Expression of Poliovirus Nonstructural Proteins in Escherichia coli Cells

MODIFICATION OF MEMBRANE PERMEABILITY INDUCED BY 2B AND 3A*

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The poliovirus nonstructural proteins 2B, 2C, 2C3A, 2C3AB, 3A, and 3AB have been cloned and efficiently expressed in Escherichia coli cells. Each individual protein, or combinations of some of them, were cloned using polymerase chain reaction techniques and correspond to the genuine poliovirus protein plus an additional methionine. The system used to express them uses PET vectors containing the promoter of gene 10 of phage T7. Expression of protein 2C in BL21(DE3) pLysS cells, which express the T7 lysozyme, is not toxic, and the bacteria synthesize this protein for several hours after induction. In contrast, the expression of proteins 2B, 3A, or 3AB is not tolerated by BL21(DE3) pLysS cells which could make them only for a limited period of time. Protein 3AB was particularly toxic and induced a rapid lysis of the recombinant clone after its induction with isopropyl-1-thio-β-D-galactopyranoside alone or with both isopropyl-1-thio-β-D-galactopyranoside and rifampicin. Further analyses showed that 3AB induced profound modifications in membrane permeability to o-nitrophenyl-β-D-galactopyranoside, labeled uridine, and nonpermeant translation inhibitors. Cloning and expression of proteins 2B, 3A, and 3AB in BL21(DE3) cells that do not contain the T7 lysozyme lead to a more sustained expression of these proteins without detectable cell lysis. Changes in permeability to low molecular weight compounds such as radioactive uridine, o-nitrophenyl-β-D-galactopyranoside, and hygromycin B readily appeared upon induction of 2B, 3A, and 3AB. Our results indicate that the poliovirus nonstructural polypeptides 2B and 3A (or 3AB) are lytic for the bacteria. In fact, both proteins 2B and 3A contain hydrophobic domains in a potential amphipathic helix; this is one characteristic shared with a number of membrane-active peptides.

The poliovirus genome is a single-stranded RNA molecule of positive polarity (1, 2). Four structural proteins (VP1, VP2, VP3, and VP4) are encoded at the 5' end of the genome, and the remaining two-thirds of the genome encode seven nonstructural polypeptides designated as 2A, 2B, 2C, 3A, 3B, 3C, and 3D (1). Elucidating the role that each of these polypeptides plays in the poliovirus replication cycle remains one of the major goals of current poliovirus research. It is known that proteins 2A and 3C are proteases, protein 3B (also known as VPg) is the genome-bound protein at the 5' end of the viral RNA, whereas protein 3D is the virus RNA-dependent RNA polymerase (1–3). Much less is known about the function of proteins 2B, 2C, and 3A. Protein 2C is involved in the replication of poliovirus genomes (4) and anchors the RNA replication complexes to the membranes of the vesicles with which they are associated (5). 3A is also a membrane-bound protein, but no catalytic activities have been reported for 2B, 2C, or 3A proteins. It is possible that some of these polypeptides are involved in the modification of membranes during poliovirus infection (6–8). Thus, poliovirus induces profound changes in the permeability of membranes to cations and low molecular weight compounds (9–11). The molecular basis of this phenomenon has been investigated in detail (7, 12–14), but the exact poliovirus protein(s) involved remains to be elucidated.

Infectious poliovirus cDNA clones opened the possibility to manipulate the genome of this important human pathogen more easily (15). Because poliovirus has only one open reading frame, all the viral polypeptides synthesized in virus-infected cells are derived from a single polyprotein (2, 3). Mature viral proteins are processed by proteolytic cleavage accomplished by the two virus-encoded proteases, 2Apro and 3Cpro, that are active as precursors and are able to cleave themselves to produce the final mature protein (3). Much effort has been concentrated on the cloning of four major regions of the poliovirus genome: 1) the 5' noncoding region (16–18); 2) the structural proteins, particularly VP1, where most antigenic determinants are located (19, 20); 3) the poliovirus proteases 2Apro and 3Cpro, which are able to cleave poliovirus protein substrates in cis or trans (21, 22); and 4) the viral polymerase (3Dpol) (23, 24). Active polymerase has been obtained by cloning a genome fragment encoding protein 3CD. The precursor made is able to cleave itself to release authentic polymerase (23). In addition, 3Dpol has been expressed in an inducible manner in Escherichia coli (24).

