Modification of late membrane permeability in avian reovirus-infected cells: viroporin activity of the s1-encoded nonstructural p10 protein *

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SUMMARY

Infection of chicken embryo fibroblasts by avian reovirus induces an increase in the permeability of the host plasma membrane at late, but not early, infection times. The absence of permeability changes at early infection times, and the dependence of late membrane modification on both viral protein synthesis and an active exocytic route, suggest that a virus-encoded membrane protein is required for avian reovirus to permeabilize cells. Further studies revealed that expression of nonstructural p10 protein in bacterial cells arrested cell growth and enhanced membrane permeability. Membrane leakiness was also observed following transient expression of p10 in BSC-40 monkey cells. Both its permeabilizing effect and the fact that p10 shares several structural and physical characteristics with other membrane-active viral proteins indicate that p10 is an avian reovirus viroporin. Furthermore, the fusogenic extracellular N-terminal domain of p10 appears to be dispensable for permeabilizing activity, since its deletion entirely abolished the fusogenic activity of p10, without affecting its ability to associate with cell membranes and to enhance membrane permeability. Similar properties have reported previously for immunodeficiency virus type I transmembrane glycoprotein gp41. Thus, like gp41, p10 appears to be a multifunctional protein that plays key roles in virus-host interaction.
INTRODUCTION

Cytolytic viruses induce profound injurious effects in susceptible host cells, resulting in morphological, structural and biochemical changes, and in cell degeneration. A widespread phenomenon during viral infection is the alteration of the permeability of the host plasma membrane (reviewed in (1-3)). Viruses can modify the membranes of their host cells in at least two ways: i) by promoting membrane fusion between virus and cell and/or between cell and cell; and ii) by altering the permeability of the plasma membrane (reviewed in (4)). The latter can occur at two different stages of infection: either during virus entry or at late infection times. Early membrane permeabilization is induced by input virus particles during the process of virus entry and uncoating, and causes structural and functional changes in the membrane. The extent and degree of these modifications vary with the virus-cell system and with the multiplicity of infection. On the other hand, enhanced permeability at late infection times requires virus gene expression and is manifested as a general increase in permeability to ions and small molecules, but not to macromolecules, suggesting the formation of hydrophilic pores in the plasma membrane. Recent evidence suggests that these pores are formed by specific viral proteins, whose ultimate function is to disorganize the membrane and kill the infected cell. These membrane-perturbing viral proteins, denominated viroporins, share several physical and structural characteristics (reviewed in (1)).

Avian reoviruses are members of the Ortheoreovirus genus, one of the nine genera of the Reoviridae family. These agents replicate in the cytoplasm, and contain a genome formed by ten segments of double-stranded RNA enclosed within a double protein capsid.
Membrane permeabilization by avian reovirus p10 protein

shell, but lack a lipid envelope (5, 6). The infection of cultured cells by avian reoviruses causes strong cytopathic effects, manifested by shrinkage, rounding and detachment from the plate. The cytopathic effects of avian reovirus infection are rather complex, involving syncytia formation and apoptosis (7-9). Previous reports have revealed that the syncytia formation activity of avian reoviruses is associated with genome segment S1 (10), a gene that contains three partially overlapping, out-of-phase, open reading frames (ORFs)1 which are highly conserved in all avian reovirus strains examined to date (Fig. 1). All three S1 ORFs are expressed in infected cells (11, 12); ORFs 1 and 2 direct the synthesis of the nonstructural proteins p10 and p17, which associate with cell membranes, while distal ORF3 expresses protein σC, a minor trimeric structural protein which is involved in virus attachment to cell receptors (13, 14). Recent evidence indicates that there is a close association between nonstructural p10 protein and the syncytial phenotype displayed by avian reoviruses (11, 15). This protein is a small transmembrane type-I (N-out) protein, and mutagenenec analysis revealed that a region of its extracellular N-terminal domain displays a sequence-dependent effect on the fusogenic activity of p10 (reference 15 and Fig. 1).

In this study we have investigated the capability of avian reovirus to alter the permeability of the host plasma membrane. We found that infection of chicken embryo fibroblasts (CEF) by avian reovirus S1133 causes membrane leakiness at late infection times, and that this alteration is dependent on both viral protein synthesis and protein translocation through the vesicular system. We also found that expression of avian reovirus nonstructural p10 protein in both prokaryotic and eukaryotic cells induces destabilization of the cell membrane, and that this function is not abolished by deletion of its extracellular
Membrane permeabilization by avian reovirus p10 protein

fusogenic domain.
EXPERIMENTAL PROCEDURES

.Cells, viruses, antibodies and cloning.

