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# Site-directed mutagenesis of hepatitis A virus protein 3A: effects on membrane interaction

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**Abstract**

Due to a stretch of hydrophobic amino acids, protein 3A of hepatitis A virus (HAV) has been suggested to act as a membrane anchor or a carrier of the genome-linked protein 3B (VPg) during viral RNA synthesis. Mutagenesis analysis was performed in order to elucidate the role of the N- and C-terminal tracts of protein 3A in cell membrane interaction. Expression of the mutated proteins in *E. coli* cells demonstrated that the presence of positively charged residues at the C-terminus is not required for membrane anchoring. Changes in the primary sequence involving charged amino acids at the N- and C-termini critically influenced the ability of the protein 3A of a cytopathic strain of HAV to change bacterial membrane permeability. This result demonstrates the strict correlation between the structure and pore-forming potential of HAV protein 3A.

*Keywords:* Hepatitis A virus; Protein 3A; Cytotoxicity; Membrane permeability; Site-directed mutagenesis

## 1. Introduction

Various viral proteins were described to be involved in the cytopathic effect in the host cell. However, the mechanisms of cell destruction, in particular those affecting the integrity of cellular membranes, have not been identified. A large variety of highly cytolytic viruses are included in the Picornavirus family [1]. Picornaviruses have a positive single-stranded RNA genome, that acts as a mRNA to direct the synthesis of a single polyprotein. Proteolytic processing by viral protease(s) is required to yield mature proteins. For some members of the family, it was shown that viral proteins 2B and 3A affect the secretory pathway of the host cell inhibiting the intracellular protein transport, and that the transmembrane domain of protein 3A can form an amphipathic helix responsible for cell lysis when expressed in bacteria [2,3]. Hepatitis A virus (HAV) is an atypical member of this group as only a few strains have been reported to induce cell damage and to shut-off host cell metabolism [4–8].

Genomic analysis of the cytopathogenic HAV strain FG ($HAV_{FG}$) suggested that the deletion of two aspartate and one serine residue near the N-terminus of protein 3A might be responsible for the altered replication phenotype, as compared to other strains [9,10]. Recombinant expression of protein 3AB of various strains showed that although 3AB binds to microsomal membranes after translation in vitro, only 3AB of strain FG ($3AB_{FG}$) induces permeability of cell membranes when expressed in *E. coli*. Analysis

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of the amino acid sequence of the N-terminal portion of $3AB_{FG}$ by the method of Schiffer and Edmundson [11] suggested that this tract can form an -helix with amphipathic properties. The cytotoxic property of $3AB_{FG}$ was likely attributed to this hypothetical secondary structure [12]. These and similar data on poliovirus [13] suggested that, due to its hydrophobic property, picornaviral 3A might function (in the form of its precursor 3AB) as an anchor for the replicating RNA to cellular membranes.

To assess the structure-function relationship of HAV protein 3A, site-specific mutations were introduced C-terminal to the transmembrane domain of the protein and near its N-terminus. The changes in the primary sequence of the protein 3A showed no effect on membrane-binding, but altered or blocked the ability to permeabilize bacterial membranes. These findings suggest that the N- and C-terminal charged residues substantially contribute to the ability of the cytotoxic protein 3A to assume a conformation with presumable pore-forming activity.

## 2. Materials and methods

### *2.1. Construction of expression plasmids*

Genes for three C-terminal and one N-terminal mutated 3A proteins were created by oligonucleotide-directed site-specific mutagenesis. Protein 3A was amplified by polymerase chain reaction (PCR) using cDNA from either pHAV/7 (HAV strain HM175) [14], or the respective region of HAV strain FG [10] as templates. The desired mutations were introduced by using appropriate oligonucleotide primers. A *Bam*HI restriction site at the 5′-end and a stop codon at the 3′-end were also introduced.