In an effort to understand the function of poliovirus proteins 2B, 2C, and 3A, we have cloned and expressed them to high levels in E. coli cells. The system we have used was first described by Studier and Moffatt (25) and uses vectors containing the gene of interest under the control of the φ10 promoter of phage T7 (25–27). The E. coli cells carry in the chromosome the integrated T7 RNA polymerase gene under the control of the IPTG1-inducible lac UV5 promoter (25–27). This system was developed to express toxic genes in E. coli based on the low levels of basal expression that occur in cells expressing T7 lysozyme, a natural and selective inhibitor of

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1 The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactopyranoside; SDS, sodium dodecyl sulfate; ONPG, o-nitrophenyl-β-D-galactopyranoside; PAGE; polyacrylamide gel electrophoresis.
T7 RNA polymerase. We considered it to be of interest to express the poliovirus nonstructural proteins in the inducible system described above and to analyze changes in membrane permeability induced by some of these viral polypeptides. Our results indicate that the poliovirus proteins, i.e. 2B and 3A, modify membrane permeability and are lytic for the bacteria.

**MATERIALS AND METHODS**

**Induction of Recombinant Bacteria—Single clones of BL21(DE3) cells (26), which inducibly express T7 RNA polymerase, or BL21(DE3) pLysS cells (26), which also express T7 lysozyme in a constitutive manner, containing the indicated plasmid, were grown overnight at 37 °C in LB medium in the presence of 100 μg/ml ampicillin and 34 μg/ml chloramphenicol in the case of pLysS cells. Then the cells were diluted 100-200-fold in M9 medium (26) supplemented with 0.2% glucose and antibiotics. When the cultures reached A600 nm of 0.6-0.8 they were induced by the addition of 0.5 mM IPTG. Rifampicin (Sigma) was used at 150 μg/ml to inhibit transcription by E. coli RNA polymerase.

**Labeling of Bacterial Proteins—** For labeling the proteins synthesized, aliquots of cultures were collected and incubated with 2 μCi/ml [35S]methionine (1.45 Ci/mmol, Amersham Corp.) for 1 h. After harvesting, the cells were pelleted and washed twice with uridine-free, prewarmed growth medium. Then the cells were resuspended in the initial volume of growth medium and incubated at 37 °C. Fifteen minutes later the cells were induced to express the target proteins. At given times 0.2-ml aliquots were removed and pelleted, and the cells were washed with 1 M salicylic acid, and after drying the gels were exposed to XAR films (Kodak) at −70 °C.

**Uridine Loading of E. coli Cultures—** Cells were grown as described above and 90 min before induction were loaded with 2 μCi/ml [3H]uridine (27.3 Ci/mmol, Amersham Corp.) for 1 h. After harvesting, the cells were pelleted and washed twice with uridine-free, prewarmed growth medium. Then the cells were resuspended in the initial volume of growth medium and incubated at 37 °C. Fifteen minutes later the cells were诱导 to express the target proteins. At given times 0.2-ml aliquots of culture were removed and pelleted, and the cells were washed with 1 M salicylic acid, and after drying the gels were exposed to XAR films (Kodak) at −70 °C.

**β-Galactosidase Assays—** At given times 0.5 ml of culture grown as described above was removed and added to 0.5 ml of growth medium supplemented with 50 μg/ml streptomycin to stop translation. The cells were centrifuged for 1 min at 12,000 rpm in an Eppendorf microcentrifuge. To measure extracellular β-galactosidase, 0.2 ml of 12 mM o-nitrophenyl-β-D-galactopyranoside (ONPG) was added to the supernatant. Reactions were carried out for 10 min at 30 °C and stopped by the addition of 0.4 ml of 1 M sodium carbonate. The absorbance at 420 nm was measured. The reaction was stopped by the scattering of light by intact cells, and absorbance at 420 nm was measured. The reaction was stopped by the scattering of light by intact cells.

**Construction of Expression Plasmids—** Construction of the vectors encoding the different poliovirus proteins to be expressed in bacteria was accomplished by digestion of plasmids from positive clones, DNAs from DH5, E. coli cells, and extensive restriction analysis and sequencing of the plasmids. The poliovirus proteins 2B, 2C, 3A, and 3AB were cloned in PET plasmids (26) using polymerase chain reaction techniques (29). In spite of the great interest in elucidating the function of these poliovirus products, to our knowledge none of these poliovirus proteins has ever been expressed efficiently in E. coli, perhaps due to toxicity.

