Genetic analysis of poliovirus protein 3A: characterization of a non-cytopathic mutant virus defective in killing Vero cells

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A mutational and genetic analysis of the poliovirus protein 3A has led to the identification of a single amino acid mutant virus with a restrictive phenotype to form plaques in Vero cells. This mutant (I46T 3A) can be grown and amplified in HeLa cells, where virus replication takes place at wild-type levels. However, Vero cells infected with this virus cannot complete the growth cycle. I46T 3A virus has a defect in the ability to kill Vero cells, as estimated by FACS analysis of propidium iodide uptake by dead cells. Since these defects are observed under conditions where no abnormalities in the rate of synthesis and processing of the mutant polyprotein occur, the inability to induce the cytopathic effect in infected Vero cells denotes the existence of a defect in the activity of 3A, but not the level of expression of the viral genome. As a consequence of this impaired capability to generate the cytopathic effect, I46T 3A mutant viruses cannot be titrated by plaque assay in Vero cells. Only revertant viruses with the wild-type sequence arise and form lysis plaques in Vero cells. Our results suggest a role for the 3A protein (or a precursor thereof) in the virus-induced cytopathic effect. The mutant virus characterized in this work may be a useful tool to understand how poliovirus kills infected cells and carries out the final step of its life-cycle, the release of virus progeny.

Introduction

Upon infection of susceptible cells, poliovirus, a single-stranded RNA virus, induces a number of biochemical and morphological changes that have been collectively named as the cytopathic effect (CPE) (Koch & Koch, 1985). Cells infected with poliovirus undergo a rapid redistribution of the cytoskeletal network, membrane vesicles appear (Bienz et al., 1983) and changes occur in the permeability of the plasma membrane (Carrasco et al., 1989, 1993). In addition, early after infection, the translational machinery is selectively inhibited by the proteolytic degradation of the translation initiation factor eIF-4G (Etchison et al., 1982). This proteolysis does not affect translation of the viral mRNAs. Virus-specific mRNAs are translated by a cap-independent mechanism that involves specific structures in the 5′ leader region of the viral mRNAs and occurs by internal initiation at an AUG located several hundred nucleotides from the 5′ end (Pelletier & Sonenberg, 1988). Poliovirus infection also inhibits cellular RNA synthesis, a process that seems to be explained by the virus-induced proteolytic degradation of cellular transcription factors (Clark & Dasgupta, 1990; Clark et al., 1991).

All these changes are dependent on the expression of the viral genome. Some, but not all of these alterations have been attributed to specific viral products. Thus, the inhibition of cellular mRNA translation is achieved by expression of the viral protease 2Apro (Krausslich et al., 1987) and the induction of membrane vesicles is associated with the expression of the viral protein 2BC (Cho et al., 1994). The inhibition of RNA synthesis can be reproduced in the presence of active viral protease 3Cpro (Clark & Dasgupta, 1990).

Initially, the appearance of the CPE was explained as a progressive deterioration of the cellular machinery (Holland, 1964). Thus, the dramatic alterations that occur within less than 5 h after infection with poliovirus and that finally lead to cell death, were explained as a consequence of the inhibition of the cellular macromolecular synthesis and the accumulation of virus progeny. In contrast to this model, conditions can be found that allow normal levels of expression of the poliovirus genome but do not lead to the appearance of the CPE. That is the case when poliovirus infection takes place in the absence of extracellular sodium and high levels of potassium ions.
The mechanisms that contribute to cell killing could reflect the action of a single specific gene product (Carrasco, 1977, 1987). The plasma membrane has been associated with the action of these putative viral cytotoxic products. After expression of the viral genome, the plasma membrane of the infected cells increases its permeability to monovalent cations (López-Rivas et al., 1987). The activity of the Na/K ATPase is inhibited and the membrane potential collapses (Carrasco, 1987). Low molecular mass translation inhibitors that are non-permeant under physiological conditions, easily cross the plasma membrane and exert their inhibitory effect upon infection with many lytic viruses, including poliovirus (Contreras & Carrasco, 1979). Despite the importance of characterizing the viral products involved in cell lysis, no genetic analysis has been carried out so far in picornaviruses to map this function.