(1) 5′-TCTCAG*GGATCC*TCAGATGATGATAATGAT-3′ (HM175, nt 4995-5024, sense primer);

(2) 5′-TCTCAG*GGATCC*TCAGATGATAATGCAGTG-3′ (FG, nt 4995-5033, sense primer);

(3) 5′-TCTCAG*GGATCC*TCAGTTGTTAATGCAGTGGCTGAG-3′ (FG, nt 4995-5033, $Asp^4$ and $Asp^5$ to Leu, sense primer);

(4) 5′-ATGATATAC*TCA*TTCAGCTGGGATTGGTTCCTCCTCTTTGCGGGAGAAATACACAAA-3′ (FG, nt 5171-5233, amino acid residues 58 and 59 deleted, antisense primer);

(5) 5′-ATGATATAC*TCA*TTCAGCTGGGATTGGTTCCTCCTCGGAGAATATCACAAA-3′(HM175, nt 5171-5233, amino acid residues 58, 59, 62 and 63 deleted, antisense primer);

(6) 5′-TACACCATGAAGCTT*TCA*TTCAGCTGGGAT-3′ (FG, nt 5209-5239, antisense primer).

PCR parameters were as described [12]. The PCR products were cloned into vector pCR II (TA Cloning system, Invitrogen Co.) and sequenced by the dideoxi-termination method. The inserts were subsequently excised by restriction with *Bam*HI and *Eco*RI, and reinserted into expression vector pET-3a [15] to obtain pET-$3A_{FG}$ (primers 2 and 6), pET-$3A_{HM}4$ (primers 1 and 5), pET-$3A_{FG}2$ (primers 2 and 4), pET-$3A_{FG}4$ (primers 2 and 5), and pET-$3A_{FG}$us (primers 3 and 6). The final constructs were sequenced to verify their identity. The amino acid sequence of all 3A proteins is shown in Fig. 1. Construction of $3AB\Delta1$, $3AB\Delta2$ and $3AB\Delta3$ mutants has been previously described [12].

### *2.2. Expression and labeling of recombinant proteins*

The expression plasmids were used to transform *E. coli* strain BL21 (DE3) pLysS, which carries the T7 RNA polymerase gene under the control of the lacUV5 promoter. This strain also carries a plasmid conferring chloramphenicol resistance and encoding for the T7 lysozyme, which is a natural inhibitor of the T7 RNA polymerase.

Expression was induced by 0.5 mM isopropyl-$\beta$-D-thiogalactoside (IPTG) as previously described [12]. At defined time points after induction, transformed bacteria were labeled with [$^{35}$S] methionine (2 $\mu$Ci/ml) for 10 min. To inhibit transcription by *E. coli* RNA polymerase, rifampicin was added at a final concentration of 150 $\mu$g/ml. Recombinant proteins were analyzed by SDS-PAGE and autoradiography.

In vitro transcription and translation were performed by using a coupled reticulocyte lysate system (TnT, Promega). Circular cDNA (0.5 to 0.8 $\mu$g) was added to 50 $\mu$l of TnT and incubated for 2 hours at 30°C following the protocol of the manufacturer. [$^{35}$S] methionine-labeled translation products were analyzed by SDS-PAGE and autoradiography. The presence of three methionine residues among the 13 N-terminal amino acids of bacterial gene 10 encoded


Fig. 1. Amino acid sequence of the 3A proteins cloned in the pET-3a vector and expressed in *E. coli*. ●, deletion; ∗, substitution of amino acid residue; +, positively charged amino acids flanking the transmembrane tract; −, negatively charged amino acids in the N-terminus of the protein. The stretch of hydrophobic transmembrane amino acids is underlined.

by all pET-3A constructs allowed the radioactive tracing of HAV protein 3A which does not contain any methionine residues.

### 2.3. Membrane binding

Each in vitro reaction (20 $\mu$l) was incubated with 2.5 $\mu$l of canine pancreatic microsomal membranes (Promega) for 15 min at 30°C. Samples were spun at 100 000 × *g* for 1 h at 4°C through a 0.5 M sucrose cushion in 40 mM Hepes-KOH, pH 7.4, 162 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM DTT, to pellet membranes [16]. Pellets were suspended in 20 $\mu$l of sample buffer and analyzed by SDS-PAGE using 15% gels.

