Deletion mapping of functional domains in the rotavirus capsid protein VP6

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VP6, the major capsid protein of rotavirus, oligomerizes into trimers that constitute the intermediate shell of the virions. In order to map functional domains in this protein, we introduced seven internal in-frame deletions within the coding region of gene 6 of human rotavirus strain Wa. Regions of homology among the VP6 proteins of group A and group C rotaviruses were targeted for deletion mutagenesis. The mutant VP6 proteins were expressed in mammalian cells using the recombinant vaccinia virus system and were examined for their ability to oligomerize into trimers as well as to assemble into double-layered virus-like particles upon coexpression with the rotavirus core protein VP2. Deletions that abolished trimerization defined a domain (residues 246 to 314) that maps within a larger region previously found to be critical for oligomerization (amino acids 105 to 328). When the capacity of each mutant to assemble into double-layered virus-like particles was analysed, three different assembly phenotypes were observed. Phenotype I was represented by two deletion mutants lacking residues 246 to 250 and 308 to 314 that produced particles with efficiencies similar to that of wild-type VP6. Phenotype II, characterized by a moderate decrease in the efficiency of particle assembly with respect to that of wild-type VP6, included two mutants with deletions at the C terminus of the protein. Phenotype III was exhibited by three mutants whose abilities to assemble into double-layered virus-like particles were drastically impaired. Two of these mutants define a previously unidentified assembly domain (amino acids 122 to 147) at the N terminus of rotavirus VP6.

Introduction

Rotaviruses, a genus of the family Reoviridae, are the most important cause of severe viral gastroenteritis in infants and young animals. The infectious virion consists of three concentric protein shells and a genome of 11 segments of double-stranded (ds) RNA (Prasad & Chiu, 1994). The innermost shell, which is made up of the RNA-binding protein VP2 (Labbé et al., 1991), encloses the dsRNA genome together with VP1 and VP3 proteins. The intermediate protein layer is composed of VP6 molecules which are present in the form of 260 trimers. The outermost shell is made up of the glycoprotein VP7 and dimeric spikes of VP4 (Shaw et al., 1993).

VP6 is the most abundant protein of the virion comprising about 51% of the total protein mass (Mattion et al., 1994). This protein bears group- and subgroup-specific epitopes (Greenberg et al., 1983), which make it the main target of diagnostic tests used for rotavirus detection. It has been shown in both insect and mammalian cells systems that coexpression of VP2 and VP6 results in the formation of double-layered virus-like particles (VLPs) (Labbé et al., 1991; González & Affranchino, 1995a). Furthermore, triple-layered rotavirus-like particles were obtained by simultaneous expression of VP2, VP4, VP6 and VP7 in insect cells (Crawford et al., 1994). The fact that VP6 constitutes the intermediate shell of the virion together with its ability to mediate the formation of recombinant double- and triple-layered particles point to a central role of VP6 in rotavirus assembly, most likely by the establishment of interactions with VP2, VP4 and VP7. Therefore, mapping of the VP6 domains involved in these protein–protein interactions will improve our understanding of the rotavirus assembly process. In this regard, Clapp & Patton (1991) have examined the ability of amino- and carboxyl-truncated species of VP6 to trimerize and bind to double-layered particles in vitro. They showed that the
trimerization domain of VP6 maps to residues 105 to 328, whereas an assembly domain is located between amino acids 251 and 397.

We have recently demonstrated the suitability of the vaccinia virus expression system to produce intracellular recombinant double-layered rotavirus-like particles in mammalian cells (González & Affranchino, 1995a). In this report, we exploit this system to analyse the interactions of VP2 and VP6 in vivo. We introduced a series of internal deletions within the open reading frame of the gene coding for VP6 of human rotavirus strain Wa. Following expression in the vaccinia virus system, the VP6 mutants were assessed for their ability to oligomerize into trimers as well as to interact with VP2 to assemble into double-layered VLPs.