As a first step to identify the viral gene products that contribute to the lysis of infected cells, the poliovirus non-structural proteins were expressed in E. coli and their membrane-permeabilizing activities analysed (Lama & Carrasco, 1992). The viral products 3A, 3AB and 2B were found to be toxic in E. coli cells and this toxicity was correlated with their ability to permeabilize bacterial membranes. Both the 3A/3AB and the 2B proteins share some similarities with membrane-active toxins and ionophore compounds (Carrasco et al., 1993; van Kuppeveld et al., 1996). These poliovirus proteins contain putative amphipathic α-helices with high helical hydrophobic moments that could permeabilize biological membranes as do other eukaryotic and prokaryotic peptides. The 3A protein contains a stretch of 22 hydrophobic amino acids that directs the binding of the polypeptide to membranes (Lama & Carrasco, 1995). The poliovirus genome is expressed as a polyprotein precursor that is processed by virus-specific proteases (Wimmer et al., 1993). As a consequence, the same mature product may form part of different precursors that may even play different roles and show different biochemical activities in the infected cell. For instance, the viral polymerase 3Dpol has RNA-dependent polymerase activity as the mature product (3Dpol) but its precursor 3CDpro is a protease which lacks any detectable polymerase activity (Harris et al., 1992). The 3AB protein seems to play multiple roles in the synthesis of virus-specific RNAs. 3AB, but not 3A, has RNA-binding activity and modulates the activity of the viral polymerase. 3AB stimulates the 3Dpol-directed synthesis of poly(U) more than 50-fold in vitro (Lama et al., 1994). Besides, mutations that abolish the 3Dpol-transactivating activity and do not bind RNA show a dramatic defect in the accumulation of viral RNAs in vivo (Lama et al., 1995). Amino acid changes in the hydrophobic domain of 3A commonly result in virus death or defective viruses with impairment in the replication of their RNAs (Giacchetti et al., 1992). The hydrophobic domain of 3A may anchor a polypeptide precursor to membranes (e.g. 3AB, 3AB3CD), an event that might be required to efficiently replicate the viral genomes. In addition, the binding of some polyprotein precursors to membranes is necessary for cleavage by the viral proteases. Thus, treatment with non-ionic detergents of bacterial membranes containing 3AB recombinant protein renders the 3A/3B cleavage site inactive as a substrate of the viral protease 3CDpro (Lama et al., 1994). 3AB may also regulate the activity of the viral protease 3C/3CDpro by a different mechanism (Molla et al., 1994). 3AB is also the most abundant precursor of the genome-bound VPg protein (Semler et al., 1982). Interestingly, Morace et al. (1993) have reported mutations in the 3A genomic region of two cytopathic strains of hepatitis A virus.

In order to understand whether the permeabilizing activity of 3A/3AB plays a role in the poliovirus-induced CPE, it was of interest to map the permeabilizing domains of these proteins and then identify residues involved in this activity which did not affect the expression of the viral genome. Here, we have carried out a mutational analysis of the 3A-coding region. A mutant poliovirus containing a single amino acid substitution in the 3A protein has been characterized. Mutant viruses expressing this defective protein show a dramatic reduction in their cytotoxic activity in Vero cells, despite the fact that they express their genomes and process their polyprotein precursors as wild-type virus does. Our results indicate for the first time a direct role for 3A in the virus-induced CPE.

**Methods**

**Cells and viruses.** Transfection with infectious RNAs was carried out in HeLa R19 cells. The viruses used in this work were obtained by transfection of HeLa cell monolayers with recombinant RNAs derived from pT7XLD constructs (van der Werf et al., 1986). This plasmid contains the full-length type 1 poliovirus (Mahoney strain) cDNA under the control of the T7 promoter. Wild-type and 3A mutant viruses were plaque-purified and amplified in HeLa cells up to titres of about 10^8 p.f.u./ml and used for subsequent experiments.

**Construction of recombinant plasmids.** The procedure used to construct the 3A mutant poliovirus cDNAs has been described elsewhere (Lama & Carrasco, 1995). Briefly, the desired 3A sequences were amplified by PCR from the corresponding pT7lac3AB plasmid. After PCR amplification, the fragments were cloned into the pT7XLD vector. The 3AB gene of the wild-type and mutant cDNAs was sequenced by the dideoxy method (Sequenase, US Biochemical). The set of pT7lac3AB mutants used in this work was obtained after screening of a random mutagenized pT7lac3AB library that has been previously described (Lama & Carrasco, 1996). These 3AB mutant proteins have reduced permeabilizing activities, as tested by different assays performed in recombinant bacteria expressing the viral 3AB protein.

**Transfection of HeLa cells.** Transfection of HeLa cells was carried out with the Lipofectin reagent (Gibco). Infectious RNA was obtained by in vitro transcription of pT7XLD with T7 RNA polymerase. Ten µg RNA without further purification was mixed with 15 µg Lipofectin and incubated at 37 °C for 2 h with a monolayer of HeLa cells previously washed with PBS to remove serum. Subsequently, the cells were kept in 2% calf serum for 3 days. At this point the viruses were recovered by three cycles of freeze–thawing and their titres were estimated by plaque assay on HeLa cells. To isolate mutant viruses for further analysis,
individual primary plaques were recovered under agar after 48 h of transfection with infectious RNA. To make sure that the introduced mutations had been retained in the genome after amplification in HeLa cells, virion RNAs were purified by proteinase K treatment in the presence of 0·5% SDS (Ortin et al., 1980) and the complete 3AB region was retrotranscribed with AMV RT and sequenced by PCR (FmødNA sequencing system, Promega) following the instructions of the manufacturer. The I46T 3A mutant that was extensively studied herein was isolated from four independent transfections and shown to contain the introduced mutation in all the cases.

