Poliovirus protein 3AB may serve as the lipophilic carrier of a protein primer (VPg or 3B) used for the initiation of genomic viral RNA synthesis. In order to study the membrane-protein interactions of 3AB required for its role in poliovirus RNA replication, we have developed an in vitro membrane association assay capable of distinguishing membrane-bound from non-membrane-bound proteins that are cotranslated together in the presence of canine microsomal membranes. This assay utilizes equilibrium sedimentation analysis in high density sucrose gradients to measure membrane association of both wild type and mutated forms of 3AB. Using this assay and other biochemical assays, we have identified the following properties of the 3AB-membrane interaction: (a) 3AB is able to post-translationally associate with microsomal membranes, (b) 3AB is able to associate with membranes in a manner consistent with that of an integral membrane protein, (c) 3AB contains a critical hydrophobic sequence within the carboxyl-terminal half of the protein that is required for membrane association, and (d) the introduction of charged residues into this hydrophobic sequence disrupts the 3AB membrane-protein interaction. Taken together, these studies indicate that poliovirus protein 3AB associates tightly with biological membranes de novo in a manner that would allow it to serve as a lipophilic anchor for the assembly of the poliovirus RNA replication complex.

Cellular membranes are complex structures composed of lipid and protein that together compartmentalize the intracellular space and serve as the physical matrix upon which numerous biosynthetic events are performed. Intracellular membranes are also utilized by some positive strand RNA viruses to organize and facilitate their viral RNA replication processes. This has been shown for members of the picornavirus family, such as poliovirus (1), as well as members of the alphavirus family (2) and potyvirus family (3). Poliovirus RNA synthesis takes place in tight replication complexes that are associated with virus-induced smooth membrane vesicles that bud from the rough endoplasmic reticulum (1, 4–8). Membrane association of both wild type and mutated forms of 3AB were initially generated by cassette mutagenesis as described by Giachetti et al. (21, 22), and cloned into a poliovirus subgenomic cDNA termed pKO3 (PV nucleotides 4154–7053 with nucleotides 6056–6516 deleted). Using the polymerase chain reaction (PCR) and the PCR primers JT3A5106 (5'-TTCAAGGACCACTCCAGTATAAAG-3') as the lipophilic carrier of VPg (3B) to the viral RNA replication machinery. The primary candidate region for the 3AB membrane association determinant is a highly conserved 22-amino acid hydrophobic domain present in the COOH-terminal half of 3A (Fig. 1) (9, 12). The presence of this hydrophobic domain corresponds with the ability to isolate the COOH-terminal amino acids of 3A, along with 3B, as part of a larger protease-protected fragment from extracts of poliovirus-infected cells (13).

Membrane-protein interactions can be typically separated into two categories, peripheral and integral (14, 15). Peripheral membrane proteins generally do not interact significantly with the hydrophobic interior of the lipid bilayer, while integral membrane proteins do interact, either through a transmembrane domain or a hairpin loop that does not pass through the bilayer (16–18). Peripheral membrane proteins associate with membranes through electrostatic interactions, typically between positively charged amino acids and negatively charged phospholipid headgroups (16). These interactions can be further strengthened by the presence of covalently bound fatty acids or phospholipids such as myristic acid or glycosylphosphatidylinositol, which provide additional hydrophobic interactions (16).

The goal of this study is to biochemically define the 3AB-membrane interaction. Previous experiments that examined the membrane association of poliovirus replication complexes indicated that the viral protein 3AB was tightly associated with smooth membranes of infected cells in a manner resistant to treatment with 0.5 M salt or 4.0 M urea (19). However, these studies were not able to demonstrate that this tight membrane interaction was the sole result of molecular determinants contained within the 3AB protein. An in-depth biochemical analysis of the determinants responsible for the 3AB membrane interaction in the absence of the viral replication complex has not been performed, nor has the inherent strength of the 3AB-membrane interaction been examined. In this study, we address the following questions. (a) Can 3AB associate with membranes when expressed in the absence of functional poliovirus replication complexes? (b) Can 3AB associate with membranes post-translationally? (c) What are the minimum molecular determinants for 3AB membrane association? (d) Is 3AB-membrane association consistent with that of a peripheral or integral membrane-protein interaction?

