Screening for membrane-permeabilizing mutants of the poliovirus protein 3AB

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Synthesis of the poliovirus polypeptide 3AB in bacterial cells results in an increase in membrane permeability. The alterations observed resemble those elicited by bacteriophage lytic proteins, which are presumed to cause pore formation in biological membranes. This property has been exploited in the development of an in vivo screening system that allows morphological differentiation of Escherichia coli clones expressing either wild-type 3AB or variant 3AB proteins lacking the ability to permeabilize bacteria. Expression of the wild-type 3AB gene in the presence of a chromogenic β-galactosidase substrate causes E. coli clones to stain dark blue. In contrast, bacterial mutants that synthesize 3AB proteins with alterations in the hydrophobic domain lack pore-forming activity and stain to a light blue colour, allowing differentiation from wild-type clones. This phenotypic property correlates with the rate of entry of the β-galactosidase substrate into the bacteria. The method developed here was used to screen more than 8000 E. coli clones after random PCR mutagenesis of the poliovirus 3AB gene. Our results show the existence of three different domains involved in the permeabilizing activity of 3AB protein. Twenty individual amino acid substitutions were identified in clones that showed the mutant phenotype and such bacteria displayed different reduced levels of permeability towards ONPG, hygromycin B, lysozyme and uridine. The procedure reported here may be of general interest to understand structure–function relationships in other eukaryotic proteins known to form pores.

Introduction

Poliovirus, like all members of the family Picornaviridae, has a messenger-sense ssRNA genome (Semler et al., 1988; Harber & Wimmer, 1993). Upon infection, the virus RNA gains access to the cytoplasm, where the genome is replicated and then encapsidated into new virus particles (Semler et al., 1988; Wimmer et al., 1987). As these events take place, various alterations are observed in the infected cell. Such changes include a number of morphological and physiological alterations that culminate in cell lysis and the release of the new progeny virions (Koch & Koch, 1985; Carrasco & Castrillo, 1987). These alterations are commonly referred as the cytopathic effect (CPE). Appearance of CPE and subsequent lysis of the host cell require expression of the virus genome (Carrasco et al., 1989) and it has been proposed that both the mechanism underlying CPE and host cell death are associated with alterations of the plasma membrane (Carrasco & Castrillo, 1987; Carrasco, 1977; Carrasco et al., 1993). According to this hypothesis, virus components are targeted to the cell membrane and alter its function, thereby causing interference with several cellular processes and resulting in cell lysis and death (Carrasco et al., 1989, 1993). In agreement with this model, profound changes in permeability towards ions and molecules of different sizes are observed in host cells after expression of the poliovirus genome (Carrasco & Smith, 1976; Carrasco, 1978; Lopez-Rivas et al., 1987).

Although the increase in membrane permeability that occurs during poliovirus infection has been well documented (Carrasco et al., 1993), there are few data regarding the identification of the virus protein(s) involved in this phenomenon. Expression of 3AB and 3A is extremely toxic to bacteria and this toxicity correlates with the ability of these proteins to make Escherichia coli cells permeable to different compounds (Lama & Carrasco, 1992a, b). These data suggest that the two poliovirus polypeptides may act as pore-forming proteins.

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when expressed in *E. coli* cells (Carrasco *et al.*, 1993). This is not a new concept for virus proteins, since it has long been known that bacteriophages permeabilize bacterial membranes to host or virus-coded lysozymes using pore-forming proteins, thus leading to lysis of the infected cells. Examples include the *E* gene product from phage *φX174* (Young & Young, 1982) and the *S* protein from bacteriophage lambda (Garret & Young, 1992). In addition, the influenza virus 10 kDa M2 protein forms ion channels when expressed in *Xenopus* oocytes (Pinto *et al.*, 1992) and alters membrane permeability upon expression in bacteria in a similar way to 3AB protein (Guinea & Carrasco, 1994). Perturbation of host cell membrane permeability has also been recognized as a major cause of the cytopathology observed in human immunodeficiency virus (HIV)-infected cells (Cloyd & Lynn, 1991). So far, the membrane-permeabilizing activity that takes place upon poliovirus infection has not been ascribed to any particular gene.

3AB is a 12 kDa membrane protein containing a hydrophobic stretch of 22 amino acids (Semler *et al.*, 1988). Its role in the poliovirus replication cycle remains largely unknown, but purified 3AB greatly stimulates the activity of the virus RNA-dependent RNA polymerase (*3Dpol*) in *vivo* (Lama *et al.*, 1994). Construction of mutant viruses encoding 3AB proteins that were inactive in the ability to transactivate *3Dpol* allowed the demonstration of a direct role for this protein in the synthesis of virus-specific RNAs (Lama *et al.*, 1995). In addition, amino acid substitutions in the 3AB hydrophobic region drastically altered the proteolytic processing pattern of the virus polyprotein (Giachetti *et al.*, 1992). The insertion of poliovirus precursor polypeptides into the membrane, a process that probably involves the 3AB domain, may be responsible for virus-specific proteases (Lama *et al.*, 1994). A role for 3AB in the initiation of RNA synthesis, as a precursor of the genome-bound protein, has also been suggested (Takegami *et al.*, 1994). Perturbation of host cell membrane permeability has also been recognized as a major cause of the cytopathology observed in human immunodeficiency virus (HIV)-infected cells (Cloyd & Lynn, 1991). So far, the membrane-permeabilizing activity that takes place upon poliovirus infection has not been ascribed to any particular gene.

