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Polypeptide 3AB of Hepatitis A Virus Is a Transmembrane Protein

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Hepatitis A virus (HAV) protein 3AB is a membrane-interacting protein containing a stretch of 21 hydrophobic amino acid residues. The nature of its membrane association was studied in detail by analysing various deletion mutants. *In vivo* and *in vitro* expression of the wild-type protein and its mutants allowed to demonstrate that the hydrophobic domain interacts with membranes and to define the portions essential for this feature. Furthermore, the results suggest that 3AB behaves as an integral membrane protein. Expression in *Escherichia coli* showed that 3AB can be isolated, in association with membranes, both in monomeric and in dimeric form. This finding was confirmed *in vitro* after post-translational incubation of the protein with microsomal membranes. Analysis of deletion mutants demonstrated that the dimerization region colocalises with the hydrophobic transmembrane domain, implicating that HAV 3AB could form oligomers mediated by the interaction of transmembrane α-helices.

Replication of picornaviruses typically occurs in close association with cell membranes (1). Poliovirus RNA synthesis proceeds in replication complexes located in the cytoplasm of the infected cell, that include virtually all virus-encoded non-structural proteins or their precursors (2). Electron microscopy has shown that the replication complexes are localised on the surface of virus-induced vesicles, and their formation has been attributed to viral proteins 2C and 2BC (3). Computer analysis has shown that the sequence of the 2C protein is the most conserved among all picornaviruses and revealed the presence of a highly conserved central region of approximately 150 residues, suggesting similar functions of this protein in the replication of the different members of the family. Recently, Teterina et al. reported that 2C and 2BC of hepatitis A virus (HAV) can efficiently interact with membranes and that expression of both proteins in mammalian cells induces a dramatic rearrangement of intracellular membranes, similar to poliovirus (4).

Association of poliovirus polypeptide 3AB with membranes has been directly demonstrated, and it has been postulated that one function of 3A (as polypeptide 3AB), is to serve as the lipophilic carrier of 3B (VPg, the primer for viral RNA synthesis) to the viral RNA replication complex (5). Subsequently, many other functions have been attributed to protein 3A in the viral replication process, all related to its ability to associate with cell membranes (6, 7). HAV 3AB shares poor homology with the corresponding proteins of the other members of the picornavirus family, and its functions have not been defined yet. As many membrane-associated proteins, HAV 3A contains a stretch of 21 uncharged amino acid residues endowed with considerable hydrophobicity and flanked by positively charged amino acids. It has recently been demonstrated that this protein can in fact interact with membranes *in vitro* and *in vivo* (8, 9). However, the nature of this interaction has not been studied in detail, and the specificity of the association has not been definitively determined.

Assuming that many of the functions of 3A in the HAV replicative cycle are exerted by its precursor form 3AB, we examined the quality of the interaction of HAV 3AB with membranes, determined the possibility of its integration into the cell membrane and defined the portion of the hydrophobic region essential for this function. Computer analysis of the primary sequence of 3AB suggested the presence of one α-helix-forming region near the N-terminus of the 3A moiety and of a second putative α-helix, bearing many charged residues, at its C-terminus. As membrane-interacting regions typically form α-helical structures, and charged domains can be involved in membrane anchorage (10-12), we decided to examine the possible contribution of

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Abbreviations used: HAV, hepatitis A virus; IPTG, isopropylthio-β-D-galactoside; 6xHis-tag, 6 consecutive hystidine residues; NP-40, Nonidet P-40; aa, amino acids.

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these two zones to the membrane association of the protein. Previous experiments of protein expression in bacteria showed the ability of 3A and 3AB to dimerize.[2] These results were confirmed in the course of this study. The presence of dimers was also demonstrated after post-translational incubation of protein 3AB with canine microsomal membranes. Mutagenesis experiments were performed to determine the residues conferring the protein this feature and their potential coincidence with the amino acids of the hydrophobic domain.

MATERIALS AND METHODS

Construction of expression plasmids. Genes coding for the mutant 3AB proteins were created by oligonucleotide-directed mutagenesis, according to the method of Higuchi et al (13). The desired mutations were introduced by using appropriate oligonucleotide primers employing pHAV/7 (14) as template. Two DNA fragments (A and B, Table 1) were synthesised by polymerase chain reaction (PCR), that contain the desired deletion and hybridise to each other. These fragments were gel-purified and, after reannealing, used as template for a second amplification using primers 57S and 67A. Primers for the first and second amplification are listed in Table 1. All nucleotide (nt) positions refer to those of HAV strain HM175 (14). PCR reagents were as described (8). First PCR was carried out for 25 cycles (30 s at 95°C, 10 s at 37°C, 1 min at 70°C) followed by 5 min at 70°C. Second PCR took place for 30 cycles (25 s at 95°C, 40 s at 50°C, 1 min at 70°C; last cycle 5 min at 70°C). PCR products were cloned into vector pCRII-TOPO (Invitrogen Co.). All the inserts were then excised by restriction with *Bam*HI and *Eco*RI, which are present in the polylinker of pCRII-TOPO, and reinserted into the respective sites of expression vector pET-3a (15). Wild-type 3AB gene was amplified by using primers 57S and 67A and then cloned in pET-3a. Wild type 3AB (3ABwt) and mutant 3ABΔid were also excised by restriction with *Bam*HI and *Hind*III (included in the primer sequences 57S and 67A) and reinserted into expression vector pQE30 (16). A PCR fragment, corresponding to the hydrophobic region of protein 3A (residues 40 to 60) was amplified by using the following primers: sense (nt 5103-5131): 5′-GTT ACT AAT *GGA TCC* TGG GTT GCT GTG GG-3′; anti (nt 5165-5198): 5′-TTT GCG GGA *AAG CTT TCA* ATA CAC AAA CCA TCC-3′.