Primary cultures of chicken embryo fibroblasts (CEF) were prepared from 9- to 10-day-old chicken embryos and grown in medium 199 supplemented with 10% tryptose phosphate broth and 5% calf serum. Monkey BSC-40 cells were grown in monolayers in medium 199 supplemented with 10% fetal bovine serum. Strain S1133 of avian reovirus was grown in semiconfluent monolayers of primary CEF, as previously described (16).

Preparation of polyclonal antibodies against the three S1-encoded avian reovirus proteins has already been described (11). Rabbit polyclonal antibodies against the purified nonstructural protein µNS were raised in our laboratory.

Cloning of ORF1 into prokaryotic and eukaryotic expression vectors has also been described (11). To generate ORF1*, ORF2 and ORF3 DNA inserts, the recombinant plasmid pBsct-S1 (11) was subjected to PCR amplification with the following primers. For ORF1*, the forward primer was 5’<CGGAATTCACGATGGCTCAGAACACGGCAGGT>3’ (EcoRI site underlined) and the reverse primer was 5’<CCCCGTCGACTCAAACGTCGTATGGCGGAG>3’ (Sall site underlined). For ORF2, the forward primer was 5’<CGGAATTCTAGCGCAATGGAATGGCTC>3’ (EcoRI site underlined) and the reverse primer was 5’<CCCCGTCGACTCATAGATCGGCGTCAAATCG>3’ (Sall site underlined). For ORF3, the forward primer was 5’<CGGAATTCTTCTTATTGGATGGCGG>3’ (EcoRI site underlined) and the reverse primer was 5’<CCCCGTCGACTTAGGTGTCGTGATGGCGG>3’ (Sall site underlined). Forward primers were designe
initiation AUG codon with a strong context for translation initiation (17). The resulting amplification products were digested and cloned into EcoRI and SalI sites of expression vectors pMalC and pCIneo. The correct orientation of the inserts was confirmed by nucleotide sequencing of the recombinant plasmids.

*Induction of recombinant protein expression in E. coli*

Overnight cultures of either BL21(DE3) or BL21(DE3)pLysE cells containing the indicated plasmid, grown at 37°C in LB medium supplemented with either 0.2% glucose and 100 µg/ml ampicillin for BL21(DE3) cells, or with 0.2% glucose, 100 µg/ml ampicillin and 34 µg/ml chloroamphenicol for BL21(DE3)pLysE cells, were diluted 100-fold in the same supplemented medium. When an absorbance of 0.6 at 600 nm was reached, cultures were induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

*Viral infections, transfections, metabolic radiolabeling and electrophoretic analysis of proteins*

Infection of CEF by avian reovirus S1133 and transient expression of viral proteins in BSC-40 cells have been described previously (11). For assessing permeabilization of eukaryotic cells to hygromycin B (HB) (Calbiochem, San Diego, CA, USA) the inhibitor was added, at a final concentration of 1.5 mM, to the cultured medium 45 min before the start of radioactive labeling. For protein radiolabeling, the cultured medium was supplemented with 100 µCi/ml of [35S]methionine-cysteine (1.45 Ci/mmol, Amersham Pharmacia Biotech) and incubated at 37°C for 45 min. Cells were washed with phosphate-buffered saline (PBS),
then lysed in 3 x Laemmli sample buffer (18), and proteins were resolved on a 10% SDS-PAGE gel and visualized by autoradiography. For metabolic radiolabeling in bacteria, cells were incubated for 20 min at 37ºC in the presence of 0.4 mM HB and 70 µCi/ml of [35S]methionine-cysteine. Labeled cells were then collected and lysed in 3 x Laemmli sample buffer.

Estimation of uridine release

Uridine preloading of cultured cells, and subsequent estimation of release were done essentially as described previously (19, 20). For eukaryotic cells, 14 h before infection or transfection, semiconfluent cell monolayers were incubated for 14 h at 37ºC in medium containing 2 µCi/ml of [5,6-3H]uridine (32 Ci/mmol, Amersham Pharmacia Biotech). The cells were then washed with PBS and subjected to either viral infection or lipofection. At the indicated times, the culture medium was collected, centrifuged at 8,000 x g for 5 min, and the supernatant was mixed with scintillation cocktail (989, DuPont-New England Nuclear). The radioactivity was then quantified in a liquid scintillation counter. For bacterial cells, 90 min before IPTG induction, cultures were incubated for 1 h in medium supplemented with 4 µCi/ml of [5,6-3H]uridine. The cells were then pelleted and washed twice with prewarmed isotope-free culture medium, resuspended in the initial volume of growth medium and incubated at 37ºC. After IPTG induction, 0.3-ml aliquots of the culture were collected and cells were pelleted. Quantification of the radioactivity in the supernatant was done as above.
Subcellular fractionation and immunoblot analysis