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We have now carried out a genetic analysis using random mutagenesis followed by screening and characterization of non-permeabilizing 3AB mutants. We describe here a sensitive and easy system for identifying key residues in 3AB that are involved in its pore-forming activity. This approach has allowed us to define those regions of the protein that are involved in the modification of membrane permeability, providing a method that could be widely used to characterize other membrane proteins endowed with such an activity.

**Methods**

**Bacterial strains and plasmids.** Expression of 3AB was carried out in BL21(DE3) *E. coli* cells (Studier *et al.*, 1990). Background cellular synthesis can be blocked by rifampicin without affecting the expression of the recombinant gene (Lama *et al.*, 1992). Cloning and expression of the wild-type 3AB gene has been described elsewhere (Lama & Carrasco, 1992a). The 3AB gene, after addition of initiation and stop codons, was cloned in plasmid pET11B (Dudendorff & Studier, 1991) to generate pT7lac3AB.

**Random mutagenesis of the 3AB gene by PCR amplification.** A PCRR-based random mutagenesis approach was used. Oligonucleotides hybridizing to complementary strands of pET11B plasmid were synthesized. Primer 5'-11B (TCGGTGATGTCGGCGATATATAGG) hybridized to a region that is located 200 nucleotides away from the Xbal site and is upstream from the T7 promoter. Primer 3'-11B (CAGGGTATTGTCTCATGAGCGG) hybridized to a region that is 172 nucleotides away from the EcoRI site and is downstream from the 3AB gene. PCR reaction conditions were: 10 mM-Tris-HCl pH 8.0, 50 mM-KCl, 1.5 or 3 mM-MgCl2 and 0.01% (w/v) gelatin, containing 200 nM each dNTP, 1-4 pg/ml of template DNA. (SphI-digested pT7lac3AB), 0.5 mM of each primer and 0.05 units/ml of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). The reaction was carried out for 30 cycles (1 min 92 °C; 1 min 37 °C; 3 min 72 °C; 10 min 72 °C last cycle). The reaction product was washed using GeneClean (Bio101) and digested with EcoRI and Xhol. The fragment containing the T7 promoter–3AB gene cassette was gel-purified and ligated into pT7lac3AB that had been previously digested with the same enzymes. Ligation products were used to transform electrocompetent BL21(DE3) cells with a Bio-Rad Gene Pulser (25 mF, 2.5 kV, 200 W). Transformation efficiencies of 107-108 per µg DNA were routinely obtained. Fresh colonies were selected for screening.

**Screening procedure to select 3AB mutants lacking pore-forming activity.** After electrotransfection of BL21(DE3) competent cells with a random mixture of 3AB poliovirus mutants (plasmid pT7lac3AB), ampicillin-resistant clones were grown overnight on LB plates (300-500 colonies/plate). These clones were transferred to nitrocellulose filters and left on fresh plates containing 10 µl of 0.2 mM- IPTG and 100 µl of 20 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Plates were incubated for 3-4 h to allow development of the blue colour. An alternative method makes use of *E. coli* cells that also express T7 lysozyme. In this case BL21(DE3)pLysS electrocompetent cells were transformed with pT7lac3AB and processed as indicated above, but this time rifampicin (5 µl of a 34 mg/ml solution) was included in the second LB plate. With this procedure a blue halo developed around wild-type clones after 6-7 h at 37 °C. DNA sequencing by the dideoxynucleotide chain termination method was used to identify nucleotide changes in the 3AB gene. Both DNA strands were sequenced. The T7 promoter region (also PCR-amplified) was also sequenced to make sure that changes in this region were not responsible for the observed phenotype.

**Assays for measurement of permeability changes.** O-nitrophenyl-β-D-galactopyranoside (ONPG) entry was tested essentially as described before (Lama & Carrasco, 1992b) by measuring the appearance of nitrophenol (by absorbance determinations at 420 nm). After induction of bacteria at the indicated times, cells were washed, ONPG was added to the medium and reaction mixtures were incubated for 15 min at 30 °C. Data were normalized for the total β-galactosidase (β-gal) activity obtained with each mutant. Total β-gal activity was expressed after lysing bacteria with chloroform-SDS. The effect of hygromycin B on translation was used to evaluate the entry of this compound into bacteria, as described previously (Lama & Carrasco, 1992b). Cells were induced with 1 mM-IPTG in the presence of rifampicin (150 µg/ml). Protein synthesis in the absence or presence of the drug was analysed by SDS–PAGE. The exit of lysozyme was analysed by
Results

Expression of 3AB in bacterial colonies elicits morphological changes

Our first aim was to develop a sensitive method to screen for non-permeabilizing mutants of E. coli expressing variants of the 3AB poliovirus protein. Expression of recombinant 3AB in bacteria produces a number of changes within the bacterial membranes that drastically alter their permeability to different compounds, probably as a consequence of a pore-forming activity of 3AB (Carrasco et al., 1993; Lama & Carrasco, 1992b). The presence of 3AB allows the unspecific diffusion of different molecules through the membrane in such a way that ONPG, a β-gal substrate, enters faster into bacteria that express 3AB. We decided to ascertain if permeability differences to ONPG or similar compounds might be useful for the characterization of 3AB-expressing bacteria. For this purpose, we took advantage of a 3AB mutant protein (Glu-75→Phe-76) that has a double change in the hydrophobic domain which spans amino acids 59–80 (Lama & Carrasco, 1995b). This mutant protein lacks permeabilizing activity and was used as a negative control in subsequent experiments.