The underlined sequences represent *Bam*HI and *Hind*III restriction sites, respectively. The stop codon is in italics. The PCR product was cloned into expression vector pGEX-2T (17) to obtain the fusion product pGEX-2T-3TM. All final constructs were completely sequenced by the dideoxy-termination method to verify their identity and the reading frame. The amino acid sequences of all mutants are shown in Fig. 1.

Expression and labelling of recombinant proteins. pET-3a constructs were used to transform *E. coli* strain BL21 (DE3) pLysS (8). Expression was induced by addition of 1 mM IPTG. After one hour, bacteria were labelled with [^{35}S]-methionine (2 μCi/ml) for 10 min. To inhibit transcription of *E. coli* RNA polymerase rifampicin was added to a final concentration of 150 μg/ml. pET-3a transformed cells induced with IPTG were used as negative control.

Membrane binding assay. For solubility studies, radiolabelled BL21 (DE3) pLysS extracts were divided into two aliquots and resuspended in lysis buffer (10% glycerol, 100 mM DTT, 160 mM Tris-HCl pH, 8) in the presence or absence of 2% SDS and then sonicated on ice (200-300 Watt). Membrane associated and cytosolic fractions

TABLE 1

Oligonucleotide Primers Used for Mutagenesis

5′	Primer sequences used to create deletion mutants	3′	nt	Primers used for amplifying fragments A and B
57S	TCTCAG<u>GGATCC</u>TCAGATGATGATAATGAT		(4995–5024)	
67A	CTATTT<u>CAAGCTT</u>*TCA*CTGAGATTCTAC		(5290–5307)	
89A	TCCTCCAACGAGCACTCCAAGAATGCCAACCTTGTGATTAGTAACAGATTGGAAAAAGCC		(5168–5088)	3ABΔid ^57S123A; *122S–67A
90S	GGCTTTTTCCAATCTGTTACTAATCACAAGGTTGGCATTCTTGGAGTGCTCGTTGGAGGA		(5088–5168)	3ABΔid1 ^57S–89A; *90S–67A
93A	GAAATGCTTATACACAAACCATCCTCCAACTGCAGCTCCCACAGCAACCCACTTGTGATT		(5189–5109)	3ABΔid2 ^57S–93A; *94S–67A
94S	AATCACAAGTGGGTTGCTGTGGGAGGATGGTTTGTGTATAAGCATTTC		(5109–5189)	3ABΔ id3 ^57S–95A; *96S–67A
95A	TGGTTCCTCCTCTTTGCGGGAGAAATGCTTGAGCACTCCAAGAATGCCAACTGCAGCTCC		(5210–5130)	3ABΔ 3.1 ^114S–67A;
96S	GGAGCTGCAGTTGGCATTCTTGGAGTGCTCAAGCATTTCTCCCGCAAAGAGGAGGAACCA		(5130–5210)	3ABΔ 5.1 ^57S–113A; *112S–67A
112S	GGAGTGCTCGTTGGAGGATGGTTTGTGTATCCAATCCCAGCTGAAGGGGTTATATCATGG		(5151–5236)	
113A	CCATGATATACCCCTTCAGCTGGGATTGGATACACAAACCATCCTCCAACGAGCACTCC		(5236–5151)	
114S	TGGTCTCAG<u>GGATCC</u>TCAGATGATGATAATGATAGTGCAGTAGAACCATCGAACTCTAAATTATCTGGC		(4992–5090)	
122S	GGCTTTTTCCAATCTGTTACTAATCACAAGAAGCATTTCTCCCGCAAAGAGGAGGAACCAATC		(5088–5213)	
123A	TGGTTCCTCCTCTTTGCGGGAGAAATGCTTCTTGTGATTAGTAACAGATTGGAAAAAGCCAG		(5210–5086)	

Note. The underlined sequences represent restriction sites. The translational stop codon is in italics. ^ and * indicate primers for fragment A and B amplification, respectively.

[2] Ciervo, Beneduce, and Morace, unpublished observation.

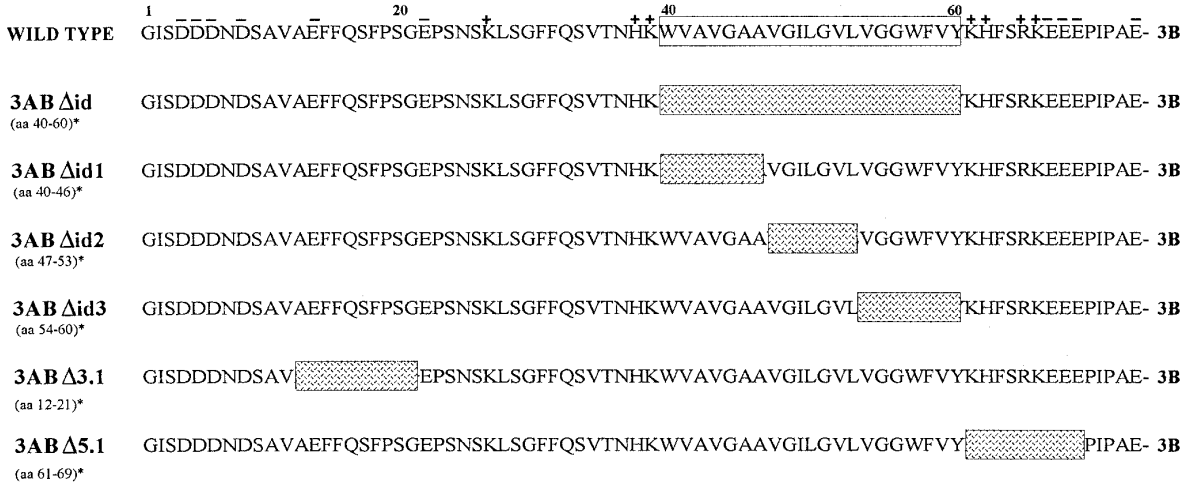

FIG. 1. Amino acid sequence of HAV protein 3A and of its deletion mutants. The open box shows the hydrophobic domain; + and - denote charged residues. Asterisks indicate the position of deleted amino acids. Dashed boxes indicate deleted regions.

were separated by pelletting through a sucrose cushion (8) and analysed by SDS-PAGE and autoradiography.

