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Folding of the rabbit hemorrhagic disease virus capsid protein and delineation of N-terminal domains dispensable for assembly

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Summary. Rabbit hemorrhagic disease virus (RHDV) and European brown hare syndrome virus (EBHSV) are caliciviruses that produce severe symptoms and are lethal to rabbits and hares. The folding of the capsid protein was studied by determination of the antigenic pattern of chimeric capsid proteins, composed of regions from RHDV and EBHSV capsid proteins. The anti-RHDV monoclonal antibody (MAb) E3, which is known to bind an external conformational epitope, recognized the RHDV C-terminal region. The anti-RHDV MAb A47, which binds a buried epitope, recognized the RHDV N-terminal region. Using a pGEX expression library, we more precisely mapped the MAb A47 epitope on a 31 residues length peptide, between residue 129 and 160 of the VP60, confirming its location in the N-terminal part of the protein. These results demonstrate that the C-terminal part of the protein is accessible to the exterior whereas the N-terminal domain of the protein constitutes the internal shell domain of the particle. With the aim of using virus-like particles (VLPs) of RHDV as epitope carriers or DNA transfer vectors, we produced in the baculovirus system three proteins, ΔN1, ΔN2 and ΔN3, truncated at the N terminus. The ΔN1 protein assembled into VLPs, demonstrating that the first 42 amino acid residues are not essential for capsid assembly. In contrast, ΔN2, from which the first 75 residues were missing, was unable to form VLPs. The small particles obtained with the ΔN3 protein lacking residues 31 to 93, located in the immunodominant region of the RHDV capsid protein, indicate that up to 62 amino acid residues can be eliminated without preventing assembly.

Introduction

Rabbit hemorrhagic disease virus (RHDV) and European brown hare syndrome virus (EBHSV) are caliciviruses, classified to the new genus *Lagovirus* of the *Caliciviridae* family [15]. These viruses cause hemorrhaging and necrotic

hepatitis, and may kill 90% of rabbit or hare populations in less than 72 hours. The calicivirus genome is a 7.5 kb positive sense single-stranded RNA with a VPg linked to its 5′ extremity [2]. The virus is 35–40 nm in diameter and has a structured surface, with regularly arranged cup-shaped depressions [12]. The virions consist of a single capsid protein with a molecular weight of 58 to 65 kDa. Although such a structure is common among plant viruses, it remains exceptional for animal viruses, making these viruses an interesting model for studying the assembly process. This structure also makes these viruses very good candidates for gene transfer or epitope carrier vectors. However, several caliciviruses, including lagoviruses and human caliciviruses, do not replicate efficiently in cell culture. The expression of cDNAs encoding the capsid proteins of lagoviruses and other caliciviruses in the baculovirus/Sf9 insect cell system has circumvented this problem. The resulting recombinant proteins were obtained in large amounts and self-assembled into virus-like particles (VLPs). The production of VLPs has been of great value in immunological and epidemiological studies of caliciviruses. Studies of the assembly process have shown that VLPs contain no nucleic acid and, therefore, that self-assembly into particles must be an intrinsic property of the calicivirus capsid protein. Electron cryomicroscopy analysis followed by three-dimensional resolution of Norwalk calicivirus VLPs [13] and RHDV VLPs [17] has shown that each of these particles consists of 90 dimers of the capsid protein. The X-ray crystallographic structure of Norwalk VLPs was recently published [14]. Resolution of the atomic structure of the Norwalk protein has led to more precise determination of the organization of domains in the protein: the shell domain (S) is constructed from the N-terminal half of the capsid protein and the protruding domain (P) from the C-terminal half. Various studies have suggested that the RHDV capsid protein probably folds in a similar way [5, 9], but no conclusive proof has yet been obtained. If we are to use calicivirus VLPs as transfer vectors, a more thorough understanding of capsid protein structure and folding is essential.

A recent investigation demonstrated that a recombinant RHDV capsid protein in which the first 30 amino acid residues had been replaced by a foreign 6-amino acid epitope, self assembled into a chimeric VLP that retained the antigenicity of the foreign epitope [10]. Although VLPs were readily produced, they were smaller (27 nm) than native VLPs (40 nm). As it has been shown that the N-terminal part of the capsid protein, between residues 31 and 250, contains an immunodominant region [9] it remains of interest to further investigate the possible deletion in this part of the protein.

Within the context of possible future use of the RHDV capsid protein as a vector, the aim of this study was to increase our understanding of the folding and assembly of the RHDV capsid protein. We first investigated the folding of the capsid protein by producing chimeric proteins composed of RHDV and EBHSV capsid proteins and analyzing their antigenic reactivity patterns with anti-RHDV MAbs. The epitope of one of these anti-RHDV MAbs (A47), which was known to bind a linear buried epitope [7], was more precisely mapped with a bacterial expression library. We then produced three N-terminally truncated recombinant proteins (ΔN) in the baculovirus system and assessed their ability to form VLP.