In vitro translation of transcripts derived from mutant cDNAs. Translations were performed with HeLa S3 cell extracts treated with micrococcal nuclease. The lysates were programmed with in vitro-transcribed poliovirus RNA. The preparation of HeLa cell extracts and the reaction conditions were essentially as described by Molla et al. (1991). Reactions were incubated at 30 °C for 15 h with 100 ng transcript RNA in the presence of 0·7 mCi/ml [35S]methionine. The synthesized proteins were analysed by autoradiography of SDS–polyacrylamide gels.

Infection of cell lines and metabolic protein labelling. Infections were performed on either HeLa R19 or Vero cell monolayers grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. To assess the expression and processing of the poliovirus polyprotein, different amounts of wild-type or mutant polioviruses were adsorbed to cell monolayers for 40 min at 37 °C. The cells were then incubated at 37 °C in DMEM supplemented with 2% calf serum and at the indicated time-points the monolayer was washed with medium without methionine and cysteine and incubated for 1 h with 12·5 µCi/ml Trans-Label. The monolayer was washed with PBS and lysed with electrophoresis buffer (1% SDS, 100 mM DTT, 17% glycerol, 0.024% bromophenol blue, 0.37 mM Tris–HCl pH 8·8). The synthesized products were analysed by autoradiography of SDS–polyacrylamide gels.

Flow cytometry assay for cell viability. Vero cells were infected as described above and incubated at 37 °C. At given times, the cell monolayer was trypsinized for 3 min at room temperature and incubated with 5 µg/ml propidium iodide (PI). The flow cytometry analysis was performed in a FACscan flow cytometer (Epics Profile Analyser, Coulter) with an argon laser tuned to 480 nm and a power emission of 25 mW. The side and forward scatter were set in log mode. PI fluorescence was collected with a BP 630/22 filter and directed to the photomultiplier (voltage of 600 V). The fluorescence signals were collected in log mode. Each sample was used to collect 10,000 signals.

Results

Mutational analysis of poliovirus 3A protein

A detailed mutational analysis in E. coli has identified domains of the 3A protein involved in the permeabilization of biological membranes (Lama & Carrasco, 1996). In that report we took advantage of the 3A/3AB permeabilizing activity and the use of β-galactosidase substrates to screen a plasmid-expressing library. This approach led to the identification of residues in 3A involved in membrane permeabilization. Here, to understand the possible role of this activity in the onset of the virus-induced CPE, the 3A mutations were cloned into the full-length poliovirus cDNA. T7 RNA polymerase was used to in vitro-transcribe the different cDNAs and the recombinant RNAs were transfected into monolayers of HeLa cells (Table 1). At different times after addition of the RNA, the CPE was estimated by microscopy. All the plates except those transfected with the K39E 3A mutant RNA showed delays in the appearance of the CPE. Interestingly, mutants with amino acid substitutions spanning the hydrophobic domain (amino acids 59–80) or in the C-terminal boundary of this region (positions 81–82) did not induce any CPE. The same results were obtained in two independent transfections. To make sure that these RNAs were non-infectious, the transfected cells were incubated for 1 week at 37 °C and lysed by three cycles of freeze–thawing. No virus was detected by plaque assay in HeLa cells. When these lysates were added to fresh HeLa cells, no CPE appeared even after another week of incubation at 37 °C, and again no virus was detected by plaque assay on HeLa cells. The transfection experiments were repeated at 39·5 °C and 32 °C with similar results. Therefore, we concluded that all of these mutations render the recombinant RNAs non-infectious and impair the replication of poliovirus. A mutation at position 42 (W42R) also resulted in a dead virus phenotype. This amino acid is very well-conserved among picornavirus 3A proteins (poliovirus, coxsackie virus, rhino-virus and bovine enterovirus) (data not shown) and must be important for a function of the protein involved in the replication of the viral genome. Amino acid changes at positions 12, 44 and 45 revealed weak delays in the onset of the CPE. Of note, mutation of residue 46 (I46T) caused a strong delay in the appearance of the CPE and a two log reduction in the titre of the viruses harvested after 72 h of transfection. The I46T 3A mutant virus has a normal plaque size phenotype when assayed in HeLa cells, suggesting that the harvested virus replicates in HeLa cells at normal levels. To further study the I46T 3A mutation, after 48 h of transfection with RNA–Lipofectins, individual plaques grown directly on agar were isolated. Four purified plaques from independent transfections were found to display identical phenotypes. Their virion RNA was extracted, retrotranscribed and sequenced by PCR. All the sequenced viruses retained the ATC to ACC mutation at codon 46 (data not shown) and showed the same mutant phenotype, which will be described later. No second-site mutation was found in the 3A region of these mutant viruses. Identical phenotypes were found when the 3A gene, retrotranscribed from mutant viral RNA, was cloned back into the polio cDNA and used to produce infectious particles by transfection with RNA–Lipofectin. Although these results do not rule out the existence of other mutations in the viral genome that may contribute to the observed phenotype, our results suggest that the I46T mutation in 3A is sufficient to induce the changes described below. This mutant virus was further characterized in this work.