Expression and purification of 3AB, 3ABΔid and GST-3TM. pQE30-3ABwt and pQE30-3ABΔid were expressed in *E. coli* strain M15 (pREP4) (Qiagen). Cells were induced with 1 mM IPTG and the recombinant proteins, carrying a 6xHis-tag at their N-terminus, were purified under denaturing conditions by using a Ni-NTA resin according to the instruction of the manufacturer (Qiagen). Protein folding was restored by dialysis against a linear gradient containing 7 to 0 M urea in renaturing buffer (10 mM Tris-HCl, pH 8, 100 mM NaCl, 10% glycerol, 1 mM DTT, 0.1% Triton X-100, 1 mM PMFS). Each dialysis step was carried out at 4°C for 8 to 12 hours. Plasmids pGEX-2T and pGEX-2T-3TM were used to transform *E. coli* strain XL1-Blue and expression of glutathione-S transferase (GST) and GST-3TM proteins, respectively, was achieved by the addition of 1 mM IPTG. The proteins were then purified by glutathione-Sepharose 4B as described by the manufacturer (Pharmacia). The concentration of all the purified proteins was determined by the method of Bradford (18).

In vitro transcription and translation—Binding to microsomal membranes. For *in vitro* transcription and translation of the pET constructs a coupled reticulocyte lysate system (TnT, Promega) was used, following the protocol of the manufacturer. After addition of cycloheximide (final concentration 300 μM) to inhibit further translation, 2.5 μl of canine pancreatic microsomal membranes (Promega) were added to 25 μl of the mixture and incubated at 30°C for 30 minutes. [^{35}S] methionine-labelled translation products were analysed by SDS-PAGE and autoradiography.

Western blotting. Proteins were resolved by SDS-PAGE and subsequently transferred to nitro-cellulose. A mixture of antibodies recognising the N-terminus of 3A and the N-terminus of 3B (a kind gift of Dr. V. Gauss-Müller) was used to detect protein 3ABwt and all the deletion mutants. GST and GST-3TM were probed with anti-GST antibody (Pharmacia). In both cases alkaline phosphatase-conjugated secondary antibodies were used. Blots were revealed by reaction with the appropriate phosphatase substrate.Proteins were resolved by SDS-PAGE and subsequently transferred to nitro-cellulose. A mixture of antibodies recognising the N-terminus of 3A and the N-terminus of 3B (a kind gift of Dr. V. Gauss-Müller) was used to detect protein 3ABwt and all the deletion mutants. GST and GST-3TM were probed with anti-GST antibody (Pharmacia). In both cases alkaline phosphatase-conjugated secondary antibodies were used. Blots were revealed by reaction with the appropriate phosphatase substrate.

Membrane association assay—Biochemical treatments. After expression, BL21 (DE3)pLysS cultures were centrifuged at 3000xg and 4°C for 10 min. Bacterial pellets were resuspended in BCM buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM PMSF) and sonicated on ice. Each pellet sample or samples from post-transcriptional TnT-microsomal membrane interaction were treated with the following reagents in BCM:4 M urea, 0.1 M Na_2CO_3,, pH 10.5, 0.5% NP-40, 1.5 M NaCl. Bacterial pellets and the protein-microsomal membrane mixture, resuspended in BCM, pH 7.5, were used as control. All reactions were incubated 30 min on ice and then centrifuged at 100,000xg and 4°C for 30 min in absence of a sucrose cushion. Samples were analysed by SDS-PAGE and autoradiography. Triton X-114 phase partitioning was performed according to the method of Bordier (19) using bacterial pellets resuspended in BCM buffer or *in vitro* translated proteins after interaction with microsomal membranes.

Immunoprecipitation. Samples from TnT-microsomal membranes and supernatants from Triton X-114 treatment were incubated with the mixture of anti 3A-anti 3B antibodies on ice for 1 hour, and added to Protein A-Sepharose in immunolysis buffer (50 mM Tris–HCl, pH 8, 1% NP-40) (20). After 1 hour of incubation at 4°C, the antibody-protein complexes were washed 6 times with immunolysis buffer and diluted in 30μl of Laemmli gel sample buffer. Each sample was boiled and subjected to SDS-PAGE. The dried gels were autoradiographed.

Chemical cross-linking. One microgram of purified 3AB and 3AB-Δid protein was incubated with different amounts of glutaraldehyde in a buffer containing 20 mM Hepes, pH 7.5 and 200 mM NaCl for 15 min at 30°C. After SDS-PAGE the samples where analysed by immunoblot with anti-3A anti-3B serum.