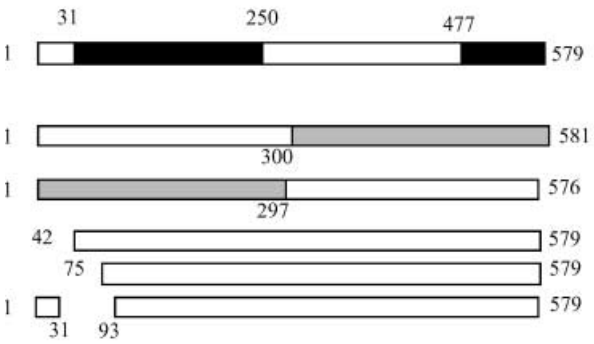
Materials and methods

Viruses and cells

Spodoptera frugiperda (Sf9) cells were maintained in suspension at 27 °C in Grace medium (Gibco BRL) supplemented with 10% fetal calf serum (Boehringer Manheim). Wild-type AcRP6-SC baculovirus and recombinant baculoviruses were grown on Sf9 cell monolayers, as previously described [11].

Construction of recombinant chimeric proteins

The chimeric proteins produced, RE and ER, are described in Fig. 1. The inserts encoding modified capsid proteins were generated by PCR with plasmids containing the VP60 gene of RHDV-SD [16] or the VP60 gene of EBHSV-GD [8], as a template, and various sets of oligonucleotides as primers. An initial ligation step was necessary to produce inserts encoding chimeric capsids. The part of the insert corresponding to the N-terminal region was produced by PCR using primer 18 TCTAGAAGATCTatgGAGGGCCAAAGCCCGCAC in association with primer 202 TCTAGA**ACGCGT**CCTCTTCGATGGTCAATGTCG for RE and primer 39 AGATCTAGATCTATGGAGGGTAAGCCTCGGGC in association with primer 200 TCT AGA**ACGCGT**CCGCTTGGATGGTCAATGTCG for ER. The fragment corresponding to the EBHSV C-terminal region of the RE insert was amplified with primer 199 TCTAGA**ACG CGT**TTCCTACCCCTCTGGCAGTGC and primer 308 TCTAGAAGATCTTCAGACATA GGAATATCCAGT whereas the fragment corresponding to the RHDV C-terminal region of the ER insert was amplified with primer 201 AGATCT**ACGCGT**AAGTTACCCTGGGAAC AACGC and primer 15 TCTAGAAGATCTAGTCCGATGAATTCAGACAT. A *Mlu*I restriction site (shown in bold typeface) was added to the end of primers 199, 200, 201 and 202. The PCR products generated with these primers were digested with *Mlu*I and ligated in standard conditions using T4 DNA ligase. As a *Bgl*II (*Bam*HI-compatible) restriction site had been added to primers 15, 18, 39 and 308, the products of this first ligation step were then digested with *Bgl*II and ligated into *Bam*HI-digested pBluescript SK (−) (Stratagene) to produce pB-RE and pB-ER.



name	characteristic	Mr
VP60	native capsid	60270
RE	chimere	60402
ER	chimere	60087
ΔN1	42aa deleted	56530
ΔN2	75aa deleted	52847
ΔN3	62aa deleted	53755

Fig. 1. Schematic representation of the 5 recombinant capsid proteins. Numbers indicate the positions of the amino acids relative to the initiating methionine of the RHDV or EBHSV capsid protein. The black boxes refer to antigenic domains defined by Martinez-Torrecuadrada et al. (1998) [9]. For chimeric proteins RE and ER, the shaded boxes represent the amino acid sequence of EBHSV capsid protein. Molecular mass (*Mr*) and characteristics of the protein are indicated on the right