Fractionation of transfected BSC-40 cells and Western blotting analysis were done as described previously (11). Isolation of bacterial membranes was essentially done as described by Lama and Carrasco (21) with minor modifications. Briefly, 30 min after IPTG induction bacteria were pelleted and washed twice with a buffer containing 50 mM Tris-HCl, pH 7.6, and 100 mM NaCl. Cells were finally resuspended in buffer A (50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 5% glycerol) and lysed by sonication. The resulting extracts were clarified by low-speed centrifugation (2,500 x g for 10 min) and the supernatant was centrifuged again (100,000 x g for 30 min). The pelleted membrane fraction was resuspended in buffer A and proteins in this fraction and in the supernatant fraction were resolved by SDS-PAGE and subsequently visualized by Coomassie-blue staining.
RESULTS

Avian reovirus causes membrane leakiness at late infection times

To analyze membrane permeability changes in response to avian reovirus infection, several assays were performed. First, the ability of hygromycin B (HB) to cross the membrane of avian reovirus-infected cells was investigated at different infection times. HB, which does not normally trespass the membrane barrier of intact cells, does penetrate cells with permeabilized membranes, causing strong inhibition of intracellular protein synthesis; it is therefore widely used to study changes in membrane permeability (1). The results shown in Fig. 2A indicate that whereas HB was unable to cross the plasma membrane of both uninfected CEF (Figs. 2A and 2B, compare lanes 1 and 2) and early-infected CEF (Figs. 2A and 2B, lanes 3-6), it readily penetrated S1133-infected CEF at late infection times, blocking protein synthesis (Figs. 2A and 2B, compare lanes 7 and 8). These results indicate that viral infection induces enhanced membrane permeability at late, but not early, infection times. In order to determine whether the perturbed plasma membrane also allows increased efflux of compounds, we next examined the leakage of radioactivity from [3H]uridine-preloaded cells. As can be observed in Fig. 2B, a substantially greater amount of radioactivity was released from infected cells at 9 hpi than at 0 or 2 hpi. These data clearly illustrate that the plasma membrane of avian reovirus-infected cells is permeabilized bidirectionally at late infection times, allowing increased entry and exit of metabolites.
Membrane permeabilization by avian reovirus requires viral protein synthesis and protein translocation through the vesicular system

We next performed experiments to assess whether newly synthesized viral proteins are required for the late permeability changes induced by avian reovirus. First of all, we checked whether the onset of viral protein synthesis and membrane modification are concurrent, by performing infections at two different multiplicities of infection (Fig. 3). When cells were infected with a multiplicity of 5 PFU/cell, viral protein synthesis and enhanced permeability to HB were both first detected at 9 hpi, as shown by \(^{35}\)S-metabolic labeling and immunoblot analysis of the nonstructural viral protein \(\mu\)NS in HB-treated and untreated cells (Figs. 3A and 3B). Increasing the multiplicity of infection up to 100 PFU/cell accelerated both processes; the onset of both viral protein synthesis and membrane leakiness were now detectable at 6 hpi (Figs. 3C and 3D). The fact that the onsets of these two events are concurrent suggests a cause-effect relationship.

To confirm this, membrane permeabilization to HB was analyzed in infected cells cultured in the presence of ribavirin. Ribavirin is a guanosine nucleoside analog that has been shown to inhibit avian and mammalian reovirus gene expression, at the level of virus transcription, without affecting the expression of cellular genes (9, 22). We have previously shown that the presence of ribavirin in the culture medium of uninfected CEF cells, at concentrations up to 400 \(\mu\)M, does not affect either the viability of the cells for at least three days or their protein synthesis capacity, analyzed 16 h after addition of the inhibitor. In the same study we also showed that ribavirin, when added to avian reovirus-infected cells at the start of the infection, does not prevent viral uncoating, indicating that the drug does not
affect the capacity of the avian reovirus to penetrate and uncoat within endosomes. The results shown in Figs. 4A and 4B reveal that ribavirin does not inhibit protein synthesis in uninfected cells (compare lanes 1 and 2), but induces a drastic reduction in the synthesis of viral polypeptides, but not cell polypeptides, in avian reovirus-infected cells (compare lanes 5 and 6). This result demonstrates that ribavirin is a specific inhibitor of reoviral, but not cellular, gene expression. Analysis of HB internalization in ribavirin-treated cells showed that, whereas ribavirin did not promote the entry of HB into uninfected cells (Fig. 4A, compare lanes 3 and 4), its presence prevented HB internalization in late-stage infected cells (Fig. 4A, compare lanes 7 and 8). Likewise, HB internalization did not take place in cells infected with UV-treated, replication-incompetent reovirions (data not shown). Together, these results demonstrate that viral gene expression is required for avian reovirus to permeabilize the host plasma membrane, and also suggest that late membrane leakiness is induced by a viroporin.