RESULTS

Secondary structure prediction by computer analysis. Membrane-associated proteins typically contain a stretch of 20-25 consecutive uncharged residues with considerable hydrophobic character flanked by positively charged amino acids (21), characteristics shared

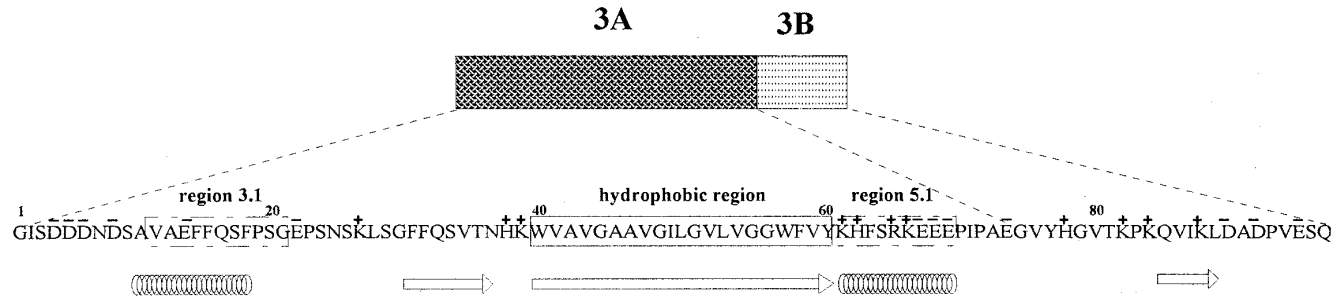

FIG. 2. Schematic representation of 3AB secondary structure prediction. Secondary structure prediction was performed by the algorithm of Chou and Fasman. Motifs are labelled as follows: α-helix (spirals), β-strand (arrows). The proposed transmembrane domain (hydrophobic region) and regions 3.1 and 5.1 are boxed.

by protein 3A of all picornaviruses (8). Computer analysis of protein 3AB of HAV performed according to the algorithm of Chou and Fasman (22) suggested the presence of two α-helices near the N- and C-termini of the 3A moiety of the protein (region 3.1 and 5.1). On the contrary, a low "intrinsic" helical propensity was attributed to the hydrophobic domain, containing a preponderance of helix-destabilising (Val, Gly, Ile) residues, Fig. 2. This suggestion prompted us to analyse in detail these regions to determine their contribution to the membrane-interacting ability of protein 3AB.

Expression of wild-type 3AB and deletion mutants in E. coli—Membrane association assay. In previous experiments we demonstrated that wild-type 3AB can interact with membranes *in vivo* and *in vitro*, and hypothesised a crucial role of the hydrophobic region (8). A deletion mutant lacking the complete hydrophobic stretch (3ABΔid) was constructed and expressed in *E. coli* BL21 (DE3) pLysS. Wild-type 3AB was used as a positive control. As HAV protein 3AB does not contain any methionine residue to allow its radioactive tracking vector pET-3a, which contains three methionines among the 13 N-terminal amino acids of gene 10, was chosen for expression. One hour after induction of protein expression in the presence of [^{35}S]-methionine and rifampicin, bacteria were harvested by centrifugation. The pellets were divided into two aliquots and extracted in the presence or absence of SDS to differentiate between membrane-bound and cytosolic proteins. SDS treatment was selected to destroy the lipid bilayer of the cell membrane, thus solubilising membrane proteins. As shown in Fig. 3 A, 3ABwt is a membrane-bound protein, since it can be found in the membrane pellet after ultracentrifugation through a sucrose cushion (lane 2), but is not detectable in the pellet when the bacterial extract is treated with SDS before sedimentation through a sucrose cushion (lane 1). On the contrary, the mutant bearing the complete deletion of the hydrophobic domain does not associate with membranes (lanes 5 and 6). Subsequently, three further mutants, which carry deletions of the first, second or third part of the hydrophobic region, (3ABΔid1, 3ABΔid2 and 3ABΔid3, respectively, see Fig. 1), were expressed to compare their membrane-binding ability with wild-type 3AB. After SDS treatment and sedimentation through a sucrose cushion, the pellets were analysed by SDS-PAGE. The results show a dramatically different behaviour of the three mutants, depending on the deleted portion of the hydrophobic domain (Fig. 3B). While mutant proteins 3ABΔId1 and ABΔId2 still interacted with membranes (lanes 1-4 and 5-8) 3AB-ΔId3 was not found in the membrane pellet after ultracentrifugation (lanes 9-12). This result demonstrates that the C-terminally third of the hydrophobic domain acts likely as a major determinant for membrane association of 3AB, through the interaction of its residues with the lipid bilayer.

Contribution of other regions of 3AB to membrane interaction. Alpha-helices have many fundamental roles in proteins and are often involved in membrane interaction (10). Computer analysis of the primary sequence of 3AB suggested that regions 3.1 (aa 12 to 21) and 5.1 (aa 61 to 69) of 3A can assume a α-helical conformation (see Fig. 2). Furthermore, domain 5.1 is characterised by the presence of many charged residues. Recently, the presence of both positively and negatively charged domains has been demonstrated to be fundamental in the interaction of some proteins with membranes (11, 12). Consequently, we decided to analyse whether these two regions could contribute to the binding of 3A to membranes. To this end, we constructed two mutants, lacking the residues of the 3.1 or 5.1 tract, and inserted them into pET-3a, thus obtaining pET-3ABΔ3.1 and pET-3ABΔ5.1, respectively. After expression and SDS treatment of the cell extracts, electrophoretic analysis showed that the absence of stretch 3.1 did not affect the membrane-binding ability of the protein (Fig. 3C, lanes 2 and 4). On the contrary, the result obtained upon expression of the mutant pET-3ABΔ5.1 was dramatically different, since the corresponding protein has lost its ability to interact with the cell membrane (Fig. 3C, lanes 5 and 6). This result shows that amino acids 61 to 69 of protein 3A contain information for membrane anchorage