For solubility studies, radiolabeled bacterial extracts were divided into two aliquots, and resuspended in lysis buffer (10% glycerol, 100 mM DTT, 160 mM Tris-HCl, pH 6.8) in the presence or absence of 2% SDS. The samples were then sonicated on ice (200–300 Watt). RNase A (10 $\mu$g/ml) and DNase (5 $\mu$g/ml) were added to the lysates and incubated at room temperature for 30 min. Membrane associated and membrane-free fractions were separated by pelletting through a sucrose cushion as described above and analyzed by SDS-PAGE.

### 2.4. Analysis of membrane permeability

Changes in permeability were observed by measuring cell lysis due to the exit of intracellular T7 lysozyme, the extracellular $\beta$-galactosidase activity, and the entry of *o*-nitrophenyl-$\beta$-D-galactopyranoside (ONPG) into bacterial cells [17].

To measure the toxicity of the different 3A proteins, cell density of bacteria expressing the constructs was determined after induction with 0.5 mM IPTG. Rifampicin (150 $\mu$g/ml) was added after 30 min to stop *E. coli* RNA polymerase. Aliquots of the cultures were taken at 30-min intervals and cell lysis was detected by measuring absorbance at 660 nm. For $\beta$-galactosidase assays, aliquots of induced cell cultures were removed at 30-min intervals, supplemented with a corresponding volume of growth medium containing streptomycin (50 $\mu$g/ml) to inhibit translation, and centrifuged. To determine the presence of extracellular $\beta$-galactosidase, 2 mM ONPG was added to the supernatant and incubated

for 10 min at 30°C. The appearance of the β-galactosidase reaction product was estimated by measuring absorbance of the supernatant at 420 nm. The pellet was used to detect the entry of ONPG into the cells. After incubation with 2 mM ONPG at 30°C for 30 min, the pellet was centrifuged to avoid interferences by bacterial cells and absorbance at 420 nm was measured.

## 3. Results

### *3.1. Membrane binding assays*

The primary structure of hepatitis A virus protein 3A includes a central hydrophobic domain which is flanked by positively charged residues, typical for a variety of membrane proteins [18,19]. In fact, membrane interaction of HAV 3AB has already been demonstrated [12]. To assess the structural role of positively charged amino acids for membrane binding, we introduced mutations into the C-terminus of 3A of two HAV strains that differ in their effect on cell metabolism. Three mutants (pET-$3A_{HM}4$, pET-$3A_{FG}2$, pET-$3A_{FG}4$) were constructed and expressed in the *E. coli* strain BL21 (DE3) pLysS. pET-$3A_{FG}$ was used as a positive control. Recombinant protein expression was detected after radiolabeling of induced bacterial cultures in the presence of rifampicin. One hour after induction, cells were harvested by centrifugation and the pellets were divided into two aliquots. To differentiate between membrane-bound and free proteins, the cells were extracted in the presence or absence of SDS. SDS-solubilized membraneous proteins were analyzed after pelleting the cell extracts through a sucrose cushion and by SDS-PAGE. As shown in Fig. 2, labeled proteins were detected in large quantity in the samples not treated with SDS (lanes 4), and only faintly in the SDS-solubilized extracts (lanes 2), indicating that 3A proteins are associated with membranes, irrespective of the deletion of two or four positively charged residues near the C-terminus or of the virus strain.

The same 3A constructs were expressed in vitro, and the radiolabeled translation products were incubated post-translationally with canine pancreatic microsomal membranes. Bound proteins were separated from unbound proteins by centrifugation on a sucrose cushion and analyzed by SDS-PAGE. Similar to what was observed in vivo, no differences among the mutated and wild-type proteins were detected (data not shown). These results demonstrate that the carboxyterminal charged amino acids do not affect the membrane-binding efficiency of HAV protein 3A.

