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INDUCTION OF MEMBRANE PROLIFERATION BY POLIOVIRUS PROTEINS 2C AND 2BC

Rafael Aldabe and Luis Carrasco

Centro de Biología Molecular (UAM-CSIC), Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

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Poliovirus infection leads to the appearance of a number of cytoplasmic vacuoles involved in the replication of virus genomes. To characterize the viral proteins involved in membrane proliferation different poliovirus proteins have been expressed in HeLa cells. Two recombinant vaccinia viruses have been obtained that express poliovirus protein 2C, one under the 5' untranslated (UTR) sequence of poliovirus and another under the leader region of EMC virus. Expression of 2C was very efficient in both cases, although better results were obtained when poliovirus 2C was expressed under the 5'UTR sequence of EMC virus. Transient expression of poliovirus proteins 2B, 2C or 2BC placed under a T7 promoter was analyzed using a recombinant vaccinia virus that contains the bacteriophage T7 RNA polymerase. The expression of 2C, or 2BC, contrary to 2B, was able to induce the proliferation of vacuoles morphologically similar to those found during poliovirus infection. These findings indicate that poliovirus protein 2C, in addition to its NTPase and RNA binding activities,is also endowed with the capacity to induce the formation of cytoplasmic vacuoles.

Poliovirus replication in susceptible cells induces a number of morphological and metabolic changes collectively referred to as the cytopathic effect (1). Amongst the morphological modifications observed, there is a great proliferation of cytoplasmic membranes, forming an intricate structure consisting of vacuoles of various sizes (2-4). The replication of poliovirus

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genomes is tightly connected with these vesicles (5-7). The physical connection between membranous vesicles and RNA replication complexes revealed by electron microscopy and fractionation procedures has also been extended to their biochemical coupling. Thus, the inhibition of phospholipid synthesis by the inhibitor cerulenin blocks poliovirus RNA replication in the infected cells (8). Moreover, brefeldin A a macrolide antibiotic that interferes with the vesicular system potently halts RNA synthesis in poliovirus-infected HeLa cells (9,10). Therefore, an intact vesicular system and correct membrane traffic are required for poliovirus to replicate its genome.

Little is known about the proteins that couple both processes, vesicle proliferation and poliovirus genome replication, and their exact functioning in biochemical terms. The involvement of protein 2C or 2BC in binding the replication complexes to membranes has been long suspected (11-14). Recently, the biochemical function of 2C has been uncovered (15,16). Protein 2C, is endowed with both ATPase and GTPase activities and RNA binding capacity (15). We have recently hypothesized that 2C could belong to a new group of animal virus proteins involved in trafficking the viral RNA through the vesicular system, a function required for viral RNA synthesis and perhaps to assemble the RNA in new virions (15). In accord, with this suggestion 2C shows similarities to the small GTP binding proteins involved in vesicular traffic and to the plant virus proteins involved in transport (17).

To determine the role of 2C in membrane proliferation and its potential interference with the vesicular system, recombinant vaccinia viruses were engineered that inducibly express protein 2C under a T7 promoter. In addition 2B, 2C or 2BC were transiently and efficiently expressed using the pTM1 plasmid. Our present findings indeed point to a role of 2C, or 2BC, in the proliferation of membranous vesicles during poliovirus infection. While our work was in progress we learnt that Cho et al. (1994) reached a similar conclusion. Their elegant electron microscopy evidence indicates that 2C or 2BC induce the proliferation of cell membranes in a similar way as occurs during poliovirus infection. Our present results confirm and extend the Cho et al. (1994) work to

indicate that: 1) 2C expression is more efficient under the untranslated region of EMC virus 2) 2C can be transiently expressed even to higher levels than from recombinant vaccinia virus 3) 2B expression and its intracellular location has been analyzed and 4) 2C (or 2BC) contrary to 2B induce vesicle proliferation in HeLa cells.