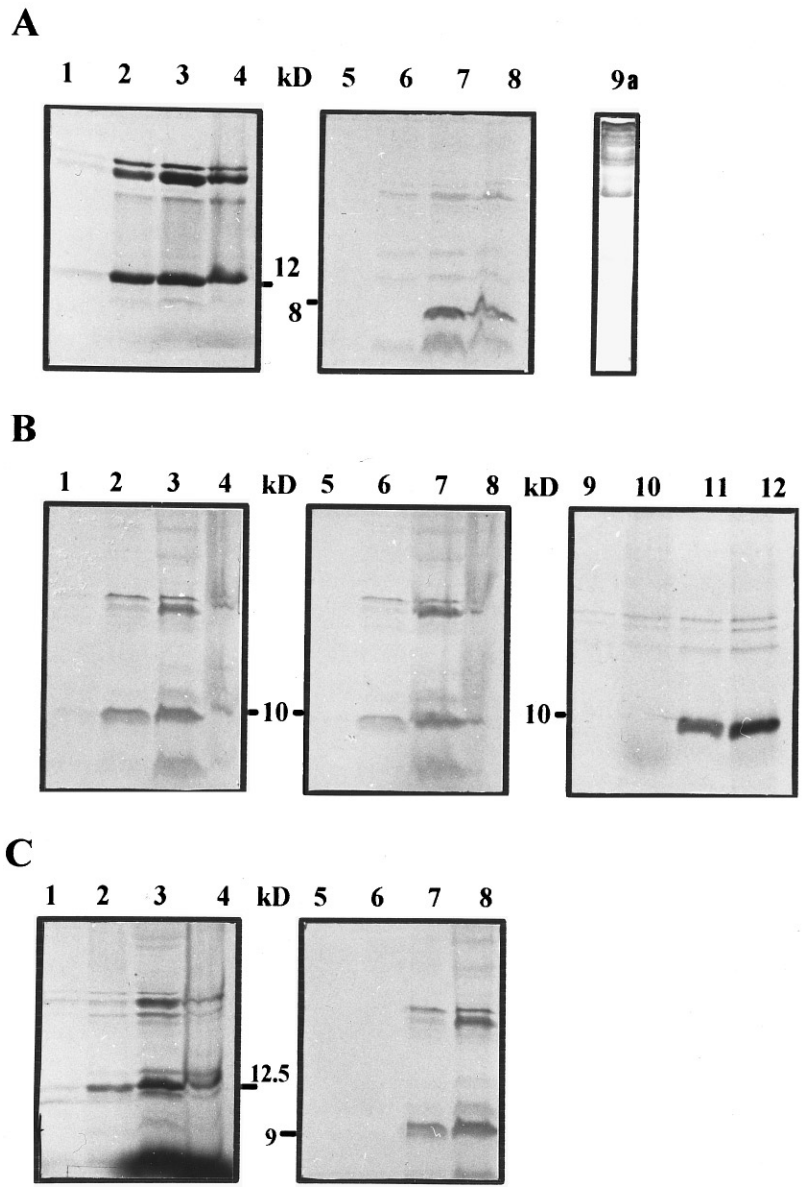

FIG. 3. Membrane association of 3ABwt and deletion mutants *in vivo*. Crude bacterial lysates were resuspended in SDS-containing or in SDS-free buffer, sonicated and subjected to ultracentrifugation through a sucrose cushion. As controls, aliquots of the same samples were analysed before ultracentrifugation. [^{35}S]-methionine-labelled proteins, expressed after IPTG induction, were resolved by electrophoresis on 15% SDS-polyacrylamide gels and autoradiographed. Pellets after ultracentrifugation (membrane fraction) of bacterial lysates were either treated with SDS-containing buffer (lanes 1, 5, and 9) or SDS-free buffer (lanes 2, 6, and 10). Crude bacterial lysates were also treated either with SDS-containing buffer (lanes 3, 7, and 11) or SDS-free buffer (lanes 4, 8 and 12). (A) Lanes 1-4 and 5-8: 3ABwt and 3ABΔid, respectively. (B) Lanes 1-4, 5-8, and 9-12: 3ABΔid1, 3ABΔid2, and 3ABΔid3 respectively. (C) Lanes 1-4 and 5-8: 3ABΔ3.1 and 3ABΔ5.1. Lane 9a shows the negative control (pET-3a transformed *E. coli* induced with IPTG).

and suggests an important role for this region in the association of the protein with membranes.

Quality of the interaction of 3AB with membranes. Depending on their method of association with the bilayer, proteins that interact with membranes have been classified as either integral (deeply inserted into the hydrophobic phase of the bilayer) or peripheral. The latter are associated with the membrane surface through electrostatic interactions and can be dislodged by treatments which do not substantially disrupt the integrity of the lipid bilayer, such as very basic pH, high ionic strength, or chaotropic agents (23). Integral proteins require the use of detergents or other disruptive agents for solubilisation (24).

To determine the quality of the interaction of 3AB with membranes different biochemical treatments were performed. Aliquots of crude bacterial extracts

