eukaryotic and prokaryotic cells (Carrasco and Vazquez, 1983; Lama and Carrasco, 1992a). Moreover, the initial finding that animal virus particles induce early membrane permeabilization and promote co-entry of macromolecules such as protein toxins into cells (Fernández-Puentes and Carrasco, 1980) has been used to introduce plasmids, without size limit, to virtually transform 100% of cells grown in tissue culture (Cotten *et al.*, 1992; Wagner *et al.*, 1992b).

Several years have elapsed since our last review on the permeabilization of cells by animal viruses (Carrasco *et al.*, 1989). Important insights into two main aspects of this area of research have been achieved since then. First, the mechanisms by which early and late membrane permeabilization occurs are now better understood. Second, we are now witnessing the identification of the viral proteins known as viroporins (Carrasco *et al.*, 1993), which are involved in modifying membrane permeability. Studies on viroporins at the molecular level will soon provide details on their mode of action and the exact function they play in the virus replication cycle.

II. Virus Entry Permeabilizes Cellular Membranes

A. Mechanisms of Virus Entry

Two different modes of virus entry into cells have been described: (1) direct penetration through the plasma membrane and (2) entry through the endocytic pathway (Hoekstra and Kok, 1989; Marsh and Helenius, 1989; Carrasco, 1994). Either of these pathways implies that the virus particle, the nucleocapsid, or at least the viral genome has to cross a membrane. This is particularly true in the case of viral particles devoid of a lipid envelope, such as poliovirus (Fig. 1). Following attachment of poliovirus to its receptor on the cell surface, the virus

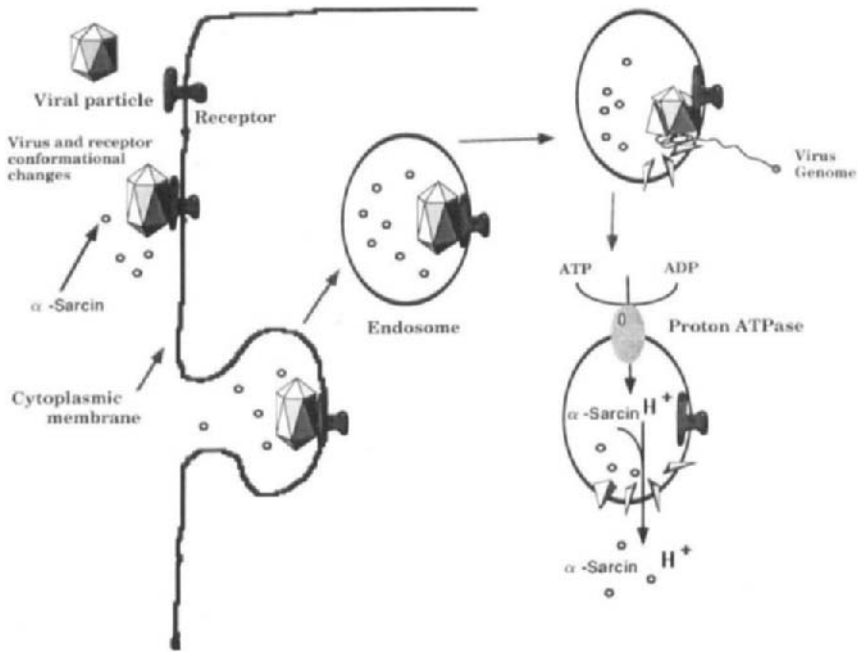

Fig. 1. Route of entry of virus particles into cells. Mechanism of early membrane permeabilization to α-sarcin molecules. The virus particle first attaches to a cell surface receptor, followed by endocytosis of virus and toxin molecules. Binding of the virion to the receptor changes its conformation and promotes interaction of viral proteins with the membrane. This interaction promotes virus uncoating and translocation of the genome to the cytoplasm. In addition, the interaction of virus proteins with the membrane leads to the formation of transient pores that dissipate the proton gradient created by the vacuolar proton ATPase, promoting the passage of toxin molecules through the endosomal membrane.

particle undergoes profound structural rearrangements (Gómez Yafal *et al.*, 1993), with the result that VP4, which is found in the interior of the particle, interacts with the cellular membrane (De Sena and Mandel, 1977), and the amino terminus of VP1, which is also located within the virion, becomes exposed at the surface, ready to interact with the membrane (Fricks and Hogle, 1990). The interaction of these virion proteins with the membrane may open a pore through which the viral genome is extruded into the cell (Fricks and Hogle, 1990). This mechanical model of genome translocation across membranes does not take into account the requirement for an energized membrane during virus infection. Thus, the interaction of poliovirus particles with receptors leads to conformational changes in virions (Mason *et al.*, 1993; Gómez Yafal *et al.*, 1993; Haywood, 1994), but uncoating and RNA release require a metabolically active cell.

Viruses that possess a lipid membrane surrounding the nucleocapsid could in principle avoid passing through cellular membranes by fusion of their envelope with the plasma or endosomal membranes (Hoekstra and Kok, 1989; Marsh and Helenius, 1989). Some viruses, such as Sendai virus, are able to fuse their lipid envelope directly with the plasma membrane, whereas other viruses, such as Semliki Forest virus, fuse their envelope with the endosomal membrane (Lamb, 1993). In all circumstances, fusion is promoted by specialized viral glycoproteins endowed with this activity, and is triggered by conformational changes in the fusion glycoprotein (Stegmann *et al.*, 1989). Two major factors can contribute to these changes in glycoprotein conformation: (1) binding of the virus particle to the receptor and/or (2) low pH (Lamb, 1993; Haywood, 1994). The fact that a low-pH step is required during the entry of some viruses into cells has been taken as evidence that acidic pH is required specifically to induce conformational changes in the viral fusion glycoprotein (Stegmann *et al.*, 1989). This model assumes that fusion necessarily leads to the delivery of the virus genome to the cytoplasma. No energy requirement has been implicated, provided that fusion takes place (Marsh and Helenius, 1989). However, this model does not explain how virus particles are able to cointernalize other macromolecules into cells (Fernández-Puentes and Carrasco, 1980; Carrasco *et al.*, 1989).

B. Co-entry of Macromolecules Promoted by Virus Particles

1. Translocation of Toxins through Membranes

Before analyzing the co-entry of toxin moieties with virus particles, it is useful to summarize the process of toxin entry into cells. Several

excellent review articles on the entry of toxins into cells have appeared (Olsnes *et al.*, 1988; Wilson and Collier, 1992; FitzGerald and Pastan, 1993; Read and Stein, 1993), and the reader is referred to these articles for additional information. Diphtheria toxin is one of the intracellularly acting toxins whose action is best understood in molecular terms (Choe *et al.*, 1992; Read and Stein, 1993). Its mode of entry into cells is briefly summarized here. Trypsinization of diphtheria toxin produces two fragments, A and B; the B polypeptide attaches the toxin to specific receptors present on the cell surface, followed by internalization of the toxin into endosomes (Wilson and Collier, 1992; Naglich *et al.*, 1992). The acidic environment of endosomes triggers interaction of a hydrophobic protein domain with the membrane, leading to the formation of an ion channel (Zalman and Wisnieski, 1984; Beaumelle *et al.*, 1992). This event is followed by the translocation of the accompanying fragment A to the cytoplasm (Olsnes *et al.*, 1988; Ariansen *et al.*, 1993), where it blocks translation (Wilson and Collier, 1992). It has been proposed that the transmembrane channel formed by fragment B aids fragment A in passing through the membrane, gaining access to the cytoplasm (Kagan *et al.*, 1981). However, the pore opened by fragment B is about 25 Å in diameter, which is incompatible with the A subunit traversing the B channel (Zalman and Wisnieski, 1984). The transient pores with cation-selective permeability resemble those formed during the entry of animal viruses (see below). Both fragments A and B interact with the membrane. The insertion of diphtheria toxin in the membrane does not suffice for the translocation of fragment A to the cytoplasm. This passage requires energy, provided by the protonmotive force, to push the protein moiety through the membrane (Beaumelle *et al.*, 1992), as occurs for translocation of other proteins across mitochondrial membranes (Pfanner and Neupert, 1990; Martin *et al.*, 1991). Energy in the form of ATP may be required to translocate other proteins, such as ricin A chain (Beaumelle *et al.*, 1993). Therefore, depending on the protein that is translocated across a particular membrane, energy in the form of a pH gradient (Olsnes *et al.*, 1988; Beaumelle *et al.*, 1992), a membrane electrical potential (Martin *et al.*, 1991), or ATP (Beaumelle *et al.*, 1993; Subramani, 1993) is necessary.

2. *Early Membrane Permeabilization by Virus Particles*

Several years ago, we found that viral particles efficiently permeabilized cells, allowing entry of protein toxins that are otherwise unable to cross the membrane because there are no receptors for them (Fernández-Puentes and Carrasco, 1980). The proteins are efficiently delivered to the cytoplasm shortly after addition of virus to the medium, and almost 100% of the cells become permeabilized within a few

minutes (Fernández-Puentes and Carrasco, 1980; Carrasco, 1981; Otero and Carrasco, 1987). The majority of animal viruses tested were able to induce this phenomenon, including Semliki Forest virus (SFV), vesicular stomatitis virus (VSV), vaccinia virus, adenovirus, and poliovirus (Fernández-Puentes and Carrasco, 1980; Carrasco, 1981; Carrasco and Esteban, 1982; FitzGerald *et al.*, 1983; Otero and Carrasco, 1987; Lee *et al.*, 1990), suggesting that the viral particle contains a component that not only promotes the entry of the viral nucleocapsid into the cell, but also translocates other macromolecules that are not physically bound to the particles across the cellular membrane to the cytoplasm (Fig. 1).

We know that not only protein toxins pass across membranes in the presence of animal virus particles, but that other proteins, such as luciferase or horseradish peroxidase (Otero and Carrasco, 1987), and even polysaccharides (González and Carrasco, 1987), are also delivered into cells by these particles. It has been shown that infectious virions are not needed for early membrane permeabilization to occur, because this phenomenon takes place with UV-inactivated (but not heat-inactivated) virions (Carrasco, 1981; Otero, 1986).

Poliovirus is one of the viruses that most efficiently promotes the entry of protein toxins, for instance α-sarcin, into HeLa cells (Fernández-Puentes and Carrasco, 1980; Carrasco, 1981; Lee *et al.*, 1990; Almela *et al.*, 1991). This permeabilization is specific for the interaction of the viral particles with receptors, because it occurs only in cells that contain the poliovirus receptor, and not in cells that cannot be infected by poliovirus, such as L cells, or BHK cells (Otero, 1987). In addition, a virion component and not a contaminant of the viral preparation promotes permeabilization, because early permeabilization is blocked by antibodies directed against poliovirus structural proteins (Otero, 1987).

A number of compounds interact with poliovirus particles and inhibit the uncoating process without interfering with attachment or the internalization process (Rossmann, 1989; Almela *et al.*, 1991). These compounds, in addition to blocking poliovirus uncoating, are also effective inhibitors of the early membrane permeabilization to α-sarcin (Almela *et al.*, 1991). Hence, uncoating of the poliovirus particle to deliver the viral genome to the cytoplasm is required to permeabilize the endosomal membrane to the accompanying proteins (Almela *et al.*, 1991).

More recently, adenovirus particle-induced cell permeabilization has been used to introduce DNA into cells by means of a receptor-mediated gene delivery system. Plasmids can bind to cells when they are complexed with transferrin–polylysine molecules (Wagner *et al.*, 1990;

Zenke *et al.*, 1990). Entry of these complexes is enhanced when adenovirus particles are present in the culture medium. Under these circumstances more than 90% of the cells express the transfected gene (Cotten *et al.*, 1992, 1993; Curiel, 1993; Wagner *et al.*, 1992a,b). Chicken adenovirus particles, which are replication defective in mammalian cells, have been successfully used to enhance receptor-mediated gene delivery into cells (Cotten *et al.*, 1993).

The system developed tends to emulate virus particles in such a way that nucleic acids (plasmid DNA) are coated with proteins (transferrin) that interact with them; these proteins contain a moiety that binds the nucleoprotein complex to receptors. Although this complex is able to bind to the cell surface, it still lacks the permeabilizing capacity of virus particles that is provided by addition of inactivated virion particles. Even the conjugation of influenza fusogenic peptides, derived from the hemagglutinin molecule, with the DNA complexes enhances gene delivery (Wagner *et al.*, 1992a), suggesting that a synthetic virus like system for efficiently transforming cells is feasible.