Synthesis of poliovirus proteins in HeLa cell extracts

It has been reported that amino acid substitutions in the 3A protein affect poliovirus RNA replication (Giachetti et al.,
Table 1. CPE and virus production after transfection of HeLa cells with infectious RNAs

<table>
<thead>
<tr>
<th>3AB gene</th>
<th>CPE* 48 h</th>
<th>CPE* 72 h</th>
<th>CPE* 96 h</th>
<th>Titre at 72 h (p.f.u./ml)</th>
<th>Plaque phenotype†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>5 × 10⁸</td>
<td>Normal</td>
</tr>
<tr>
<td>I12V</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>6 × 10⁸</td>
<td>Normal</td>
</tr>
<tr>
<td>K39E</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>2 × 10⁸</td>
<td>Normal</td>
</tr>
<tr>
<td>W42R</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>V44A</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>1 × 10⁸</td>
<td>Normal</td>
</tr>
<tr>
<td>N45D</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>3 × 10⁸</td>
<td>Normal</td>
</tr>
<tr>
<td>I46T</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>1 × 10⁸</td>
<td>Normal</td>
</tr>
<tr>
<td>I62K</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>I63P</td>
<td>–</td>
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<tr>
<td>V75E/V76F</td>
<td>–</td>
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<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Y77H</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>M79K</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>K81E</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>L82P</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Assigned by estimating the percentage of cells showing drastic CPE (rounding). —, No CPE; +, less than 20% of cells affected; +, 20–40%; ++, 40–80%; ++++, total CPE.
† Plaque phenotype was determined for those viruses harvested after 72 h of transfection.

1992; Lama et al., 1995). Transfections with RNA genomes containing mutations that abrogate lysis of the infected cells might produce a dead virus phenotype, indistinguishable from that observed in dead viruses with no RNA replication. However, in single rounds of infections cells inoculated with the first type of virus would produce normal levels of viral products and virions, whereas the second class would show defects in expression of the viral genomes. Both the detection of the CPE after transfection of cells and the detection of lysis plaques rely on several rounds of replication that may not take place in the case of a lysis-defective virus. Thus, we wanted to address whether the constructed poliovirus mutants could faithfully express their genomes in an in vitro translation system. Cytoplasmic HeLa cell extracts were prepared and treated with micrococal nuclease to eliminate the endogenous mRNAs. These lysates were programmed with either wild-type or mutant recombinant RNAs synthesized with T7 RNA polymerase (Fig. 1). These cell extracts translate the poliovirus RNA into a polyprotein precursor that is processed to give mature products with a similar pattern to that observed in infected cells (Fig. 1, compare lanes 1 and 3). Most mutations in the hydrophobic domain (L63P, T67P, V75E/V76F, Y77H, M79K) and also that at position 82 (L82P) caused an aberrant proteolytic processing of the polyprotein. Introduction of charged or α-helix-breaking residues in the hydrophobic domain produced this effect. The 3AB/3CD cleavage site, which after processing by the viral protease 3C/3CD leads to the appearance of the 3AB product, was not cleaved in most of these mutants. In addition, very little 2C protein was found in translations programmed with these mutant RNAs. Translation of Y77H 3A mutant RNA resulted in an aberrant pattern of processing, where most of the signal accumulated in a high molecular mass protein and no mature 2C, 2A, 3AB or 3A appeared in the lysates. Our results corroborate previous findings which showed that amino acid substitutions in the 3A hydrophobic domain can drastically alter the general pattern of processing of the polyprotein and suggest that membrane binding (probably through the 3A domain) of some of the precursors may be required for efficient proteolytic processing. These data also demonstrate that the dead virus phenotype found with these mutants must be ascribed to the absence of virus-specific products required for virus replication, rather than to the knock-out of a single 3A function (e.g. cell death or lysis). Changes at positions 42 (W42R), 62 (I62K) and 81 (K81E) did not alter the proteolytic pattern of the polyprotein. Therefore, these RNAs could synthesize 3A proteins defective in a function required for the virus to replicate.