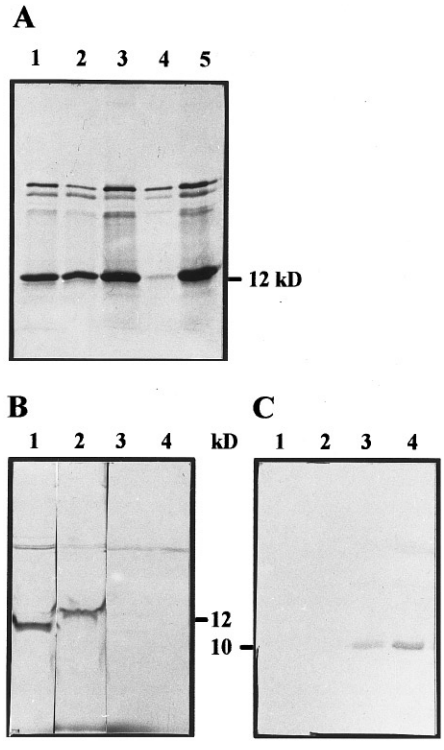

FIG. 4. Quality of the interaction of 3AB with membranes. Biochemical analysis and Triton X-114 phase-partitioning. Aliquots of crude bacterial lysates from BL21(DE3)pLysS expressing [^{35}S]-methionine-labelled 3ABwt or deletion mutants were tested and analysed on 15% SDS-polyacrylamide gel. (A) Extraction treatments of 3ABwt with 4M urea (lane 2), 0.1M Na_2CO_3 pH 10.5 (lane 3), 0.5% NP-40 (lane 4) and 1.5 M NaCl (lane 5). An aliquot of crude bacterial lysates resuspended in BCM, pH 7.5 was used as a control (lane 1). 3ABΔ3.1, 3ABΔid1 and 3ABΔid2 are not shown as the result of their treatments were similar to 3ABwt. Triton X-114 phase-partitioning: (B) detergent phase; (C) aqueous phase after immunoprecipitation. Lanes 1 and 2: 3ABwt and 3ABΔ3.1, lanes 3 and 4: 3ABΔid1 and 3ABΔid2.

from cultures expressing wild-type 3AB and mutants 3ABΔ3.1, 3ABΔid1 and 3ABΔid2, labelled with radioactive methionine, were tested. All treated proteins remained associated with the membrane fraction after extraction with 1.5 M NaCl, 0.1 M Na_2CO_3, pH 10.5 or 4M urea. On the contrary, treatment with Nonidet P-40, a non-ionic detergent, extracted 3AB from the lipid bilayer (Fig. 4A).

Phase partitioning, that physically separates integral membrane proteins from peripheral proteins, was also used to define the kind of association of 3AB with cell membranes. This application ensues from the unusual properties of Triton X-114, which is soluble in aqueous buffers at 0°C and separates out as a distinct detergent phase above 20°C. In general, peripheral proteins are found in the aqueous phase and hydrophobic species in the detergent phase (16). As shown in Fig. 4, treatment of wild-type 3AB and 3ABΔ3.1 (panel B, lanes 1 and 2) resulted in their partitioning to the detergent phase after low-speed centrifugation, while 3ABΔid1 and 3ABΔid2 were found in the aqueous phase (panel C, lanes 3 and 4). Anomalous partitioning of 3ABΔid1 and 3ABΔid2 can be explained by similar observations on integral membrane proteins presenting a pronounced hydrophilic character (25), which is also a feature of HAV 3A. We hypothesise that deletion of seven residues from the hydrophobic domain in the mutants 3ABΔid1 and 3ABΔid2 increases their hydrophilicity, in a manner consistent with their behaviour in this experiment.

To test the interaction with eukaryotic membranes, genes coding for 3ABwt and for 3ABΔ3.1, 3ABΔid1 and 3ABΔid2 were transcribed and translated *in vitro* and, after blocking any further reaction, added to microsomal membranes. After incubation for 30 minutes, the proteins were exposed to the biochemical treatments described above. The results of this experiment confirmed the observations *in vivo*, as only treatment with Nonidet P-40 extracted 3AB from the microsomal membranes (Fig. 5, lane 4 and data not shown). Furthermore, 3ABwt was recovered into the detergent phase after Triton-X 114 treatment (Fig. 5, lane 6). Taken together, these results clearly demonstrate that 3AB of HAV is an integral membrane protein, that its association can occur also post-translationally, and that the hydrophobic domain is responsible for the membrane-binding.

Analysis of the dimer-forming region. The membrane-spanning portions of many integral membrane proteins consist in one or more transmembrane α-helices, that can associate with one another in lipid bilayers through side-by-side interactions (26). During previous expression experiments we noted the presence of dimeric forms of 3A and 3AB. This finding was confirmed in the course of the present study. Expression of the wild-type 3AB in bacteria and electrophoretic separation showed a band corresponding to the ex-

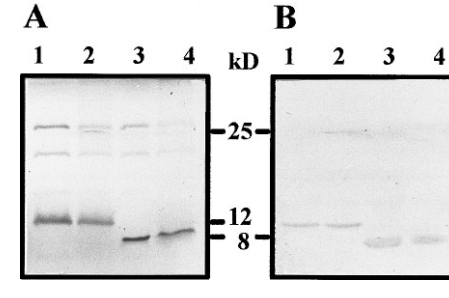

FIG. 5. Post-translational interaction of 3ABwt with microsomal membranes—Biochemical analysis. Thirty μl of TnT-canine microsomal membranes mixture was treated with the following reagents and analysed by 15% SDS-polyacrylamide gel: 4 M urea (lane 2), 0.1M Na_2CO_3 pH 10.5 (lane 3), 0.5% NP-40 (lane 4) and 1.5M NaCl (lane 5). TritonX-114, detergent phase (lane 6) and immunoprecipitation of aqueous phase (lane 7). An aliquot of the mixture resuspended in BCM pH 7.5 was used as control (lane 1).

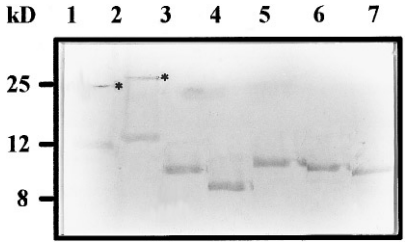

FIG. 6. Analysis of *in vivo* formed dimers. Western blot of bacterial crude lysates, run on a 20% SDS-gel. Lanes 1 to 7: 3ABwt, 3ABΔ3.1, 3ABΔ5.1, 3ABΔid, 3ABΔid1, 3ABΔid2 and 3ABΔid3, respectively. Dimers are indicated with an asterisk.

pected molecular weight, and a second band of double size. Immunoblotting, with a mixture of antisera recognising the N-terminus of 3A and the N-terminus of 3B, confirmed the identity of both bands (Fig. 6).