3. Inhibitors of Vacuolar Proton-ATPase

The use of several macrolide antibiotics that powerfully block proton-ATPase pumps in mammalian cells promises to be crucial in elucidating the mechanisms by which animal virus particles or genomes pass into the cell interior (Bowman *et al.*, 1988). In addition, the forces that govern early membrane permeabilization by viruses is now better understood thanks to these antibiotics. Bafilomycin A (BFLA) and concanamycins are selective inhibitors of endosomal proton-ATPase (Dröse *et al.*, 1993). BFLA and concanamycin A strongly inhibited the entry of several animal viruses that possess a lipid envelope, such as SFV, VSV, and influenza virus (Perez and Carrasco, 1993, 1994; Guinea and Carrasco, 1994b,c). On the other hand, the infectivity of poliovirus was not blocked by BFLA, indicating that poliovirus uncoats and releases its genome by a mechanism that is independent of an acidic environment (Perez and Carrasco, 1993). We are currently investigating the requirement of membrane potential for poliovirus entry into cells. Because entry of SFV and poliovirus was affected differentially by BFLA, they constituted good models to test the involvement of the proton-ATPase activity in the cointernalization process. Curiously enough, BFLA inhibited the co-entry of α-sarcin promoted by both SFV and poliovirus particles (Perez and Carrasco, 1993). This result clearly indicates that the exit of α-sarcin from endosomes requires a proton gradient and is coupled to the action of the proton-ATPase. These findings prompted us to suggest that the low-pH requirement previously reported for the entry of some animal viruses

could be due to a requirement for a pH gradient, rather than to low pH per se (Carrasco *et al.*, 1993; Carrasco, 1994). The pH gradient would provide the energy required in this process, as occurs during the entry of toxins into cells (see Section II,B,1). If so, a virion component would be able to couple the proton gradient to the release of macromolecules from the endosome. Therefore, the mechanism of the co-entry of protein toxins is more specific than previously thought and does not involve the physical rupture of endosomes by animal virus particles; rather the toxin would be translocated across the intact endosomal membrane by an activity present in virion particles (Carrasco *et al.*, 1989, 1993; Perez and Carrasco, 1993).

C. A Proton Motive Model to Account for Virus Entry and Early Membrane Permeabilization

Current models that explain the entry of viruses into cells do not account for all the existing experimental evidence (see Section II,A). The requirement of a metabolically active cell for delivery of virus genome into the cytoplasm is not rationalized. In addition, these models do not explain the mechanism by which early permeabilization, observed with virus particles, to other macromolecules takes place. We have proposed that the proton-motive force generated in endosomes by the activity of the vacuolar proton-ATPase pump can be used to promote uncoating and to drive the viral genome through the lipid barrier of the membrane (Carrasco *et al.*, 1993; Perez and Carrasco, 1993; Carrasco, 1994). Therefore, the energy accumulated in endosomes is required for the viral nucleocapsid to enter cells. In this model the same route and energy can be used by macromolecules to pass across the membrane and would thus account for the co-entry process. A brief account of the different steps of virus entry according to the proton-motive model, would be as follows:

1. Viral proteins can alter their conformation on receptor binding and insert into the cellular membrane (Flynn *et al.*, 1990; Meyer *et al.*, 1992). Acidic pH could also influence these conformational changes (Marsh and Helenius, 1989), but this may not be strictly required for fusion (Haywood and Boyer, 1985; Edwards and Brown, 1991; Haywood, 1994). In fact, binding of virus to its receptor is not strictly required for foot-and-mouth virus infection (Mason *et al.*, 1993).
2. Once viral proteins are inserted in the membrane they would open a pore through which protons and other ions pass down their concentration gradient. Increasing evidence indicates that virus

particles open pores on interaction with membranes (Spruce *et al.*, 1991; Schlegel *et al.*, 1991; Tosteson *et al.*, 1993). In fact, some virus particles contain "textbook examples" of ion channels (Kalko *et al.*, 1992).

3. The viral proteins inserted in the membrane may couple the energy liberated by the movement of protons (or ions) to the cytoplasm down a concentration gradient, to the translocation of the viral genome (or protein toxins) in the same direction (see Fig. 1).

This model predicts that there are viral proteins that open pores in membranes and, together with receptors, are able to use energy to translocate substrates in a nonfavorable thermodynamic direction. In contrast to an uncoupler, which simply dissipates the energy stored in ionic gradients, this "virus-transducing complex" is able to couple the energy to genome translocation. The pore size would permit the passage of protons and other ions, but prevent diffusion of macromolecules.

Additional support for this concept is provided by the fact that virions modify membrane potential during entry (Fuchs and Kohn, 1983; Rosenthal and Shapiro, 1983; Seth *et al.*, 1985; Bashford *et al.*, 1985), probably as a consequence of the capacity of virion proteins to form ion channels (Schlegel *et al.*, 1991; Spruce *et al.*, 1991; Tosteson *et al.*, 1993). In addition, SFV does not enter cells when the membrane potential is abolished by modifying the concentration of monovalent cations, even under acidic conditions (Helenius *et al.*, 1985). As indicated above, viruses are able to monitor and infect only cells that are metabolically active (Fuchs and Kohn, 1983). Even though viruses attach to dead cells (and even to isolated membranes, or truncated receptors) (Lentz, 1990; Haywood, 1994), they effectively fuse and enter only cells that possess an energized membrane (Fuchs and Kohn, 1983).

In addition to virus entry, the early permeabilization phenomenon observed with virus particles can also be easily rationalized by this proton-motive model. Thus, viral proteins involved in the translocation of the viral particle across the membrane would also translocate other macromolecules, depending on an existing pH gradient or membrane potential. Therefore, this model accounts for the observation that the toxin moieties involved in translocation through membranes are exchangable with virus particles (Fernández-Puentes and Carrasco, 1980).

The mechanical and the proton-motive models can be easily differentiated by experimental tests. The classical low-pH model predicts that a low pH suffices for virus fusion and entry in a mechanistic way, i.e., low pH changes the conformation of a protein that is inserted into

the cellular membrane and fusion ensues. The proton-motive model predicts that a pH gradient is required, thus low pH is not sufficient (Guinea and Carrasco, 1994b,c; Perez and Carrasco, 1994). Moreover, in principle, the pH gradient may not be necessary for entry of those virus species that could use either the membrane potential or the pH gradient. Future studies in this exciting field of virus entry directed to elucidating the exact molecular basis of early membrane permeabilization should decide which of these models is closer to reality.

III. Modification of Membrane Permeability as a Result of Virus Gene Expression

A. Mechanism of Late Membrane Permeabilization

The molecular mechanisms by which animal viruses modify membrane permeability late during infection will be considered in this section. Because the work done in this field has been previously reviewed in depth (Carrasco *et al.*, 1989), the late modifications of the membrane induced by animal virus infection will be briefly summarized and work done in the last few years will be highlighted. One of the most salient characteristics of the late membrane modifications is that they require viral gene expression (Carrasco *et al.*, 1989), suggesting that one or several virus gene products are responsible for these changes. Figure 2 shows the pathway for synthesis and membrane insertion of virus proteins with membrane-active capacity and the postinfection alterations that influence membrane function.

Two fundamental aspects are of interest in considering the late membrane leakiness induced by animal virus infection: (1) the nature of the cell membrane modifications at the molecular level and (2) the identity of the viral products responsible for these modifications. Considering the large number of cytolytic animal viruses identified, we have only a limited understanding of these two aspects in a few viral systems.

Each cytolytic animal virus modifies the permeability of the plasma membrane with different kinetics, most probably reflecting not only the different timing of virus gene expression but also the intrinsic activity of each particular viral protein involved in cell lysis. Moreover, the particular cell line considered and the culture conditions also influence these alterations in membrane permeability. The specific phenomenology of plasma membrane permeabilization is thus a characteristic not only of the animal virus considered but also of the cell it

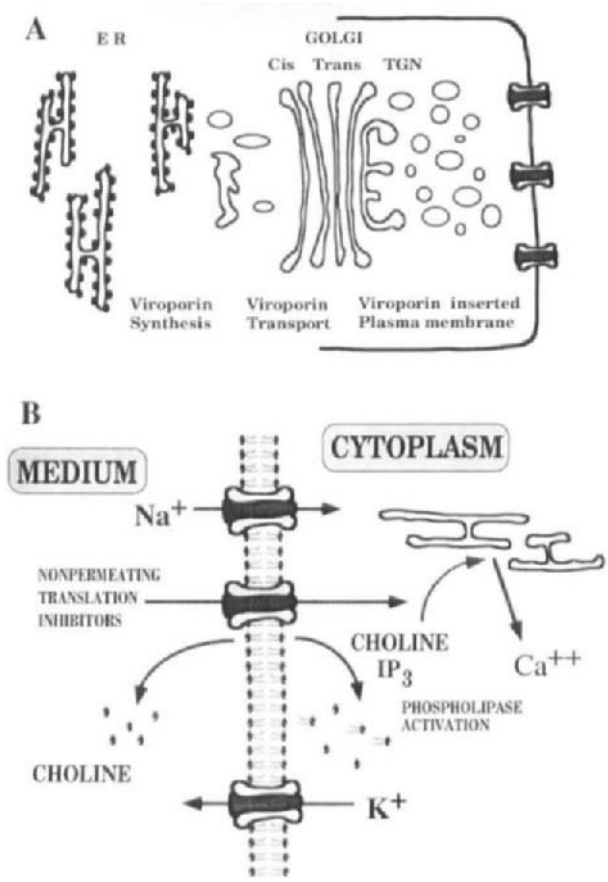

FIG. 2. (A) Synthesis and transport of viroporin molecules. After synthesis in the endoplasmic reticulum (ER), the viroporin is transported through the Golgi apparatus and sorted to the plasma membrane, where it forms hydrophilic channels; TGN, trans-Golgi network. (B) Late membrane permeabilization by viroporin molecules. Alteration of monovalent and divalent ion content is depicted. Low-molecular-weight hydrophilic molecules diffuse through the pore. Phospholipase activation modifies the integrity of membrane phospholipids and releases a number of phospholipid moieties.

infects. Nevertheless, some general features describe the changes that accompany the disruption of the cell membrane.

1. Monovalent Cations and Membrane Potential

The most common change that occurs in the plasma membrane during infection of a susceptible cell by a cytolytic virus is enhanced permeability to monovalent cations. This effect is obviously accom-

panied by a drastic drop in membrane potential (see Fuchs and Kohn, 1983; Carrasco *et al.*, 1989). Sodium ions that are pumped outside the cell by Na^+,K^+-ATPase readily enter into infected cells, whereas potassium ions that accumulate inside the cell leak out. In the case of picornaviruses or togaviruses these modifications in ion distribution are initially observed 2 to 3 hr after infection (Carrasco and Smith, 1976; Nair *et al.*, 1979; Nair, 1981, 1984; Garry *et al.*, 1979; Egberts *et al.*, 1977; Muñoz *et al.*, 1985a; Lacal and Carrasco, 1982; Ulug *et al.*, 1984; Lopez-Rivas *et al.*, 1987), so that the majority of viral macromolecular synthesis takes place in a cytoplasm in which the concentrations of monovalent ions are continuously changing (Muñoz *et al.*, 1985b; Carrasco and Castrillo, 1987; Ulug *et al.*, 1987). Hence, the synthesis of poliovirus or Semliki Forest virus proteins is carried out in a cytoplasm that contains a higher concentration of sodium and a lower concentration of potassium ions compared to uninfected cells. Consequently, viral mRNAs that are translated late during virus infection have adopted special structures that cause them to be optimally translated under these altered conditions (Saborio *et al.*, 1974; Nuss *et al.*, 1975; Carrasco and Smith, 1976; Carrasco *et al.*, 1979). Hence, the resistance of some viral mRNAs to translational inhibition by high concentrations of sodium ions in the external medium is taken as a reflection of the alterations that take place in ion concentration in the cytoplasm of virus-infected cells. This resistance is a widespread phenomenon observed for the translation of mRNAs from cytolytic animal viruses (Nuss *et al.*, 1975; Saborio *et al.*, 1974; Cherney and Wilhelm, 1979; Garry *et al.*, 1979; Alonso and Carrasco, 1982a; Carrasco *et al.*, 1979; Nair, 1981, 1984; Mizzen *et al.*, 1987; Castrillo *et al.*, 1987). On the other hand, these ionic concentrations are inhibitory for the translation of most cellular mRNAs, although the synthesis of a few cellular proteins, such as the heat-shock proteins, is resistant to such modifications (Muñoz *et al.*, 1984; Macejak and Sarnow, 1991).