### *3.2. Effect of 3A mutants on bacterial growth and membrane permeability*

In a previous study we hypothesized the formation of a putative amphipathic helix in the N-terminal

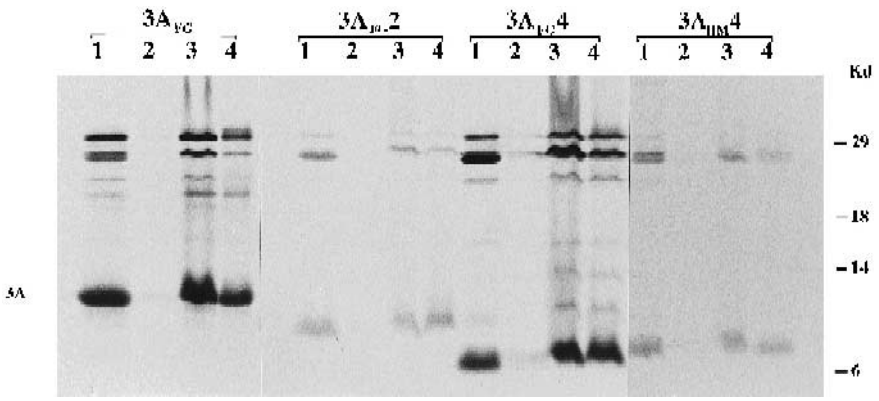


Fig. 2. Expression of HAV 3A protein in *E. coli*. BL21 (DE3) pLysS cells carrying constructs encoding $3A_{FG}$ or different 3A mutants were induced with IPTG. Labeling was performed by addition of [$^{35}$S] methionine. Lane 1: bacterial crude extract resuspended in SDS-containing buffer; lane 2: pellet from the same extract of lane 1 after ultracentrifugation; lane 3: bacterial crude extract resuspended in buffer without SDS; lane 4: pellet from the same extract of lane 3 after ultracentrifugation. The mol wt. of 3A decreases with the number of deleted residues.