BL21 (DE3) E. coli cells expressing either wild-type or the 3AB mutant were grown and mixed in a 1:1 ratio. Bacteria were grown overnight on LB plates and subsequently transferred to fresh plates containing both X-Gal, a chromogenic substrate for β-gal, and IPTG for induction of the recombinant protein. After 3 h of induction at 37 °C, two different types of clones were easily differentiated by their colour (Fig. 1a). Ten dark blue and 10 light blue clones were selected from the master plate and grown in M9 liquid medium. Proteins were labelled with [35S]methionine (2 μCi/ml) and analysed by SDS-PAGE. These colonies were originally chosen by their colour differences. Those with a dark blue tone were designated as W and those showing a light blue colour as M. The positions of the wild-type and the Glu-75→Phe-76 mutant protein are shown.

measuring cell lysis (drop in OD600). When T7 lysozyme leaks out from the bacterial cytoplasm to the periplasmic space a rapid lysis is observed. The exit of [3H]uridine was monitored as described previously (Lama & Carrasco, 1992b).

Fig. 1. Procedure to detect pore-forming 3AB mutants. (a) BL21 (DE3) E. coli cells containing the pT7lac3AB plasmid coding for either wild-type (W) or Glu-75→Phe-76 variant protein (M) were mixed in a 1:1 ratio and spread onto LB Petri plates. Cells were grown overnight and transferred to nitrocellulose filters, which were then placed onto fresh LB plates containing IPTG and X-Gal (see Methods). After 4 h of incubation at 37 °C, colonies were photographed. The genotype of the labelled colonies was assessed by analysis of the proteins synthesized upon induction (see c). (b) BL21 (DE3)pLysS cells expressing either wild-type or Glu-75→Phe-76 mutant protein were processed as described before, but in this case rifampicin was also added to freshly-prepared agar plates. A detail of the plate is shown. The genotype of the colonies was assessed by protein and restriction enzyme analyses. (c) Twenty clones from the master plate used in (a) were selected, grown and induced with 1 mM IPTG in M9 liquid medium. Proteins were labelled with [35S]methionine (2 μCi/ml) and analysed by SDS-PAGE. These colonies were originally chosen by their colour differences. Those with a dark blue tone were designated as W and those showing a light blue colour as M. The positions of the wild-type and the Glu-75→Phe-76 mutant protein are shown.
Table 1. ONPG entry and total β-gal activity of individual clones isolated from nitrocellulose filters after induction with IPTG

<table>
<thead>
<tr>
<th>3AB</th>
<th>Total β-gal (A_{420})</th>
<th>ONPG entry (A_{420})</th>
<th>ONPG entry/total β-gal</th>
<th>Stimulation (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.807 ± 0.032</td>
<td>0.596 ± 0.035</td>
<td>74</td>
<td>3.2</td>
</tr>
<tr>
<td>Glu-75-Phe-76</td>
<td>0.406 ± 0.020</td>
<td>0.094 ± 0.001</td>
<td>23</td>
<td>1.0</td>
</tr>
<tr>
<td>Lys-62</td>
<td>0.933 ± 0.047</td>
<td>0.500 ± 0.03</td>
<td>53</td>
<td>2.3</td>
</tr>
</tbody>
</table>

sure that clones showing phenotypic variations had undergone membrane permeability changes, we measured the entry of ONPG into individual clones that had been previously induced on nitrocellulose filters under the same conditions employed for colour screening. The entry of ONPG into the different clones was then immediately measured. As shown in Table 1, the ONPG entry level was six times greater in bacteria that express wild-type 3AB than in the Glu-75-Phe-76 mutant. Even though total β-gal activity was almost twofold greater in wild-type bacteria, normalization to this value still gave a threefold stimulation in the entry of ONPG in wild-type clones as compared with the Glu-75-Phe-76 mutant. This differences can easily account for the observed colour differences between the stained colonies. Another 3AB variant (Lys-62) that developed an intermediate blue colour when processed for the X-Gal assay (data not shown) showed a twofold increase in ONPG entry.

In control experiments where mutant bacteria were mixed with a 1000-fold excess of wild-type clones, the mutant colonies were easily recovered from the Petri plate. Therefore, we conclude that the procedure described above can be readily used to screen for non-permeabilizing 3AB mutants.