These ionic alterations have been described not only for picornavirus- or togavirus-infected cells, but also for other systems, including cells infected by vesicular stomatitis virus (Garry and Waite, 1979), reovirus (Muñoz *et al.*, 1985a), coronavirus (mouse hepatitis virus) (Mizzen *et al.*, 1987), rotavirus (del Castillo *et al.*, 1991), Tacaribe virus (Rey *et al.*, 1988), vaccinia virus (Norrie *et al.*, 1982), influenza virus (Lopez Vancell *et al.*, 1984; Carrasco and Lacal, 1983), herpesviruses (Hackstadt and Mallavia, 1982; Nokta *et al.*, 1988), and SV40 (Eggleton and Norkin, 1981).

In summary, progressive membrane damage induces a collapse of ionic gradients and disrupts membrane potential in virus-infected cells (Fig. 2). Cell morphology at this time of infection usually appears

normal under the phase-contrast microscope; cell rounding and shrinkage take place later, at a time when the membrane is highly damaged (Lopez-Rivas *et al.*, 1987). The phenomenology of these changes is similar to that observed with membrane-active toxins (Bernheimer and Rudy, 1986; Dempsey, 1990; Bevins and Zasloff, 1990; Guihard *et al.*, 1993), or ionophores (Alonso and Carrasco, 1982b; Heitz *et al.*, 1989; Katsu *et al.*, 1989).

2. Divalent Cations and pH

The concentrations of protons and calcium ions also change at about the same time that monovalent ion concentrations are affected. Despite the importance of protons and divalent cations as regulators of a plethora of cellular functions (Carafoli, 1987; Whitaker, 1990; Madshus, 1988), few studies have been performed on this subject in animal virus-infected cells.

Intracellular alkalinization of about 0.3 pH units has been reported in poliovirus-infected cells (Holsey *et al.*, 1990; Holsey and Nair, 1993). Virus gene expression is necessary for this modification to occur, but only the translation of the input viral RNA is required. These results contrast with the finding that Sindbis virus infection causes an acidification of about 0.5 pH units in BHK cells (Moore *et al.*, 1988). A similar decrease in the cytoplasmic pH is observed in MDCK cells infected by influenza virus (Ciampor *et al.*, 1992b). In addition, variations in the pH of cellular compartments in influenza virus-infected cells are thought to be mediated by the M2 protein (Ciampor *et al.*, 1992a). Differences in the methodology used to estimate the cytoplasmic pH may account for the contradictory results obtained in poliovirus- and togavirus-infected cells. It is also possible, although unlikely, that different viruses have opposite effects on cytoplasmic pH.

Initial attempts to measure variations in the concentration of calcium ions in virus-infected cells showed that calcium uptake by mitochondria increased during the initial hours of Semliki Forest virus infection, perhaps reflecting a rise in cytosolic calcium (Peterhans *et al.*, 1979). The increased uptake declined at late stages of infection, suggesting mitochondrial injury by virus infection (Peterhans *et al.*, 1979). As with poliovirus-infected cells (Lopez-Rivas *et al.*, 1987), no significant differences were observed in total cellular calcium as determined by radioactive calcium (Peterhans *et al.*, 1979). Using this technique, Nokata *et al.* (1987) noted a greater influx of calcium into cytomegalovirus-infected human cells during the initial hours of infection, leading to the suggestion that a viral gene product altered membrane permeability to this cation (Nokta *et al.*, 1987). The use of fluorescent probes to estimate free cytosolic calcium provides a more

accurate method than measurements based on radioactive calcium (Cobbold and Rink, 1987). The use of both radioactive calcium and fluorescent probes in rotavirus-infected cells clearly indicates that permeability to calcium is drastically altered and the concentration of this cation increases by severalfold in the infected cells (Michaelangeli *et al.*, 1991). These modifications are clearly apparent from about the fourth hour postinfection and are dependent on virus gene expression (Michelangeli *et al.*, 1991). The expression of individual rotavirus proteins in insect (*Spodoptera frugiperda*) cells points to NSP4 expression as the nonstructural glycoprotein responsible for elevating calcium levels during rotavirus infection (Tian *et al.*, 1994). Influenza virus infection of neutrophils causes a rise in intracellular calcium very early during infection (Hartshorn *et al.*, 1988). This effect has been related to a functional deactivation of neutrophils by influenza virus infection.

Curiously, to my knowledge, no studies measuring free cytoplasmic calcium in picornavirus-infected cells have been published. Attempts to estimate the concentrations of calcium in poliovirus-infected HeLa cells by means of radioactive calcium revealed almost no difference from concentrations in uninfected cells (Lopez-Rivas *et al.*, 1987). However, these studies did not discriminate between free or bound calcium (Lopez-Rivas *et al.*, 1987). More recent results using fluorescent probes indicate that calcium levels in the cytoplasm of poliovirus-infected cells rise by 5- to 10-fold from the third hour postinfection (A. Irurzun, J. Arroyo, and L. Carrasco, unpublished observations).

3. Enhanced Permeability to Other Compounds

In addition to ions, other low-molecular-weight compounds readily diffuse through the membranes of virus-infected cells (Carrasco *et al.*, 1989). Nucleotides and sugars leak from cells and a number of hydrophilic antibiotics that do not permeate the plasma membrane of normal cells selectively enter virus-infected cells (Carrasco, 1978; Contreras and Carrasco, 1979; Carrasco and Vazquez, 1983). The use of these nonpermeating antibiotics provides a sensitive assay for changes in membrane permeability induced by viruses or other compounds (Alonso and Carrasco, 1981, 1982b; Lama and Carrasco, 1992a), and also gives an accurate indication as to the timing of changes in permeability.

It could be speculated that changes in membrane permeability occur only in a fraction of "dead" cells, whereas metabolically active cells maintain intact membranes while synthesizing viral macromolecules. This is not so, because the normally nonpermeating translation inhibitors totally block viral protein synthesis, indicating that cells actively

synthesizing viral proteins lose the permeability barrier to these compounds (Carrasco, 1978; Contreras and Carrasco, 1979). Still, it is possible that the viral proteins made in leaky cells are useless for the virus, i.e., these proteins will not participate in the formation of virions and are made after the virus progenies have escaped from the cells. This is unlikely, because the inhibition of protein synthesis by hygromycin B, a nonpermeating inhibitor, reduces progeny virus formation by several orders of magnitude (Benedetto *et al.*, 1980; Lacal and Carrasco, 1983).

The conclusion reached from these studies is that membrane permeability nonspecifically increases at the beginning of the late phase of virus infection. Ions and other low-molecular-weight hydrophilic molecules readily diffuse through the plasma membrane in both directions. Phenomenologically, it seems that pores appear in a gradual fashion after virus infection, as previously predicted (Carrasco, 1977, 1987).

B. Modification of Lipase Activity

Apart from the characteristic permeability properties of the membrane with respect to different solutes, an important question relates to the physical integrity of the membrane phospholipids. The most surprising aspect of this area of research is the paucity of data regarding the molecular basis of membrane lysis by cytolytic viruses. The identification of virus gene products that enhance membrane permeability will promote more interest in analyses of phospholipid integrity and lipase activation in animal virus-infected cells.

HeLa cells infected with poliovirus undergo a marked stimulation of phospholipase C, whereas the activity of other phospholipases does not increase (Guinea *et al.*, 1989; Irurzun *et al.*, 1993b). As a result of this stimulation, choline and phosphorylcholine appear in the culture medium from the third hour postinfection, and the levels of inositol triphosphate (IP_3) in the cytoplasm of poliovirus-infected cells is several times higher than in uninfected cells (Guinea *et al.*, 1989). Thus, it is not surprising to find elevated concentrations of divalent cations in the cytoplasm of these cells (see Section III,A,2). In conclusion, phospholipase C is activated in poliovirus-infected cells, leading to the release of choline to the medium and the formation of high amounts of IP_3 (Fig. 2). Perhaps this inositol metabolite, in turn, triggers the release of membrane-bound calcium into cytosolic free calcium (Berridge and Irvine, 1989; Whitaker, 1990). The consequences that these changes may have for cellular metabolism and for viral functions, such as assembly, have not been determined.

Not all viruses induce the activation of phospholipase C alone. In cells infected with other animal viruses there is an increase in choline and arachidonic acid release to the medium, suggesting that in addition to phospholipase C, phospholipase A_2 is also activated in these systems (Perez *et al.*, 1993). Arachidonate metabolism is modified by a number of animal viruses, including orthopoxviruses (Palumbo *et al.*, 1993), herpesviruses (AbuBakar *et al.*, 1990), Semliki Forest virus and vesicular stomatitis virus (Perez *et al.*, 1993). During the infection of BSC-1 cells by cowpox virus or vaccinia virus there is an enhanced arachidonate product formation from the cyclooxygenase and lipoxygenase pathways. Possibly a virus gene product is involved in mediating the alterations of arachidonate metabolism (Palumbo *et al.*, 1993). These alterations seem to be necessary for orthopoxvirus replication, because inhibitors of lipoxygenase pathway block virus replication (Palumbo and Buller, 1991). During the infection of HeLa cells with SFV or BHK cells with vesicular stomatitis virus (VSV), there is a significant increase in phospholipase C and A_2 activity. Entry of hygromycin B was prevented by zinc ions or chloroquine, indicating that the increase in membrane permeability in SFV-infected cells may be mediated in part by lipase activation (Perez *et al.*, 1993). Respiratory syncytial virus infection of differentiated U937 cells induces a sustained stimulation of 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, a potent mediator of inflammation, suggesting that this compound plays an important part in RSV-induced inflammatory response in lungs (Villani *et al.*, 1991). Even the addition of purified gp120 from HIV-1 to human monocytes results in the activation of the cyclooxygenase and lipoxygenase pathways (Wahl *et al.*, 1989). These results lead to the suggestion that the induction of arachidonic acid metabolites is required for the replication of some animal viruses (Palumbo and Buller, 1991) and may also be important for virus pathogenesis.

Apart from the stimulation of lipase activity as a result of virus gene expression, animal viruses can also modify lipase activity during entry into cells, as observed with influenza virus (Harshorn *et al.*, 1988) and cytomegalovirus (AbuBakar *et al.*, 1990).

The actual picture that emerges from these results is that the activation of these phospholipases depends very much on the virus and the cell type used in these studies. Two consequences may be predicted if these two phospholipases are activated during the course of virus infection: (1) the formation of prostaglandins and (2) the formation of diacylglycerol and lysophosphatidylcholine. These molecules are effectors of many cellular and viral functions (Exton, 1990; Billah, 1993). In addition, the presence of these phospholipid moieties in the plasma membrane destabilizes the lipid bilayer and enhances membrane permeability (Felix, 1982).

It is not surprising to find that cellular membranes containing embedded pore-forming proteins display increased phospholipase activities. Many membrane-active compounds have a similar effect (Katsu *et al.*, 1989; Young, 1992). It seems plausible to suggest that pore-forming proteins disturb membrane integrity, leading to membrane permeabilization that in turn increases phospholipase activity, which further destabilizes the membrane. The membrane might be permeabilized even in the absence of phospholipase activation, as occurs with phage systems (Hardaway *et al.*, 1975; Young, 1992). Future studies will define more precisely the role that each of these changes plays in the increased membrane permeabilization induced by animal virus infection.

Finally, infection of chicken embryo fibroblasts by Rous sarcoma virus, a nonlytic transforming virus, stimulates the release of arachidonic metabolites (Barker *et al.*, 1989), an effect involved in cell transformation by tyrosine kinase-containing retroviruses (Barker *et al.*, 1989).