MATERIALS AND METHODS

Cells and viruses

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

Plasmid construction

The expression vector pSC11 L2C was constructed following the next steps: 1) polymerase chain reaction (PCR) with the oligonucleotides that hybridize with the regions 1-23 (primer 5´Lead.E1A: CCC GGG AAT TCG TTA AC TTAAAACAGCTCTGGGGTTGTAC), 722-743 (primer 3´Lead.E1A: GGG CCC AAT ATTATGATACAATTGTCTGATTG), 3833-3853 (primer 5´2B.E1A: GGG CCG GCC CG GGCATCACCAATTA CATAGAG), (primer 5´2C.E1A: GGC CGG CCCG GGTGACAGTTGGTT GAAGAAG) and 4991-5110 (primer 3´2C.E1A: GGG CCC GAG CTC AGG CCT TAC TA TTGAAACAAAGCCTCCATAC) of poliovirus type 1 cDNA cloned in vector pT7XLD (the poliovirus sequence is underlined); 2) the sequences corresponding to poliovirus 5´ leader region, 2BC and 2C were inserted into pBluescript (Stratagene, La Jolla, CA) to generate pSKL2BC and pSKL2C; 3) pSKL2BC was digested with SphI-SacI and the fragment inserted in pSKL2C to generate pSKL2C_2; 4) this plasmid was digested with XhoI-SpeI and the fragment obtained was treated with Klenow enzyme and inserted into pAR2529 digested with BamH I and incubated with Klenow generating pAR2529L2C; 5) Finally, to obtain pSC11 L2C the fragment Bgl II of pAR2529 L2C, Klenow-treated, was inserted into pSC11 digested with Sma I. The construction with the poliovirus 2C sequence in the same orientation than the promotor p7.5 was selected.

The expression plasmids pTM1 2B, pTM1 2BC and pTM1 2C were constructed using PCR techniques. Oligonucleotides were designed to hybridize with the regions 3833-3853 (primer 5´2B.B2: GGC CGG CCC GGGATCACCAATTACATAGAG), 4104-4123 (primer 3´2B.B1: GGC CCG GAT CCT TAT TA TTGCTTGATGACATA AGGTA), 4124-4144 (primer 5´2C.E2A: GGG GCC ATG GGTGACAG TTGGTTGAAGAAG) and 4991-5110 (primer 3´2C.E1A) of poliovirus type 1 cDNA cloned in vector pT7XLD (poliovirus sequence is underlined). Amplified products were cloned into pTM1 (kindly givento us by Dr. B. Moss, NIH, Bethesda) digested with Nco I to subclone the poliovirus 2C amplified fragment or with Nco I blunt-ended-BamH I to subclone 2B. The pTM1 2C 4124-4728 subclone obtained was digested with the endonucleases Sph I-Sac I as the subclone pSKL2BC to reconstitute the wild-type sequence corresponding to poliovirus 2C in the vector pTM1. The plasmid pTM1 2BC was generated using the fragment Afl II-Pst I from pSKL2BC_2 that was inserted into pTM1 2B. Recobombinant vaccinia viruses vTM 2C and vSC L2C were prepared essentially as described (18).

Transfection of DNAs with the VT7 expression system

For transfection experiments, cells were plated in 24-well dishes (Nunc) 24 hours before infection with VT7 (m.o.i. 5) (kindly given by Dr. B.Moss, NIH, Bethesda). After 45 minutes of virus adsortion, a mixture of DNA (0.5 μg/ well) and Lipofectin (2μg/ well) was added to cells in DMEM as described by the manufacturer (GIBCO, BRL). Cells were harvested at the times indicated in each figure legend.

Protein analysis by SDS-PAGE. Immunoblot assays

To estimate protein synthesis cells were labelled with 25 μCi/ml (^{35}S)methionine (1.45Ci/mmol, Amersham) in methionine-free medium. To examine the radiolabelled proteins, cell monolayers were dissolved in sample buffer (62.5 mM Tris-HCl (pH6.8), 2% sodium dodecyl sulfate, 0.1M DTT, 17% glycerol and 0.024% bromophenol blue as indicator), loaded onto 15% SDS-PAGE gels and electrophoresed at 80 V for 16 hours.