**RESULTS**

**Expression of Nonstructural Poliovirus Proteins in E. coli—** The poliovirus proteins 2B, 2C, 2C3A, 2C3AB, and 3AB were cloned in PET plasmids (26) using polymerase chain reaction techniques (29). In spite of the great interest in elucidating the function of these poliovirus products, to our knowledge none of these poliovirus proteins has ever been expressed efficiently in E. coli, perhaps due to toxicity.

**Induction of Recombinant Clones—** The plasmid is similar to the T7 expression plasmids used by us for the recombinant clones bearing plasmid pT7.2C leads to the synthesis of a very prominent polypeptide that does not migrate with authentic protein 2C from poliovirus-infected HeLa cells. Addition of IPTG and rifampicin (a selective inhibitor of bacterial, but not T7, RNA polymerase) gives rise to almost the exclusive synthesis of 2C-related polypeptides after 30 min of induction and stops 3 h later. Immunoblot analysis of the polypeptides made in the presence of IPTG using an anti-2C antiserum detected, in addition to 2C, other smaller proteins corresponding to the labeled bands (results not shown). The origin of these smaller products is still unknown and may involve premature termination. This possibility is supported not only by the immunoblot analyses, but also by the fact that similar proteins are detected in the recombinant clones that express 2C3A and 2C3AB but not in the clones that synthesize another protein (see below). The expression of 2C3A and 2C3AB follows kinetics similar to 2C.

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Fig. 1. Induction of 2C, 2C3A, and 2C3AB proteins in BL21(DE3) pLysS E. coli cells. BL21(DE3) pLysS cells containing the plasmids pT7.2C (2C), pT7.2C3A (2C3A), or pT7.2C3AB (2C3AB) were grown, induced with 0.5 mM IPTG, and labeled at the postinduction hours (h) indicated with [35S]methionine as described under "Materials and Methods." 150 μg/ml rifampicin (+RIF) was added to the cultures after 20 min of addition of IPTG. Labeled proteins from infected HeLa cells (P) were used as molecular weight markers. Electrophoresis was carried out in 15% polyacrylamide gels with 0.1% SDS (SDS-PAGE). Target proteins have been underlined.

Fig. 2. Induction of 2B, 3A, and 3AB in BL21(DE3) pLysS E. coli cells. BL21(DE3) pLysS cells containing the plasmids pT7.2B (2B), pT7.3A (3A), or pT7.3AB (3AB) were grown, induced with 0.5 mM IPTG, and labeled at the postinduction hours (h) shown with [35S]methionine as described in the legend of Fig. 1. Electrophoresis was carried out in 20% polyacrylamide gels with 0.1% SDS (SDS-PAGE). Labeled proteins from infected HeLa cells (P) were used as molecular weight markers. The position of recombinant proteins is shown. RIF, rifampicin.

Modification of E. coli Membrane Permeability by the Expression of Protein 3AB—Poliovirus protein 3AB is associated with membranes and is thought to act as a donor of protein 3BV₃ (30, 31). In fact, 3AB is one of the most hydrophobic and basic proteins encoded by poliovirus, a property typical of membrane-active proteins (32). Although 3AB is a good candidate to account for the permeability changes observed in human cells infected with poliovirus (7, 8, 12), the protein(s) responsible for those changes has not yet been identified. Therefore, we were interested in determining whether the expression of 3AB in E. coli cells leads to permeability changes akin to those reported for cultured mammalian cells (6, 7). To this end bacteria were loaded with labeled uridine, and the release of radioactivity was monitored after induction of 3AB synthesis (Fig. 4A). Clearly enough, substantial amounts of radioactivity released into the medium are detected soon after induction of 3AB synthesis in the absence of rifampicin. As a control, the clone expressing 2C does not show that behavior (Fig. 4A).

Poliovirus modifies the membrane of the infected cells in such a way that not only are metabolites and ions released from the cytoplasm (9, 12, 33, 34), but compounds excluded by normal membranes, such as the antibiotics edeine and hygromycin B, enter the cell interior (10, 11, 35). To test this possibility in the bacterial clones expressing 3AB, two different assays were carried out. After removing the growth medium, bacteria were resuspended in medium containing ONPG. Therefore, the β-galactosidase activity measured was dependent on the entry of the substrate into the bacteria. The galactose analog ONPG enters cells that contain the pT7.3AB construction more readily after induction as compared with a control of cells expressing a truncated form of a T7 phage hydrophobic protein p10 (25) that accounts for more than 30% of the cellular protein (Fig. 4B), indicating that the...
Poliovirus Proteins 2B and 3A Induce Cell Lysis