C. Vesicular Traffic in Cells Infected with Animal Viruses

In addition to modifications in the structural and functional properties of the plasma membrane, animal viruses can also modify the vesicular system. However, these modifications may not be so relevant to the enhanced permeabilization effect but may instead be connected with other viral functions such as genome replication (Guinea and Carrasco, 1990, 1991; Perez *et al.*, 1991; Irurzun *et al.*, 1992, 1993a) or virus assembly (Sodeik *et al.*, 1993; Schmelz *et al.*, 1994). Therefore, modification of the vesicular system by animal viruses will not be reviewed here. I will only point out that some animal viruses, such as poliovirus, not only use the vesicular system to transport and sort their proteins, but also to replicate their genomes. The replication of poliovirus genomes takes place in close association with newly made membrane vesicles (Caliguiri and Tamm, 1969, 1970; Semler *et al.*, 1988). During infection with a number of animal viruses, membrane traffic is profoundly modified in such a way that vesicles of various sizes appear in the cytoplasm. These distinctive structures were termed "viroplasm" in the case of poliovirus (Dales *et al.*, 1965), "cytopathic vacuoles" in togaviruses (Acheson and Tamm, 1967; Grimley *et al.*, 1968; Froshauer and Kartenbeck, 1988), or "factories" in vaccinia virus-infected cells (Cairns, 1960; Dales and Siminovitch, 1961; Moss, 1990). The macrolide antibiotic brefeldin A is active against animal viruses that contain glycoproteins and also shows antiviral activity against poliovirus (Maynell *et al.*, 1992; Irurzun *et al.*, 1992; Tucker *et al.*, 1993; Molla *et al.*, 1993), a virus lacking a lipid envelope and that

does not encode glycoproteins (Urzainqui and Carrasco, 1988). The results obtained with brefeldin A indicate that the poliovirus replication complexes must pass through the Golgi apparatus in order to be functional (Irurzun *et al.*, 1992), a process that may be mediated by protein 2C (Rodríguez and Carrasco, 1993). Apart from poliovirus, the synthesis of viral RNA by other animal viruses is also depressed by inhibition of lipid synthesis or membrane traffic (Perez *et al.*, 1991; Perez and Carrasco, 1991; Irurzun *et al.*, 1993a).

IV. Viroporins: Viral Proteins That Modify Membrane Permeability

The great majority of animal viruses are cytolytic. During their growth cycle they lyse cells by mechanisms that are as yet poorly understood. Animal virus replication is not necessarily bound to cytopathogenicity. Thus, mutant animal viruses with reduced cytopathic properties are able, in many instances, to replicate and express their genomes to normal levels (Kowalski *et al.*, 1991; Schlesinger *et al.*, 1993), suggesting that at least in some viruses a single gene product is the major cause of the cytopathic effect. The concept advanced that the cytopathic effect is produced by the expression of one or a (limited) number of virus genes (Carrasco, 1987) is gaining support, as opposed to the idea that the bulk of virus gene expression, or the formation and accumulation of viral particles, is cytopathic. The virus products involved in cytopathicity most probably have the plasma membrane as the primary target of their toxic effects. The suggestion that animal viruses encode proteins "that form small pores in the lipid bilayer," acting as ionophores (Carrasco, 1977), is now leading to the recognition of a new family of virus proteins that enhance membrane permeability: the viroporins. With increasing knowledge of viral genomes and the application of cloning techniques, a number of proteins involved in modifying membrane permeability have been identified in recent years, and we are beginning to understand the function they play in cytopathogenicity and virus growth.

In many respects cell lysis induced by animal viruses is similar to the lysis induced by membrane-active compounds or phages (see Section VI). The knowledge accumulated from these systems may benefit our understanding of the molecular-level processes involved in cell lysis during animal virus replication. Characterization of animal virus products involved in cell lysis is a major goal toward understanding their mode of action. Progress in elucidating the putative viral proteins involved in modification of membrane permeability has been

achieved through their cloning and individual expression in bacteria and eukaryotic cells.

A. Picornavirus 3A Protein

Members of the Picornaviridae family have a positive, single-stranded RNA molecule as genome (Semler *et al.*, 1988; Harber and Wimmer, 1993). Translation of this RNA yields a polyprotein that is proteolytically cleaved to produce the mature picornavirus proteins (Fig. 3). Growth of poliovirus in susceptible cells leads to drastic changes in membrane permeability, as described in Section III,A. Picornavirus-infected cells perhaps represent the animal virus system in which modifications of membrane permeability have been most thoroughly analyzed. Despite a wealth of information, however, little is known about the picornavirus genes involved in these changes.

As a first step toward the identification of poliovirus proteins involved in increased cellular permeability, most of the poliovirus nonstructural proteins were individually cloned in *E. coli* (Lama *et al.*, 1992), and their expression was regulated by the use of a powerful inducible system (Studier *et al.*, 1990; Dubendorff and Studier, 1991). These studies led to the identification of two toxic proteins with lytic

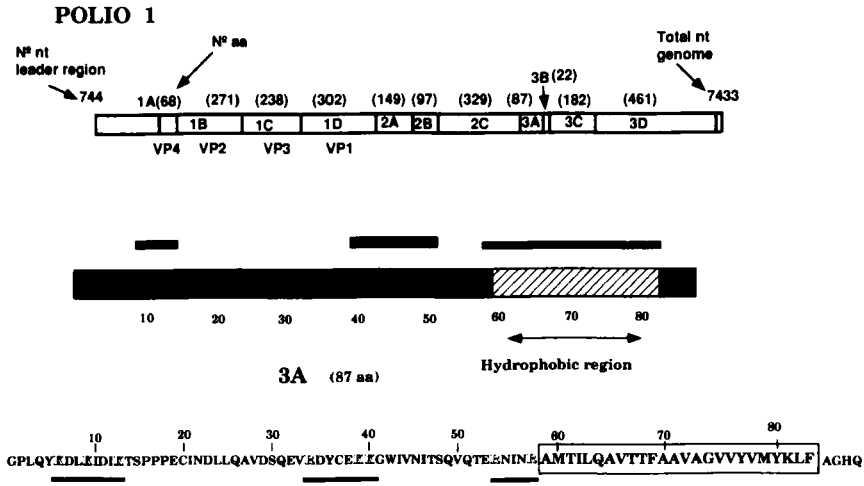

FIG. 3. Schematic representation of the poliovirus genome (top) and poliovirus protein 3A (bottom). The hydrophobic region of protein 3A is depicted. The shaded rectangles above the protein indicate the regions of the molecule that participate in membrane permeabilization (for details see text). Underlined are sequences containing basic amino acids.

capacity for bacteria: poliovirus proteins 2B and 3A (Lama and Carrasco, 1992a,b). Induction of elevated synthesis of either of these polypeptides permeabilized prokaryotic cells, whereas none of the other poliovirus nonstructural products produced this effect (Lama and Carrasco, 1992a). Polypeptide 3A, and its precursor 3AB, are known to interact with cellular membranes in infected human cells (Takegami *et al.*, 1983a,b). Computer analyses of these proteins indicated that the hydrophobicity of 3A extended over a region of 20–22 amino acids that formed an amphipathic helix. Moreover, 3A is a basic protein that shows some homology with other lytic proteins (Carrasco *et al.*, 1993). In contrast, protein 2B lacks an amphipathic helical domain.

Because membrane-active proteins act on all types of cells, irrespective of whether they are eukaryotic or prokaryotic (Andreu *et al.*, 1985; Bernheimer and Rudy, 1986; Dempsey, 1990; Kokryakov *et al.*, 1993), their expression and analysis of their mode of action in *E. coli* cells provide a powerful model system. The inducible system developed by Studier *et al.* (1990; Dudendorff and Studier, 1991) has already proved to be suitable for the characterization of these toxic and highly lytic virus proteins, because their expression is tightly regulated (Lama and Carrasco, 1992b). In addition, these viral proteins modified membrane permeability in a manner similar to that observed using poliovirus in mammalian cells (Lama and Carrasco, 1992a). In this respect, changes in permeability to different small molecules (lactose analogs, lysozyme, nucleosides, nonpermeating inhibitors) induced by poliovirus 3A occurred in both directions (influx and efflux), at a time when other larger molecules were effectively excluded by the plasma membrane. This suggests the formation of hydrophilic pores of a defined size (Lama and Carrasco, 1992a; Lama, 1994).

The prokaryotic system used in these studies was also useful for characterizing 3A proteins with mutations affecting their permeabilizing properties. Because synthesis of poliovirus polypeptide 3A in bacterial cells elicits an increase in membrane permeability, the host cells can be morphologically differentiated from *E. coli* clones expressing wild-type 3A in the presence of a chromogenic substrate (Lama, 1994). Characterization of the mutants indicated that lysis was a highly specific property of the protein, because changes in a single amino acid abolished the permeabilizing activity and hence the lytic properties of 3A. Even though some 3A mutants still encode a 3A basic protein that is synthesized in cells, usually to higher levels than wild-type 3A, they do not increase membrane permeability (Lama, 1994). Some of the mutations were located in the hydrophobic domain of protein 3A, giving rise to partially soluble viral polypeptides, whereas other mutations were located in two different domains of 3A, outside of the hy-

drophobic region (see Fig. 3). An important concept that arises when considering the action of the different 3A mutants is that the ability of a protein to modify membrane permeability should be envisaged in a very specific way. As with enzymes, wherein a single amino acid change may lead to the loss of enzymatic activity, protein 3A loses its pore-forming potential as a result of a small change, for example, the modification of a single amino acid.

Although poliovirus protein 3A is lytic for *E. coli* cells in the presence of phage T7 lysozyme, and this lytic capacity is specific and does not simply arise from accumulation of 3A in cells, it has not been proved that this protein is responsible for the cytolytic effects of poliovirus. The findings described thus far favor the view that 3A is the poliovirus protein involved in cell lysis, but to reach this conclusion two fundamental predictions still need to be proved. One is that poliovirus protein 3A increases membrane permeability when it is individually expressed in uninfected mammalian cells. Second, poliovirus strains containing mutations in the 3A gene should be unable to induce membrane alterations. A definitive answer to the identity of the poliovirus protein involved in permeability changes must await these results.

B. Several HIV Proteins Modify Membrane Permeability

Human immunodeficiency virus (HIV) is a retrovirus that causes AIDS (Gougeon *et al.*, 1993; Levy, 1993b). Great efforts have been devoted to understanding AIDS and HIV biology, giving rise in a rather short period of time to a detailed picture of the mechanism by which HIV infection modifies membrane permeability. As with other lentiviruses, HIV infection of susceptible cells leads to a number of cytopathic effects, including cell death (Levy, 1993b). Progression to disease in HIV-infected patients is accompanied by loss of $CD4^+$ T lymphocytes and has been correlated with the emergence of more cytopathic virus variants (Levy, 1993a,b). These HIV variants replicate more rapidly, are more cytopathic for infected cells, and may contribute to lymphocyte depletion and the severity of HIV-induced pathogenesis (Gougeon *et al.*, 1993).

The cytopathic effects of HIV infection are rather complex, involving syncytia formation, cell ballooning, and individual cell lysis (Garry *et al.*, 1988). The severity of these effects depends on the HIV variant and on the cell type analyzed. As usually occurs with cytolytic animal viruses, the cytopathic effects are the outcome of interaction of a given virus with a given host cell; other conditions also modulate and influence the cytopathic effect (Bablanian, 1975; Schrom and Babla-

nian, 1981; Fraenkel-Conrat and Wagner, 1984). Due to this complexity it is not surprising to find that different viral molecules, or different regions of a given protein, participate to different extents in the cytopathic effect. The proteins of fusogenic viruses, such as HIV, are involved in the formation of multinucleated cells (syncytia) and must be distinguished from the proteins that modify individual cell morphology. Both events might be classified as cytopathic effects, but the proteins contributing to them might be different. This distinction is not usually made in studies devoted to analysis of virus cytopathogenicity, sometimes creating confusion. Syncytia formation by HIV is related to the fusion properties of glycoproteins, whereas cell killing may be effected by a different gene product, although the molecular events underlying both phenomena may have in common the disturbance of membrane function (Garry *et al.*, 1988; Cloyd and Lynn, 1991). In conclusion, changes in membrane permeability by HIV, and perhaps by other enveloped animal viruses, may be produced not only by a typical viroporin, but also by several glycoprotein domains.

The major HIV-induced cell injury has been correlated with perturbation of cell membrane permeability (Lynn *et al.*, 1988; Garry *et al.*, 1988; Cloyd and Lynn, 1991), but not with membrane fusion (Somasundaran and Robinson, 1987). Alterations of membrane permeability by HIV infection are observed early during infection, induced by virus particles (Garry *et al.*, 1988; Fermin and Garry, 1992), and late in the infectious cycle, as a result of gene expression. These late alterations include nonspecific modification of the transport of low-molecular-weight compounds and monovalent and divalent cations (Lynn *et al.*, 1988; Cloyd and Lynn, 1991). Sucrose enters HIV-infected cells, whereas inulin is excluded, suggesting that the pores formed by the expression of HIV genes have a specific diameter that allows passage of hydrophilic molecules up to a given size (Cloyd and Lynn, 1991; Miller *et al.*, 1993). Lipid synthesis is also affected in HIV-infected cells, as in other animal virus-infected cells (Carrasco *et al.*, 1989; Perez and Carrasco, 1991; Perez *et al.*, 1991). In HIV infection, synthesis of phospholipids decreases, whereas synthesis of neutral lipids is enhanced (Lynn *et al.*, 1988).