For immunoblot analysis the proteins were transferred to a nitrocellulose membrane (trans-blot transfer medium, Bio-Rad) overnight at 200 mA in a transfer buffer (25 mM Tris-HCl (pH 8.3), 90 mM glyine, 20% methanol). The membrane was blocked with 5% non-fat dry milk in PBS before incubation for 2 hours with the specific antibodies in 1% dry milk. The blot was then washed with four changes of PBS, containing 0.05% Tween 20 (Sigma). A second incubation with biotinylated goat anti-rabbit antibody (Vector laboratories) was carried out for 1 hour before washing four times with the same solution. Blots were incubated with streptavidine-peroxidase conjugate (Boehringer) for 30 minutes. After washing, the blot was developed by the luminol-liciferin system (19). Briefly, 10 ml 2.5 mM luminol (Boehringer), 78 mM luciferin (Boehringer) were mixed with 10 ml 100 mM Tris-HCl (pH 8) 0.018% H_2O_2. The filter was soaked for 1 or 2 minutes, dried at room temperature and exposed to X-Ray films for 10 seconds to 15 minutes depending on the signal obtained.

Electron microscopy

Vaccinia infected cells were washed twice with cold PBS, removed and fixed for 60 minutes in 6% paraformaldehyde, 0.1 M phosphate (pH 7.2), 6% sucrose, pelleted and washed in 0.1 M phosphate (pH 7.2), 6% sucrose. The free-aldehyde groups were quenched by inmersion in 50mM ammonium chloride in PBS for 60 minutes at room temperature (two changes of 30 minutes each), rinsed in PBS and, finally, processed for embedding in Lowicryl K4M (Chemische Wercke Lowi, Waldkraiburg, Germany) according to the manufacturer instructions. The ultrathin sections were preincubated 5 minutes in a PBS solution containing 0.1% BSA and 1% gelatine and incubated for 1 hour with anti-2C antibody diluted in the same buffer. After several washes with PBS and distilled water the sections were incubated 45 minutes with protein A-gold and after with PBS and distilled water. The samples were stained for 7 minutes in 2% uranyl acetate and for 45 seconds in lead citrate. The sections were viewed with a Jeol 1010 electron microscope at 80 kV.

For immunofluorescence microscopy HeLa cells were washed three times with PBS and fixed with 4% paraformaldehide in PBS for 15 minutes. After fixation the cells were washed with PBS and the free aldehyde groups were reacted with 0.1 M glycine in PBS for 10 minutes. Then, the cells were permeabilized with 0.1% saponine, 0.1% BSA in PBS for 1 hour. After rinsing the cells three times, primary antibody binding was visualized with goat anti-rabbit FITC or goat anti-mouse RITC diluted in 0.1% saponine, 0.1% BSA-PBS for 1 hour (for double immunofluorescence the polyclonal primary antibody was added first). After three washes in PBS the cover slips were mounted on glass slides in moviol and viewed with a Nikon Fluophot microscope.

RESULTS

Expression of poliovirus protein 2C from recombinant vaccinia virus. Comparison of poliovirus and EMC virus untranslated leader sequences.

Recombinant vaccinia viruses bearing poliovirus protein 2C placed under the control of a T7 promoter were obtained as described in Materials and Methods. Two different types of recombinant viruses were made, one that has the poliovirus 5'UTR before the 2C sequence (VSCL 2C) and another that contains the EMC virus 5'UTR (VTM 2C). HeLa cells were infected with each recombinant vaccinia virus and protein synthesis analyzed at 8-9 h.p.i. by labelling with [^{35}S]methionine. A clear band migrating as poliovirus 2C appears when the vaccinia recombinants are co-infected with vaccinia VT7, a virus that expresses the bacteriophage T7 RNA polymerase (Fig. 1). It is apparent that there is more 2C synthesized when the EMCV 5'UTR is placed before 2C, than when the poliovirus 5'UTR is utilized. To analyze that the 2C band synthesized corresponds to authentic poliovirus 2C, the proteins were transferred to a nitrocellulose membrane and immunoreacted against anti-2C antiserum. Figure 1B shows a prominent band in the region of 2C that immunoreacts with anti-2C antiserum. This band appears when cells are co-infected with VT7, although in the case of VTM 2C a faint band is also apparent even when VT7 is absent, suggesting that transcripts are generated from an upstream promoter containing the EMCV leader region that can direct internal initiation of