Methods

**Construction of recombinant vaccinia viruses.** All rotavirus sequences were derived from the human strain Wa (G serotype 1 and P serotype 8; Matton et al., 1994) The construction and characterization of the recombinant vaccinia viruses vv-Wa-2 and vv-Wa-6 expressing VP2 and VP6 of rotavirus strain Wa, respectively, have been described before (González & Affranchino, 1995a). The cDNA clone of gene 6 was used as the parental gene for all mutagenesis. Seven internal in-frame deletions were introduced into the coding region of gene 6 by asymmetric PCR-based site-directed mutagenesis as described before (González et al., 1993; González & Affranchino, 1995b). The mutated VP6 genes were subjected to DNA sequencing to confirm the presence of the corresponding deletions and the absence of nucleotide misincorporations due to the gene amplification steps. In all cases, the mutated VP6 genes were cloned into the vaccinia transfer vector pMY601 (Davison & Moss, 1990). Recombinant vaccinia viruses were obtained, selected and purified as previously described (González et al., 1993). The recombinant vaccinia viruses expressing the VP6 deletion mutants were designated vv-D1 to vv-D7.

**Protein expression and metabolic labelling.** The recombinant vaccinia viruses expressing VP2, VP6 and the VP6 deletion mutants were used to infect confluent monolayers of CV-1 cells (35 mm diameter Petri dishes) at a multiplicity of 5 p.f.u. per cell for each virus. At 13 h post-infection, the cells were starved for 1 h in methionine- and cysteine-deficient DMEM containing 3% foetal bovine serum and metabolically labelled for 4 h as described above, harvested and lysed in 100 µl of TND buffer. The lysate was clarified by centrifugation for 10 min at 12000 g to remove nuclei and cell debris. VLPs were purified from the post-nuclear supernatants by ultracentrifugation through a 30% (w/v) sucrose cushion as previously described (González & Affranchino, 1995a). The resulting pellet was immunoprecipitated with the anti-Wa serum and viral polypeptides were resolved on SDS–10% polyacrylamide gels.

**Electron microscopy.** CV-1 cells coinfected with vv-Wa-2 and each of the vaccinia viruses expressing the VP6 mutants (vv-D1 to vv-D7) at a multiplicity of 5 p.f.u. per cell for each virus were harvested at 24 h post-infection. Particles were purified from cell lysates as described above and resuspended in 10 mM Tris–HCl (pH 7.5). Negative staining of the VLPs and electron microscopy analysis of the samples were performed as previously described (González & Affranchino, 1995a).

Results

**Construction of VP6 deletion mutants and analysis of expression**

To study the functional domains of VP6, seven deletions were constructed in which five to thirteen codons were removed from within the VP6 coding region. The location of the deletions (D1 to D7) is shown in Fig. 1. The regions targeted for deletion comprise domains of high sequence homology among the VP6 proteins of group A and C rotaviruses (Table 1). The mutated VP6 genes were used to generate recombinant vaccinia viruses expressing the corresponding deleted VP6 molecules, whose phenotypes were then characterized.

To determine whether the deletions introduced into VP6 had any effect on the synthesis or stability of this protein, CV-1 cells were infected with the recombinant vaccinia viruses and metabolically labelled for 4 h as described in Methods. VP6 was immunoprecipitated from the cell lysates with a polyclonal anti-Wa serum. As shown in Fig. 2, the recombinant vaccinia viruses expressing the VP6 deletion mutants directed the synthesis of essentially wild-type levels of VP6 protein. The relative electrophoretic migration of the recombinant VP6 proteins expressed by the deletion mutants correlated with the extent of the deletion introduced into each construct (Fig. 2). The sole exception was mutant D4, carrying one of the largest deletions, which migrated slightly faster than expected. The possibility of an error in the VP6 coding sequence of the D4 mutant was excluded on the basis of the sequencing data of mutant D4 DNA. It is therefore likely that the electrophoretic mobility observed for this mutant reflects not only the extent of the deletion introduced into the molecule but also the absence of the specific residues that were removed from the protein.

**Effect of VP6 deletions on trimer formation**

To investigate the ability of the VP6 deletion mutants to oligomerize into trimers, samples of 35S-labelled lysates from

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The document is a research paper discussing the construction and characterization of recombinant vaccinia viruses expressing different deletion mutants of the VP6 protein of human rotavirus strain Wa. The study aimed to analyze the interactions of VP2 and VP6 and the ability of VP6 deletion mutants to assemble into double-layered VLPs. The results showed that the recombinant vaccinia viruses expressing the deleted versions of VP6 (vv-D1 to vv-D7) had wild-type levels of VP6 protein and were able to form trimers, demonstrating the suitability of the vaccinia virus expression system for studying rotavirus protein interactions.