Since viroporins are transmembrane proteins that exert their permeabilizing effect when inserted into the cell membrane, we next investigated whether an active exocytic route is required for membrane permeabilization in avian reovirus-infected cells. With this end, we investigated the effect of brefeldin A on the capacity of HB to penetrate late-stage infected cells (Fig. 4C). Brefeldin A is a macrolide antibiotic that has been shown to inhibit vesicle transport to the cell surface, by causing the resorption of the Golgi complex into the endoplasmic reticulum (23-25). At the concentration of 5 µg/ml, brefeldin A did not inhibit protein synthesis in uninfected CEF (compare lanes 1 and 2), and caused only a minor reduction in protein synthesis in avian reovirus-infected cells (compare lanes 5 and 6).
Furthermore, the presence of brefeldin A only induced a slight decrease in the intracellular production of infectious viral particles (data not shown), indicating that this antibiotic hardly affects virus replication and reovirion assembly. Similar results regarding the effect of brefeldin A have been reported previously for the replication of avian reovirus 176 in the continuous quail fibrosarcoma cell line QT6 (26). However, the presence of brefeldin A abrogated the ability of the virus to induce both membrane permeabilization to HB (Fig. 4C, compare lanes 7 and 8) and syncytia formation (data not shown), indicating that an active exocytic system is required for avian reovirus to induce these alterations.

Modification of membrane permeability by avian reovirus p10 protein

We have recently shown that the two nonstructural proteins, p10 and p17, encoded by the tricistronic avian reovirus S1 gene become associated with cell membranes in both prokaryotic and eukaryotic cells (11). A priori, then, these two proteins appear to be good candidates for involvement in membrane permeabilization. To assess the permeabilizing activity of the S1-encoded proteins, we first used an inducible Escherichia coli expression system that has been shown to be suitable for the characterization of other membrane-active viral proteins. In this study we used E. coli BL21(DE3)pLysE cells, which contain an IPTG-inducible T7 RNA polymerase gene within the chromosome as well as the pLysE plasmid that constitutively expresses high levels of the T7 phage lysozyme (27). This system, therefore, allows us to test for permeabilization of the inner bacterial membrane, since when intact, intracellular lysozyme cannot reach the cell wall, whereas a perturbed membrane will allow lysozyme to gain access to and degrade the cell wall peptidoglycan, giving rise to rapid
Membrane permeabilization by avian reovirus p10 protein

cell lysis.

As a first approach to analyzing the effect of the three S1-encoded proteins on bacterial membranes, their open reading frames were individually cloned into the maltose-binding protein (MBP) gene fusion vector, pMalC, and the resulting recombinant plasmids were used to transform BL21(DE3)pLysE E. coli cells. Electrophoretic analysis of extracts from recombinant bacteria showed that MBP and all three MBP-fused polypeptides were expressed in IPTG-induced bacteria (Fig. 5C, odd lanes), but not in uninduced bacteria (data not shown). The growth rates of the transformed bacterial cells were analyzed spectrophotometrically by measurement of optical density at 600 nm. The results shown in Fig. 5A revealed that, while bacteria transformed with pMalC, pMalC-ORF3, or pMalC-ORF2 grew exponentially during the 180-min period following IPTG induction, drastic arrest of cell growth followed IPTG induction of pMalC-ORF1-transformed bacteria. This finding indicates that expression of p10 enhances the permeability of the inner membrane to intracellular lysozyme, giving rise to rapid cell lysis. On the other hand, the expression of MBP-p10, but not of the other two MBP-fused proteins, promoted the release of substantial amounts of radioactivity from [3H]uridine-preloaded cells (Fig. 5B) and rendered cells susceptible to HB inhibition (Fig. 5C, compare odd lanes with even lanes).

To gather more evidence on the permeabilization activity of p10, we next compared the capacity of p10 and p17 to induce membrane leakiness in eukaryotic cells. First of all, the p17- and p10-encoding ORFs were individually cloned into the eukaryotic expression vector pCIneo, and the resulting recombinant plasmids were lipofected into monolayers of BSC-40 monkey cells. The Western blot analysis shown in Figs. 6A and 6B demonstrates
that the two proteins were synthesized in transfected cells, as previously reported (11). A subsequent electrophoretic analysis of $^{35}$S-radiolabeled cell extracts revealed that, while HB could readily penetrate cells transformed with pClneo-ORF1 (Fig. 6C, lane 3), this inhibitor was unable to cross the plasma membrane of cells lipofected with either the empty vector or the pClneo-ORF2 recombinant vector (Fig. 6C, lanes 1 and 2). Furthermore, the amount of radioactivity released from $[^3H]$uridine-preloaded cells was much greater in pClneo-ORF1-transformed cells than in cells lipofected with either pCineo or pClneo-ORF2 (Fig. 6D). These data demonstrate that p10, but not p17, possesses permeabilizing activity in both eukaryotic and prokaryotic cells.