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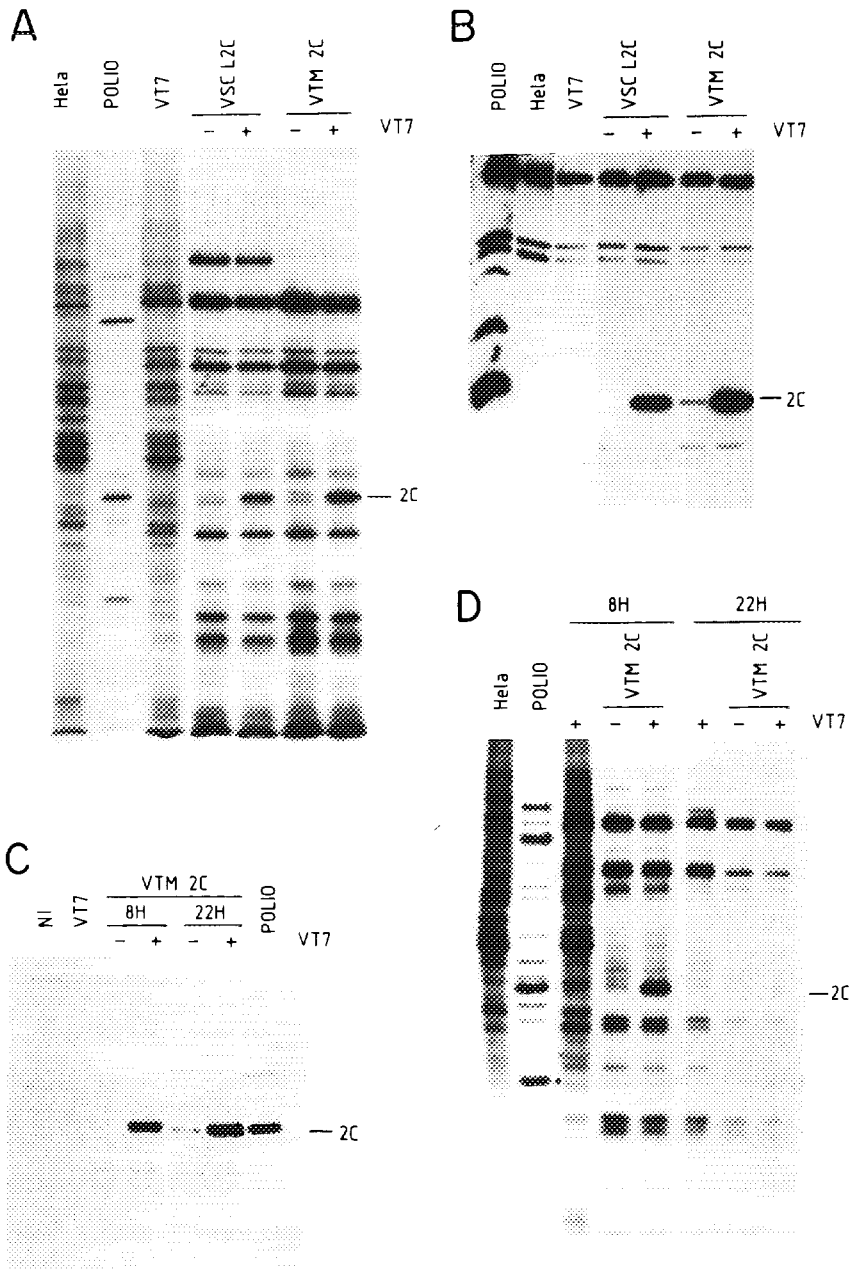
Figure 1. Expression of poliovirus protein 2C. Panel A. Protein synthesis in HeLa cells infected with vSC L2C or vTM 2C. HeLa cells grown in 24-well dishes were infected (m.o.i. 5 pfu/cell) with three different recombinant vaccinia viruses (vT7, vSC L2C and vTM 2C) or co-infected with vT7-vSC L2C or vT7-vTM 2C. At 7 h.p.i. cells were labelled with [^{35}S]Methionine (25μCi/ml) for 1h. Proteins were analyzed by 15% SDS-PAGE. **Panel B**: Immunoblot assay of the samples shown in panel A. **Panel C:** Immunoblot assay of the samples shown in panel D. **Panel D:** Kinetics of protein 2C synthesis in HeLa cells infected with vTM 2C. Cells were individually infected or co-infected with vT7 or vTM 2C (5 pfu/cell). Cells were labelled for 1h with [^{35}S]Methionine (25μCi/ml) at the indicated times and analyzed by 15% SDS-PAGE.