Restrictive phenotype of I46T 3A mutant virus

Viruses harvested from HeLa cells transfected with I46T 3A RNA were amplified to high titres and used to analyse the growth kinetics in one-step growth curves. Either HeLa or Vero cells were infected and at different times after infection both the intracellular and the extracellular virus were titrated by plaque assay in the same producer cell lines (Fig. 2). The mutant virus grew at wild-type levels in HeLa cells and no difference in the amount of infectious particles was found (Fig.
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2A, B). However, the I46T 3A virus was produced with delayed kinetics in Vero cells. Thus, after 5 h of infection a 1000-fold reduction was found in the number of infectious particles titrated in Vero cells. These differences were still found even when the amount of wild-type input RNA was reduced to 10 p.f.u. per cell as compared to 50 p.f.u. per cell used with the I46T 3A virus. Curiously, despite these differences in the rate of production of wild-type and mutant virus, both viruses showed identical plaque size phenotypes in Vero cells.

To understand better the restrictive phenotype shown in Vero cells, both wild-type and I46T 3A viruses were used to infect either HeLa or Vero cells (producer cells) and the viruses harvested from these cells were titrated in both cell lines (Table 2). Titres of about $10^9$ p.f.u./ml were found for the wild-type virus harvested from either HeLa or Vero cells, independently of the cell line used to perform the plaque assays. As shown in Table 2, I46T 3A virus grown in HeLa cells showed a 1000-fold reduction in the amount of infectious particles detected in Vero cells. Interestingly, the same stock of 3A mutant virus grew in HeLa cells similar to the wild-type virus. On the other hand, the virus produced in Vero cells infected with I46T 3A showed less than 10-fold inhibition as compared to the wild-type virus when titrated in Hela cells, but a drastic inhibition was found when the viruses were assayed in Vero cells. These results demonstrate that I46T 3A mutant virus must replicate its genome and produce mature infectious particles at normal rates in Vero cells, since almost normal amounts of infectious viruses are detected when the lysates are tested on HeLa cells. Normal levels of synthesis and proteolytic processing of the I46T 3A polyprotein were also found in infected Vero cells (see later, Fig. 4). One explanation for these results is that Vero cells contain or lack a factor that makes I46T 3A virus undetectable by the plaque lysis assay. These viruses may have a lower capability to kill Vero cells and spread their virus progeny.

Two predictions from this hypothesis are: (i) those viruses detected on plaque lysis assays performed in Vero cells must be revertant viruses which have recovered their capability to lyse these cells; and (ii) I46T 3A mutant viruses must show a defect in their capability to induce CPE in Vero cells.

To test our first prediction, I46T 3A viruses were used to infect Vero cells at a high multiplicity and the intracellular viruses were harvested and plaqued on Vero cells. Five independent lysis plaques were isolated and the 3A-coding region of their virion RNAs was retrotranscribed and sequenced. All of them had reverted to the wild-type
agar. Infections were carried out at 37 °C with either wild-type or I46T 3A poliovirus. Then cells were trypsinized, spun down and resuspended in a buffer containing PI. Mock-infected cells treated in the same way were used to set the FACscan experimental parameters. Fig. 3(A, C and E) shows the size distribution of mock, wild-type and I46T 3A-infected cells, respectively. More than 95% of the mock-infected cells were located in region A (Fig. 3A); however, after infection with wild-type virus about half of the cells became smaller and displaced to region B (Fig. 3C). Fig. 3(D) shows the PI uptake of cells distributed in regions A plus B of Fig. 3(C). Window 2 in this plot was set to contain no more than 3% of the mock-infected cells stained with PI (Fig. 3B). However, 34% of the cells infected with wild-type poliovirus were strongly stained with PI. This population of dead cells is a consequence of the virus-induced CPE. These PI-positive cells mostly represented the population of cells contained in region B, Fig. 3(C) (data not shown), suggesting that before dying, Vero cells undergo a size reduction. Surprisingly, this size reduction was not found after infection with the single amino acid 3A mutant (I46T 3A) (Fig. 3E) and

However, when the pool of virion RNA coming out of the first Vero infection was sequenced, the viral RNA retained the original mutation (ACC for threonine). This result is expected since the fraction of revertant (wild-type) viruses in this sample must be less than 1%, therefore undetectable by sequence analysis (the ratio of infectious viruses detected in HeLa and Vero cells resulted in a 150-fold difference; see Table 2). Furthermore, the ability to detect I46T genomes in the pool of RNAs from virions harvested after infection of Vero cells indicates that these genomes are fully competent for packaging in these cells. As expected, three out of three independent lysis plaques isolated after infection of HeLa cells retained the I46T mutations in their genomes (data not shown). Taken together, these results indicate that those viruses with an I46T 3A genotype cannot be detected in a plaque assay performed in Vero cells. In addition, the recovery of revertant viruses with the wild-type sequence in codon 46 from Vero cells demonstrates that this mutation is the cause of the observed phenotype.