Construction and selection of recombinant baculoviruses

The recombinant baculovirus encoding the entire capsid protein of RHDV (BacG320) has been described elsewhere [6]. The three RHDV proteins with N-terminal deletions produced by recombinant baculoviruses are described in Fig. 1. All recombinant baculoviruses were produced with the Bac-to-BacTM system (Gibco-BRL), according to the manufacturer's instructions. The various inserts encoding modified RHDV capsid proteins were generated by PCR with plasmids containing the VP60 gene of RHDV-SD [16] as a template, and various sets of oligonucleotides as primers. For production of the ΔN1, ΔN2 primer 15 was used for PCR, in association with a specific primer, primer 170 TCTA GAAGATCTatgACTGCAGAGAACTCA for ΔN1 and primer 171 TCTAGAAGATCTatg GACGTTTTCACTTGG for ΔN2 (start codons are indicated in lower case). For ΔN3 an initial ligation step was necessary to produce insert. The 5′ part of the insert was produced by PCR by using primer 18 and primer 173 TCTAGA**ACGCGT**GCCATCGGTTGTGGTT while the 3′ part of the insert was produced with primer 15 in association with primer 172 TCTAGA**ACGCGT**CAACATTCTCCACAGAACAA. The PCR products generated were digested with *Mlu*I (restriction site added to the end of primer 172 and 173, indicated in bold) and ligated in standard conditions using T4 DNA ligase. As a *Bgl*II (*Bam*HI-compatible) restriction site had been added to primers 15, 18, 170, and 171 all inserts were digested with *Bgl*II and ligated into the *Bam*HI-digested pFastBac plasmid provided with the Bac-to-BacTM system (GIBCO-BRL). Positive recombinant baculoviruses were identified by indirect immunofluorescence staining with monoclonal antibody as previously described [7]. They were plaque-purified twice, and used to prepare virus stocks.

Production and immunodetection of recombinant capsid proteins

The chimeric capsid genes cloned into plasmid pB-RE and pB-ER were transcribed and further translated with the TNT T7 coupled reticulocyte lysate system (Promega) in the presence of L-(^{35}S)PRO-MIX (Amersham), according to the manufacturer's instructions. Western-blot analysis of recombinant capsid proteins and the immunoprecipitation of radiolabeled proteins were carried out with anti-RHDV MAbs as previously described [7].

Electron microscopy

Particles present in the supernatant of Sf9 cells infected with recombinant baculovirus were ultracentrifuged. Pellets were directly adsorbed onto a grid. All grids were examined in an electron microscope (Philips) after negative staining with 2% uranyl acetate.

pGEX expression library

The pGEX expression library was constructed using a method adapted from that described by Delmas et al. (1990) [3]. In brief, random fragments of the RHDV capsid gene were obtained by sonication of the capsid gene of the RHDV-SD strain [15]. The ends of these fragments were filled in with the Klenow fragment of DNA polymerase I and the fragments were then ligated into the pGEX-3 expression vector (Pharmacia), cut with *Bam*HI and blunted with the Klenow fragment. The pGEX library was first screened for the presence of the VP60 insert by Southern blotting with the ECL system, as described by the manufacturer. Thirteen other constructs, pGEX I to pGEX XIII were generated by inserting defined PCR products into the *Bam*HI-*Eco*RI digested pGEX-3 vector according to standard procedures. Inserts were obtained by PCR with a plasmid containing the VP60 gene of RHDV-SD [16]

as the template and primers 557 (GAATTCACTGACTAGTGTGGGAACAAGGGC) and 556 (GGATCCAACATTCTCCACAGAACAACCCA) for pGEX I, 557 and 840 (GGATC CGTGGCATGCAGTTTCGCTTCA) for pGEX II, 557 and 841 (GGATCCTAGTTGCCG GATCGGGTGTG) for pGEX III, 557 and 579 (GGATCCTGGTGGGCGGTTGGTTGCGG) for pGEX IV, 557 and 558 (GGATCCCGGCCGTGATACCACCGGGCATC) for pGEX V, 556 and 843 (GAATTCACTGATGGTGACAGGTTCAAGTG) for pGEX VI, 556 and 842 (GAATTCACTACGAGCGTCGATGACAACATGG) for pGEX VII, 556 and 580 (GAATTC GGGAATTGCCTGACCTCCAGCCC) for pGEX VIII, 556 and 559 (GAATTCCCCTG GTCCAATCTCGATGCCCGG) for pGEX IX, 840 and 843 for pGEX X, 841 and 842 for pGEX XI, 579 and 580 for pGEX XII, 579 and 842 for pGEX XIII. As *Bam*HI and *Eco*RI restriction sites were present at the end of primer, PCR products were digested by both enzymes before cloning into the pGEX3 vector. MAb A47 was used for immunoscreening of the bacterial colonies as previously described [3] and for western blot characterization of the product isolated from the bacterial pellets.