translation. 22 hours post-infection with VTM 2C no detectable synthesis of protein 2C is apparent (Fig. 1D), although the protein synthesized remains in the cells (Fig. 1C). In conclusion, recombinant vaccinia viruses can efficiently direct the synthesis of poliovirus 2C protein, the level of expression of this



protein is comparable to the more efficiently expressed gene after vaccinia infection of HeLa cells.

Transient expression of poliovirus 2B, 2C and 2BC.

One of the objectives of this work was to analyze the effects of poliovirus 2B, 2C and 2BC expression on HeLa cells, particularly focusing to the action of these proteins on the vesicular system. Before to obtain recombinant vaccinia viruses expressing poliovirus 2B and 2BC we sought to compare the efficiency of expression of protein 2C from a recombinant vaccinia virus and transiently upon transfection with pTM1-L2C. For this purpose cells were infected with VT7 and transfected with pTM1-L2B, pTM1-L2BC or pTM1-L2C, the proteins were labelled at 8-9 h.p.i. Strikingly, transient expression of 2C using this system gives even better results for poliovirus 2C expression than from recombinant vaccinia virus. In the case of 2C expression, the majoritary protein synthesized in vaccinia virus-infected HeLa cells corresponds to poliovirus 2C. The expression of 2BC is also quite efficient, although it takes place at lower levels than 2C, while synthesis of poliovirus 2B was barely detected by labelling with [^{35}S]methionine (Fig. 2A). Contrary to the results obtained with the recombinant VTM 2C, synthesis of protein 2C continued to significant levels, even after 22 hours of transfection (Results not shown). To test that the proteins synthesized corresponded to authentic poliovirus 2B, 2C or 2BC they were immunoblotted using specific antisera against 2B or 2C. Figure 2B shows that a band migrating as 2B in the case of pTM1-2B transfection, or as 2BC (pTM1-2BC transfection) immunoreacted with the anti-2B antiserum, while 2BC and 2C reacted with the anti-2C antiserum. In conclusion, our findings indicate that the three poliovirus proteins 2B, 2C and 2BC are detected after transient expression. Particularly striking is the level of 2C synthesized in this system that is superior to the expression obtained with the recombinant VV containing poliovirus 2C. Due to these results it was also of interest to analyze the percentage of cells that expressed poliovirus 2C using the two expression systems. About 80% of the cells gave a clear immunofluorescence to poliovirus 2C at 8 hours p.i. when they were infected with the recombinant VV bearing 2C, while less than 30% transiently expressed 2C as detected by immunofluorescence (Results not shown).

Intracellular localization and effects on membrane proliferation by poliovirus proteins 2B, 2C and 2BC in HeLa cells.