Expression of 3AB induces cell lysis in E. coli strains, such as BL21(DE3)pLysS cells, that contain a plasmid encoding for phage T7 lysozyme. Under normal conditions, the inner bacterial membrane retains the T7 lysozyme within the cytoplasm without affecting cellular growth. However, the permeabilization caused by 3AB allows the leakage of the lysozyme from the cytoplasm to the periplasmic space, causing rapid cell lysis (Lama & Carrasco, 1992b). We tested whether this phenomenon could also be used to differentiate bacteria phenotypically. In these experiments rifampicin was added to the plate to allow expression of the 3AB protein. T7 lysozyme is an inhibitor of T7 RNA polymerase and when both systems (the lac operon and T7 lysozyme) are used together to control expression no recombinant protein is detected after addition of IPTG alone (data not shown). Addition of rifampicin blocks expression of both T7 lysozyme and the lac repressor, and therefore de-represses the system and leads to 3AB expression.

BL21(DE3)pLysS cells that expressed either wild-type or Glu-75-Phe-76 variant proteins were mixed, grown, transferred to nitrocellulose and induced with IPTG, X-Gal and rifampicin. Fig. 1(b) shows a detailed portion of a plate. Bacteria expressing the wild-type protein developed a halo around the colony 3–5 times larger than the mutant counterpart (note also the differences in colony size between both clones). The genotype of the clones was assessed by protein and sequence enzyme analyses (results not shown). The results clearly show that X-Gal entry into cells (Fig. 1a) or the release of β-gal into agar plates (Fig. 1b) can both be used for the in vivo screening of 3AB variants.

Random PCR mutagenesis and screening for 3AB mutants

We used PCR to generate a random distribution of mutations throughout the 3AB gene. A number of variables control the rate of error of Taq DNA polymerase including, particularly, the number of reaction cycles and the concentrations of magnesium ions and dNTPs (White et al., 1989). Very high mutation rates would produce multiple changes within the 3AB gene, thus making it difficult to identify regions involved in permeability changes. Thus, the PCR reaction was carried out employing relatively high-fidelity conditions (1.5 mM-MgCl₂, 200 mM-dNTPs and 30 reaction cycles). After PCR, the amplified product was digested with EcoRI and XhoI restriction enzymes and cloned into the pT7lac3AB plasmid that had been digested with the same enzymes. Dephosphorylation was avoided during cloning. Plasmid pT7lac3AB was used instead of pET11B, the parental plasmid, to avoid negative false results that would be caused by pET11B-carrying bacteria that do not bear the insert (clones with light blue phenotype). In control experiments using the pET11B plasmid, the percentage of negative false results was above the frequency of mutants (data not shown). The ligation
Table 2. Mutations found in light blue colonies

<table>
<thead>
<tr>
<th>Expt</th>
<th>PCR conditions*</th>
<th>Screened clones</th>
<th>Mutant frequency†</th>
<th>Nucleotide changes‡</th>
<th>Nucleotide substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 mM-Mg²⁺</td>
<td>418</td>
<td>0.71</td>
<td>2:3</td>
<td>1 1 1 0</td>
</tr>
<tr>
<td>2</td>
<td>5 mM-Mg²⁺</td>
<td>2650</td>
<td>0.50</td>
<td>ND</td>
<td>ND ND ND ND</td>
</tr>
<tr>
<td>3</td>
<td>1.5 mM-Mg²⁺</td>
<td>3832</td>
<td>0.28</td>
<td>1:27</td>
<td>8 2 0 1</td>
</tr>
<tr>
<td>4</td>
<td>1.5 mM-Mg²⁺</td>
<td>8600</td>
<td>0.37</td>
<td>1:22</td>
<td>27 1 1 3</td>
</tr>
</tbody>
</table>

* PCR amplification was carried out for 30 cycles as described in Methods.
† Percentage of colonies with mutant phenotype.
‡ Average number of nucleotide substitutions per 3AB gene. ND, Not determined.
§ All deletions found were caused by single nucleotide substitutions.

Fig. 2. Effect of hygromycin B on the synthesis of wild-type and 3AB variants. BL21(DE3) cells carrying pT7lac3AB plasmids, expressing different variant 3AB proteins, were induced in the presence of rifampicin (see Methods). After addition of 2 mM-hygromycin B (+) or nothing (−), proteins were labelled with [35S]methionine and analysed by SDS-PAGE. 'Random' mutants analysed here come from experiment 4 shown in Table 2 and were obtained following the screening procedure shown in Fig. 1 (o). All these mutants express single amino acid substitutions in 3AB except for Met-60 → stop*, which contains a stop codon at position 60 generated from an out-of-frame deletion of one nucleotide. The latter mutant also expresses amino acid substitutions at positions 57, 58 and 59 of 3AB. For comparison, bacteria expressing wild-type and Glu-75–Phe-76 and Lys-62 variant proteins were also included. Three randomly chosen clones with a dark blue phenotype were also analysed.

mixture was utilized to transform electrocompetent BL21(DE3) cells and the X-Gal entry method (see Fig. 1a) was used to screen for mutants.