Results

Folding of the RHDV capsid protein

To investigate the folding of the RHDV capsid protein, we constructed chimeric genes encoding fusion proteins derived from the N- and C-terminal parts of the RHDV capsid protein and the EBHSV capsid protein (Fig. 1). The RE protein consisted of the N-terminal region of the RHDV capsid protein (first 300 amino acid residues) fused to the C-terminal region of the EBHSV capsid protein (last 281 amino acid residues). The ER protein consisted of the N-terminal region of the EBHSV capsid protein (first 297 amino acid residues) fused to the C-terminal region of the RHDV capsid protein (last 279 amino acid residues). The chimeric genes encoding these two proteins were inserted into the pBS-SK-vector under control of the T7 promoter. Proteins were produced by in vitro transcription and translation in rabbit reticulocyte lysate in the presence of ^{35}S methionine (Fig. 2A). They were then immunoprecipitated with specific anti-RHDV MAbs. We used MAb A47, which is known to bind an internal epitope of RHDV [7], and the anti-RHDV MAb E3, which is known to bind a conformational epitope on the external dimeric arch of the VLPs [17]. The RE protein and the positive control (pBG3), corresponding to the entire RHDV protein, were readily immunoprecipitated by MAb A47. In contrast, no signal was detectable for the ER protein and the negative control (pBH3), corresponding to the entire EBHSV protein (Fig. 2B). MAb E3 was found to bind the entire RHDV protein (pBG3) and the ER protein, but not the RE and EBHSV (pBH3) proteins (Fig. 2C).

Mapping of the MAb A47 epitope

To confirm the N-terminal location of the MAb A47 epitope, we mapped the A47 epitope, using a VP60 pGEX expression library. This library was produced by random fragmentation of the VP60 gene, followed by insertion of the fragments into the pGEX expression vector such that expression resulted in a chimeric protein consisting of the sequence encoded by the gene fragment fused to the glutathione

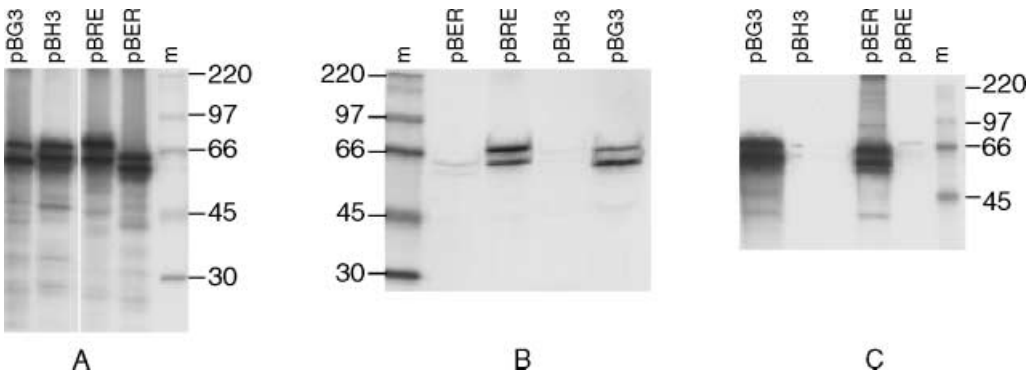
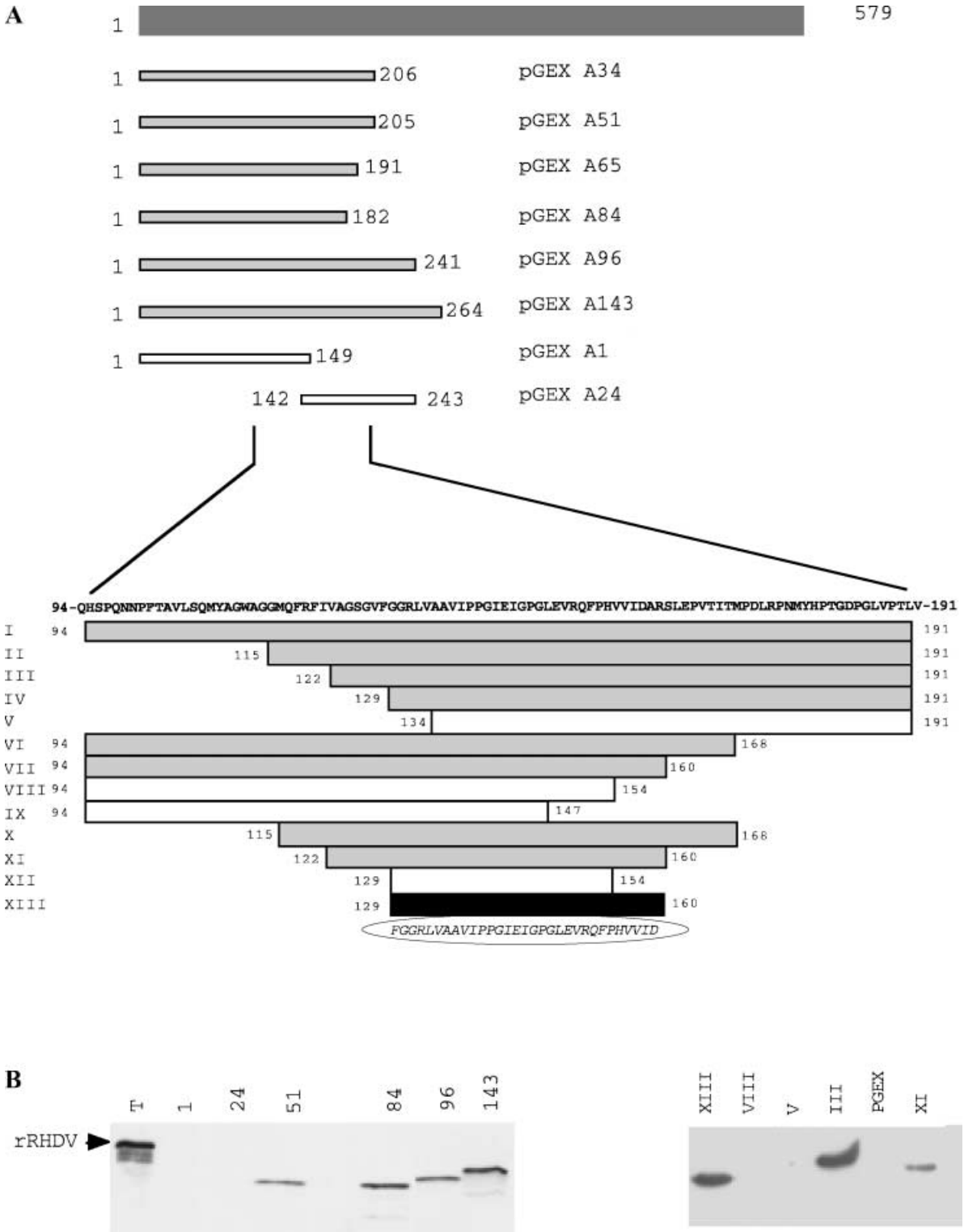