MATERIALS AND METHODS
Plasmids and Cloning—All amino acid substitutions and deletions were ultimately cloned into pTM1 (20) for generation of RNA transcripts to be used for in vitro translations. Amino acid substitutions in 3AB were initially generated by cassette mutagenesis as described by Giachetti et al. (21, 22), and cloned into a poliovirus subgenomic cDNA termed pKO3 (PV nucleotides 4154–7053 with nucleotides 6056–6516 deleted). Using the polymerase chain reaction (PCR) and the PCR primers JT3A5106 (5'-TTCAAGGACCACTCCAGTATAAAG-3') and
The codon for 3AB was amplified such that an in-frame stop codon was placed after the ultimate glutamine residue in 3B (VPg). The amplified 3AB sequence was then digested with AsuII and blunt-ended using the Klenow fragment of *Escherichia coli* DNA polymerase. The 3AB DNA-containing fragments were then digested with BamHI, gel-purified, and ligated into purified pTM1 (digested with EcoRI and BamHI) along with the annealed oligonucleotides encoding the FLAG epitope in-frame and 5′-proximal to the entire 3AB coding sequence (see Fig. 2, top). When the wild type 3AB sequence was cloned into pTM1 as described above, the resulting plasmid was called pTM1FG3AB-Dwt. Deletions of domains I and II within 3AB were initiated by digesting pTM1FG3AB with either BglII or HindIII. The ends of the digested DNA were then repaired using T4 DNA polymerase, followed by digestion with *Eag*I (EagI cuts once in the pTM1 vector sequence). The *Eag*I to BglII and HindIII to *Eag*I fragments were isolated and ligated into a purified *Pst*I to *Pst*I fragment of pTM1FG3AB using T4 DNA ligase. This procedure was used to generate pTM1FG3AB-DIA and pTM1FG3AB-DII, respectively. The plasmid pTM1FG3AB-DI-A was generated by digesting pKO3 with BglII (blunt-ended by incubation with the Klenow fragment of *E. coli* DNA polymerase) and BamHI, and the resulting fragment was ligated into purified BamHI- and HindIII-cut pKO3 DNA (the HindIII site was blunt-ended). The resulting pKO3 was used as the DNA template for the PCR amplification and subsequent cloning of pTM1FG3AB-DIA into pTM1 (along with the FLAG epitope sequence) as described above, resulting in the construct pTM1FG3AB-DIA.

The plasmid encoding rabbit cytochrome *b* was generously provided by Dr. Alan Steggles. The cytochrome *b* cDNA was first digested with EcoRI and HindIII to release the entire cytochrome *b* coding region including the UGA termination codon. This DNA fragment was then digested with *Hind*III and blunt-ended. The cytochrome *b* cDNA fragment was ligated, along with oligonucleotides encoding the FLAG epitope described above, into gel-purified pTM1 vector sequence cut with EcoRI and *Stu*I. This strategy removes the nucleotides encoding the first two amino acids of cytochrome *b* and places the sequences encoding the FLAG epitope in frame and 5′ proximal to the sequences encoding human β-globin. The resulting plasmid was designated pTM1FGβ-GL.

In Vitro Transcription—Before transcription, all plasmids were linearized (using *Stu*I for pTM1FG3AB and HindIII for pTM1FGβ-GL and gel-purified. RNA was synthesized as described by Charini et al., (23) with the following exceptions: (a) the reaction mixtures were in 100-μl reactions, (b) the final concentration of all four NTPs was 0.5 mM, (c) the amount of transcription template was 1–2 μg of appropriate transcription template, (d) the incubations were carried out at 37 °C for 1.5–2.0 h, and (e) the RNA transcripts were not subsequently treated with DNase I. Following transcription, the RNAs were extracted twice with phenol/chloroform and ethanol-p precipitated two times using ammonium acetate followed by one time using sodium acetate. The RNAs were then resuspended in diethyl pyrocarbonate-treated water. Finally, the RNAs were quantitated by either ethidium staining next to known quantities of similar sized RNAs or by trace labeling using 12.5 μCi of [α-32P]UTP/100-μl transcription.