### Table 2. Restrictive phenotype of I46T 3AB mutant poliovirus

Either HeLa or Vero cells (producer cells) were infected with 100 p.f.u. per cell of wild-type or I46T 3A mutant poliovirus. The intracellular viruses were harvested and titrated on target cells overlaid with 0.7% agar. Infections were carried out at 37 °C.

<table>
<thead>
<tr>
<th>Assayed virus (producer cells)</th>
<th>Titre (p.f.u./ml) on target cells</th>
<th>Ratio H/V*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (HeLa)</td>
<td>6.4 × 10^8</td>
<td>2-8 × 10^8</td>
</tr>
<tr>
<td>I46T (HeLa)</td>
<td>6.8 × 10^8</td>
<td>5.0 × 10^5</td>
</tr>
<tr>
<td>Wild-type (Vero)</td>
<td>6.6 × 10^8</td>
<td>3.2 × 10^5</td>
</tr>
<tr>
<td>I46T (Vero)</td>
<td>8.0 × 10^7</td>
<td>5.2 × 10^6</td>
</tr>
<tr>
<td>Revertant (Vero)</td>
<td>1.0 × 10^8</td>
<td>1.0 × 10^8</td>
</tr>
</tbody>
</table>

* The ratio between the titres in HeLa and Vero cells (H/V) was estimated by dividing these values.

The kinetics of replication of these revertant viruses were identical in both HeLa and Vero cells (Table 2).
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**Fig. 3.** Flow cytometry analysis of Vero cells infected with wild-type or I46T 3A poliovirus. Incorporation of PI in dead cells. Vero cell monolayers were infected with 50 p.f.u. per cell of either wild-type or mutant poliovirus. At 7 h p.i., the cells were trypsinized and incubated with PI to determine the number of dead cells. (A), (C) and (E) show the size distribution of mock-, wild-type- or I46T 3A-infected cells, respectively (LSS, light side scatter; LFS, light forward scatter). Smaller cells move to the left in these plots. Mock-infected cells were localized into the A region. More than 95% of the cells included in this region were alive, as estimated by PI uptake, whereas about 60% of the cells contained in the B region (C) were dead (high PI content). The percentage of the total number of cells included in region B was 10% (A), 55% (C) and 13% (E). (B), (D) and (F) show the logarithm of the fluorescence (LFL) produced by the uptake of PI in mock-, wild-type- or I46T 3A-infected Vero cells, respectively. 10,000 cells from regions A and B (A, C and E) were analysed in these plots. Window 2 in (B), (D) and (F) was set to contain no more than 3% of the cells that had been mock-treated (B).

Only 5% of the infected cells became PI-positive. A revertant with the wild-type sequence at position 46 behaved as wild-type virus (data not shown). Taken together, these results indicate that I46T 3A mutant virus has a severe defect in the induction of the CPE in infected Vero cells.

**I46T 3A mutant virus processes its polyprotein at normal levels**

Poliovirus protein 3A is involved in replication of the viral RNAs. 3A or 3A-containing precursors may play different roles in replication.
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Fig. 4. Synthesis and processing of the poliovirus polyprotein in infected cells. Vero cells were infected with 100, 25 or 10 p.f.u. per cell of wild-type poliovirus, or with 100 p.f.u./ml of I46T 3A mutant virus. At different times after infection, the synthesized proteins were labelled for 1 h with a mixture of [35S]methionine and [35S]cysteine. The synthesized proteins were separated in polyacrylamide gels and visualized by autoradiography. HeLa cells were infected with 100 p.f.u. per cell of either wild-type or 3A mutant virus. The position of some of the poliovirus proteins is denoted.

roles in the synthesis of viral genomes (Giachetti et al., 1992; Lama & Carrasco, 1995). Thus, we cannot rule out that amino acid substitutions in protein 3A impair the ability of the virus to replicate its genome. Mutation I46T 3A is located close to position 42, where a Trp to Arg change produces a dead virus phenotype (Table 1). Therefore, to test whether I46T 3A virus had any defect in the expression of its genome, we studied the synthesis and processing of the poliovirus polyprotein in Vero and HeLa cells infected with 100 p.f.u. per cell (Fig. 4). Infection was followed up to 8 h after adsorption of the virus. After infection with I46T 3A, no difference was found in the general pattern of viral proteins, as compared to wild-type virus, indicating that this virus correctly processes its polyprotein precursor. Normal levels of processing of the 3A/3B cleavage site were also observed. In addition, the same level of virus-induced inhibition of cellular protein synthesis was found in both infections. The appearance of viral proteins was slightly delayed in the 3A mutant. Thus, from 3 to 5 h after infection more viral products were synthesized in wild-type- than in I46T 3A-infected cells. This fact may indicate a defect in the replication of the I46T genomes, perhaps at the RNA level. However, these differences disappeared later in the infection cycle. Of note, at 7 h after infection, when measurements of PI-stained cells were performed (Fig. 3), 3A mutant virus synthesized even more viral products than its wild-type counterpart (compare 2C or 3A proteins). No differences were found in the total levels of 3A/3AB products as estimated by Western blot analysis (data not shown). These results suggest that the differences found in the capabilities of wild-type and 3A mutant viruses to induce CPE cannot be ascribed to differences in the level of viral products, but rather to defects in the activity of the 3A protein. In addition, small differences in the kinetics of protein synthesis were also observed in HeLa cells infected with either the wild-type or the mutant virus. These results reinforce the above conclusion, since these cells allow growth of I46T 3A virus to wild-type levels (Table 2 and Fig. 2). However, our results do not rule out the existence of weak viral RNA synthesis defects in the I46T 3A mutant virus. These differences might become more apparent at low multiplicities of infection and could explain the delay observed in the onset of the CPE after transfection of HeLa cells with infectious I46T mutant RNAs (Table 1).