Fig. 4. Permeability changes in BL21(DE3) pLysS cells after induction of 3AB expression. Panel A, BL21(DE3) pLysS cells containing pT7.3AB (Δ) or pT7.2C (○) were preloaded for 1 h with 2 μCi/ml [3H]uridine and at zero time were induced with 0.5 mM IPTG. At the postinduction times shown, the level of radioactivity in the culture medium was measured. Total radioactivity incorporated in the culture was 23,880 cpm. The 16,900 cpm released after 120 min represents about 71% of the total radioactivity. Panel B BL21(DE3) pLysS cells containing pT7.3AB (closed symbols) or pET3XA (open symbols) were induced with 0.5 mM IPTG at zero time, and at the times shown the entry of ONPG (□, ●) or β-galactosidase activity recovered from the extracellular medium (○, ●) was analyzed. Measurements are given as absorbance at 420 nm. Panel C, a culture of BL21(DE3) pLysS-pT7.3AB cells was induced with IPTG at zero time and treated (●) or not treated (○) with 0.4 mM hygromycin (Hygr.) from the beginning of induction. Cell density (absorbance at 660 nm) was measured at different postinduction times.

simple overexpression of a heterologous protein does not modify membrane permeability. The differences in the total amount of β-galactosidase activity between the two clones were not greater than 10% (data not shown). Moreover, induction of 3AB expression gives rise to increased β-galactosidase activity in the extracellular medium, probably due to cell lysis. Finally, we carried out the test for the entry of nonpermeant inhibitors. For this purpose two known nonpermeant translation inhibitors, i.e. edeine and hygromycin B (10, 11), were used. Inhibition of protein synthesis by edeine or hygromycin B was observed; however, these inhibitors did not affect translation of control or uninduced bacteria (results not shown). Interestingly, when hygromycin B is added with IPTG to E. coli cells that express 3AB, cellular lysis is prevented (Fig. 4C). The conclusion of these experiments is that the synthesis of 3AB induces a rapid permeabilization of the bacterial membrane in both directions. The influx of hygromycin B causes inhibition of 3AB synthesis and prevents lysis of the cells.

Expression of Proteins 2B, 3A, and 3AB in BL21(DE3) Cells Lacking the T7 Lysozyme—Because the BL21(DE3) pLysS cells used in the previous experiments were lysed by the expression of 3AB and to a lesser extent 3A and 2B, we reasoned that lysis could be the result of permeability changes induced by the expression of these proteins followed by lysis of the cell wall induced by the lysozyme present in pLysS cells (26, 27). Therefore, we first tried to use the BL21(DE3) cells that do not express the T7 lysozyme to analyze the synthesis of 2B, 3A, and 3AB proteins. However, the basal level of T7 RNA polymerase made it impossible to establish clones expressing these proteins. Then we used the system recently described by Dubendorff and Studier (36) that employs the lac operator-repressor system to reduce the basal levels of transcription. Addition of IPTG in this system increases the level of T7 RNA polymerase and also relieves the inhibition of the T7 promoter by lac repressor. The genes encoding proteins 2B, 3A, and 3AB were cloned in the pET11B vector and placed under the control of a hybrid T7/lac promoter-operator system. Fig. 5 shows that the expression of protein 3AB in the absence of rifampicin is sustained for at least 4 h, whereas in the presence of rifampicin synthesis of 3AB declines after 3-4 h of synthesis, as occurred with 2C synthesis in pLysS cells (see Fig. 1). Coomassie Blue staining showed that 3AB was made to higher levels in BL21(DE3) cells as compared with the BL21(DE3) pLysS cells (not shown). After 3 h of expression 3A and 2B synthesis stops and cellular protein synthesis ensues (Fig. 5). At the time when 2B is expressed, cellular protein synthesis is very much reduced, a phenomenon that cannot be explained simply by competition of the newly made 2B mRNAs. Compare the proteins synthesized by cells containing pT7lac3AB and pT7lac2B induced with IPTG alone (Fig. 5).

Analysis of cell density shows that none of these proteins was lytic for BL21(DE3) cells that lack the T7 lysozyme (Fig. 6A). Moreover, β-galactosidase activity cannot be found in the extracellular medium after induction of any of the clones (results not shown). Because the expression of these proteins did not lyse the cells, this constituted a good system to analyze permeability changes of the membrane without cell wall digestion. Thus, we first tested the leakage of radioactivity from [3H]uridine-preloaded cells (Fig. 6B) or ONPG entry (Fig. 6C). Compared with the control bacteria containing the parental vector pET11B, expression of the three proteins 2B, 3A, and 3AB drastically modified membrane permeability as assessed by these tests.