1. Portions of the HIV gp41 Increase Membrane Permeability

Initial studies with HIV-1 mutants suggested that cytopathicity of T lymphocytes was affected by the carboxy region of the transmembrane (TM) glycoprotein (gp41) (Fisher *et al.*, 1986; Sodroski *et al.*, 1986). Cytopathogenicity induced by other retroviruses, such as feline leukemia virus or simian immunodeficiency virus (SIV), is also affected by the TM glycoprotein (Bosch *et al.*, 1989; Thomas and Overbaugh,

1993), indicating that the interaction of an HIV protein with the membrane is the basis of the cytopathicity.

Several regions in HIV gp41 have been implicated in membrane interactions (see Fig. 4). One region is located at the amino terminus of the protein and is involved in cell fusion. The amino terminus of orthomyxovirus and paramyxovirus TM glycoproteins contains a region of hydrophobic amino acids involved in membrane fusion; this region has been termed "fusion peptide." Similarities between the amino terminus of HIV gp41 and these fusion peptides have been noted (Gallaher, 1987). Point mutations in the amino-terminal region reduced syncytium formation, without affecting glycoprotein processing (Freed *et*

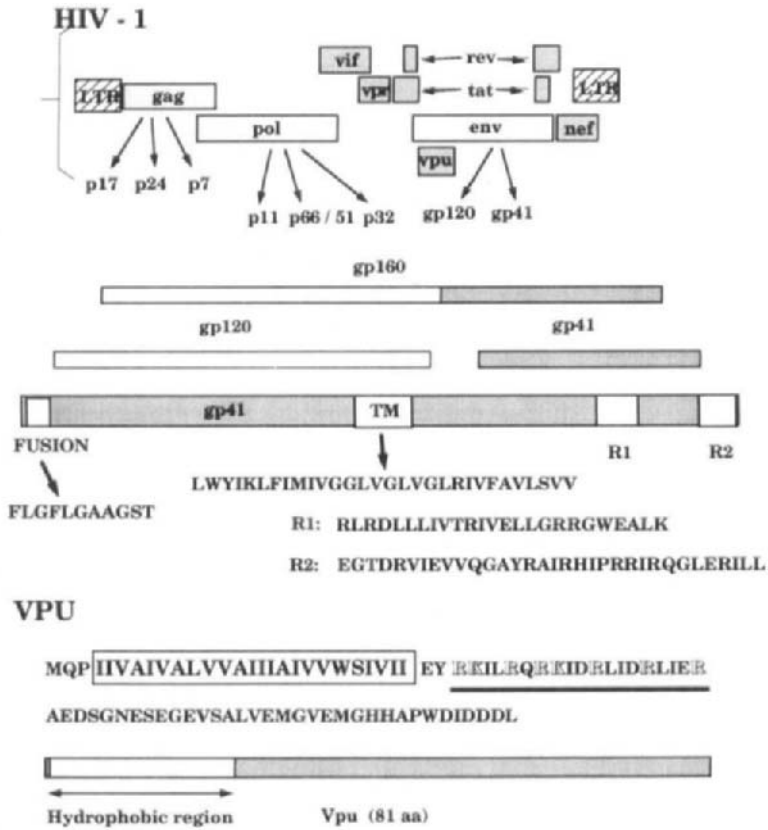

FIG. 4. Schematic representation of the HIV-1 genome (top). The structures of the two glycoproteins, gp120 and gp41, as well as protein Vpu are depicted. In the Vpu molecule the box outlines the hydrophobic region; the basic amino acids are shown in open-face font and sequences containing basic amino acids are underlined.

al., 1990; Kowalski *et al.*, 1991). Mutations of individual amino acids located in the amino-terminal hydrophobic stretch of gp41 as well as polar amino acids that flank this region profoundly block syncytium formation (Freed *et al.*, 1990). A similar result is observed with two mutants that contain several amino acid insertions in this region (Kowalski *et al.*, 1991). Curiously, the replication ability and virion production by these mutants do not differ from that of wild-type HIV-1 (HXBC2) in Jurkat lymphocytes, indicating that the levels of HIV gene expression (Somasundaran and Robinson, 1988), or virus particles, are not sufficient to induce cytopathic effects (Kowalski *et al.*, 1991). Single-cell lysis of Jurkat cells was also affected by modifying the hydrophobic amino terminus of gp41 after 5–10 days of infection, whereas maximal expression of p24 peaked at day 5 for wild-type and mutant viruses (Kowalski *et al.*, 1991).

Membrane fusion and syncytia formation are also effected by the membrane-spanning domain (Owens *et al.*, 1994) and the cytoplasmic moiety (carboxy terminus) of HIV-1 gp41 (Lee *et al.*, 1989). Deletion of the carboxy-terminal 6–14 amino acids of HIV-1 gp41 alters the infectivity and cytopathogenity, although, surprisingly, a larger deletion of 76 amino acids did not abolish viral replication (Lee *et al.*, 1989). The conclusion reached was that conformational determinants, rather than protein length, are important for gp41 function. Deletion of 146 amino acids of the cytoplasmic domain of SIVmac (plus several point mutations throughout the protein) produces a nonpathogenic variant, SIVmac 1A11, in monkeys. Curiously, the expression of the recombinant TM from SIVmac 1A11 produces much larger and more numerous syncytia in rhesus macrophages compared to the parental TM (Ritter *et al.*, 1993). Clearly, cell killing is unrelated to fusion ability or pathogenicity, in both SIV and HIV (Somasundaran and Robinson, 1987; Chakrabarti *et al.*, 1989; Ritter *et al.*, 1993).

There are two other regions in the gp41 glycprotein of HIV that display high hydrophobic moment, suggesting that they may also associate with membranes (Venable *et al.*, 1989). These two regions span approximately amino acids 772–790 and 828–848 (Fig. 4). Theoretically, these regions could form highly amphipathic α-helices that may reside in the membrane as aggregates, forming hydrophilic pores (Venable *et al.*, 1989). The hydrophobic faces of these peptides would be in contact with membrane lipids; the hydrophilic faces would form the pore in contact with water (Venable *et al.*, 1989; Eisenberg and Wesson, 1990). Indeed, gp41 tends to aggregate preferentially to form tetramers, although trimers are also detected (Pinter *et al.*, 1989). Similarities between these regions and those in other lytic peptides, such as melittin, have also been noted (Eisenberg and Wesson, 1990).

Apart from structural considerations of gp41, two lines of evidence suggest that gp41 alters membrane permeability. Peptide homologs of the region spanning amino acids 828–855 are very toxic for prokaryotic and eukaryotic cells, eliciting membrane permeability changes and cell lysis (Miller *et al.*, 1991, 1993). It was suggested that the lytic peptides form pores of defined size in the plasma membrane (Miller *et al.*, 1993).

In addition to the regions of gp41 discussed above, the transmembrane domain of this protein could, in principle, also participate in modifying membrane permeability. This region shows similarities to other viroporins, including the presence of an adjacent region containing basic amino acids. Future studies on the membrane-active properties of gp41 could be directed to the expression of this protein in model systems to analyze the capacity of the whole protein to increase membrane permeability and the capacity of truncated versions of gp41 to affect the membrane. These analyses would map with more precision each portion of gp41 that plays a role in enhancing membrane permeability.

2. *HIV Vpu Protein*

The *vpu* gene of HIV-1 is located 3′ to the first exon of *tat* and overlaps with the 5′ and of *env* (Strebel *et al.*, 1988; Cohen *et al.*, 1988). HIV-2 and SIV do not contain an analogous protein. Vpu is a typical viroporin molecule; it is an integral membrane protein containing 81 amino acids and is phosphorylated on a serine residue (Strebel *et al.*, 1989). It contains a hydrophobic stretch of 28 amino acids at the amino terminus (Fig. 4) embedded in the membrane, with the hydrophilic carboxy terminus oriented toward the cytoplasm (Maldarelli *et al.*, 1993). Vpu is produced late in replication and it has not been found in virions; the protein is localized in a perinuclear region, most likely the Golgi complex (Klimkait *et al.*, 1990).

Genetic analysis with Vpu mutants suggests that this protein participates in late steps of infection related to virus release from infected cells (Terwilliger *et al.*, 1989; Klimkait *et al.*, 1990; Yao *et al.*, 1992; Göttlinger *et al.*, 1993). The absence of Vpu results in an accumulation of cell-associated viral proteins. T cells infected with Vpu-positive virus release three- to fivefold more virions than do cells with Vpu-negative virus. Virions from Vpu-negative provirus accumulate at the cell surface and in endosomes (Klimkait *et al.*, 1990). The mutant virion particles released are not homogeneous particles, displaying various sizes and shapes, and some of them contain several nucleoids (Klimkait *et al.*, 1990). Vpu complements Vpu-negative mutants in trans, but it has no effect on production of SIV that is devoid of a Vpu

counterpart (Terwilliger *et al.*, 1989). In contrast, more recent evidence indicates that the Vpu protein of HIV-1 not only facilitates the release of virus particles from the surface of infected cells, but also enhances capsid release from other retroviruses, such as HIV-2, visna virus, and Moloney murine leukemia virus (Göttlinger *et al.*, 1993).

In addition to enhancing release of virus particles, Vpu also affects cell killing. Less cytopathic Vpu mutants have been isolated and show a decrease in syncytium formation and cell killing in $CD4^+$ human T cells (Terwilliger *et al.*, 1989; Kishi *et al.*, 1992), although it is unclear if these mutant viruses contain additional mutations in other genes that are responsible for this phenotype. Apart from these effects on virus release and cell killing, Vpu induces the degradation of CD4 molecules in the infected cells, suggesting multiple roles for Vpu (Willey *et al.*, 1992).

Similarities of Vpu with other amphipathic viral proteins, including influenza M2, have been noted (Maldarelli *et al.*, 1993). Apart from these structural similarities, I would draw attention to the functional resemblance of Vpu to togavirus 6K and influenza M2, because these three proteins are involved in efficient release of virus particles from cells (see below). To my knowledge, there have been no studies on the modification of membrane permeability by the expression of individual Vpu molecules. Such studies would indicate the extent to which Vpu qualifies as a real viroporin molecule.

C. Influenza M2 Protein Forms Ionic Pores

Influenza virus is a negative-sense stranded RNA virus that contains a segmented genome. Segment 7 encodes at least two proteins (M1 and M2) synthesized from two different mRNAs derived by transcription of segment 7 and differential splicing (Lamb *et al.*, 1981). The structure of M2 resembles that of poliovirus 3A, togavirus 6K and HIV-1 Vpu (see above). M2 is also an integral membrane protein associated with both the Golgi complex and the cell surface (Lamb *et al.*, 1985; Zebedee *et al.*, 1985). In polarized epithelial cells (MDCK), M2, like hemagglutinin HA, is preferentially expressed at the apical surface in close proximity to budding virions (Hughey *et al.*, 1992). Although early studies indicated that M2 was absent from virions, as with Vpu and togavirus 6K, it was later suggested that M2 is also present in virions (Zebedee and Lamb, 1988; Hughey *et al.*, 1992). M2 contains 97 amino acid residues, with a hydrophobic membrane-spanning region of about 20 amino acids that may form an amphipathic helix. M2, as well as HIV-1 Vpu, can form homotetramers that, in the case of M2, are disulfide linked (Sugrue and Hay, 1991). In

addition to tetramers, higher molecular-weight structures containing 10–12 molecules of M2 are also detected (Sugrue and Hay, 1991). The amino terminus of the M2 protein is located in the extracellular space, whereas the carboxy terminus is cytoplasmic (Zebedee *et al.*, 1985). Hence, M2 is a typical type I integral membrane protein (Holsinger *et al.*, 1994). The M2 proteins from various influenza virus strains contain palmitate moieties, most probably attached to cysteine 50, whereas threonine 65 is phosphorylated (Veit *et al.*, 1991; Sugrue *et al.*, 1990b).