It could be hypothesized that a threshold level of a virus factor is necessary to start a CPE. Infection with the I46T 3A virus might require a longer time to accumulate this factor, leading to a different response in the host cell. In order to rule out this possibility, Vero cells were infected with decreasing amounts of wild-type poliovirus and the number of dead cells after infection was estimated by PI uptake. As shown in Fig. 4, infection of Vero cells with either 25 or 10 p.f.u. per cell of wild-type virus blocked cellular protein synthesis but the rate of synthesis of viral products was significantly lower as compared to Vero cells infected with 100 p.f.u. per cell of I46T 3A. Nevertheless, even as little as 10 p.f.u. per cell of wild-type virus produced a stronger CPE than 100 p.f.u. per cell of I46T 3A (Fig. 5). Thus, wild-type virus induced the death of three to six times more Vero cells than the 3A mutant virus, despite the higher levels of expression of the mutant viral products,
replication cycle. Here, we have analysed mutations that have substitutions in the 3A protein. This protein is endowed with membrane-permeabilizing activity that it is revealed upon infection (Young, 1992). Late in the bacteriophage replication cycle, a membrane-permeabilizing protein is expressed at the inner membrane. These proteins, which have collectively been named ‘holins’, permeabilize the inner bacterial membrane and allow leakage of the lysozyme that leads to the catalytic degradation of the bacterial wall and subsequent lysis of the infected cell. The possibility that membrane-permeabilizing proteins might play a role in the final steps of the growth cycle of lytic animal viruses has long been hypothesized (Carrasco, 1987; Carrasco et al., 1989).

In this report, we have characterized single amino acid substitutions in the 3A protein. This protein is endowed with membrane-permeabilizing activity that it is revealed upon expression in E. coli cells (Lama & Carrasco, 1992). Poliovirus 3A protein has also been implicated in different steps of the replication cycle. Here, we have analysed mutations that have been previously associated with reductions in the permeabilizing activity of 3AB (Lama & Carrasco, 1996). Fourteen different mutations were introduced into the full-length poliovirus cDNA. Most of these changes produced dead viruses, in agreement with previous reports involving this protein in the replication of the RNA genomes (Giachetti et al., 1992; Lama et al., 1995). However, an interesting mutant has been characterized in this analysis. A single amino acid substitution of residue 46 of 3A (I46T 3A) produced an infectious virus that replicated at regular levels in HeLa cells. No difference was found in the kinetics of production of infectious viruses in HeLa cells. However, this mutant virus showed a restrictive phenotype in Vero cells. I46T 3A viruses do not develop lysis plaques on agar-embedded Vero cells. Thus, these viruses could not be titrated in these cells, since only revertant viruses that had acquired the wild-type sequence at codon 46 completed enough cycles to develop detectable lysis plaques. Curiously, no significant defect was found in the synthesis and processing of the viral polyprotein in either Vero or HeLa cells. Since this mutant virus synthesized a full set of viral proteins at wild-type levels, we concluded that the studied amino acid change at position 46 causes a defect in a 3A activity. Further analyses with infected Vero cells demonstrated that I46T 3A mutant virus has a reduced capability to kill Vero cells, as estimated by PI uptake. The ability of this mutant to kill Vero cells is inhibited over 80% and even lower levels of expression of wild-type 3A protein were still more effective in the induction of the CPE. These results explain the inability of this mutant virus to be titrated in Vero cells and suggest a role for 3A in either the onset or the maintenance of the CPE in Vero cells. An interesting aspect of our findings is that mutant viruses produced in Vero cells have successfully completed all the steps required for the formation of mature infectious particles, which are detected only upon infection of HeLa cells. I46T viruses produced in Vero cells are not unstable or immature, since these viruses are fully competent for infection of HeLa cells. It could be hypothesized that I46T mutant viruses have a cell-type-specific defect in virus entry. However, I46T poliovirus expresses its genome at good levels after infection of Vero cells. These results argue against defects in virion entry, which would result in poor levels of expression of the mutant genomes. Transfection of HeLa cells with I46T RNA led to a delay in the appearance of CPE and a significance reduction of virus titre compared to wild-type, yet these viruses were indistinguishable from wild-type (in HeLa cells) in terms of plaque size and growth curves. This apparent contradiction may be explained by the fact that the I46T 3A mutation affects RNA replication in infected cells. A slight delay in protein synthesis was observed in both infected HeLa and Vero cells. This delay was more evident in Vero cells, where the peak of protein synthesis occurs at 5 h post-infection (p.i.) with wild-type virus and 6–7 h p.i. in I46T 3A-infected cells (Fig. 4). Minor effects on the expression of the viral genome may be revealed after transfection of cells with infectious RNAs, leading to the appearance of delayed CPEs,
but would not be apparent when cells are infected at high multiplicities of infection. Minor defects in expression of the mutant genomes were ruled out as the cause of the observed phenotypes, since conditions were found where wild-type virus genomes are expressed to much lower levels but still exert higher levels of CPE than those found in Vero cells infected with I46T 3A.