To test the localization of 2B, 2C and 2BC upon transient expression in HeLa cells, immunofluorescence studies were conducted, labelling the cells with fluorescein-antibodies against the poliovirus proteins Initially, these studies were performed in HeLa cells infected with poliovirus. Control HeLa cells do not react with anti-2B (Results not shown) or anti-2C antibodies (Fig. 3A), while there is a slight reaction (with anti-2C antiserum) in cells infected with vaccinia

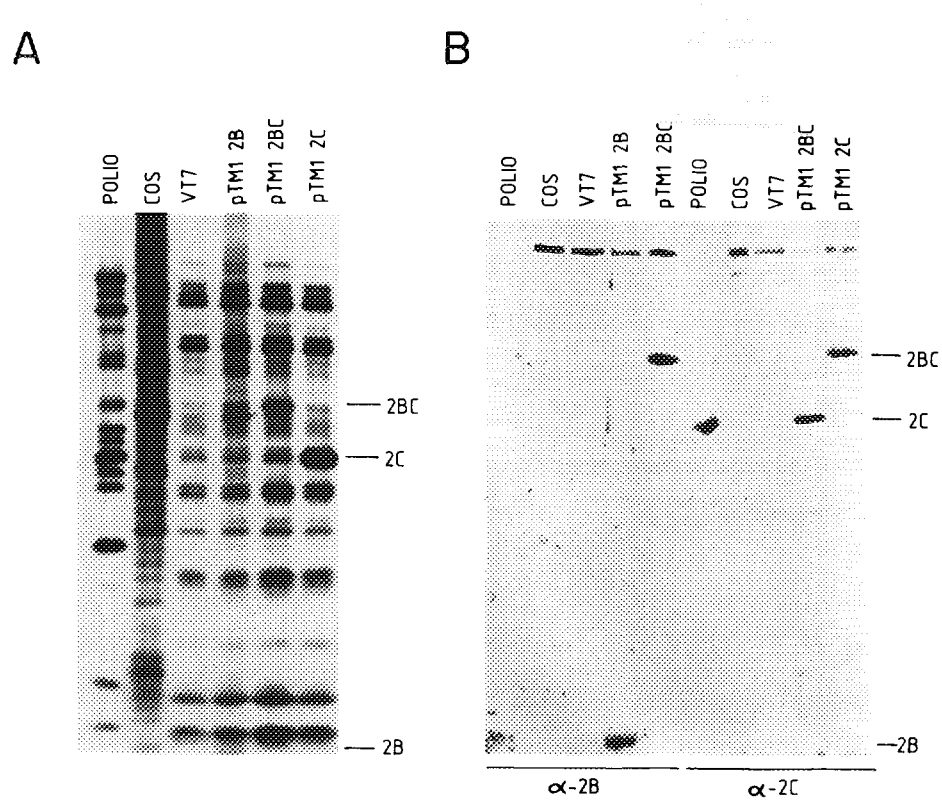


Figure 2. Transient expression of poliovirus proteins 2B, 2BC or 2C in COS cells. Cells were infected with vT7 (5 pfu/cell) and transfected with plasmids pTM1 2B, pTM1 2BC or pTM1 2C by the lipofectin method, as described in Materials and Methods. Cells were labelled for 1h with [^{35}S]Methionine (25μCi/ml) at the indicated times and analyzed by 15% SDS-PAGE (Panel A) or by immunoblot assay (Panel B). Protein 2BC was assayed against anti- 2B antibody (α-2B) and against anti-2C antibody (α-2C).