M2 is able to modify membrane permeability when expressed in *Xenopus* oocytes (Pinto *et al.*, 1992) or *E. coli* cells (Guinea and Carrasco, 1994a). Patch-clamp studies of *Xenopus* oocytes expressing the natural influenza M2 protein show an increased ionic flux (sodium and potassium) through the plasma membrane (Pinto *et al.*, 1992; Wang *et al.*, 1993). This increased membrane permeability to monovalent cations probably extends to protons and other low-molecular-weight molecules, although direct evidence for higher permeation to other solutes, including protons, has not been presented. Enhanced membrane permeability induced by M2 increased at low pH (6–6.5) in *Xenopus* oocytes (Pinto *et al.*, 1992). Mutants of M2 with an altered transmembrane region were unable to permeabilize amphibian cells (Pinto *et al.*, 1992).

The use of *E. coli* cells expressing high levels of M2 protein in an inducible manner, as discussed in Section IV,A, indicated that this protein was lytic for prokaryotic cells that express lysozyme, and modified membrane permeability to a number of hydrophilic molecules (Guinea and Carrasco, 1994a). The nonspecific increase in permeability in prokaryotic cells indicates that M2, like other viroporins, forms hydrophilic pores in the membrane. These pores allow the passages of ions and other low-molecular-weight molecules. This membrane disruption was similar to the effects observed in infected cells (Lopez Vancell *et al.*, 1984; Carrasco and Lacal, 1983). Therefore, M2 is a strong candidate to account for the changes in membrane permeability in influenza virus-infected cells. MDCK cells infected with influenza virus show enhanced passive permeability to sodium and potassium ions 5 hr postinfection (Lopez Vancell *et al.*, 1984). Permeability toward to nonpermeating translation inhibitors was observed before the onset of synthesis of influenza virus proteins (Carrasco and Lacal, 1983). A more detailed kinetic analysis of permeability changes in influenza virus-infected cells is required to assess more precisely the nature and the timing of membrane disruption that occur in these cells. Finally, influenza M2 mutants and the individual expression of M2 in mammalian cells can now be exploited to explore the capacity of M2 to promote permeability changes in mammalian cells.

In view of these findings, it is tempting to suggest that M2 is a viroporin that nonspecifically increases membrane permeability and participates in the influenza virus replication cycle as other viroporins do, i.e., by facilitating virus release from the infected cells. An alternative view suggests that M2 has proton translocation activity; as such, M2 would participate at two different steps during the influenza growth cycle, early during infection when the virus enters cells, by promoting the entry of protons into the viral nucleocapsid to destabilize or collapse this structure, and later in the infectious cycle during the synthesis of viral glycoproteins by modulating the pH in the Golgi apparatus (Sugrue *et al.*, 1990a; Hay, 1991). In this model, M2 would raise the pH in the Golgi complex, such that the integrity of HA1/HA2 during transport through the vesicular system to the plasma membrane would be maintained (Hay, 1991; Takeuchi and Lamb, 1994). As yet, there is no evidence that M2 has proton translocation activity. Rather, this protein nonspecifically increases membrane permeability. Clearly, experiments employing M2-deficient mutants are necessary to assess the exact role played by M2 during the influenza virus life cycle.

Further clues to the action of M2 come from studies with the antiviral compound amantadine. This agent blocks the growth of a number of animal viruses, including some strains of influenza virus (Tominack and Hayden, 1987; Hay, 1991). The exact mode of action of amantadine in influenza virus replication remains unclear. It blocks two steps of infection. Early inhibition by amantadine is related to inhibition of virus uncoating (Hay, 1991). This effect is rather nonspecific because high concentrations of the drug are required and replication of other animal viruses is inhibited by these amantadine concentrations (Nicholson, 1984; Rosenthal *et al.*, 1982, 1988; Schulman, 1982; Wittels and Spear, 1991). The molecular basis of this early inhibition by amantadine relies on the accumulation of the drug in endosomes, an event that leads to increased endosomal pH. It was suggested that amantadine binds to the pore region of the channel formed by M2 (Sugrue and Hay, 1991). The M2 protein present in virions would permit the flow of ions, including protons, from the endosome into the virus particle interior to disrupt macromolecular interactions, freeing the nucleocapsid from the M1 protein. This activity of M2 would be blocked by amantadine. More recent experiments suggest that amantadine acts as an allosteric blocker that binds to another portion of M2 not directly involved in the pore region (Wang *et al.*, 1993).

At low concentrations amantadine blocks a very late step of influenza virus maturation; viruses are able to bud from the cell surface, but they are unable to pinch off from the cell (Ruigrok *et al.*, 1991). This situation closely resembles HIV-1 viruses defective in Vpu

(Klimkait *et al.*, 1990) or togaviruses that lack the 6K protein (Liljeström *et al.*, 1991). Mutants of influenza virus resistant to low concentrations of amantadine have been isolated and characterized. They map in protein M2 and, more precisely, in amino acid residues 27–35 (Hay *et al.*, 1986). Inhibition of M2 function by amantadine causes a reduction in the pH of vesicles of the Golgi complex triggering the conformational change in HA (Ciampor *et al.*, 1992a). Interestingly, this effect of amantadine is reversed by monensin (Sugrue *et al.*, 1990a); this is not surprising in view of the idea that viroporins have ionophore-like activity (Carrasco, 1987). Thus, it was suggested that amantadine brings about a premature conformational change in HA that occurs in the trans-Golgi complex during the transport of HA to the cell surface (Sugrue *et al.*, 1990a). This change in HA would block virus release.

In support of the view that M2 is involved in cytopathicity is the recent finding that expression of M2 increased the cytopathic effect in insect Sf9 (*Spodoptera frugiperda*) cells infected with recombinant baculovirus. Addition of amantadine to these cells increased the yield of M2 by 10-fold (Black *et al.*, 1993). These results are similar to our findings that expression of mutated poliovirus 3A protein that is unable to permeabilize bacterial cells is enhanced by more than 20-fold (J. Lama and L. Carrasco, unpublished results).

D. Togavirus 6K Protein

Togaviruses are plus-stranded RNA-containing viruses that possess a lipid envelope (Schlesinger and Schlesinger, 1993). During the late phase of Semliki Forest virus or Sindbis virus infection there is a drastic shut-off of host protein synthesis, and only the translation of a viral subgenomic mRNA takes place (Ulug *et al.*, 1987), producing a polyprotein precursor that contains several proteins in the order C–p62–6K–E1 (Garoff *et al.*, 1980). This precursor is proteolytically cleaved to generate the mature viral proteins: C, the capsid protein, is autocatalytically cleaved and interacts with the genomic RNA to form the nucleocapsid, whereas the rest of the proteins are processed by the cellular signal peptidase (Liljestrom and Garoff, 1991). The p62 precursor is hydrolyzed to generate E3 and E2 that, together with E1, comprise the three glycoproteins present in the lipid bilayer of virion particles (Schlesinger and Schlesinger, 1993; Barth *et al.*, 1992). Togaviruses induce drastic changes in membrane permeability during infection (Carrasco, 1978; Garry *et al.*, 1979; Ulug *et al.*, 1984; Muñoz *et al.*, 1985b; Garry *et al.*, 1986; Ulug *et al.*, 1987). These modifications lead to an imbalance of ions in the cytoplasm, thus blocking host

protein synthesis (Garry *et al.*, 1979; Ulug *et al.*, 1987). The translation of Semliki Forest virus subgenomic 26S mRNA is not inhibited by increasing the concentrations of monovalent ions either *in vitro* or in the infected cells (Carrasco *et al.*, 1979; Alonso and Carrasco, 1982a). We have suggested that some of the changes in membrane permeability observed during togavirus infection could be explained by the membrane-active characteristics of the 6K protein (Sanz *et al.*, 1994). This protein is a heavily acylated polypeptide of 60 amino acids that interacts with membranes (GaedigK-Nitschko *et al.*, 1990; GaedigK-Nitschko and Schlesinger, 1990; Lusa *et al.*, 1991). Although the 6K protein is initially associated with p62 and E1 and travels through the ER to the plasma membrane as a complex with these proteins, it is not encapsidated in virions (Lusa *et al.*, 1991). Thus, most 6K is excluded during budding of new virions, although some 6K can still be detected in these particles (GaedigK-Nitschko and Schlesinger, 1990).

Once again the 6K protein shows structural similarities with other viroporins (Carrasco *et al.*, 1993). The togavirus 6K protein is a hydrophobic membrane protein that increases membrane permeability and induces cell lysis in the bacterial model system described in Section IV,A (Sanz *et al.*, 1994). Inducible expression of the Semliki Forest virus protein 6K is very toxic for *E. coli*, causing increased membrane permeability and cell lysis. Synthesis of 6K led to a sudden release of choline from preloaded cells and to the entry of the hydrophilic antibiotic hygromycin B (Sanz *et al.*, 1994).

The exact role played by the 6K protein in the Semliki Forest virus replication cycle remains largely unknown. Complete removal of the 6K sequence from the genome had no effect on virus RNA replication, gene expression, or glycoprotein transport, but there was a reduction in virus release (Liljeström *et al.*, 1991). Mutants that contain an underacylated 6K protein also have defects in budding of new virions (GaedigK-Nitschko *et al.*, 1990). Recently, a Sindbis virus 6K mutant containing an in-frame insertion of 15 amino acids has been generated (Schlesinger *et al.*, 1993). This mutant was viable and the virions formed had no detectable 6K, but they were as infectious as wild-type Sindbis virus (Schlesinger *et al.*, 1993). Synthesis of viral proteins occurred in the 6K-deficient mutants, but there was some interference with the proteolytic processing at the junction between p62 and 6K. Notably, this Sindbis virus mutant was defective in its ability to block host translation (Schlesinger *et al.*, 1993), suggesting that synthesis of the 6K plays a part in the shut-off of host protein synthesis by togaviruses. These results agree well with the recent finding that hy-

potonic medium reverses the shut-off of cellular translation induced by Sindbis virus (Garry, 1994).

No studies have analyzed the permeabilization of cells during infection with the mutant viruses defective in 6K. In agreement with the idea that the 6K protein modifies membrane permeability, thus facilitating virus release from cells, is the finding that the alterations in ion transport in Sindbis virus-infected cells modulate the release of progeny viruses (Ulug *et al.*, 1989). As may occur with HIV, it is possible that alteration of membrane function by togaviruses is accomplished by several viral proteins, including the 6K protein and some glycoprotein moieties.

E. Other Putative Viroporins

Analyses of Kyte and Doolittle (1982) hydrophobicity plots and the actual hydrophobic sequence of poliovirus 3A indicate that a portion of this protein has an index above 2.5 and a hydrophobic stretch of about 20 amino acids (Carrasco *et al.*, 1993). Close to this sequence there are basic amino acids, whereas acidic residues are not present. In addition to the viral proteins discussed above, i.e., poliovirus 3A, HIV-1 Vpu, influenza M2, and togavirus 6K, several known virus proteins also have such characteristic sequence elements (see Figs. 5 and 6). Some paramyxoviruses, such as respiratory syncytial virus, contain a small hydrophobic (SH) polypeptide that is an integral membrane protein (Olmsted and Collins, 1989). The RSV SH protein is composed of 64 amino acids and contains a very hydrophobic stretch of amino acids in the center of the protein, followed by a basic sequence, typical of membrane-active proteins. The SH protein is expressed at the cell surface (Olmsted and Collins, 1989), but the function of SH in the virus replicative cycle remains unknown. Several species of SH have been distinguished, including one that is glycosylated (Olmsted and Collins, 1989). The protein forms aggregates of various monomers, including species of about 8–10 monomers (Collins and Mottet, 1993). Infectious bronchitis virus, a coronavirus, encodes a small protein of 110 amino acids with a hydrophobic region of about 20 residues (Smith *et al.*, 1990). This protein is membrane associated and is located in the Golgi apparatus and at the cell surface (Smith *et al.*, 1990).