The fusogenic domain of p10 is dispensable for its membrane permeabilizing activity

It has been recently shown that p10 is a type I transmembrane protein that causes cell-cell fusion when transiently expressed in mammalian cells, and that deletion of a region of its extracellular N-terminal domain abrogates the fusogenic activity of p10, without affecting its ability to associate with cell membranes (15). As a first approach to assessing whether this “fusogenic sequence” is also involved in membrane permeabilization, a 5’-truncated version of the ORF1 (ORF1*) was cloned into the prokaryotic expression vector pMalC or the eukaryotic vector pClneo. The resulting recombinant vectors pMalC-ORF1* and pClneo-ORF1*, as well as their corresponding empty vectors and the recombinant vectors containing nontruncated ORF1 inserts, were then introduced into prokaryotic or eukaryotic cells.
Membrane permeabilization by avian reovirus p10 protein

The experiments carried out with bacterial cells are shown in Fig. 7. Both electrophoretic and immunoblot analysis of cell extracts demonstrated that MBP, MBP-p10 and MBP-p10* were all expressed in the corresponding transformed bacteria after IPTG induction (Figs. 7A and 7B, lanes 2, 6 and 10), but not in uninduced bacteria (lanes 1, 5 and 9). Fractionation of the extracts from induced bacteria (I) into supernatant (S) and pellet (P) fractions revealed that while most MBP is associated with the soluble fraction (Figs. 7A and 7B, compare lanes 3 and 4), both MBP-p10 and MBP-p10* segregated almost exclusively with the pelleted fraction (Figs. 7A and 7B, compare lanes 7 and 11 with lanes 8 and 12), suggesting that they become associated with the bacterial membrane. Furthermore, IPTG induction of bacteria transformed with pMalC-ORF1 and with pMalC-ORF1*, but not with the empty plasmid pMalC, caused arrest of cell growth (Fig. 7C), suggesting that both p10 and p10* facilitate access of the intracellular lysozyme to the cell wall, by permeabilizing the inner membrane. Finally, both HB internalization and leakage of radioactivity from [3H]uridine-preloaded bacteria were observed in cells transformed with the two recombinant plasmids, but not in cells transformed with the empty plasmid pMalC (Figs.7D and 7E). From these results, we conclude that both p10 and p10* have the ability to permeabilize bacterial membranes.

A similar conclusion was reached from the results of the experiments performed with eukaryotic cells (Fig. 8). Thus, while p17, p10 and p10*, but not σC, segregate exclusively with the membrane fraction when transiently expressed in BSC-40 monkey cells (Fig. 8A), only p10 and p10* are able to promote both HB internalization and uridine release (Figs. 8B and 8C). Taken together, these results demonstrate that the fusogenic amino-terminal
Membrane permeabilization by avian reovirus p10 protein

fragment of p10 is dispensable for membrane association and for membrane permeabilization. However, this fragment is indeed required for the fusogenic activity of p10, since its deletion abrogated the syncytia formation capacity of p10 (Fig. 8D), as previously reported (15).
DISCUSSION

The alteration of membrane permeability is a common feature of infection by cytolytic animal viruses, and such alterations not only exert profound effects on the metabolism of the infected cell, but also contribute to the development of the cytopathic effect. Early alterations of the membrane allow low-molecular-weight compounds as well as macromolecules to enter cells together with viral particles. Late membrane leakiness requires virus gene expression and allows the passage of ions and small molecules, but not macromolecules (reviewed in (1)).

In the first part of the present study we examined host membrane alterations in response to avian reovirus infection. We found that the plasma membrane of S1133-infected CEF becomes permeabilized at late, but not early, infection times, and that the perturbed membrane allows increased influx and efflux of metabolites. Enhanced membrane permeability at late infection times has been also observed in cells infected with flaviviruses (28), herpexviruses, rhabdoviruses and paramyxoviruses (29), papovaviruses (30), picornaviruses (31), retroviruses (32, 33) and togaviruses (34).

The absence of increased permeability at early infection times, and the dependence of membrane leakiness on both virus gene expression and intracellular protein trafficking, strongly suggest that viroporins are involved in avian reovirus-induced permeabilization. Accordingly, in the second part of our study we tried to identify avian reovirus proteins displaying viroporin activity. We focused on the proteins encoded by the tricistronic S1 genome segment because two of them, the nonstructural proteins p10 and p17, have been...
Membrane permeabilization by avian reovirus p10 protein