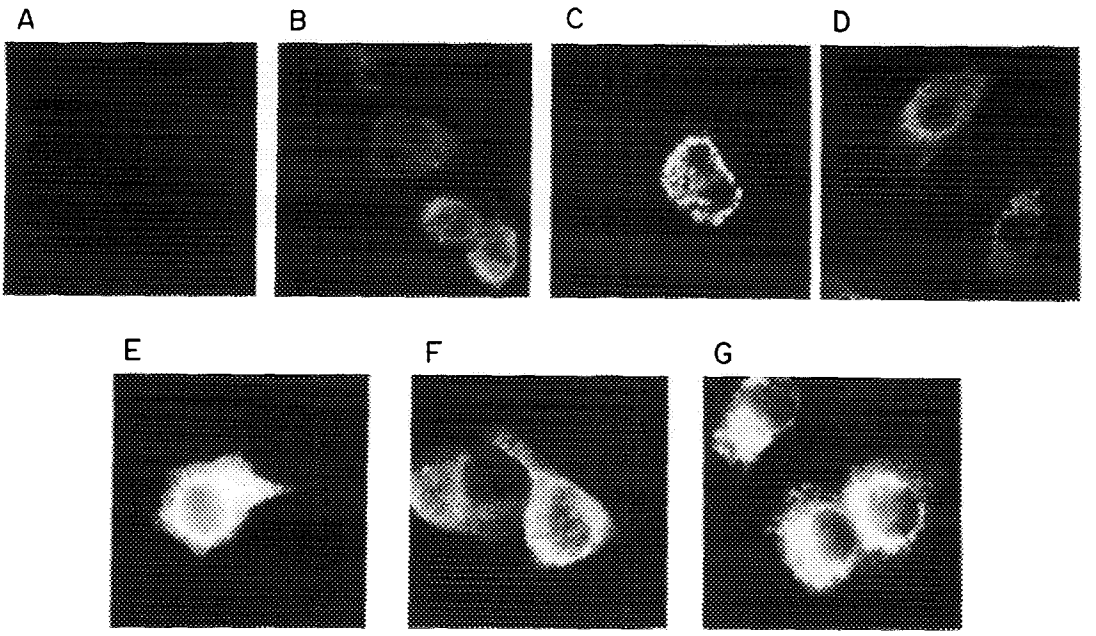


Figure 3. Intracellular localization of poliovirus proteins 2B, 2C and 2BC. HeLa cells were mock-infected (panel A) or infected with poliovirus (panels C and D) and 4 h post-infection immunoreacted with anti-2B antiserum (panel C) or anti-2C antiserum (panels A and D). HeLa cells were infected with vaccinia VT7 (5 pfu/cell) (panels B, E, F and G) and transfected with pTM1-2B (panel E), pTM1-2C (panel F) or pTM1-2BC (panel G) by the lipofectin method. After 8 h cells were immunoreacted with anti-2C antiserum (panels B, F and G) or anti-2B antiserum (panel E).

virus (Fig. 3B). The localization of poliovirus proteins immunoreactive with anti-2B antibodies (2B and 2BC) shows a vesiculated pattern around the nucleus; the distribution of proteins that react with anti-2C antibodies (2C and 2BC) is also pernuclear and is clearly distributed in vesiculae (Fig. 3D). Transient expression of 2B upon transfection with pTM1-2B and vaccinia infection gives a disseminated distribution of this protein throughout the cytoplasm (Fig. 3E) while the distribution of 2C (Fig. 3F) or 2BC (Fig. 3G) after transient expression is localized in vacuolated areas around the nucleus.

Electron microscopy analysis of HeLa cells infected with VT7 shows the presence of several vaccinia virions in the cytoplasm (Fig. 4A). On the other