The mechanism of the 3A-induced CPE is at present unknown. The I46T 3A mutation was originally characterized after screening a random mixture of 3AB-expressing plasmids. Recombinant E. coli expressing this protein displays reduced levels of permeability to ONPG, uridine and hygromycin B as compared to bacteria expressing the wild-type protein (Lama & Carrasco, 1996). Vero cells infected with I46T 3A virus are as sensitive to hygromycin B as cells infected with wild-type virus (data not shown). These results suggest that permeabilization of the plasma membrane may not be linked to the induction of the 3A-induced CPE. Also, a requirement for 3A to permeabilize the infected cell is that this protein reaches the plasma membrane. So far, our attempts to localize 3A/3AB at the plasma membrane have not been successful. Our results cannot distinguish between 3A or a precursor thereof as the causative agent of the virus-induced CPE. Poliovirus infection enhances the concentration of cytoplasmic calcium (Iruzun et al., 1995). To date, the viral product eliciting these changes has not been identified. No direct experimental evidence has linked the increase of intracellular calcium and the poliovirus-induced cytotoxicity; however, these factors seem to be associated in infections with other lytic viruses, such as rotaviruses (Michelangeli et al., 1991). One could speculate that the increase in calcium concentration may lead to the onset of the CPE in poliovirus-infected cells. Preliminary FACscan analyses have shown that upon infection with poliovirus, those Vero cells that become smaller (and subsequently incorporate PI) have increased levels of intracellular calcium. In addition, infection with I46T 3A mutant poliovirus does not increase the intracellular calcium (data not shown). Whether the increase in intracellular calcium is the cause or the effect of the virus-induced CPE is currently under study in our laboratory.

Another interesting finding from our results on the incorporation of PI is the mechanism by which Vero cells die. FACscan analysis showed that Vero cells undergo a size reduction after infection with poliovirus. This population of smaller cells has a high fraction (about 60% in Fig. 3C) of PI-stained cells, suggesting that this cell population has been engaged in a death pathway. However, when these experiments were repeated with HeLa cells, the uptake of PI was not a reliable parameter to estimate the number of dead cells. Infection of HeLa cells with poliovirus induced an increase in the average size of the population, but these cells hardly incorporated PI before dying (data not shown). Unfortunately, we have not been able to use the PI-staining method to monitor the kinetics of the CPE in infected HeLa cells. Currently, other methods are being tested to study the mechanism of cell death in these cells. Commonly, cell death by lytic viruses takes place via a process that involves an increase in size before the cells virtually explode due to osmotic shock (Koch & Koch, 1985). The morphological changes found in the way that HeLa and Vero cells die suggest that the same agent (poliovirus) may use different mechanisms to cause cell death (e.g. apoptosis versus osmotic lysis).

Interestingly, both induction and prevention of apoptosis has been reported in specific cell lines infected with poliovirus (Tolskaya et al., 1995). Apoptosis has also been proven as a major cause of cytopathogenicity in some virus systems (Jeuriissen et al., 1992; Laurent-Crawford et al., 1993; Levine et al., 1993; Hinshaw et al., 1994; Esolen et al., 1995). In addition, enhancement of cytosolic calcium can trigger the apoptotic response (Bowen, 1993). We are currently testing whether poliovirus infection induces apoptosis in Vero cells. The use of animal models will be necessary to understand if the mechanism of cell killing adopted in poliovirus-infected Vero cells is physiologically relevant and whether 3A plays a role in the induction of the CPE in vivo.

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