Poliovirus, like other cytoplasmic viruses, increases membrane permeability to the so-called nonpermeant translation inhibitors (10, 11). Hygromycin B is the most widely used nonpermeant antibiotic because this compound selectively enters cells in which membrane permeability has been altered and thus blocks translation (6, 7). Fig. 7 shows that hygromycin B does not inhibit protein synthesis in control cells containing the pET11B vector, whereas protein synthesis was strongly inhibited in the recombinant clones that express the poliovirus proteins 2B, 3A, and 3AB. The conclusion from these experiments is that expression of any of the proteins 2B, 3A, and 3AB in BL21(DE3) cells strongly modifies membrane permeability but does not induce cell lysis.
### DISCUSSION

Picornaviruses are simple viruses that encode only a few mature proteins (1-3). In spite of this simplicity the purification and elucidation of the biological properties of a number of poliovirus proteins have remained elusive (1, 3, 30). This is the case for the three poliovirus proteins known as 2B, 2C, and 3A. The results presented in this contribution represent a first step toward the cloning and efficient expression of these three polypeptides to start their purification to analyze their biological activities and assay their direct effects on cells in culture. The expression of proteins 2C, 2C3A, 2C3AB (this work), 3D (24), 2APro (31), and 3CP (37, 38) to high levels is not lytic for E. coli cells in contrast to the high toxicity and lytic properties of proteins 2B, 3A, and 3AB. In spite of that toxicity, these proteins can be cloned and expressed to high levels using the PET vectors. A recent report describes the synthesis and partial purification of 2C3AB to be used as a substrate for 3CPro, but the effects of the expression of this protein in bacteria were not analyzed (38).

A number of processes during the replication cycle of poliovirus still remain obscure (1, 2, 30). One such process is the mechanism involved in the modification of membrane permeability and the viral protein(s) involved (8, 9, 12, 39). With regard to the mechanism, it seems that a selective increase in phospholipase C activity occurs at the time when permeability is enhanced (13). However, to fully understand the way in which poliovirus modifies the membrane leading to the lysis of the infected cells we need to know which protein(s) is involved (8, 9, 12, 35). Of the 11 mature poliovirus proteins, 2 contain the most hydrophobic domains according to the hydrophobicity plot of Kyte and Doolittle, i.e. proteins 2Bootnote{F. Martinez-Abarca and L. Carrasco, unpublished results.}.
and 3A. The latter is the most basic protein according to its behavior in two-dimensional gels (40, 41). These two characteristics are typical of peptides and proteins that distort cellular membranes (32). Moreover, protein 3A contains an amphipathic helix between residues 60 and 85 as predicted by computer analyses. In fact, 3AB is associated with membranes in the infected cells (31, 42). A model has been suggested in which 3A would serve to anchor the precursor 3AB to membranes; thus 3BPr would be attached and ready to be coupled to the 5’ end of viral genomes (31). Apart from this simple physical function of protein 3A as an anchor for 3B, no other activity for this protein has been suggested. Although there is a great evolutionary distance between bacteria and membranes, expression in bacteriophage may be involved in the induction of membrane leakiness that occurs during poliovirus infection (9, 12). It must be kept in mind that membrane-active proteins such as melittin, me-gainins, etc. are active in both prokaryotic and eukaryotic cells (43, 44). The lytic activity of several phage proteins has been well documented (45–48). For instance, heterologous expression in E. coli of the protein encoded by gene E of phage φX174 was shown to be necessary and sufficient to cause lysis of the culture (46). In a similar report the lytic action of bacteriophage λ lysis genes S, R, and Rz were studied (48). We wish to emphasize that we have examined all the poliovirus nonstructural proteins, and only 2B and 3A (or 3AB) modified membrane permeability. It may be that expression of any hydrophobic protein to high levels in bacteria would lead to cell lysis in a nonspecific manner. A number of observations indicate that this is not so. Thus, although protein 2C is also a hydrophobic protein that is tightly bound to membranes in the infected cells, its expression to high levels in this system does not modify the entry or exit of compounds excluded by the membrane. On the other hand, in some of our experiments, proteins 2B and 3A have been expressed to lower levels with a dramatic change in permeability. Experiments are now in progress to clone these proteins in mammalian cells using inducible vectors to analyze their effects on those cells.

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