**Methods**

- **Construction of recombinant vaccinia viruses.** The construction and characterization of recombinant vaccinia viruses expressing VP2 and VP6 of rotavirus strain Wa were described previously (González & Affranchino, 1995a).
- **Protein expression and metabolic labelling.** The recombinant vaccinia viruses were used to infect CV-1 cells, and the VP6 deletion mutants were metabolically labelled with [35S]methionine/cysteine mix for 4 h.

**Results**

- **Construction of VP6 deletion mutants and analysis of expression.** The ability of the deletion mutants to form trimers was assessed, showing wild-type levels of VP6 protein.

**Effect of VP6 deletions on trimer formation.** Samples of [35S]labelled lysates were analyzed to assess the ability of the VP6 deletion mutants to oligomerize into trimers.
Fig. 1. Location of deletions in the Wa VP6 protein. The position and length of each deletion is indicated by a line below the sequence. Deletions were numbered D1 to D7 from the amino to the carboxyl terminus of the protein.

Table 1. Deletion mutagenesis of group A rotavirus VP6

<table>
<thead>
<tr>
<th>Deletion</th>
<th>Residues*</th>
<th>Group A versus Group C†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>122–134 I K F K R I N F D N S S E 122–134 S---T---N---Q</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>137–147 E N W N L Q N R R Q R 137–147 K---A-S--EN</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>246–250 T W F F N 246–250 ---L---</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>260–271 E V E F L N G Q I I N 260–271 T I--YN---L-D</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>308–314 F P Q A Q P F 307–313 ---G-G--Y</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>332–337 E S V L A D 331–336 ---C---</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>378–384 R E D N L Q R 377–383 Q----E-</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in the upper line refer to the positions of the deleted amino acids in the Wa VP6 protein. The lower line indicates the location of the corresponding region in group C rotavirus VP6.
† Sequence homology between the deleted domains in group A rotavirus VP6 (upper line) and the corresponding regions of group C VP6 (lower line). Dashes indicate identical amino acids.

cells infected with the recombinant vaccinia viruses were immunoprecipitated with the subgroup II-specific monoclonal antibody 631/9 and incubated at 37 °C rather than 100 °C prior to analysis by gel electrophoresis. The monoclonal 631/9 reacts only with the trimeric form of VP6 (Gorziglia et al., 1988). Therefore, if a VP6 mutant fails to oligomerize, it will not be recognized by this monoclonal antibody. Samples were treated at 37 °C before electrophoresis to preserve VP6-specific quaternary protein interactions and allow the detection of trimers. Fig. 3(a) shows the oligomerization capacity of wild-type and mutant VP6 proteins. As expected, wild-type VP6 was only detected in its trimeric form. Indeed, a single band of approximately 140 kDa was observed with the monoclonal 631/9 (Fig. 3a, lane 0). Trimers were also detected in the case of D1, D2, D6 and D7 mutants, although bands corresponding to the VP6 monomers were also present, indicating that these mutants were able to form trimers but a fraction of them was unstable (Fig. 3a). As measured by
densitometry, the efficiency of trimer formation was 60%, 81%, 64% and 53% for D1, D2, D6 and D7 mutants, respectively. By contrast, mutants D3, D4 and D5 failed to oligomerize as inferred from the absence of both trimeric and monomeric forms of VP6 (Fig. 3a). This interpretation is based on the fact that the toxicity of the 631/9 monoclonal antibody depends on the integrity of the trimeric form of the protein. The possibility existed that mutants D3, D4 and D5 formed trimers but that they were not detected by the monoclonal antibody used in our assays. To rule out this possibility, the immunoprecipitation step was omitted and the 35S-labelled lysates from cells infected with vv-Wa-6, vv-D3, vv-D4 and vv-D5 were directly incubated at 37 °C for 15 min and analysed by gel electrophoresis (Fig. 3b). Under these conditions, a band corresponding to the trimeric form of VP6 was present only in the case of cells expressing wild-type VP6, whereas the lysates from cells infected with vv-D3, vv-D4 and vv-D5 only exhibited the monomeric form (Fig. 3b). These results therefore indicate that the deletions D3, D4 and D5 prevent VP6 trimer formation.