Fig. 2. Immunoprecipitation of the chimeric proteins ER and RE with anti-RHDV VP60 MAbs. Chimeric proteins were cloned under control of the T7 promoter into pBS-SK- vector. Efficient production of [^{35}S]radiolabelled in-vitro translated proteins was monitored by electrophoresis in a 10 % polyacrylamide gel of 3 μl of samples (**A**). Radiolabelled proteins (12.5 μl of the sample) were immunoprecipited with MAb A47 (**B**) or MAb E3 (**C**) and further analysed in a 10% polyacrylamide gel. Molecular mass markers (*m*) are in kDa on the side of each panel

S-transferase gene (GST). The library was first screened by Southern blotting to select clones with inserts corresponding to VP60 gene fragments. The induced proteins were then screened by Western blotting with MAb A47 and the inserts of the positive clones were sequenced. Six of the 143 bacterial colonies analyzed were found to be positive (Fig. 3A). The six proteins giving a positive signal began at the first N-terminal residue of the RHDV protein and ended at residue 264 for the largest (pGEXA143) and residue 182 for the smallest (pGEXA84) (Fig. 3B). Thus, the A47 epitope is located in the first 182 residues of the capsid protein. More precisely, the epitope must be centered around the residues 142 to 149, because the proteins encoded by clones pGEX A1, ending at position 142, and pGEX A24, beginning at position 149, were not recognized by MAb A47 (Fig. 3). To confirm this, we generated thirteen clones, from pGEX I to pGEX XIII, by

Fig. 3. Epitope mapping of the anti-RHDV MAb A47 with the bacterial pGEX expression system. (**A**) Schematic representation of the 21 pGEX-VP60 clones. The dark grey bar at the top refers to the entire RHDV capsid protein. Inserts corresponding to random fragment s of RHDV-VP60 gene (clone 1 to 143) or to specifically PCR amplified fragments of the VP60 gene (clone I to XIII) were cloned into the pGEX-3 expression vector. Proteins were produced in fusion with the GST protein after induction with IPTG of the *E. coli* bacteria containing the recombinant pGEX expression vectors. Numbers refer to the positions of amino acids relative to the initiating methionine of RHDV capsid protein. The grey boxes represent proteins recognised by the MAb A47 while open boxes correspond to proteins not recognised by MAb A47. (**B**) Two examples of immunoblot of induced proteins screened with the MAb A47. Numbers at the top of the blot refer to the pGEX clones while T sample corresponds to rRHDV VLPs produced in the baculovirus system and pGEX to the vector without VP60 insert


A
1
579
pGEX A34
206
pGEX A51
205
pGEX A65
191
pGEX A84
182
pGEX A96
241
pGEX A143
264
pGEX A1
149
pGEX A24
142
243
94-QHSPQNNPFTAVLSQMYAGWAGGMQFRFIVAGSGVFGGRLVAAVIPPGIEIGPGLEVRQFPHVVIDARSLEPVTITMPDLRPNMYHPTGDPGLVPTLV-191
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
FGGRLVAAVIPPGIEIGPGLEVRQFPHVVID
B
rRHDV
T
1
24
51
84
96
143
XIII
VIII
V
III
PGEX
XI

cloning PCR products covering this region (Fig. 3A). As expected, the larger protein encoded by clone pGEX I was recognized by MAb A47, demonstrating that the A47 epitope is located between residues 94 and 191. A more precise delimitation of the binding domain was realized by truncation of pGEX I: from N terminal end for constructions II to V and from C terminal end for constructions VI to IX. Four other constructions were centered around residues 142 to 149 (X to XIII) (Fig. 3). Analysis of the reactivity of each clone against MAb A47 allowed us to delimit the epitope of A47 on a 31 aa length peptide between residues 129 and 160 of the VP60 protein (pGEX XIII) (Fig. 3).