To determine the protein domain involved in oligomerization different 3AB deletion mutants were expressed in bacteria in the presence of [^{35}S]-methionine. The same cell extracts were electrophoresed and subjected to immunoblotting. As shown in Fig. 6, while proteins 3ABwt and 3ABΔ3.1 dimerized, the mutants 3ABΔid, 3ABΔid1, 3ABΔid2, 3ABΔid3 and protein 3ABΔ5.1 had lost this ability. Other mutants, carrying large deletions in different regions of 3A were also assayed for dimerization and showed to behave similar to 3AB wt (data not shown). *In vitro* transcription and translation of plasmids pET-3ABwt and 3ABΔid was also performed with the subsequent incubation with canine microsomes. Each sample was subjected to immunoprecipitation to verify the identity of the observed bands. As shown in Fig. 7, dimer formation was observed when 3ABwt was exposed post-translationally to microsomal membranes; on the contrary, in the lane corresponding to 3ABΔid, or in absence of the membranes, the proteins were present only in monomeric form.

To study the oligomerization ability of 3AB the genes coding for proteins 3ABwt and 3ABΔid were cloned into the expression vector pQE30 to obtain fusion proteins with a His-tag at their N-termini, that can be

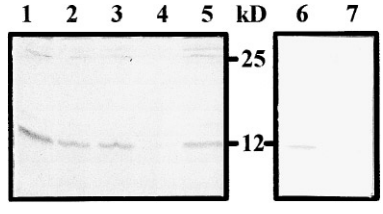

FIG. 7. Analysis of *in vitro* formed dimers. (A) Transcription and translation was carried out either in the absence (lanes 1 and 3) or in the presence (lanes 2 and 4) of canine pancreatic microsomal membranes. Lanes 1 and 2: 3ABwt; lanes 3 and 4: 3ABΔid. (B) The same samples after immunoprecipitation.

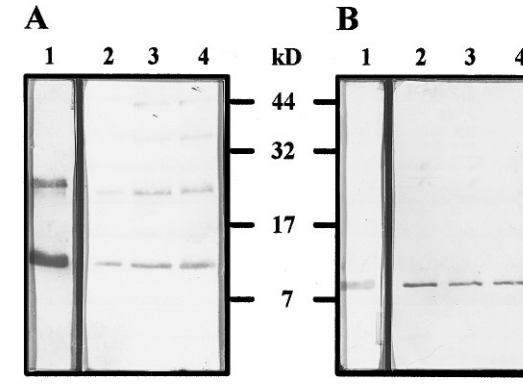

FIG. 8. Chemical cross-linking of purified 3ABs. Purified proteins 3ABwt (A) and 3ABΔid (B) were loaded on a 15% SDS-polyacrylamide gel either silver-stained (lanes 1) or immunoblotted after chemical cross-linking with 0 mM (lanes 2), 0.5 mM (lanes 3), and 1 mM (lanes 4) glutaraldehyde.

affinity-purified by Ni-NTA resin. Chemical cross-linking experiments employing these proteins were performed. As expected, the results show the formation of oligomers by 3ABwt but not by 3ABΔid (Fig. 8), indicating that the hydrophobic domain is crucial for dimerization of 3AB.

To verify the role of the hydrophobic transmembrane region in dimer formation, the portion of 3A gene corresponding to the hydrophobic domain was cloned into expression vector pGEX-2T. The aim of this experiment was to assess whether this domain would be able to induce dimerization of a monomeric protein, such as GST. Following expression of the pGEX-2T-3TM fusion product in *E. coli*, the GST-3TM fusion protein was purified by glutathione, and subjected to SDS-PAGE including either SDS or 50 mM DTT in the sample buffer and in the gel loading buffer. Western blotting of the purified protein, using an antibody directed against GST, showed both the monomeric and dimeric form of the protein in the sample run in the presence of SDS (Fig. 9, lane 4), and of the dimer alone in the absence of detergent (Fig. 9, lane 6). These results indicate that the hydrophobic domain of 3A is responsible for oligomerization of HAV 3AB in the presence of membranes, and that maintenance of its complete amino acid sequence is required for this feature.

DISCUSSION

Little information is available on the role of HAV protein 3AB in the virus life cycle. Protein 3AB of poliovirus has been demonstrated to exert several fundamental functions in the viral replication process, all related with its ability to associate with cell membranes. It acts as an anchor of the downstream encoded protein 3B, the primer for viral RNA synthesis, serves as co-factor for the viral polymerase 3D^{pol}, and stimu-

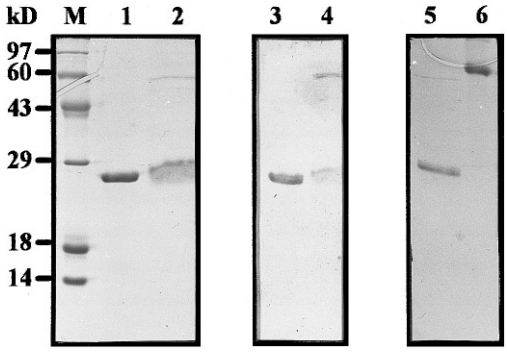

FIG. 9. Analysis of the dimer-forming transmembrane domain of 3A. Proteins GST and GST-3TM were expressed, purified by glutathione affinity chromatography, and separated by 15% SDS-PAGE. Lanes 1 and 2: protein GST (negative control) and GST-3TM, loaded and electrophoresed in the presence of SDS. Lanes 3 and 4: Western blot of the same proteins, performed with anti-GST antibody. Lanes 5 and 6: proteins GST and GST-3TM loaded in sample buffer containing 50 mM DTT, and electrophoresed in the absence of SDS in the Laemmli buffer. Gels were stained with Coomassie brilliant blue.

lates primer- and template-dependent poly(U) and RNA synthesis (5). Furthermore, 3AB aids in the RNA-interaction of $3CD^{pro}$ with the 5′ and 3′ terminal sequences of poliovirus RNA (6).