After screening 8600 clones (Table 2), 32 clones presenting the mutant phenotype were isolated (light blue colour). The mutant frequency of 0.37% was similar to that (0.28%) obtained in an independent experiment where 11 mutants were isolated from 3830 clones. This frequency is rather low in comparison with data reported elsewhere. Thus, Liebig et al. (1991) reported a mutant frequency of 5% for a PCR-based random mutagenesis method. However, in those experiments only 50% of the mutants had a single amino acid substitution as opposed to 27/32 (84%) obtained under our PCR conditions (Table 2). Raising the magnesium ion concentration from 1.5 to 5 mM increased the mutation frequency to 0.6% (Table 2). However, under these conditions most of the isolated mutants...
Table 3. Phenotypes of 3AB mutants

<table>
<thead>
<tr>
<th>Mutation</th>
<th>ONPG entry (%)</th>
<th>Lysis (%)</th>
<th>Hygromycin B inhibition (%)</th>
<th>Cell division (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys-9 → Glu</td>
<td>45</td>
<td>44</td>
<td>54</td>
<td>51</td>
</tr>
<tr>
<td>Ile-10 → Val</td>
<td>49</td>
<td>65</td>
<td>81</td>
<td>41</td>
</tr>
<tr>
<td>Ile-12 → Val</td>
<td>45</td>
<td>59</td>
<td>91</td>
<td>28</td>
</tr>
<tr>
<td>Lys-13 → Glu</td>
<td>45</td>
<td>47</td>
<td>84</td>
<td>48</td>
</tr>
<tr>
<td>Lys-39 → Glu</td>
<td>37</td>
<td>40</td>
<td>61</td>
<td>67</td>
</tr>
<tr>
<td>Trp-42 → Arg</td>
<td>46</td>
<td>40</td>
<td>60</td>
<td>64</td>
</tr>
<tr>
<td>Val-44 → Gly</td>
<td>23</td>
<td>41</td>
<td>69</td>
<td>40</td>
</tr>
<tr>
<td>Val-44 → Ala</td>
<td>37</td>
<td>36</td>
<td>70</td>
<td>50</td>
</tr>
<tr>
<td>Asn-45 → Asp</td>
<td>32</td>
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<td>38</td>
<td>69</td>
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<td>Ile-46 → Thr</td>
<td>25</td>
<td>48</td>
<td>67</td>
<td>89</td>
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<tr>
<td>Gln-49 → stop</td>
<td>16</td>
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<td>2</td>
<td>55</td>
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<tr>
<td>Val-50 → Ala</td>
<td>43</td>
<td>59</td>
<td>68</td>
<td>49</td>
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<tr>
<td>Met-60 → stop§</td>
<td>21</td>
<td>ND</td>
<td>18</td>
<td>56</td>
</tr>
<tr>
<td>Leu-63 → Pro</td>
<td>29</td>
<td>28</td>
<td>46</td>
<td>64</td>
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<tr>
<td>Thr-67 → Pro</td>
<td>18</td>
<td>ND</td>
<td>88</td>
<td>47</td>
</tr>
<tr>
<td>Tyr-77 → His</td>
<td>24</td>
<td>19</td>
<td>33</td>
<td>68</td>
</tr>
<tr>
<td>Met-79 → Lys</td>
<td>28</td>
<td>1</td>
<td>35</td>
<td>68</td>
</tr>
<tr>
<td>Tyr-80 → stop</td>
<td>29</td>
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<td>66</td>
<td>60</td>
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<td>Lys-81 → Glu</td>
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<td>88</td>
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<td>Leu-82 → Pro</td>
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<tr>
<td>Ile-62 → Lys</td>
<td>44</td>
<td>60</td>
<td>89</td>
<td>72</td>
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<tr>
<td>Val-75 → Glu, Val-76 → Phe</td>
<td>20</td>
<td>0</td>
<td>14</td>
<td>88</td>
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<tr>
<td>No protein</td>
<td></td>
<td></td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Wild-type</td>
<td>61</td>
<td>80</td>
<td>100</td>
<td>11</td>
</tr>
</tbody>
</table>

* Data represent the percentage of the total β-gal activity that was achieved without lysing the cells.
† Measured in pLysS cells in the presence of rifampicin after 2 h of induction. ND, Not determined.
‡ Protein synthesis was measured by densitometric scanning of SDS-polyacrylamide gels, as shown in Fig. 2. Percentage inhibition is shown.
§ The fraction of the culture undergoing cell division after 2 h of induction was estimated by measuring the absorbance at 660 nm. The percentage of the culture at time zero is shown.
|| This mutant also carried amino acid substitutions at positions 57, 58 and 59.
\* Bacteria carrying plasmid pET11B alone. Hygromycin B inhibition was measured in the absence of rifampicin.

contained two or three amino acid substitutions (data not shown).