Production and characterization of recombinant lagovirus capsid proteins with N-terminal deletions

To identify N-terminal domains not essential for capsid assembly, we produced three recombinant capsid proteins in the baculovirus expression system (Fig. 1). ΔN1, ΔN2 and ΔN3 correspond to RHDV capsid proteins lacking various numbers of amino-acid residues at the N-terminal end. Proteins ΔN1 and ΔN2 lacked the first 42 and 75 amino acids respectively. These two proteins were designed so as to determine more precisely the number of residues that could be deleted from the N-terminal end of the protein without preventing self-assembly. The ΔN3 protein lacked the 62 amino acids between residues 31 and 93 in the RHDV immunodominant region described by Martinez-Torrecuadrada et al. (1998) [9]. All modified capsid genes inserted into baculovirus were verified by PCR amplification and sequencing. No modification or mutation with respect to the predicted sequence was identified. We determined the titers of the recombinant baculoviruses, which were then used to infect cells at a m.o.i. of 5 pfu/cell. We investigated the efficient production of recombinant capsid proteins by immunofluorescence with the anti-RHDV MAbs E3, A47, C36, E29, which have been described elsewhere [7] (Table 1). We also evaluated the production of recombinant proteins by western blotting with MAbA47 (Fig. 4). All the new recombinant capsid proteins included the MAb A47 epitope and were recognized, whereas no signal was detected with the negative control, Bac+, corresponding to AcRP6-Sc lysate. The signals obtained with the ΔN1 and ΔN2 proteins seemed to be stronger than those obtained with the ΔN3 protein and the positive control, corresponding to the entire VP60 (BacG320), possibly demonstrating a better production efficiency of ΔN1 and ΔN2. The observed molecular masses of all of the proteins detected were as predicted (Fig. 1). The ΔN1, ΔN2 and ΔN3 proteins were also immunoprecipitated with MAb E3 and, as in western blots, the molecular mass of the protein immunoprecipitated was as predicted (Table 1).

Assembly of VLPs from the recombinant ΔN capsid proteins

We investigated the self-assembly of the recombinant proteins into VLPs. Particles present in the supernatant of Sf9 cells infected with recombinant baculovirus were

Table 1. Immunodetection of recombinant deleted capsid proteins

	Western blot									
	SDS-PAGE	TGA[a]			Immunofluorescence				Immunoprecipitation	
	A47[b]	E29[b]	E3[b]	SL10[b]	E3	E29	C36[b]	SL10	E3	SL10
Bac G320[c]	+	+	+	+	+	+	+	+	+	+
ΔN1	+	+	+	+	+	+	+	+	+	+
ΔN2	+	−	−	−	+	+	+	+	+	+
ΔN3	+	+	+	+	+	+	+	+	+	+
Bac+[d]	−	−	−	−	−	−	−	−	−	−

[a]Tris-Glycin Agarose gel electrophoresis
[b]Anti-RHDV antibodies used in immunodetection tests have been already described [5]
[c]BacG320 correspond to the baculovirus producing the entire RHDV VP60 (positive control)
[d]Bac+ correspond to the wild baculovirus AcRP6-Sc and was used as negative control

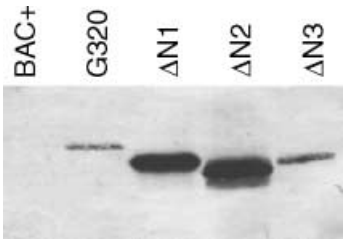


Fig. 4. Immunoblot of the ΔN recombinant capsid protein with anti-RHDV MAb A47. The Sf9 cells infected with each recombinant baculovirus were harvested 48 h post-infection. Proteins in cellular lysate were separated by SDS-PAGE (10 %) transferred onto a nitrocellulose membrane and further reacted with anti-RHDV MAb A47 (1/1000) and alkaline phosphatase conjugated secondary antibodies