Among DNA-containing viruses several viroporin candidates can be described, based only on sequence data. However, I shall mention only those putative viroporins for which there is at least some experimental evidence for membrane association. Human papillomavirus expresses an E5 protein that is associated with the membrane and interacts with

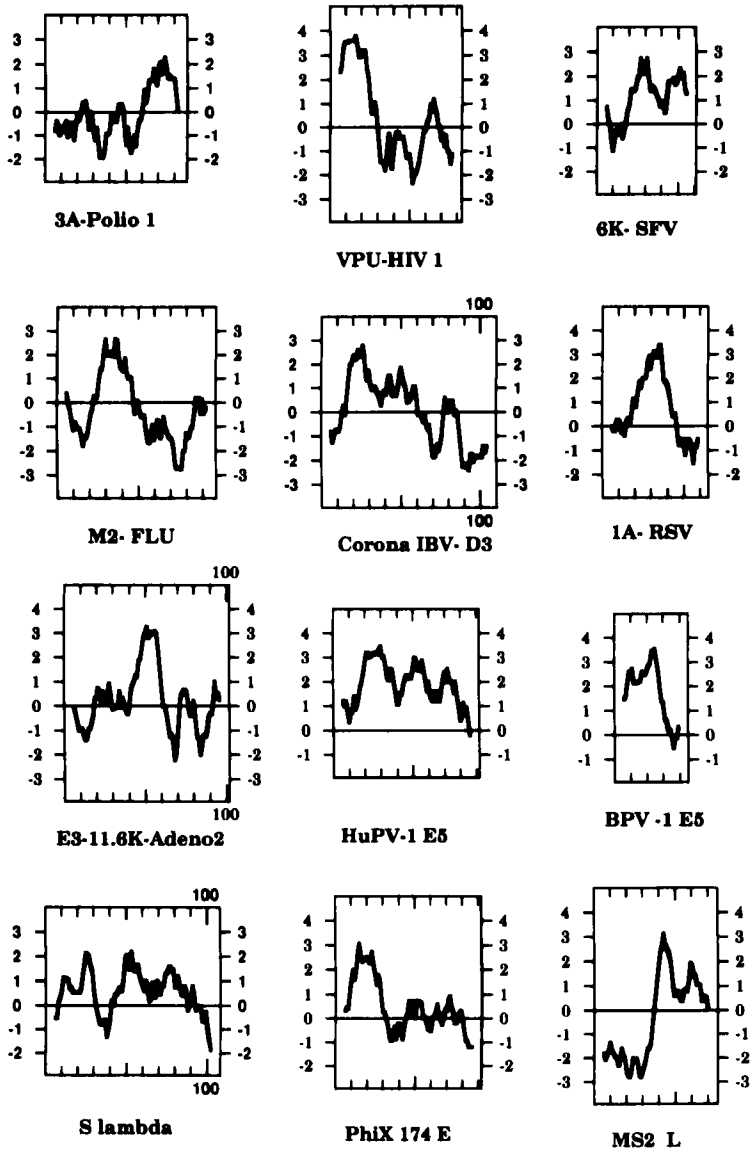

FIG. 5. Hydrophobicity plots (Kyte and Doolittle, 1982) of putative membrane-active molecules.

the pore-forming protein of the vacuolar proton ATPase (Goldstein *et al.*, 1991; Conrad *et al.*, 1993). The E5 proteins from several human papillomaviruses and bovine papilloma virus 1 also contain a very

FIG. 6. Sequences of several membrane-active proteins from viruses and other natural sources. The boxes in the sequences show the hydrophobic regions; the basic amino acids are shown in open-face font and sequences containing basic amino acids are underlined.

hydrophobic region of amino acids that most probably span the lipid bilayer. Adenoviruses encode a 11.6K protein that is glycosylated and localizes in the Golgi apparatus and the nuclear membrane (Scaria *et al.*, 1992; Tollefson *et al.*, 1992). This protein is of typical size and contains the hydrophobic region and basic amino acids of viroporins (Figs. 5 and 6). As yet, there is no evidence that these proteins are involved in altering membrane function.

V. General Properties of Viroporins

A. Viroporin Structure

With the increasing availability of viral genome sequences, the putative viroporins encoded by animal viruses may eventually be predicted. Moreover, the ease of genetic manipulations that allow individual cloning and expression of these proteins will promote more interest in elucidating the mechanisms of action of this group of viral proteins. In a few years, further work in this field should provide us with a more complete picture of viroporin activity in the infected cells and could provide additional targets of antiviral intervention.

Although we are only beginning to glimpse the existence of this new family of animal virus proteins that are involved in cell lysis, some generalities of their structure and function can be summarized. Structurally, viroporins are in general short proteins containing around 50–120 amino acid residues. Analysis of the amino acid content of several viroporins shows that they contain a higher than average content of leucine plus isoleucine residues and a lower content of glycine. All of virporins possess a hydrophobic stretch of about 20 amino acids, which in some instances may form an amphipathic helix. Thus, they are integral membrane proteins with at least one membrane-spanning domain. Viroporins tend to form oligomers, most frequently tetramers, thus leaving a hydrophilic pore through which ions and low-molecular-weight hydrophilic compounds pass in a nonspecific manner. In addition to tetramers, other oligomeric structures containing multiple viroporin molecules may be formed. In addition to the hydrophobic region, viroporins usually contain basic amino acids, as seen with membrane-active toxins (see Section VI). These regions of basic amino acids may participate in membrane permeabilization by destabilizing the lipid bilayer. In summary, the structure of viroporins allows formation of oligomeric structures spanning the membrane to form hydrophilic pores.

B. Viroporin Function

The main function of viroporins is to help in the release of progeny virus from the infected cells. Spread of virus to other surrounding cells or tissues is facilitated by cell lysis. Apart from this function, it seems logical to think that the expression of proteins that lead to cell lysis would also have detrimental effects for the infected cells, even before lysis occurs. These effects could be manifested as modifications in normal cell morphology and metabolism, including the synthesis of cellular macromolecules.

It may still be argued by some virologists that changes in membrane permeability are the result of the exit of whole virus particles from cells. I would stress that the opposite view is more plausible, i.e., that viruses exist the cell because the membrane is being lysed by specific membrane-active proteins encoded by the viral genome. Therefore, a new concept arises—namely, that animal viruses contain in their genomes one or more genes specifically devoted to lysing the plasma membrane. This may be particularly important for viruses that possess naked virion particles. In order to leave the cells, such viruses, which are devoid of a lipid membrane, need to lyse the cell membrane. In the case of enveloped virion particles, this envelope is acquired in the last step of assembly, by budding from the plasma membrane, which is modified by a specific viral product that produces pores. The increased membrane permeability due to pore formation would be required for efficient budding of enveloped viruses. In the absence of viroporins animal viruses are still made, but the number of virus progeny is greatly diminished. Little is known about the forces that induce a virus particle to bud and be released from the cell. Membrane budding could also be regulated by alterations that virus-infected cells suffer in calcium homeostasis. Apart from calcium, the levels of other cations and pH also change drastically during infection. Several years ago, it was indicated that the ionic milieu in the cytoplasm of a virus-infected cell during virus assembly is quite different from that in the early stages of infection, when the virus decapsidates (Carrasco, 1977). Virus assembly is known to be affected by cations and pH *in vitro,* so why not also in the infected cell?

Therefore, viroporin action at the molecular level may be directed to deenergizing the membrane and disrupting its physical integrity. Formation of pores leads to the dissipation of the membrane potential and the ionic gradients that would be gradually destroyed as infection progresses. Not only the ionic gradients maintained by the plasma membrane, but also those present in intracellular compartments, including the proton gradient, would disappear. The physical integrity of

the membrane could be disrupted initially by the formation of hydrophilic pores and by the insertion in the membrane of viroporin regions containing basic amino acids that destabilize the lipid bilayer in a detergent fashion. The focal distribution of viroporins in the bilayer would promote preferential virus release through these regions. Subsequently, stimulation of phospholipases may complete the physical disappearance of the lipid bilayer in certain regions of the plasma membrane, leading to cell lysis.

Finally, viroporin activity may also have consequences for cellular metabolism and morphology, as occurs with membrane-active toxins that affect an array of cellular functions by simply modifying membrane function. Viroporins could affect cellular metabolism, including macromolecular synthesis. It was initially proposed that the modifications in membrane permeability to monovalent ions in virus-infected cells is responsible for the virus-induced shut-off of host translation (Carrasco and Smith, 1976; Carrasco, 1977). Overwhelming evidence indicates that this may be the case of EMC virus-infected cells. Togaviruses block host translation by a similar mechanism (Garry *et al.*, 1979; Ulug *et al.*, 1984, 1987; Muñoz *et al.*, 1985a). The translation of cellular mRNAs will be rapidly inhibited by the action of the lytic protein, whereas viral RNA translation tolerates these adverse conditions and is still translated. Perhaps shut-off of host translation is a by-product of a viral process, such as cell lysis, a requirement to liberate the virus particles made during infection.

All experimental evidence on the potential activity of viroporins has been obtained with whole, wild-type viruses; few virus mutants have been assessed for viroporin function. As yet, only the togaviruses defective in 6K and HIV mutated in Vpu have been examined. Furthermore, effects of the individual expression of viroporins in mammalian cells are still an unexplored field. At present we have evidence that the individual expression of poliovirus 3A, SFV 6K, and influenza M2 in *E. coli* and amphibian cells modifies membrane permeability in a nonspecific way. The inducible prokaryotic system described in Section IV,A promises to be of great value not only for identifying other putative viroporins, but also for obtaining mutants to explore the portions of the molecule most relevant to viroporin activity. The identification of pore formation by M2 expressed in amphibian cells constitutes another promising model system to characterize the animal virus proteins that modify membrane permeability.

C. Pore-Forming versus Ion-Channel-Forming Proteins

Resolution of three-dimensional structures of proteins, and more particularly of integral membrane proteins, has revealed the architec-

ture of some proteins that form pores and ionic channels in membranes (Eisenman and Dani, 1987; Aschroft and Röper, 1993; Cowan, 1993; Catterall, 1993; Imoto, 1993; Hoshi and Zagotta, 1993; Massotte *et al.*, 1993). The most common feature of proteins that form ion channels is the presence of domains containing α-helices that span the membrane (Eisenman and Dani, 1987; Eisenberg, 1984; Lear *et al.*, 1988; Imoto, 1993). The association of several amphipathic helices builds up a pore in such a way that the hydrophobic region of the α-helix is in contact with the membrane lipids, whereas the hydrophilic residues form part of the hydrophilic pore lumen (Oblatt-Montal *et al.*, 1993; DeGrado *et al.*, 1989; Segrest *et al.*, 1990). Visualization of botulinum neurotoxin-induced channels in vesicles has been achieved (Schmid *et al.*, 1993). The channel is formed by interaction of four neurotoxin molecules. Pores can also be formed by amphipathic β-sheets, which have a tendency to associate and can traverse the membrane if they contain at least 10 amino acids. The best-known members of this group of membrane proteins are the bacterial porins (Cowan, 1993; Schulz, 1993), which are proteins located in the outer membrane of gram-negative bacteria and which allow the passive passage of solutes (Cowan, 1993). The antiparallel β-barrel structure of porins allows their insertion into the membrane, leaving a pore that can be gated by other regions of the molecule (Cowan, 1993; Schulz, 1993; Weiss and Schulz, 1993). Lymphocyte perforins may also form similar structures responsible for cell lysis (Lichtenheld *et al.*, 1988; Shinkai *et al.*, 1988; Lowrey *et al.*, 1989; Ojcius *et al.*, 1991). Selective ion channels present a funnel-shaped architecture, with at least one narrow region within the channel pore, where ion selection takes place (Eisenman and Dani, 1987; Imoto, 1993). In contrast to this selectivity of ion channels, proteins involved in cell lysis allow the nonspecific passage of ions and of low-molecular-weight hydrophilic compounds, with size being the only constraint on permeability.

With respect to viroporins, it is of interest to point out the identification of a rather small cellular protein containing only 130 amino acid residues; this protein is involved in voltage-gated potassium channels (Takumi *et al.*, 1988; Hausdorff *et al.*, 1991; Murai *et al.*, 1989). It differs from other known ion channel proteins; it is still unknown if this protein induces membrane depolarization by forming aggregates in membranes, but it constitutes a good model system to analyze fundamental aspects of cellular proteins endowed with ion channel activity.

In conclusion, ion channels have been defined as aqueous pores through which selected ions can move from one side of the membrane to the other. These channels are usually selective for a given ion and control the flux of ions by gating this pore in response to various

factors. Ion channels are integral components of the membrane involved in the normal functioning of cells. Their channel-gating characteristics ensure that the ion channel remains open or closed, depending on the cellular functional state. Thus, ion channels are nonlytic for cells.

Membrane pores are discussed here in the context of their relationship to cell lysis. They are formed by alien proteins, whose ultimate function is to disorganize the membrane and kill the cell. Pores formed by these lytic proteins do not have a determined pore size, which may vary according to the number of proteins that interact to form a given pore. In this sense the formation of pores is plastic. The initial pore is formed by oligomerization of a determined number of pore-forming proteins. However, as infection progresses and the number of viroporins in the plasma membrane increases, other proteins can be recruited to widen the pore size. Pores are not selective with regard to the molecules that can pass through them. Ions, in the first instance, because of their smaller size, and low-molecular-weight compounds can pass through the pores. As the pore size increases, however, macromolecules, including enzymes, may leak through them. This is exactly the phenomenology observed in animal virus-infected cells: initially the permeability of ions and of low-molecular-weight compounds increases in both directions, and finally enzymes leak out of the infected cells.