Characterization of 3AB mutants: modification of membrane permeability

Membrane permeability changes in 3AB mutants displaying the light blue phenotype were characterized in detail. Firstly, the 3AB gene from each of these mutants was totally sequenced to determine the amino acid change introduced by PCR and the mutants were then analysed for modifications in membrane permeability. All these mutants showed different levels of permeabilizing activity relative to cells expressing the wild-type 3AB protein. We studied the effect of hygromycin B on translation within recombinant clones. Hygromycin B is an effective inhibitor of eukaryotic and prokaryotic cell-free protein-synthesizing systems (Carrasco & Vazquez, 1983) but enters animal cells only slowly under normal conditions. Infection with animal viruses makes such cells readily permeable and sensitive to this antibiotic (Carrasco, 1978; Carrasco & Vazquez, 1983; Contreras & Carrasco, 1979). As previously demonstrated (Lama & Carrasco, 1992b, 1995) and also shown in Fig. 2, translation in bacteria expressing wild-type 3AB was completely blocked by addition of the drug (more than 99% inhibition). However, the Glu-75-Phe-76 mutant was resistant to hygromycin B (14% inhibition only). Indeed, when hygromycin B sensitivity was determined for the other isolated mutants, all of them showed higher levels of resistance to the drug than bacteria expressing the wild-type 3AB protein (Fig. 2). Translation in control bacteria which do not express 3AB was not inhibited under these experimental conditions (data not shown). Changes located in the hydrophobic domain of 3A, as typified by a tyrosine to histidine substitution at position 77, resulted in strong resistance to the drug. Translation stop codons introduced before the hydrophobic region of 3AB (amino acids 59-80) rendered bacteria...
completely resistant to hygromycin B (i.e. positions 49 or 60). In contrast, a stop codon introduced at position 80, just at the C terminus of the hydrophobic domain of 3A, rendered bacteria partially sensitive to the drug. Mutations at position 82 (leucine to proline) or 45 (asparagine to aspartic acid), both of which are outside the hydrophobic domain, also conferred strong resistance to hygromycin B. These results corroborate previous findings (Lama & Carrasco, 1995) showing that the integrity of the hydrophobic domain spanning amino acids 59–80 of 3AB is important for eliciting permeability changes and also demonstrate that other regions of the protein participate in this activity. Of particular importance is another hydrophobic region that contains basic amino acids and is located between positions 40–50.

The entry of the β-gal substrate ONPG into bacteria expressing the different forms of 3AB was also analysed. Under our reaction conditions, 60% of the total β-gal activity in wild-type clones was accessible when the ONPG substrate was added to the medium without lysing the cells (Table 3). When either no recombinant protein or mutant Glu-75-Phe-76 was expressed, only 20% of the total activity was accessible without lysing cells. Mutants obtained by X-Gal screening showed values of ONPG entry varying from 20% (leucine 82 to proline, tyrosine 77 to histidine, methionine 60 to stop, valine 44 to glycine, etc.) to almost 50% (e.g. isoleucine 10 to valine).

We also studied the exit of compounds from induced cells to determine if the increase in membrane permeability was bidirectional. To this end, E. coli cells were preloaded with [3H]uridine and the exit of the compound was estimated by measuring the radioactivity released to the medium. Fig. 3 shows the behaviour of some of the 3AB mutants, as compared with bacteria expressing the wild-type protein and with bacteria expressing no recombinant protein. The release of radioactivity from the mutants varied between those observed for bacteria expressing the Glu-75–Phe-76 3AB protein (where loss of uridine was 15 times slower than in wild-type clones) to those for bacteria expressing the mutant protein with a valine to alanine change at position 50 (where loss of uridine was four times slower than in wild-type clones). No cellular lysis was detected during the course of the experiment shown in Fig. 3, either by measuring the OD660 or by measuring the appearance of β-gal activity in the culture medium.

We also studied the toxicity of the different 3AB mutants by assessing the abilities of the expressed variant proteins to arrest cell division. After induction of the recombinant wild-type protein, only 10% of the culture underwent cell division after 2 h of induction, compared with 90% of cells expressing the Glu-75–Phe-76 variant. Screened mutants gave values ranging between both extremes (Table 3).

Expression of wild-type 3AB in BL21(DE3)pLysS cells causes cell lysis (Lama & Carrasco, 1992b). These cells contain T7 lysozyme (Studier, 1991) and expression of the virus protein allows leakage of the enzyme to the peptidoglycan layer, thereby causing cell lysis. In this respect the 3AB protein resembles the mode of action of bacteriophage ‘holins’, which permeabilize the inner bacterial membrane to host or virus-coded lysozymes and thus cause cell lysis (Young, 1992). Mutations directed to the hydrophobic domain of any of the screened mutants greatly reduced the lytic activity (Fig. 4). In addition, other mutations located in the N terminus of the virus protein also reduced the lytic activity to varying degrees.

**Discussion**

To gain further insight into the role of 3AB in the modification of cellular membranes, the generation and analysis of viable mutant viruses that express variants of this protein would be of great value. Apparently, 3AB performs several functions in the poliovirus growth cycle (Giachetti et al., 1992; Lama et al., 1994, 1995), and we anticipate that interpretation of data obtained with 3AB-mutated full-length poliovirus genomes may not be straightforward. For instance, any mutation that abolishes the binding of 3AB to cellular membranes would destroy the pore-forming activity of the protein and such mutations might also interfere with the proteolytic processing of the polyprotein. Therefore, it is of
Fig. 4. Lytic effect of variant 3AB proteins. BL21 (DE3)pLysS cells, expressing different 3AB proteins, were induced with 1 mM IPTG. Rifampicin was present from 30 min post-induction. The number of cells at different times post-induction was estimated by turbidimetry (OD$_{660}$). Mutants shown in this experiment come from experiment 4 (Table 2). Wild-type, Glu-75–Phe-76 mutant and control bacteria, expressing no recombinant protein, were also included. For clarity data obtained from the same experiment have been divided into two plots. The single-letter amino acid code is used.