ultracentrifuged. On the immunoblot of VLPs separated by electrophoresis in a Tris-glycine agarose gel in non-denaturing conditions, samples corresponding to rRHDV (recombinant VP60 of RHDV expressed in baculovirus, corresponding to BacG320), ΔN1 and ΔN3 displayed a strong signal whereas the ΔN2 sample gave no signal (Table 1). The pattern of reactivity was similar if the E3 MAb or the rabbit convalescent polyclonal SL1/10 serum [6] was used. The presence of antigenic VLPs was further confirmed by negative-contrast electron microscopy (Fig. 5). Pseudo-particles similar in size (38-nm) and morphology to those of the control rRHDV were observed with ΔN1. The particles formed in the ΔN3 sample were similar in morphology to those of rRHDV but were smaller, with an estimated diameter of 27 nm. No particles were observed in the ΔN2 sample or in the negative control AcRP6-SC sample corresponding to the wild-type baculovirus.

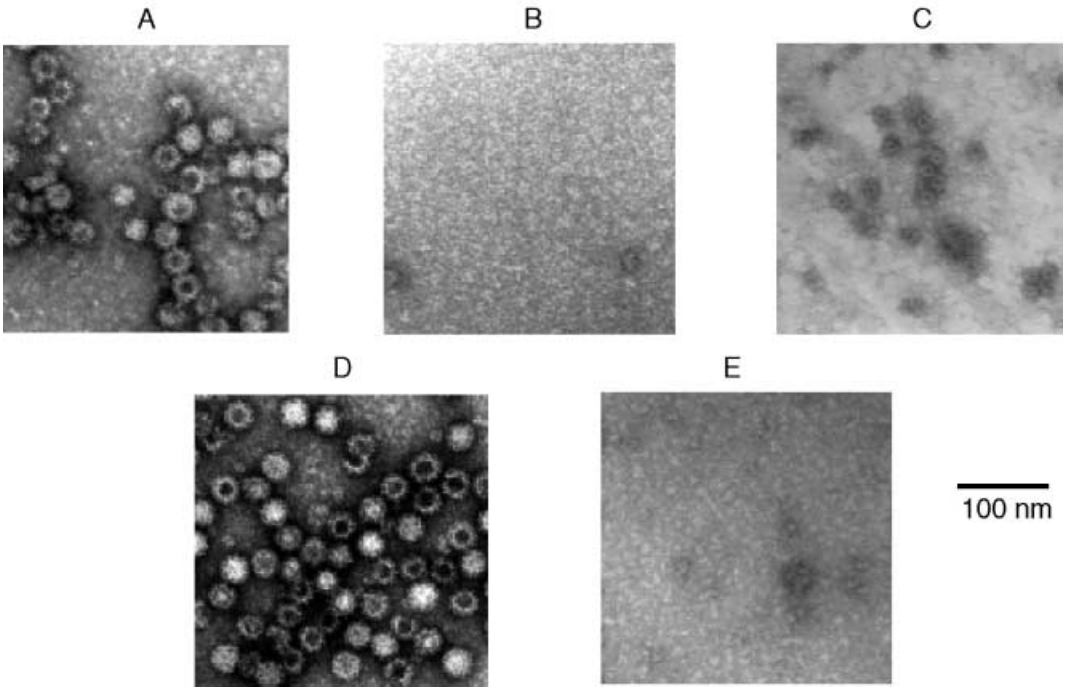


Fig. 5. Electron micrographs of ΔN recombinant RHDV particles. The Sf9 cells were infected with each recombinant baculovirus and supernatant were harvested 5 days post-infection. Supernatants were ultrancentrifuged and stained with uranyl acetate (2 %). Each micrograph corresponds to a recombinant protein: **A** ΔN1, **B** ΔN2, **C** ΔN3, **D** full length rRHDV protein (Bac G320), **E** Negative control: AcRP6-Sc (wild baculovirus)

Discussion

The RHDV capsid protein is thought to fold in similar manner to human calicivirus, with the N-terminal 250 amino-acid residues comprising the shell domain of the particle (S) and the remaining C-terminal residues forming a protruding domain (P) [5, 9, 17, 20]. Our results with the chimeric proteins RE and ER, composed of regions of the RHDV and EBHSV capsid proteins, were consistent with this hypothesis. MAb E3, which binds specifically to an external conformational epitope on RHDV capsid protein dimers [7, 17], bound the ER protein but not the RE protein, demonstrating that the exposed E3 epitope is constituted from sequences located in the C-terminal half of the RHDV protein. MAb A47, which binds an internal epitope of RHDV [7], recognized the RE protein and not the ER protein, implying that the A47 epitope is located in the N-terminal part of the protein. We mapped precisely the A47 epitope with the pGEX expression system to a 31 aa length peptide between residues 129 and 160 of the VP60. This epitope is located in the immunodominant region described by Martinez-Torrecuadrada et al. (1998) [9].