Effect of VP6 deletions on the assembly of double-layered VLPs

We have previously shown that coexpression in mammalian cells of recombinant VP2 and VP6 by means of the vaccinia virus system results in the assembly of intracellular particles resembling native double-layered rotavirus particles (González & Affranchino, 1995a). To determine whether the VP6 deletion mutants retained the ability to form double-layered VLPs by their interaction with VP2, cells were coinfected with each of the recombinant vaccinia viruses expressing the VP6 mutants and vv-Wa-2, which directs the synthesis of Wa VP2. Cells were metabolically labelled for 4 h and lysed as described in Methods. Particles were purified from the clarified supernatants of cell lysates by ultracentrifugation through a 30% (w/v) sucrose cushion. The pelleted material was immunoprecipitated with a polyclonal anti-Wa serum and analysed by gel electrophoresis. In addition, the particulate fraction from cells infected with vv-Wa-6 alone was also purified. To analyse the efficiency with which the VP6 deletion mutants assembled into double-layered VLPs, the amount of particle-associated mutant VP6 was quantified by densitometric scanning of the autoradiograms. The values measured were normalized to the content of VP2 detected in the particulate fraction. The resulting VP6:VP2 ratios were compared with that measured in wild-type double-layered VLPs.

A representative experiment of double-layered VLPs production is illustrated in Fig. 4. Moreover, Table 2 shows the quantitative analysis of the results from three independent experiments. In the case of cells coexpressing wild-type VP6 and wild-type VP2, both proteins were detected in the particulate fraction (Fig. 4b, lane 0). According to electron cryomicroscopy studies the ratio of VP6 to VP2 in rotavirus particles is 3:9 (Prasad et al., 1988). Taking into account the number of methionines and cysteines present in Wa VP6 and VP2, the VP6:VP2 ratio expected to be found in double-layered particles after metabolic labelling should be corrected to 1:6. The VP6:VP2 ratio in the particulate fraction of cells expressing wild-type VP6 and VP2 was found to be 1:6, which is in agreement with the stoichiometry expected. When the pellet fraction of cells expressing wild-type VP6 was analysed, only trace amounts of VP6 were detected (Fig. 4b, lane 8), indicating that in our system, in the absence of VP2, VP6 is not recovered as particulate material.

The phenotypes of VP6 deletion mutants could be arranged into three groups with respect to particle assembly (Fig. 4b and Table 2). Group I phenotype consisted of a pattern of particle production similar to that of wild-type VP6 and was represented by mutants D3 and D5. Group II mutants exhibited a moderate reduction (42 to 55%) in the efficiency of particle
formation with respect to that of wild-type VP6. This group included D4 and D7 deletion mutants. In contrast, group III mutants (D1, D2 and D6) showed a major defect in double-layered particle production. The levels of particle-associated VP6 were reduced by 70 to 83% with respect to those of wild-type VP6 as a consequence of these deletions. Electron microscopy analysis of the different particulate fractions from the coexpression experiments showed mainly core-like structures in the case of mutants D1, D2 and D6 (data not shown). By contrast, double-layered VLPs were observed in the case of the other deletion mutants (data not shown). These particles were morphologically similar to those obtained upon co-expression of wild-type VP6 and VP2 (González & Affranchino, 1995 a).

**Discussion**

Rotaviruses have the unique property of maturing intracellularly by budding through the rough endoplasmic reticulum. Single-layered (core) particles assemble from VP1, VP2 and VP3 within characteristic perinuclear viroplasmic inclusions. Following their assembly, the core particles acquire the middle-layer protein VP6. During rotavirus morphogenesis, double-layered particles interact with NSP4, a virus-encoded transmembrane protein that functions as an endoplasmic reticulum receptor for VP6 and VP4 (Au et al., 1989, 1993; Meyer et al., 1989). This step allows the budding of the particles into the lumen of the endoplasmic reticulum where their maturation is completed by the incorporation of the outer capsid protein VP7 (Poruchynsky et al., 1991). It is clear from this evidence that VP6 plays a central role in rotavirus morphogenesis, since it may not only establish interactions with other structural proteins but also mediates the binding of the double-layered particles to the nonstructural protein NSP4. Therefore, elucidation of the molecular nature of these interactions is necessary to gain a better understanding of the process of rotavirus assembly.