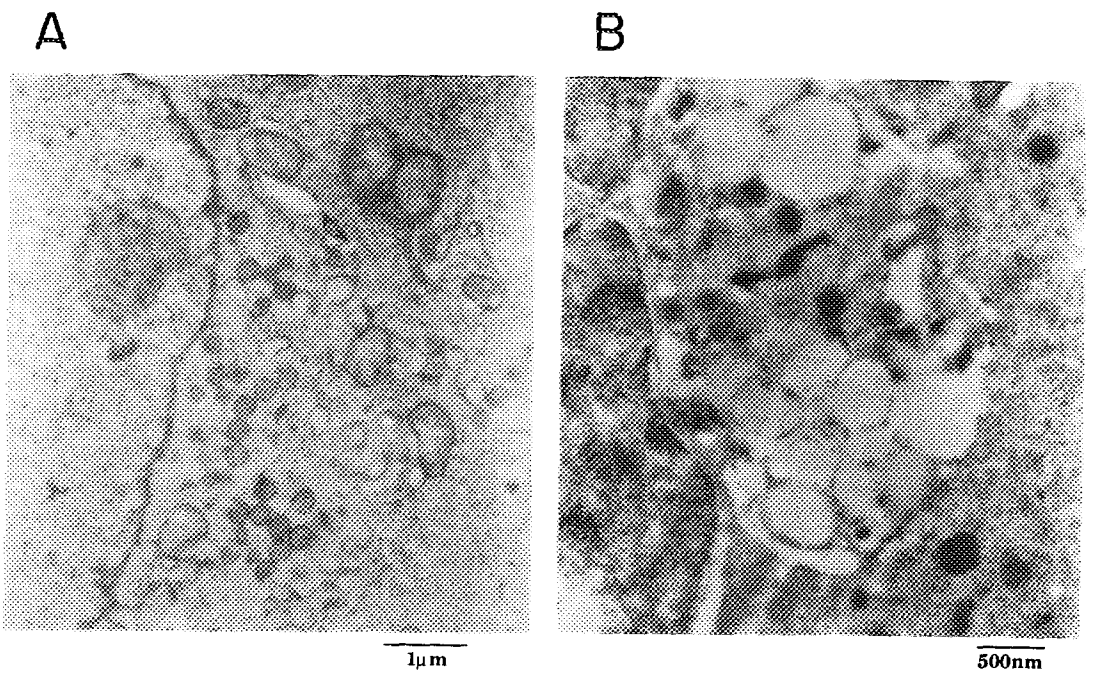


Figure 4. Electron microscopy and immunogold staining of HeLa cells that express poliovirus protein 2C. HeLa cells were infected with vaccinia VT7 virus (5 pfu/cell) and transfected (panel B) or not (panel A) with pTM1-2C. After 16 h of infection cells were observed under the electron microscope. In panel B the cells were immunoreacted with anti-2C antiserum and gold (spheres)-labelled protein A.

hand, HeLa cells co-infected with VT7 and VTM2C clearly show the appearance of vesicles about 200-500 nm diameter in the cytoplasm of the infected cells. Immunogold labelling of these cells shows the presence of poliovirus 2C associated with them (Fig. 4B). Our results agree well with those recently described by Cho et al. (1994), who also observed membrane proliferation after expression of poliovirus 2C or 2BC.

DISCUSSION

Knowledge to understand the function of protein 2C during the poliovirus life cycle has been gained by two different approaches. The first was by means of guanidine, a selective inhibitor of poliovirus RNA synthesis (20), guanidine acts on poliovirus protein 2C and immediately halts viral RNA synthesis (20,21).

Effects of guanidine on virus assembly have also been described (20). Another line of evidence involving poliovirus 2C in genome replication comes from the isolation of ts mutants in this protein (22). Curiously, some 2C mutants have uncoating defects, perhaps reflecting the action of 2C during virus assembly (23). Certainly, 2C must play crucial functions during poliovirus replication, because a number of mutants in this gene recently described are non-infectious (24,25).

Not only physical, but also functional connections between membrane proliferation and poliovirus RNA synthesis have been described in the infected cells. Both, the inhibition of phospholipid synthesis or vesicular traffic induces a profound inhibition of poliovirus genome replication (8,9,26). Therefore, candidate poliovirus proteins involved in these connections have been sought. Our findings that poliovirus 2C was an NTPase that binds RNA and is devoid of helicase activity (15), led us to suggest that 2C shows similarities with the small GTP-binding proteins that participate in vesicular trafficking. Therefore, 2C could couple the induction of membrane proliferation to the traffic of viral genomes through membranes. Recent findings (27) and our present results lend support to the idea that poliovirus 2C and its precursor 2BC, are capable to induce vesicle proliferation in the absence of other poliovirus proteins. In addition, we have extended these findings to indicate that 2B alone is devoid of this effect. A potential drawback of these studies is that membrane proliferation induced by poliovirus 2C or 2BC has been analyzed in the context of vaccinia virus infection. Vaccinia virus is also a cytoplasmic animal virus that modifies vesicular traffic (28) and could help or complement poliovirus protein 2C to induce membrane proliferation. At present we are directing our efforts to get high levels of 2C expression in the absence of vaccinia virus.

In summary poliovirus protein 2C (or 2BC) expression, but not 2B modifies the intracellular distribution of membranes, constituting a new function associated with this protein, apart from its NTPase and RNA binding capabilities (15). These functions of poliovirus 2C make this protein a very attractive model to further studies trying to understand its exact role during poliovirus RNA replication.

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