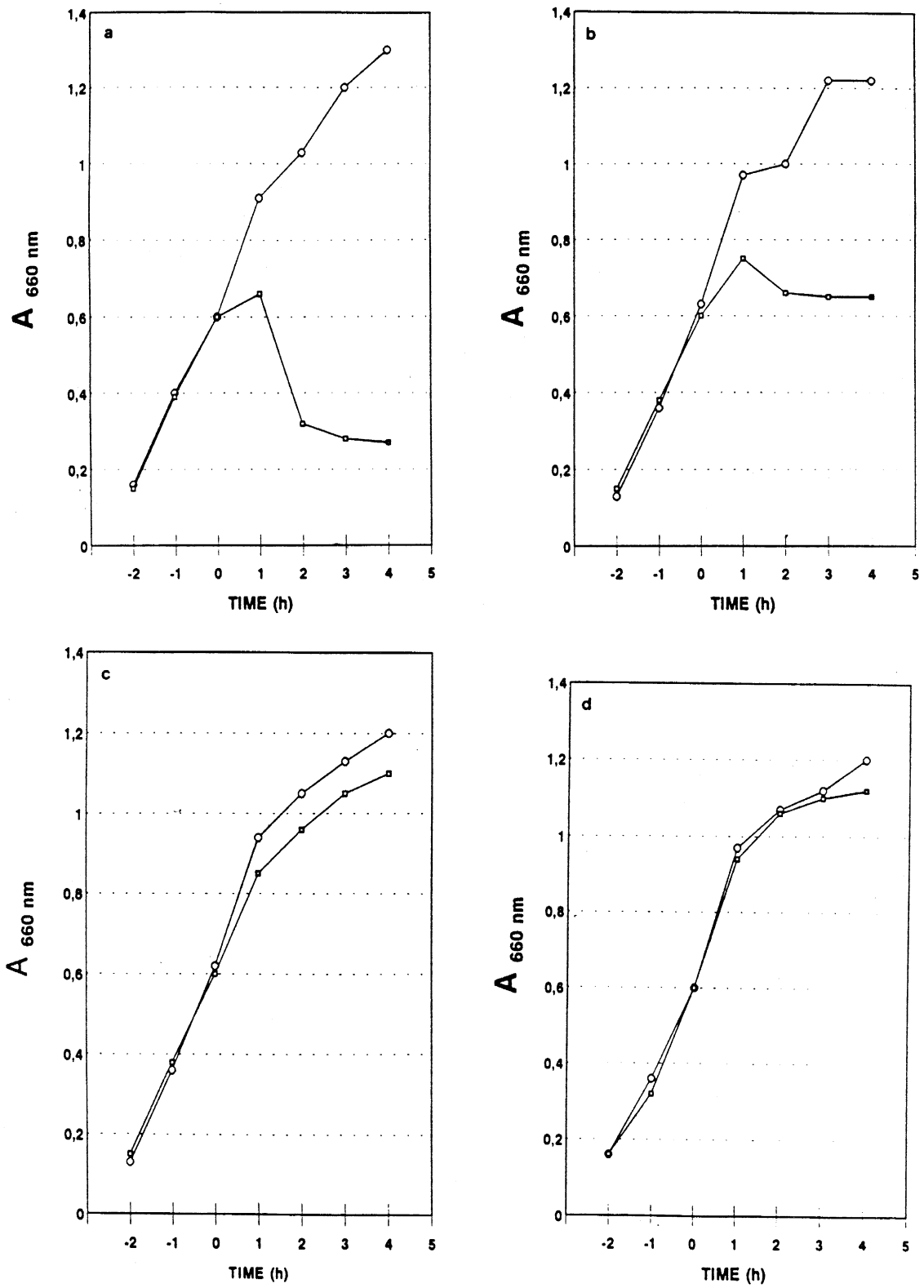


Fig. 3. Cell density of *E. coli* BL21 (DE3) pLysS cells expressing $3A_{FG}$ (a), $3A_{FG}2$ (b), $3A_{FG}4$ (c) and $3A_{FG}$us (d). At zero time, cells were induced with IPTG (□) or not induced (○). Optical density (absorbance at 660 nm) of the cell cultures was measured at the times indicated.

tract of $3A_{FG}$, and observed that mutations affecting the helix (mutants 3AB$\Delta$1, 3AB$\Delta$2 and 3AB$\Delta$3, see Fig. 1) led to the loss of the protein's ability to influence bacterial membrane permeability [12]. To test whether the permeabilizing effect can be influenced by other mutations in this tract or in other portions of the protein, we expressed in bacteria $3A_{FG}$, $3A_{FG}2$, $3A_{FG}4$ and a third mutant (pET-$3A_{FG}$us), encoding a modified protein $3A_{FG}$ where the negative charge of the N-terminus was totally abolished by replacing the aspartic acid residues with two leucines. The optical density of transformants expressing the recombinant proteins was determined at various time points and compared with uninduced cells. The expression of protein $3A_{FG}$ in BL21 (DE3)pLys cells caused a rapid decrease in absorbance at 660 nm, indicating cell lysis, due to the leakage of T7 lysozyme from the bacterial cytoplasm (Fig. 3a). Expression of $3A_{FG}2$ resulted in reduced bacterial growth (Fig. 3b). Growth was unaffected in cells carrying pET-$3A_{FG}4$ and pET-$3A_{FG}$us (Fig. 3c and d). To determine whether this effect on bacterial growth was due to changes in membrane permeability, the intake (ONPG) and release ($\beta$-galactosidase) of macromolecules was determined after the induction of 3A. While high levels of extracellular activity were found when $3A_{FG}$ and $3A_{FG}2$ were expressed, the extracellular enzyme activity was minimal upon induction of $3A_{FG}4$ and $3A_{FG}$us (Fig. 4). Similarly, the uptake of ONPG was higher in cells expressing wild-type $3A_{FG}$ and $3A_{FG}2$ (Fig. 4).