In this report, we used the expression of VP6 deletion mutants in mammalian cells by the vaccinia virus system to characterize functional domains in this protein. The differential ability of the VP6 mutants to oligomerize into trimers delineated a trimerization domain between amino acids 246 and 314. This region maps within a larger domain (amino acids 105 to 328) that has previously been identified as important for trimerization by *in vitro* transcription-translation analysis of truncated forms of VP6 genes (Clapp & Patton, 1991). Furthermore, it has been reported that a single amino acid change from proline to glutamine at position 309 in the VP6 product of a group A lamb rotavirus reduced trimer stability (Shen et al., 1994). In keeping with this observation, mutant D5, which has proline-309 deleted, was impaired in trimerization. The oligomerization domain defined by Clapp & Patton (1991) begins at residue 105. However, two of our N terminus deletion mutants, D1 and D2, were still competent in trimerization, suggesting that the region spanning residues...
122 to 147 is not necessary for oligomerization. This result, together with the fact that removal of amino acids 246 to 250 in mutant D3 blocks trimer formation, indicate that the N-terminal end of the trimerization domain may be mapped between residues 147 and 246.

We showed that deletions D3, D4 and D5 delineate a region between residues 246 to 314 that is involved in trimer formation. Interestingly, this domain exhibits amino acid sequence conservation between group A and group C rotavirus VP6, suggesting that a similar region may mediate the oligomerization of this protein in both viruses. However, Tosser et al. (1992) did not observe formation of chimeric trimers upon coexpression of recombinant VP6 proteins of group A and group C rotaviruses. This evidence led the authors to propose that within the trimerization domain there may be group-specific structural elements that prevent hetero-trimerization. It is therefore likely that oligomerization of VP6 in group A and group C rotaviruses involves a similar domain, although it may be strongly dependent on group-specific residues.

In an attempt to map the VP6 assembly domains, the deletion mutants of this protein were examined for their ability to assemble into double-layered VLPs upon coexpression with the core protein VP2. The introduction of deletions within the coding sequence of the VP6 gene led to three different assembly phenotypes. Deletions D3 and D5 did not significantly affect double-layered particle formation, whereas D4 and D7 mutants exhibited a moderate decrease in the yield of double-layered particles with respect to wild-type VP6. In contrast, deletions D1, D2 and D6 severely affected the ability of VP6 to assemble into particles. Clapp & Patton (1991) have reported that the VP6 region spanning residues 251 to 397 is essential for the binding of VP6 to double-layered particles. In agreement with their observation, we found that the deletions causing a moderate decrease in particle formation (deletions D4 and D7) as well as one of the mutations that drastically diminishes the efficiency of this process (deletion D6) map within this region. However, deletions D1 and D2, which also cause a major defect in particle formation, delineate a previously unidentified assembly domain at the N terminus of the protein. The region affected by these mutations (amino acids 122 to 147) is characterized by its richness in charged and polar amino acids. The fact that this assembly domain located at the N terminus of VP6 was not found in the study of Clapp & Patton (1991) may be attributed to the approach used by these authors to assess the ability of the VP6 mutants to assemble into particles. In the Clapp & Patton study, truncated versions of VP6 were examined for their capacity to associate in vitro with purified double-layered particles, whereas our analysis relied on the ability of the VP6 deletion mutants to assemble in vivo into double-layered VLPs upon coexpression with VP2 in mammalian cells.

It has been reported that two temperature-sensitive mutations at the N terminus of rotavirus SA11 VP6 (positions 10 and 13) interfere with the ability of the virus to assemble into particles at the non-permissive temperature (Mansell et al., 1994). Evidence was provided that the defect was not at the level of binding to VP2, since a VP6 mutant lacking residues 6 to 18 was still able to interact with VP2 (Mansell et al., 1994). The phenotype of this mutant clearly differs from that described in our study for deletion mutants D1 and D2. Indeed, deletions D1 and D2 impaired the interaction of VP6 with the core protein VP2.

It is noteworthy that the assembly domains defined by deletions D1, D2 and D6 are highly conserved in both group A and group C rotavirus VP6. In this regard, the finding that chimeric particles can be obtained by coexpression of group C VP6 and group A VP2 (Tosser et al., 1992) supports the notion that group A and group C rotaviruses share a common VP2-interacting VP6 domain.

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References


Functional domains of rotavirus VP6


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