Our results confirm that the N-terminal part of the RHDV capsid protein is buried within the particle whereas the C-terminal part is exposed. They were

obtained by biochemical and immunological approaches and were consistent with the results obtained in studies of the X-ray crystallographic structure of Norwalk virus VLPs [14]. However, the two subdomains, P1 and P2, that subdivide the P domain of the Norwalk capsid, have not yet been shown to exist in the RHDV capsid protein. In the Norwalk virus capsid, the P1 subdomain consists of 166 residues including the first and last residues of the P domain whereas the P2 subdomain, located at the surface of the capsid, consists of the remaining 127 residues of the P domain. In this context, fine mapping of the E3 epitope would be of value to characterize and to localize the potential P2 domain on the RHDV capsid protein.

The production of three recombinant RHDV capsid proteins with deletions in the N-terminal region made it possible to identify domains in the N-terminal part of the protein that were not essential for VLP formation. The ΔN1 protein self-assembled, demonstrating that the first 42 amino acids were not essential for the self-assembly of VLPs. In contrast, a larger deletion corresponding to the first 75 amino acids prevented assembly as ΔN2 formed no particles. Nagesha et al. (1999) [10] produced VLPs with a truncated RHDV protein lacking the first 30 amino-acid residues, fused to a foreign epitope. Our work extends the domain known not to be required for self-assembly by 12 residues. The VLPs produced by Nagesha et al. (1999) were small whereas the VLPs we obtained with the ΔN1 protein were similar to the VLPs produced with entire rRHDV protein. It is possible that the foreign peptide inserted into the VLPs produced by Nagesha et al. (1999) affected the RHDV capsid protein, leading to capsid misassembly, whereas the VLPs obtained from capsid protein with a straightforward deletion may have assembled like native VLPs (data not shown by the authors). The ΔN3 protein we produced, in which the 62 amino acids between positions 31 and 93 had been deleted, also self-assembled into small VLPs. Thus, in the N-terminal part of the RHDV protein, amino acid composition, the original location of the sequence deleted, and the foreign sequence inserted seem to affect the folding and assembly of the capsid protein as much as the length of the sequence deleted.

The ΔN3 VLPs and the small particles described by Nagesha et al. (1999) are similar in size (27 nm diameter) and morphology. However, their characteristics differ from those of the core-like particle (CLPs) isolated from RHDV samples corresponding to assembly of the N-terminal region of the RHDV protein into small smooth particles [1]. So, like Nagesha et al. (1999), we suggest that the 27 nm VLPs were constructed from 60 RHDV capsid dimers (rather than the 90 present in a normal particle), forming particles of $T = 1$ symmetry similar to the Norwalk VLPs isolated in the baculovirus system [19]. It is unlikely that the small particles correspond to CLPs because MAb E3 bound ΔN3, demonstrating that at least the part of the C-terminal end of the protein containing the MAb E3 epitope was present in the small particle. The misassembly of capsid particles from $T = 3$ normal symmetry to $T = 1$ has also been reported for other icosahedral viruses with capsids composed of a single protein and, in all cases, $T = 1$ particles are produced if the N-terminal residues of the capsid are deleted or disordered [19, 21].

Nevertheless, the production of ΔN1 and ΔN3 particles opens up new possibilities for the use of VLPs as epitope carriers or gene transfer vectors. Fusion of a foreign sequence to the N-terminal end of the RHDV capsid protein presents advantage that the foreign peptide is buried inside the VLPs. For gene transfer, this facilitates the packaging and protection of the DNA within the particle. The insertion of the DNA packaging sequence of a human papillomavirus at the N terminus of the ΔN1 protein has already been tested with success [4], with the production of VLPs able to transfer DNA to several cell lines. For use as an epitope carrier, the internal location of the foreign epitope protects it from proteolysis. Furthermore, studies of the antigenic structure of the RHDV capsid protein have demonstrated that an immunodominant domain of the RHDV capsid protein is localized in the N terminal part of the protein [9, 18]. Thus, the foreign epitope fused to ΔN1 or ΔN3 protein would be present in a region that would increase the immunogenicity of the peptide. Further investigations are required to test the immunogenicity of foreign epitopes fused to the ΔN1 and ΔN3 proteins and to identify other suitable domains for deletion, especially in the C-terminal part of the protein, to improve the efficiency of these VLPs as epitope carriers.

In conclusion, our results confirm that the RHDV capsid protein folds such that its N-terminal part forms the internal shell and the C-terminal part of the protein is exposed. We produced two RHDV capsid proteins, one deleted of the 42 N-terminal residues and the other deleted of 62 residues located in an internal immunodominant region of the N-terminal part of the RHDV protein, which displayed self-assembly. These proteins are of potential interest for the development of new eukaryotic vectors.

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