Fig. 5. (a) Schematic representation of the 3AB protein showing the positions of the different amino acid changes. The length of the line showing each variant represents the degree of change as measured by the lack of permeabilization to hygromycin B. The largest line corresponds to the least permeabilizing capacity. The amino acid sequence of poliovirus 3A protein is represented. (b) Two alternative models to explain the mode of action of 3AB as a membrane permeabilizer are represented.
interest to determine if certain residues in 3AB are involved exclusively in permeabilizing activity. Accordingly, we developed a new approach to identify and analyze 3AB mutants that lack the ability to permeabilize bacterial membranes. Our method relies on the in vivo expression of the 3AB recombinant protein in E. coli and allows an assessment of how this expression affects the inner bacterial membrane. Expression of the 3AB poliovirus protein produces a threefold increase in the uptake of the β-gal substrate ONPG relative to the level observed before induction. This property was used to develop a procedure where phenotypic differences in 3AB-expressing clones were related to differences in the rate of entry of X-Gal.

**Permeabilizing 3AB mutants**

A schematic representation of protein 3AB illustrating the locations affected by the mutations is depicted in Fig. 5(a). The detected mutations map in three separate regions of the gene for the 3A protein. Mutations conferring the lowest permeabilizing activity introduce amino acid changes in the C terminus of the hydrophobic domain of the protein. Two additional regions were identified by X-Gal screening. The first one, located around amino acids 9–13, was responsible for the appearance of weak mutant phenotypes. Another domain around residues 39–50 was also involved in the permeabilizing phenomenon, with amino acid substitutions in this region greatly affecting the permeabilizing activity. Our screening procedure was sensitive enough to detect very subtle substitutions. For instance, removal of a methyl group far away from the hydrophobic region (isoleucine to valine substitutions at either position 10 or 12) reduced by four- to fivefold the rate of leakage of radioactivity from [3H]uridine-preloaded cells.

A total of 20 different single amino acid substitutions were found in an experiment where more than 8000 clones were screened. This number indicates that one-fifth of the 3AB codons and almost one quarter of the 87 3A codons are susceptible to mutate to a less permeabilizing phenotype. These data are consistent with the hypothesis that 3AB does not perform its permeabilizing activity as an enzyme and hence has no active site which would be shown by a small number of mutated amino acids. The available data suggest that 3AB probably forms pores, although we do not have direct evidence to demonstrate the presence of such structures in E. coli cells expressing 3AB. It can be speculated that mutations affecting the permeabilizing properties of 3AB could be grouped into two classes: (i) those directly affecting the insertion of the virus protein into the bacterial membranes; and (ii) mutations altering the correct ‘membrane–protein’ or protein structure that is required for 3AB function. Mutations located in the hydrophobic domain and causing the introduction of charged groups into the membrane-spanning region (e.g. Lys-79 or His-77) would belong to class (i). The hydrophobic region (amino acids 59–80) is likely to span the bilayer by way of an α-helix, as predicted by computer analysis (Carrasco et al., 1993). Mutations that introduce proline residues into this region (e.g. Pro-63 and Pro-67) should also be grouped into class (i). By studying the differential sensitivity to solubilization by 5 M-guanidine, we have determined that mutants with either charged amino acids or proline residues introduced in the hydrophobic domain failed to correctly associate with membranes (data not shown). In addition, immunogold analysis localizes wild-type 3AB protein, but not the Glu-75–Phe-76 mutant, to the inner membrane of E. coli cells (data not shown).

Interestingly, a leucine to proline substitution at position 82, outside the hydrophobic domain, rendered 3AB soluble in 5 M-guanidine (data not shown) and caused one of the lowest permeabilizing activities (Table 3). This finding suggests that either α-helix structures placed beyond the hydrophobic domain are also involved in protein function or that the region needed to associate with membranes goes beyond amino acid 80 and also includes the highly charged residue at position 81 (lysine). Mutations affecting protein–protein or membrane–protein interactions or those altering the structure of the hydrophilic lumen of the pore would belong to class (ii). Protein 3AB forms high molecular weight oligomeric structures when expressed in E. coli (J. Lama & L. Carrasco, unpublished results). The formation of oligomers by this 12 kDa protein, as diagrammatically illustrated in Fig. 5(b, model 2), should give a macromolecular structure large enough to permit the release of small proteins like T7 lysozyme. It is interesting to note that some of the mutant proteins failed to induce lysozyme exit (e.g. Met-79 to lysine and Tyr-80 to stop) but the effect of these mutations on uridine exit and hygromycin entry were limited (Table 3), suggesting that these proteins can still associate with membranes and permeabilize them to small molecules but the pore-forming activity is selectively blocked. Specific membrane–protein interactions may also be important for 3AB function. The protein may partially exert its permeabilizing action by interacting with phospholipids, thereby disorganizing the membrane in a detergent-like manner (model 1, Fig. 5b). In this model, charged amino acids might interact with polar phospholipid heads. Nevertheless, under our expression conditions where recombinant proteins are synthesized to high levels, a model where the membrane is disorganized in an unspecific way would finally lead to complete and total permeabilization of the cells. Note, however, that 3AB permeabilizes bacteria to T7 lysozyme but not to the β-gal. Some of the detected mutations involve positively charged lysine groups (positions 9, 13, 39 and 81) which are substituted by negatively charged glutamic acid residues. Such residues may interact directly with the negatively charged phospholipid heads. The simplified model 2 (Fig. 5b), where the virus protein crosses the lipid bilayer once, may not be truly representative. In some bacteriophage lytic proteins, the polypeptide chain is thought to traverse the membrane two or three times (Young, 1992). The structures of such membrane-spanning regions are difficult to predict by computer analysis because of the existence of charged or polar groups inside the membrane region. These groups are
accommodated into the bilayer because they are cancelled by in-ternal-eral or interhelical salt bridges. This situation applies to rhodopsin, whose three-dimensional structure is known, and where multiple charged residues are found spanning the membrane (Eisenberg et al., 1982). Mutations expressed between positions 9-13 and 39-50 might provide the potential to indicate whether these positions in 3AB are in membrane-imbedded regions.