shown recently to associate with the membranes of both prokaryotic and eukaryotic cells (11), and also because p10 has been shown both to display fusogenic activity (11, 15) and to share structural characteristics with other membrane-active viral proteins (see below). Our results demonstrate that whereas both nonstructural proteins p10 and p17, but not σC, are entirely associated with prokaryotic and eukaryotic cell membranes, only the expression of p10 is able to induce enhanced membrane permeability to HB and uridine. Furthermore, p10, but not p17 or σC, inhibited cell growth in the E. coli system, suggesting that p10 induces disorganization of the inner bacterial membrane, and hence allows cytoplasmic lysozyme to reach and digest the peptidoglycan layer of the outer cell wall. Taken together, these results indicate that p10 is an avian reovirus viroporin, and also strongly suggest that p10 is the protein involved in modifying membrane permeability in avian reovirus-infected cells. In support of this possibility, we found that the onsets of membrane permeabilization to HB and p10 synthesis, in cells infected with different avian reovirus multiplicities, were concurrent (data not shown). Several proteins from different viruses have also been reported to possess viroporin activity, including poliovirus 2BC and 3AB (19, 21, 35), adenovirus E3-11.6K (36), togavirus 6K protein (37), coxsackie B3 virus 2B (38), hepatitis C virus E1 protein (39), influenza M2 (40), picornavirus 3A and 2B (41), hepatitis A virus 3A, 2B and 2BC (42, 43), human immunodeficiency virus type-1 gp41 and Vpu (32, 33), rotavirus NSP4 (44), African horsesickness virus NSP3 (45), human respiratory syncytial virus small hydrophobic protein (20), and small hydrophobic nonstructural proteins of Japanese encephalitis virus (28).

In contrast to most nonenveloped viruses, including mammalian reoviruses, avian
reoviruses induce syncytia formation, an activity related to the nonstructural p10 protein. Furthermore, this fusion activity appears to be located at the extracellular amino terminus of p10, since deletion of its extreme N-terminal fragment abrogates the ability of p10 to cause cell-cell fusion, without affecting its capacity to associate with membranes (15). Therefore, it was important to assess the implication of this fragment in the permeabilizing activity of p10. The results of the present work indicate that the fragment containing the initial N-terminal 22 amino acid residues of p10, although necessary for fusogenic activity, is not required for membrane permeabilization, since its deletion does not affect the capacity of p10 to associate with cell membranes or to trigger permeability changes in either prokaryotic or eukaryotic cells. This finding suggests that syncytium formation and membrane destabilization are associated with different domains of the p10 protein, and therefore that they are unrelated phenomena. Furthermore, these two events are not concurrent, since membrane permeabilization always precedes syncytia formation in infected and transfected cells (data not shown). This situation resembles that of the immunodeficiency virus type 1 transmembrane glycoprotein gp41, which contains an amino-terminal domain involved in membrane fusion and syncytia formation, while both the membrane-spanning region and sequences located at the carboxy terminus are involved in increasing membrane permeability (32).

In addition to its permeabilizing activity, p10 also shares several structural and physical characteristics with other viroporins. Thus, p10 and all reported viroporins are small hydrophobic integral membrane proteins of 50-120 amino acid residues containing at least one transmembrane hydrophobic domain of 20-30 residues and an intracellular region rich
Membrane permeabilization by avian reovirus p10 protein

in basic residues that is adjacent to the transmembrane domain (reviewed in (1)). Furthermore, p10 and some viroporins contain cysteine residues in their cytoplasmic regions, often positioned proximal to their membrane-spanning domains, and several of these cysteine residues have been found to be modified by palmitoylation (46-50). Interestingly, mutagenesis of the two contiguous cysteines 63 and 64 to alanines, and of lysine 69 to methionine, was reported to abolish the capacity of avian reovirus p10 protein to induce syncytia formation but not to associate with membranes (15). Finally, most viroporins have been shown to form hydrophilic pores by oligomerization, thus allowing ions and low-molecular-weight hydrophilic compounds to diffuse through the membrane. (reviewed in (1)). Whether p10 likewise oligomerizes, whether the two contiguous cysteines are palmitoylated, and whether these cysteines and/or the basic residues play a role in the permeabilizing activity of p10 merit further studies.

The hydrophilic channels formed by viroporin activity allow low-molecular-weight hydrophilic molecules to cross the membrane, and hence membrane potential is disrupted, ionic gradients collapse and essential compounds are released from the cell (1, 2, 51). The alterations in ion concentration occurring in the cytoplasm of virus-infected cells could favor virus replication, by enhancing the intracellular concentration of sodium ions. This might promote translation of viral versus cellular mRNAs, since translation of mRNAs from many cytolytic animal viruses, including reoviruses, is fairly resistant to high sodium concentrations, whereas high sodium concentrations are inhibitory for the translation of most cellular mRNAs (52-57). On the other hand, progressive membrane damage is thought to promote cell lysis and virus release, facilitating virus spreading to surrounding cells (36, 58).
Curiously, our results and those of Duncan et al (26) demonstrate that membrane permeabilization and syncytia formation are not essential steps in the avian reovirus replication cycle, since their inhibition by brefeldin A was not accompanied by a significant reduction in either viral protein synthesis or infectious progeny virus production. However, the presence of brefeldin A retards and partially inhibits avian reovirus-induced cytopathic effects and virus release (26), suggesting that membrane permeabilization and syncytia formation, although not strictly required, can accelerate the production of a lytic-type infection. This, and the fact that both the p10 ORF and the syncytial phenotype are conserved among all known avian reovirus isolates as well as two atypical mammalian reoviruses, Nelson Bay virus and baboon reovirus, suggests that expression of the p10 protein could confer some selective advantages to the virus, such as enhanced virus spreading in infected animals. Assessing the role that the p10 protein plays in the replication cycle of avian reovirus is problematic, since no method has yet been developed to manipulate the reoviral genome in order to generate mutant viruses that do not express a particular protein or that express mutated proteins.