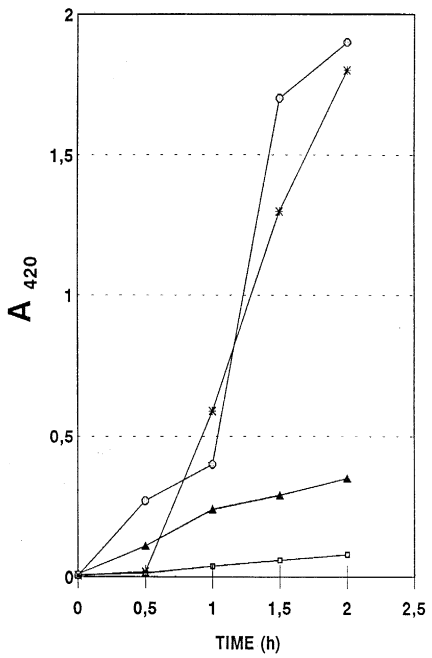


Fig. 4. Modification of membrane permeability of BL21 (DE3) pLysS cells expressing $3A_{FG}$ (∗ and ○) and $3A_{FG}4$ (□ and ▲). Entry of ONPG (∗, □) and $\beta$-galactosidase activity in extracellular medium (○, ▲) were measured at subsequent times after induction with IPTG (zero time). Expression of $3A_{FG}$us gave results similar to $3A_{FG}4$.

The result of the expression of 3A mutants on bacterial growth and cell membrane permeability suggests that both the N- and C-terminal charged amino acids are involved in conferring protein $3A_{FG}$ its cytotoxicity.

## 4. Discussion

Due to its slow replication rate in most host cell systems, great interest has been directed towards elucidating the role of HAV nonstructural proteins in virus replication. Attention has been focused on proteins 2B and 2C and their involvement in virulence and attenuation [20]. In contrast to most other picornaviruses which induce host cell protein shutoff, the growth of the majority of strains and variants of HAV does not give rise to a cytopathic effect [1,4]. Replication of other picornaviruses results in a series of metabolic modifications leading to the death of the host cell. Proteinases 2A and 3C are implicated in the degradation of a component of the cellular cap binding complex and in the inactivation of transcription factors required for transcription by cellular RNA polymerases II and III [21,22]. Poliovirus proteins 2B and 3A have been shown to inhibit the cellular protein secretion pathway [2]. No data are available to define the molecular basis of the cytopathic phenotype exerted by some strains of HAV. The analysis of the function and properties of protein 3AB of HAV showed that, due to its hydrophobicity, 3A may function as an anchor of the precursor 3AB to cell membranes and thus probably orient the replication complex to the endoplasmic reticulum [12]. In addition, the toxic potential to bacteria of protein 3AB of the cytopathic strain FG has been demonstrated, similar to what was observed for poliovirus [3,12].

To investigate the correlation between the secondary structure of protein $3A_{FG}$ and its lytic poten-

tial, we engineered amino acid substitutions or deletions in the putative amphipathic helix near the N-terminus or in the C-terminus of the protein, and tested their effect on membrane interaction and cytotoxicity. In a first group of mutants, two or four positively charged residues C-terminal to the hydrophobic transmembrane region were deleted. Neither deletion influenced the ability of protein 3A to interact with membranes in vivo and in vitro. The finding that these positively charged amino acids can be deleted with no apparent effect on anchoring suggests that they are not required for stable interaction with the membrane.

Expression of 3A mutants in *E. coli* cells revealed a consistent effect of the deletions on the toxicity of protein 3A of the cytopathic strain FG. Lack of two positive residues (aa 62 and 63) reduced the toxicity of the protein for *E. coli*, whereas the deletion of four positively charged amino acids (aa 58, 59, 62, and 63) caused the loss of its toxic potential. Expression of another mutant, where the negative charge of the N-terminus of protein $3A_{FG}$ was totally suppressed, replacing aspartic acids 4 and 5 with two leucines, had no effect on the bacterial growth nor on the integrity of the cell membrane. In a previous study we reported the construction of 3 mutants (3AB$\Delta$1, 3AB$\Delta$2 and 3AB$\Delta$3) carrying deletions of individual charged residues in the N-terminal tract of 3A leading to abrogation of the putative amphipathic helix present at the N-terminus of $3A_{FG}$, and whose expression had no effect on the permeability of the bacterial membrane [12]. Similar results were obtained upon expression of $3A_{FG}$us, whose N-terminus could form an amphipathic helix, as determined by the method of Schiffer and Edmundson [11] (Fig. 5), but lacks negative charges. These data indicate that both the formation of a putative amphipathic helix and the presence of negative charges at N-terminus