Immuno precipitation—Immuno precipitations were carried out by adjusting each sample to 25 mM Tris (pH 7.4), 0.865 mM NaCl, 5% glyceral, 0.096% SDS, 0.85% Triton X-100, 30% β-mercaptoethanol, and 85 units/ml aprotinin (950 μl total volume) followed by the addition of 10 μg of anti-FLAG M2 monoclonal antibody (Kodak-IBI). After incubating each sample at 4 °C (>1 h), antibody-protein complexes were collected by either Protein A- or Protein G-agarose, washed once with lysis buffer (24) (25 mM Tris (pH 7.4), 300 mM NaCl, 1 mM CaCl2, 1% Triton X-100) and diluted in 40 μl of Laemmli gel sample buffer (LSB) (25). Each sample was then boiled, vortexed, and subjected to SDS-polyacrylamide gel electrophoresis.

Gradient Membrane Association Assay—For each form of 3AB to be analyzed in the presence and absence of canine microsomal membranes, a 200-μl in vitro translation was set up on ice to contain the following: 0.8 volume (180 μl) of rabbit reticulocyte lysate (Promega; precentrifuged at 50,000 × g for 30 min to remove any protein aggregates), 0.075 volume (15 μl) of potassium buffer that yields 15 mM KCl and 5 mM KSCN at final concentration, 0.05 volume (10 μl) of [35S]methionine (Amersham, >1000 Ci/mmol), 0.025 volume (5 μl) of amino acids minus methionine (Promega), 0.05–0.1 volume of RNA (10–20 μl) (1.5–2 μg of mRNA (–9–4 pmol) for cytochrome *b*, –3–4 μg (–6–8 pmol) of mRNA for β-globin, and –8.0 μg (–12 pmol) of mRNA for each form of 3AB). The translation mixture was then divided in half (100 μl each), and to one half 0.25 eq/ml canine microsomal membranes (Promega) were added. An equivalent volume of membrane diluent (buffer B; Ref. 26) was added to the other half. The translations were allowed to proceed for 30 min at 30 °C, followed by the addition of cycloheximide to a final concentration of 300 μl to stop all protein synthesis. Each sample was then subjected to equilibrium centrifugation in high density sucrose gradients essentially as described by Caligiuri and Tamm (1), with the volumes proportionately scaled down to be used in a 2.7-ml (total volume) gradient. Briefly, the in vitro translations were diluted to 0.27 volume (600 μl) with 36% w/v sucrose that was 1.2 × reticulocyte standard buffer (RSB; 1 × RSB yields 10 ml Tris (pH 7.4), 10 mM KCl,
Membrane Association of Poliovirus Protein 3AB

In Vitro 3AB Membrane Association Assay—In order to analyze the molecular determinants for membrane association, poliovirus protein 3AB was generated by in vitro translation in the absence of other poliovirus proteins. The mRNAs encoding 3AB and the model proteins (discussed below) contained an internal ribosome entry site from encephalomyocarditis virus genomic RNA and coded for an epitope tag (DYKDDDDK; termed FLAG) at the amino terminus of each protein (Fig. 2). The membrane association properties of the wild type and mutated forms of poliovirus protein 3AB were measured by sucrose density gradient analysis as described under “Materials and Methods” and outlined in Fig. 2. Membrane-protein interactions were detected as the differential association of 3AB with the rough endoplasmic reticulum (RER). The rationale for using gradient analysis was to separate aggregated protein from that which is truly associated with the RER. The conditions for centrifugation were similar to those described by Caligiuri and Tamm (1), in which the authors showed by phospholipid analysis and [3H]uridine labeling that the rough endoplasmic reticulum reaches equilibrium in the 45%–60% sucrose interface (density >1.2 g/ml) following a 16–18-h spin at 86,000 × g. A direct interaction of a protein with the RER would be indicated by protein fractionation into the high density sucrose portion of the gradient following centrifugation when the translation was carried out in the presence of microsomal membranes.