Protein 3AB by itself shows a potent permeabilizing activity in E. coli, even though all the mutants screened map to the 3A portion of 3AB. This finding suggests that 3A, and not 3AB, is the protein involved in the production of membrane permeability changes in poliovirus-infected cells. More extensive mutagenesis studies might detect key amino acids that are involved in this activity in the VPg moiety, although this is perhaps an unlikely possibility since 3A alone is endowed with full permeabilizing capacity (Lama & Carrasco, 1992b) and 3B is not a hydrophobic protein. The roles of the 3A and 3AB proteins in the picornavirus replication cycle remain largely unknown. 3A-containing polypeptides have been localized in the membranous vesicles that are induced after infection and where active genome replication takes place (Takegami et al., 1983 a, b). So far, we have failed to reproduce the permeabilizing activity of 3A/3AB proteins on the plasma membrane of eukaryotic cells. Rather than a lack of permeabilizing activity in animal cells, these results may reveal an impediment for these proteins to localize to the cytoplasmic membrane. Locations for either 3AB or 3A on the cell surface of infected cells have never been reported. In addition, a recent report has shown that poliovirus infection inhibits protein transport to the plasma membrane and this effect can be reproduced by individual expression of either 3A or 2B proteins (Doedens & Kirkegaard, 1995). Therefore, synthesis of any of these proteins may impede their trafficking to the plasma membrane. The possibility remains that 3AB protein might exert CPE by forming pores after it has been localized in cytoplasmic vesicles and thereby increasing the intracellular calcium concentration. Such an increase has been implicated in the cytotoxic effects induced by rotavirus (Tiang et al., 1993). Preliminary data have shown that most of the mutations characterized in this work are lethal for virus growth, specially those affecting the association of 3AB with membranes. Nevertheless, substitutions in the amino acid 44-46 region greatly affect the CPE of the virus without altering the synthesis or processing of the virus polyprotein (data not shown). Further experiments will be required to ascertain whether the decreased CPE is actually due to a lack of permeabilizing activity in 3A/3AB.

Applications of the screening procedure

The method described in this work for the selection of variants of pore-forming proteins is rapid, easy and sensitive. Mutant frequencies around 1/300 were obtained in two independent experiments. Moreover, the procedure was qualitative, thereby allowing detection of mutants with a broad range of phenotypic properties. We have developed two different methods for the screening of mutants. The X-Gal entry method was chosen to screen for 3AB mutants. Analysis of light blue colonies against a background of dark blue wild-type colonies provided a more sensitive method than the one based on cellular lysis. The latter, however, allows a more useful approach for detection of wild-type clones in a background of non-permeabilized bacteria. This would be the case when searching for proteins with pore-forming activity in cDNA libraries of complex virus genomes, or when searching for second-site revertants that reactivate a non-functional mutant.

There are several potential limitations to the methodology. Since the assay takes place in bacterial cells, certain post-translational modifications (e.g. glycosylation or phosphorylation) or even proper folding of the recombinant protein may not occur in these prokaryotic cells.

E. coli clones that had been co-transformed with wild-type and variant 3AB proteins were found with a low frequency in our screenings. Some of these clones showed a mutant phenotype (bacteria were not permeabilized upon induction) suggesting that wild-type and mutant products interact with each other (J. Lama & L. Carrasco, unpublished results). Screening and identification of such trans-dominant mutants would allow the construction of genetically engineered viruses designed to interfere with the replication and spreading of wild-type viruses. This kind of strategy was called 'intracellular immunization' (Baltimore, 1988) and has been proved to be useful against in vitro HIV-1 replication (Trono et al., 1989; Sullenger et al., 1990). Another interesting strategy would be to adapt the X-Gal procedure to screen for thermosensitive 3AB mutants in E. coli.

We believe that the approach described in the present work may be extended to other proteins with known pore-forming activity. Structure-function relationship studies on perforins, complement proteins and a great number of lytic peptides from eukaryotic and prokaryotic origin are suitable candidates for analysis using our procedure. Our approach could also be invaluable for the identification and characterization of other virus proteins involved in membrane damage in animal virus-infected cells.

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