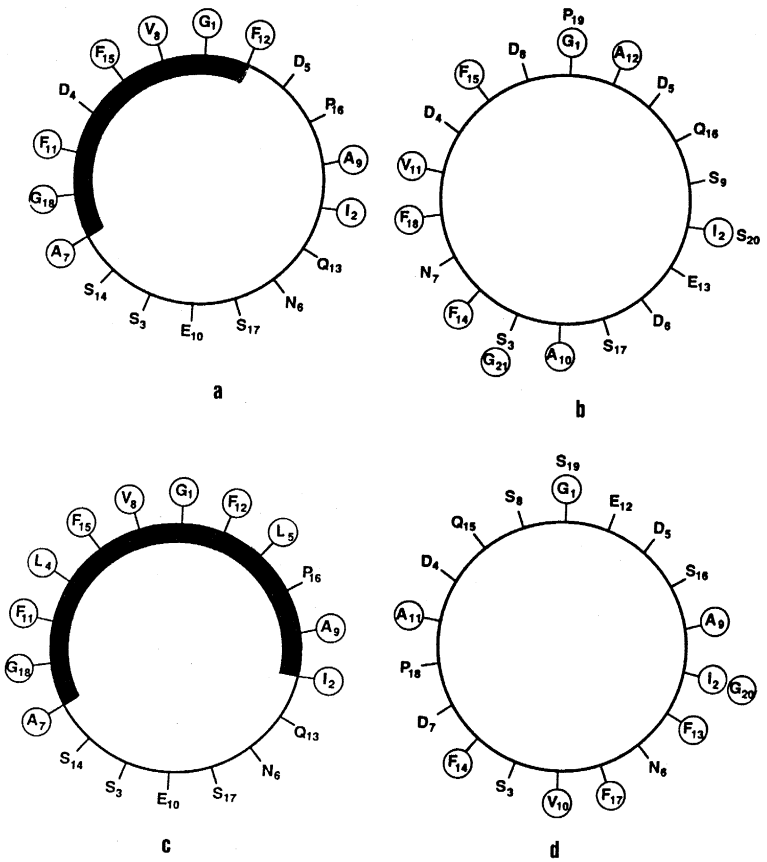


Fig. 5. Putative helical wheels of the N-terminal portion of proteins: (a) $3A_{FG}$ (residues 1–18), (b) $3A_{HM}$ (residues 1–21), (c) $3A_{FG}$us (residues 1–18) and (d) 3A$\Delta$1 (residues 1–20). Hydrophobic amino acids are circled. The shaded area indicates the nonpolar face.

are crucial for the cytotoxic feature of $3A_{FG}$. Furthermore, analysis of our data suggests that protein 3A is cytotoxic to bacteria if the hydrophobic phase of the putative helical wheel at its N-terminus consists of a stretch of at least seven hydrophobic residues interrupted by not more than one non-hydrophobic amino acid.

On the other hand, deletion of all the positive charges located C-terminal of the transmembrane region renders $3A_{FG}$ non toxic and thus similar to the respective protein of HAV strain HM175. As the same deletion had no effect on the properties of $3A_{HM}$ (data not shown) it can be supposed that the noncovalent association of the negative charges present in the N-terminal amphipathic helix with the positive charges at the C-terminus of $3A_{FG}$ is necessary in order for it to assume its functional conformation.

The $3A_{FG}$ peptide shares characteristics with other small hydrophobic proteins capable of permeabilizing membranes [13,23–25]. These peptides (e.g., poliovirus protein 3A, Semliki Forest virus 6k protein, magainins, cecropins) contain sequences which are known to or are suggested to form pores in the cell membrane.

All steps of the picornavirus life cycle occur in the cytoplasm of the host cell. However, some nuclear proteins have been shown to play an important role in virus replication and to be relocalized to the cytoplasm of the infected cells [26,27]. The cytopathic HAV strain FG shows a fast-growing phenotype, as compared to other HAV strains, which could correlate with an acceleration of viral protein and RNA synthesis. Hypothetical pore-formation into the nuclear membrane induced by $3A_{FG}$ could account for the accelerated growth by promoting a more efficient relocalization of nuclear proteins to the cytoplasm with respect to other strains. As a consequence, this hypothetical pore-formation in nuclear membranes could lead to a depletion of nuclear proteins needed for cell replication and to cell death (cytopathic effect).

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