Acknowledgments
Membrane permeabilization by avian reovirus p10 protein

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Membrane permeabilization by avian reovirus p10 protein


Membrane permeabilization by avian reovirus p10 protein


Membrane permeabilization by avian reovirus p10 protein


Membrane permeabilization by avian reovirus p10 protein

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Membrane permeabilization by avian reovirus p10 protein

FOOTNOTES

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1 The abbreviations used are: ORF, open reading frame; CEF, chicken embryo fibroblasts; HB, hygromycin B; IPTG, isopropyl-thio-β-D-galactoside; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PFU, particle forming units; UV, ultraviolet light.
Membrane permeabilization by avian reovirus p10 protein

FIGURE LEGENDS

Figure 1 - Structure of the S1 genome segment and of the p10 protein of avian reovirus S1133.

(A) Schematic representation of the ORF distribution of the tricistronic S1133 S1 gene. The positions of the start and stop codons are indicated at the ends of the corresponding ORFs. The name, number of amino acid residues and molecular mass of the proteins encoded by each ORF are indicated within boxes.

(B) Amino acid sequences of p10 protein and of a deleted version of p10 lacking its first 22 N-terminal amino acid residues (p10*). The predicted transmembrane, extracellular and intracellular domains of these proteins are indicated within boxes in the middle of the two sequences. Basic amino acids located in the intracellular region adjacent to the transmembrane domain are dotted and cysteine residues marked with an asterisk.

Figure 2 - Membrane permeability assays.

(A) Mock-infected (CEF) and avian reovirus-infected cells (50 PFU/cell; S1133-CEF) were incubated, at the indicated times after infection, for 45 min in the presence (even lanes) or absence (odd lanes) of 1.5 mM hygromycin B (HB). Cells were then labeled with [35S]methionine-cysteine for another 45 min in the presence of HB. Cell extracts were prepared and analyzed by SDS-PAGE and autoradiography. Positions of molecular size markers are indicated on the left and positions of the three size classes of avian reovirus polypeptides are indicated on the right.
(B) Cells were pre-incubated in medium containing 2 μCi/ml of [5,6-3H]uridine for 14 h, then infected. At the indicated infection times, the radioactivity released to the medium was measured. The values shown are means of four independent experiments, and error bars indicate standard deviations of the mean.

Figure 3 - **Effects of viral multiplicity on viral protein synthesis and membrane permeability.** Cells were infected with either 5 PFU/ml (panels A and B) or 100 PFU/ml (panels C and D) of avian reovirus S1133, and incubated with (even lanes) or without (odd lanes) 1.5 mM HB for 45 min, at the times indicated on top.

(A and C) Cells were then labeled with [35S]methionine-cysteine for another 45 min in the presence of the inhibitor, then lysed. Total cell extracts were analyzed by SDS-PAGE, with visualization of proteins by autoradiography. Positions of molecular mass markers are indicated on the left of panel A, and positions of the three size classes of avian reovirus polypeptides are indicated between the two panels.

(B and D) Nonradiolabeled cell extracts were subjected to immunoblot analysis with anti-μNS rabbit polyclonal antibodies.

Figure 4 - **Effects of ribavirin and brefeldin A on membrane permeability.** Mock-infected cells (CEF) and avian reovirus-infected cells (S1133-CEF) were incubated for 9 h in the presence (even lanes) or absence of 200 μM ribavirin (Rib.; panels A and B) or 5 μg/ml brefeldin A (BA; panel C). The culture medium was then supplemented (lanes +) or not
(lanes -) with 1.5 mM HB, and incubated for another 45 min.

(A and C) Cells were subsequently labeled with $[^{35}\text{S}]$methionine-cysteine for 45 min, then lysed, and total cell extracts were analyzed by SDS-PAGE and autoradiography. Positions of molecular mass markers are indicated on the left of panel A and positions of the three size classes of avian reovirus polypeptides are indicated between the two panels.

(B) Nonradiolabeled cells were lysed, and the resulting cell extracts were subjected to immunoblot analysis with a rabbit polyclonal anti-µNS antiserum.