VI. Other Lytic Proteins of Natural Origin

It is beyond the scope of this work to review in depth the different proteins that possess lytic activity. However, detailed studies accomplished with lytic agents can greatly benefit our understanding of the action of viroporins at the molecular level.

A. Lytic Proteins from Bacteriophages

Identification of phage proteins involved in the lysis of bacteria is far more advanced than is the case for analogous proteins from animal viruses (Young, 1992). Extensive studies have been carried out on the molecular basis of phage-induced lysis in bacteria. In all cases analyzed, the bacteriophage encodes a specific protein responsible for lysing the bacterial membrane (Young, 1992). These lytic proteins should not be confused with lysozyme, an enzyme also encoded by some phages (and by cells) that is able to digest peptidoglycans, and hence the bacterial cell wall. Some virologists still may have the idea that

lysis of bacteria by phages is due to lysozyme activity. In fact, bacterial lysis by phage infection results from permeabilization of the lipid bilayer by specific lytic proteins, thus allowing lysozyme to act on the external cell wall (Garret and Young, 1992).

These proteins are integral membrane proteins and lyse bacteria by making holes; for this reason they were christened "holins" (Young, 1992). There is a strong structural (and perhaps functional) similarity between holins and viroporins (see Figs. 5 and 6). At present there is evidence that about seven proteins may act as holins. These proteins are S and S^{21} from λ phage and the lambdoid phage 21, respectively; protein t from phage T4; gp17.5 from T7 phage; LydA encoded by phage P1; the ϕ29-encoded p14; and p10 from phage ϕ6 (Young, 1992). As with viroporins, these proteins are unrelated in terms of primary structure, but they have in common one or two putative membrane-spanning domains of hydrophobic amino acids. It has been speculated that the two transmembrane domains can interact with each other to form a hole in the membrane. Perhaps, as in the case of viroporins, holins can form multimeric aggregates to generate pores in the membrane, even if they possess a single transmembrane domain. Bacteriophage lysis can be prevented without inhibiting the accumulation of intracellular phage particles (Josslin, 1971). Phospholipase activity occurs as a consequence of hole formation by phage T4, suggesting a role for lipases in lysis (Bennett *et al.*, 1971; Buller *et al.*, 1975). However, phage release still occurs even if phospholipase activation is inhibited (Hardaway *et al.*, 1975).

For historical and technical reasons, phage lysis has been more frequently studied in temperate phages, particularly in lambdoid phages. The proteins encoded by gene *S* of phage λ and phage 21 contain two membrane-spanning domains separated by a β-turn region with a charge-rich carboxy terminus (Young, 1992). The protein encoded by gene *S* (107 codons) is a basic 8.5-kDa protein (Altman *et al.*, 1983). According to a proposed model, 12–15 S monomers could participate in the formation of a hole with a 5-nm diameter, allowing the passage of small proteins. Theoretical calculations indicate that the number of holes per cell may be as low as 200, making their detection by electron microscopy difficult (Young, 1992). In the absence of *S* gene function, the product of gene *R*, a transglycosidase that degrades the peptidoglycan of *E. coli*, accumulates intracellularly, without lysis or apparent cytotoxicity (Reader and Siminovitch, 1971). Expression of the *S* gene in the absence of activity of gene *R* (S^+ R^- phage) does not lead to cellular lysis by phage, but permeabilization of the membrane still occurs, leading to inhibition of host macromolecular synthesis and cell death (Reader and Siminovitch, 1971; Garret and Young, 1992). Ex-

pression of the *S* lysis gene in *Saccharomyces cerevisiae* led to its accumulation in the plasma membrane and cytotoxicity (Garrett *et al.*, 1990), further indicating that activity of holins or viroporins can be observed in both prokaryotic and eukaryotic cells (see Section IV,A).

The nature of the lesion produced by holins in the cytoplasmic membrane has been analyzed in some detail. As in animal virus-infected cells, a nonspecific increase in membrane permeability is observed in both directions across the membrane. Even low-molecular-weight proteins can cross the damaged membrane; lysozyme leaks out, whereas RNase I can enter the cytoplasm (Young, 1992). This situation resembles infection of mammalian cells by picornaviruses, wherein different molecules cross the membrane in both directions (Carrasco *et al.*, 1989). Even the entry of protein toxins can be detected to a small extent, suggesting that the pores formed are heterogeneous in size or that small proteins can, to a limited extent, cross damaged membranes by unknown mechanisms. The efficiency of membrane penetration by a given compound would depend primarily on the size and the chemical characteristics of the molecule, whereas detection of its passage depends on the sensitivity of the assay employed. Thus, labeled ions or sugars can easily be shown to cross a leaky membrane, whereas no protein traffic is detected with labeled protein, unless enzymatic action is assayed.

Some genetically simpler phages, such as ϕX174 or MS2, lyse cells without the participation of a phage-encoded lysozyme activity. In this case, too, the action of a single species of holin protein is required. The general features of these proteins conform to the features integral membrane proteins, with a highly hydrophobic stretch of amino acids (see Figs. 5 and 6). Digestion of the bacterial cell wall is most probably accomplished by cellular enzymes.

Bacteriophage ϕX174 contains gene *E*, which is involved in cell lysis (Barrell *et al.*, 1976). As with viroporins from animal viruses, the 91-amino acid protein contains a transmembrane domain and several regions of basic amino acids. E protein is also able to form oligomers (Bläsi *et al.*, 1989). Inducible expression of E from a plasmid leads to a sudden collapse of Na^+ and K^{4+} gradients with concomitant cell lysis (Witte *et al.*, 1987; Witte and Lubitz, 1989). The formation of transmembrane tunnels has been observed by electron microscopy (Witte *et al.*, 1990). Curiously, there is only one E hole per cell in a specific location of the cell envelope. It remains to be demonstrated that a similar structure appears in ϕX174-infected cells.

RNA phages such as MS2 or f2 contain an *L* gene that encodes a membrane-bound protein (Model *et al.*, 1979; Beremand and Blumenthal, 1979). L protein is involved in cell lysis, allowing the release

of viral particles. The structure of L is again typical of a virus-encoded membrane-active protein. It contains 75 amino acids with a hydrophobic stretch of about 27 residues and a region of basic amino acids at the amino terminus. Even after deletion of a 40-amino acid N-terminal region, the L protein is competent for lysis (Berkhout *et al.*, 1985). This led to the synthesis of a number of peptides comprising the hydrophobic region of L; these peptides are lytic for bacteria, dissipate the ionic gradients, and increase membrane permeability in model membrane vesicles (Goessens *et al.*, 1988). L protein localizes in special cellular structures, the membrane adhesion sites, to form defined pores (Walderich and Höltje, 1989). Defects in lytic genes from bacteriophages lead to an impairment in release of virus particles, whereas the synthesis of viral macromolecules and virion particles is normal, or even increases.

B. Nonviral Lytic Proteins from Natural Sources

To kill cells, nature has evolved compounds targeted to destruction of the plasma membrane. Apart from ionophores, a number of protein toxins act directly on membranes. This is the case, for example, for melittin (Bernheimer and Rudy, 1986; Dempsey, 1990), bacterial hemolysins (Felix, 1982; Suttorp *et al.*, 1993), magainin (Bevins and Zasloff, 1990), cecropins (Andreu *et al.*, 1985; van Hofsten *et al.*, 1985), attacins (Hultmark *et al.*, 1983), perforins (Podack, 1992), complement (Podack, 1986; Muller-Eberhard, 1988), defensins (Ganz *et al.*, 1990; Cociancich *et al.*, 1993), protegrins (Kokryakov *et al.*, 1993), and *Entamoeba histolytica* pore-forming peptide (Leippe *et al.*, 1992).

Understanding the mode of action of the increasing number of proteins discovered to lyse cells is simplified by analysis of two paradigmatic peptides: melittin, which modifies membrane permeability by disrupting the lipid bilayer of membranes, and alamethicin, a peptide able to form aggregates that span the membrane-forming aqueous pores. It is likely that viroporins, or some moieties of viral glycoproteins, could injure the plasma membrane by similar mechanisms. Moreover, some viroporins may contain different domains, one involved in pore formation and another in membrane disruption. The reader is referred to reviews on permeabilizing agents for additional details on these compounds (Felix, 1982; Bernheimer and Rudy, 1986; Kaiser and Kezdy, 1987; Vaara, 1992).

Melittin is the main toxic component of honey bee venom (Dempsey, 1990). It is composed of 26 amino acids, of which residues 1–19 are very hydrophobic and can form an amphipathic α-helix, whereas the domain containing amino acids 20–26 is very basic. Both domains are

required for efficient lysis (Dempsey, 1990). Several agents, including divalent cations, have been reported to protect against the lytic effects of melittin and other toxins (Bashford *et al.*, 1989). However, this finding has not been reproduced by others (Portlock *et al.*, 1990). Melittin has been used in numerous studies as a model to investigate the interaction of peptides and membranes in order to elucidate the molecular basis of lytic activity. The action of melittin on membranes is complex and depends on a number of parameters, including the composition of the membrane, the membrane potential, and the melittin concentration. On interaction of melittin with membranes, there is an increase in ion conductance, membrane depolarization, membrane fusion, phospholipase activity, etc. (Dempsey, 1990). The initial effects of melittin on membranes may include the formation of pores that are permeable to ions but still impermeable to proteins (Dempsey, 1990). These pores probably form discrete channels before the more generalized effect on membrane lipid disorganization takes place. This leads to the breakdown of membranes into micelles, similar to the action of some detergents.

Alamethicin, like gramicidin A, is a linear peptide antibiotic that inserts into membranes and produces voltage-gated ion channels. The major component of alamethicin is composed of a peptide containing 20 amino acids; it forms an amphipathic α-helix, leading to aggregates that form pores in the membrane (Fox and Richards, 1982; Kaiser and Kezdy, 1987). Recently, high-resolution analysis of dimeric gramicidin A inserted into lipid bilayers has revealed a structure that is consistent with channel formation by this peptide (Ketchem *et al.*, 1993).

Melittin and gramicidin S, a cyclic peptide antibiotic, stimulate the release of phospholipids from erythrocyte membranes at concentrations that modify membrane permeability, indicating that membrane moieties are released with consequent enhancement in permeability (Katsu *et al.*, 1989). The detailed molecular actions of melittin and alamethicin that are responsible for lysis may be different (Portlock *et al.*, 1990).

VII. Future Prospects

The modifications in membrane permeability produced during virus infection are now clearly defined. Early membrane permeabilization is caused by the viral particles during the process of virus entry and uncoating. Insights into the molecular mechanisms of this process suggest that animal viruses require a proton-motive force to enter cells and to permeabilize them. Future work in this field would benefit from the use of selective inhibitors of vacuolar proton ATPase and from

experiments aimed at elucidating the exact role that the pH gradient and/or the membrane potential play in the process of virus entry.

Late membrane leakiness requires virus gene expression and is manifested as a general increase in permeability to ions and low-molecular-weight compounds, suggesting that physical pores in the membrane are generated during virus infection. These pores would arise by oligomerization of particular viral proteins designed to lyse the infected cells. Efforts should be made to understand the structural characteristics of these aqueous pores.

Knowledge gained in the past few years on animal virus proteins that enhance membrane permeability is delineating a new family of proteins, the viroporins, which are necessary for efficient exit of virus particles from infected cells. The general structural characteristics of these viroporins indicate that they are relatively small (40–120 residues) basic proteins that usually contain a hydrophobic region of about 20 amino acids. It can be anticipated that the future characterization of new viroporins will give a better idea of how general this structure is among virus proteins that are endowed with lytic activity.

Apart from the function of viroporins in the virus growth cycle, the way is now open to analyze the effects that these proteins have on different cellular functions, such as inhibition of host macromolecular synthesis and the cytopathic effect. At least two avenues of research could be followed. One is the cloning and expression of individual viroporins to assay their effects on the host cell. The second approach is to generate viruses with mutant viroporin function. Characterization of these viruses and the actions of the variant viroporins in cellular metabolism would provide more definitive answers to the questions concerning membrane modification and the inhibition of host macromolecular synthesis by virus infection.

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