Protein 3AB of HAV is a membrane-interacting protein, containing a stretch of 21 consecutive uncharged hydrophobic residues (8). The results of the present study show that it behaves as an integral membrane protein, as it is dislodged only by treatments that destroy the native membrane structure, and that the hydrophobic domain is the transmembrane (TM) segment. TM segments exist characteristically as α-helices in the lipid bilayer, yet computer analysis of the hydrophobic domain of HAV 3A suggests that it cannot form an α-helix due to the high content of helix-breaker (Gly) and β-sheet promoter (Ile, Val) residues. However, it has been demonstrated that peptide helicity is greatly influenced by the molecular environment, and that the hydrophobic interactions between peptide side-chains and lipids appear to be the primary forces determining the conformation of a transmembrane segments (27). These results, showing that the helical propensity of a given amino acid stretch cannot be defined *a priori*, give support to our conclusions.

Expression of mutants bearing deletions of the first, second or third part of the hydrophobic region, indicated that the most C-terminal 7 residues give probably the main contribution to membrane association of 3AB, through their interaction with the lipid bilayer. Deletion of the first and second group of residues did not seem to affect membrane binding of the protein, as the corresponding mutants retain the ability to interact with membranes and are as susceptible to biochemical treatments as is the wild-type protein. The third part of the hydrophobic region (aa 54 to 60) differs from the rest of the domain in its content of a small cluster of aromatic residues. Aromatic amino acids are also present in domain II (aa 73 to 80) of poliovirus 3A, that has recently been demonstrated to be crucial for membrane association of 3AB (28). Many surveys indicate that aromatic amino acids have a special propensity to localise at the interface region of the lipid bilayer (29). It has also been suggested that the amphipathic residues Try and Trp, with their polar groups facing the aqueous face and their non-polar regions dipped into the lipid bilayer, may serve to position and to stabilise the helix with respect to the membrane environment (30). As a consequence, we speculate that the third part of the hydrophobic domain of 3A of HAV may be fundamental for a stable interaction of the protein with membranes.

The interaction with canine microsomal membranes showed that 3AB is able to associate with membranes post-translationally. As in all picornaviruses, the genomic RNA of HAV contains a single open reading frame encoding a long polyprotein that is post-translationally cleaved to yield the mature proteins (31). Therefore, the observed post-translational interaction of HAV 3AB with the membranes fits with this model and seems to be essential for the function of 3AB and its precursors.[3]

Computer analysis of the predicted secondary structure of protein 3AB suggests that a α-helix could be formed near the C-terminus of 3A (amino acids 61 to 69), a region including a high percentage of charged residues. A mutant protein lacking this domain failed to interact with membranes, suggesting that those residues might have a role in membrane association of 3A. In fact, it has been reported that charged domains, including both positive and negative residues, can be crucial for membrane anchorage (11, 12). In 3AB, the charged C-terminal region could contribute, together with amino acids 54 to 60 of the hydrophobic domain, to the stabilisation of the membrane interaction of 3AB. The stable interaction of 3AB with membranes is therefore fundamental for its role in HAV RNA replication.

Another interesting result we observed upon expression of HAV 3AB in the presence of membranes, both *in vivo* and *in vitro*, is the finding of dimeric forms of the protein. These dimers were shown to be SDS-resistant when samples were boiled in Laemmli buffer before SDS-polyacrylamide electrophoresis. This behaviour is not surprising as it has been already observed that, whereas most proteins completely lose their native structure in 2% SDS, some intrinsic membrane proteins retain a significant level of secondary structure in the presence of the detergent (32). In this study we demonstrate that the hydrophobic domain is responsible for dimerization, also in absence of other

[3] V. Gauss-Müller, personal communication.

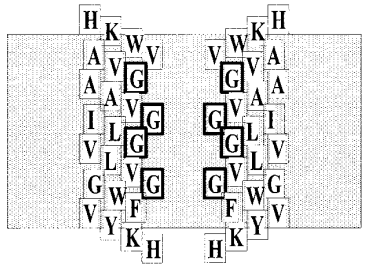

FIG. 10. Two-dimensional representation of 3A transmembrane domain. Transmembrane domain is represented in α-helical configuration. Facing Gly residues are in bold. The membrane is represented as a dotted surface.

regions of the protein and that the complete domain is needed for this feature. This observation has been corroborated by chemical cross-linking of the purified proteins 3ABwt and 3ABΔid. Transmembrane α-helices can associate with one another in the membrane by means of side-by-side interactions, and it has been demonstrated that such association is important in protein folding and oligomerization (26). Conserved glycine residues have been observed in transmembrane helices formed by different proteins, and it has been suggested that their distinct pattern would permit the close approach of the helices (33). The hydrophobic domain of 3AB contains five Gly residues, four of which could be implicated in α-helical pairing, and therefore be crucial in dimerization of the protein (Fig. 10). The results obtained upon expression of the mutants bearing deletions of different portions of the hydrophobic domain suggest that all four glycine residues on the same face of the helix is necessary for oligomerization of 3AB.

In conclusion, the data presented in this paper, showing that protein 3AB can associate post-translationally with membranes as an integral protein, are in agreement with the hypothesis that 3A acts as a stable anchor of 3B to the membranes of the vesicles during HAV replication cycle. Furthermore, it could be hypothesised that the ability of protein 3AB to dimerize into the membranes is required for some function(s) in the viral replicative cycle.

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