Figure 5 - Effects of the three S1-encoded proteins on bacterial membrane permeability. E. coli BL21(DE3)pLysE cells carrying the plasmids indicated within the box were induced with 1 mM IPTG.

(A) The optical densities of cell cultures at 600 nm were measured at the indicated times post-induction.

(B) Cells that had been preloaded with [5,6-$^3$H]uridine were IPTG-induced, and at the indicated times post-induction the radioactivity released to the medium was measured. The values shown are means of four independent experiments.

(C) One hour after induction, cells transformed with pMalC (lanes 1 and 2) or with one of the pMalC recombinant plasmids containing the inserts shown on top (lanes 3-8) were incubated for 20 min with $[^{35}\text{S}]$methionine-cysteine in the absence (odd lanes) or presence (even lanes) of 0.4 mM HB. Cells were then lysed, and the extracts analyzed by SDS-PAGE and autoradiography. Positions of MBP and of MBP-fused proteins are indicated on the
sides of the figure.

Figure 6 - Permeabilization activity of p10 and p17 expressed in eukaryotic cells.
Semiconfluent monolayers of BSC-40 cells were lipofected with the recombinant expression vectors pCIneo, pCIneo-ORF1 or pCIneo-ORF2, and incubated for 20 h.
(A and B) Cells were then lysed and subjected to immunoblot analysis with polyclonal antibodies raised against the recombinant proteins MBP-p10 (panel A) and His-p17 (panel B).
(C) Cells were then incubated for 45 min in the presence of 1.5 mM HB, and for another 45 min in the presence [35S]methionine-cysteine. Total cell extracts were then prepared and analyzed by SDS-PAGE and autoradiography. Positions of molecular mass markers are indicated on the left.
(D) The radioactivity released to the medium from [5,6-3H]uridine-preloaded cells was measured. The values shown are means of four independent experiments, and error bars indicate standard deviations of the mean.

Figure 7 - Permeability changes induced by expression of p10 and p10* in bacterial cells.
(A and B) Overnight cultures of E. coli BL21(DE3) cells carrying the plasmids pMalC (-), pMalC-ORF1 (ORF1) or pMalC-ORF1* (ORF1*) were induced with IPTG (lanes I) or not (lanes U), and 30 min later cells were lysed and the extracts fractionated into supernatant (S) and pellet (P) fractions by centrifugation. Samples were then analyzed by SDS-PAGE and...
proteins were either visualized by Coomassie blue staining (panel A) or transferred to a PVDF membrane and subjected to immunoblot analysis with a polyclonal anti-MBP antiserum (panel B).

(C) The optical densities at 600 nm of cultures of *E. coli* BL21(DE3)pLysE transformed with the plasmids indicated within the box were measured at the indicated times post-induction. Values shown are means of three independent experiments.

(D) One hour after induction, transformed *E. coli* BL21(DE3)pLysE cells were incubated for 20 min with \([^{35}\text{S}]\)methionine-cysteine in the presence (even lanes) or absence (odd lanes) of 0.4 mM HB. Cells were then lysed, and the extracts analyzed by SDS-PAGE and autoradiography. Positions of MBP-p10 and of MBP-p10* are indicated on the right.

(E) Transformed *E. coli* BL21(DE3)pLysE cells that had been preloaded with [5,6-\(^3\)H]uridine were induced with IPTG and the radioactivity released to the medium was measured at the indicated times post-induction. Values shown are means of three independent experiments.

Figure 8 - **Permeabilization and fusogenic activities of p10 and p10* expressed in eukaryotic cells.**

(A) Immunoblot analysis of soluble (S) and membrane (M) fractions prepared from extracts of BSC-40 cells lipofected with pCIneo-ORF3 (σC), pCIneo-ORF2 (p17), pCIneo-ORF1 (p10) or pCIneo-ORF1* (p10*). Protein σC was detected with anti-σC, protein p17 with anti-His-p17 and proteins p10 and p10* with anti-MBP-p10 polyclonal antiserum.
Membrane permeabilization by avian reovirus p10 protein

(B) At 20 h post-transfection, lipofected cells were incubated for 45 min with 1.5 mM HB, and for another 45 min in the presence [\(^{35}\)S]methionine-cysteine. Cells were then lysed and total cell extracts were analyzed by SDS-PAGE and autoradiography. Positions of molecular mass markers are indicated on the left.

(C) Cells that had been lipofected with the plasmids shown on top were visualized at 36 hpt by microscopy under visible light after staining with Giemsa. Lanes (-): cells were mock-transfected with lipofectin.
A

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hpi
HB (1.5 mM)

B

Extracellular cpm x 10^4

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Modification of late membrane permeability in avian reovirus-infected cells: viroporin activity of the s1-encoded nonstructural p10 protein

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