To validate the assay, our analyses included two “model” proteins with known membrane association characteristics. As a positive control, rabbit cytochrome b5 was chosen for its characteristics as an integral membrane protein that spontaneously associates with microsomal membranes via a monotopic anchor sequence (18, 27, 28). For the negative control, the non-membrane-associated protein human β-globin was used. Unless otherwise indicated, the model proteins were cotranslated along with wild type and mutated forms of 3AB. Each gradient fraction was subjected to immunoprecipitation analysis as described under “Materials and Methods” using a monoclonal antibody directed toward the FLAG epitope present at the amino terminus of each in vitro translated protein.

The results of a typical sucrose density gradient analysis are described below.

RESULTS

In Vitro Translation (+) or (-) Microsomal Membranes

Overlay Each Over Sucrose step Gradient

and Centrifuge at 86,000 x g for 16-18 hrs.

EMCV IRES

FLAG

3AB or Model Protein

In Vitro Translation

Analyze Samples by SDS-PAGE

Membrane Associated Proteins

Harvest Gradient

Immunoprecipitate Fractions with anti-FLAG mAb

Fig. 2. Protocol outlining the membrane association assay using high density sucrose gradient centrifugation. The line diagram at the top represents the mRNAs used for in vitro translations followed by a brief flow chart of the gradient membrane association assay. The box adjacent to the FLAG sequence corresponds to the coding sequence for 3AB (and its mutated derivatives), cytochrome b5, or β-globin. Following centrifugation, the gradient was harvested from the bottom as described under “Materials and Methods” and each fraction was subjected to immunoprecipitation using the anti-FLAG monoclonal antibody. The immunoprecipitations were then analyzed on 15% polyacrylamide-SDS gels, and the gels were fluorographed and visualized by autoradiography.

lanes 8–16), with minimal radiolabeled protein found toward the bottom. However, when the wild type form of 3AB, cytochrome b5, and β-globin were translated in the presence of canine microsomal membranes, cytochrome b5 and 3AB were found in the bottom half of the gradient (Fig. 3, middle panel, lanes 3–7). In contrast, the model non-membrane protein, β-globin, localized within the gradient in a manner identical to that when it was translated in the absence of membranes (lanes 8–16). This result indicated that this assay allowed the discrimination between membrane and non-membrane proteins when translated together in vitro, and that poliovirus protein 3AB behaved like the model integral membrane protein cytochrome b5 and associated with the RER. Additionally, this analysis demonstrated biochemically that 3AB is able to associate with microsomal membranes in the absence of other poliovirus proteins.

One additional question that was addressed using this assay was whether the wild type form of 3AB could associate with microsomal membranes post-translationally. All three proteins, 3AB, cytochrome b5, and β-globin, were first translated in the absence of microsomal membranes, and, following the addition of cycloheximide to inhibit further translation, equivalent amounts of microsomal membranes were added to the in vitro translation mixture. As shown in Fig. 3 (bottom panel, lanes 4–7), both the cytochrome b5 and poliovirus protein 3AB were found in the high density sucrose fractions, indi-
Deletions and substitutions within the hydrophobic domain of 3AB that were tested for their effects on membrane association are shown in Fig. 4. Based on restriction sites engineered into the poliovirus cDNA (21, 22) that lie within the nucleotide sequence encoding amino acids 59–81, the hydrophobic domain of 3AB has been divided into two subdomains, domains I and II, corresponding to amino acids 64–72 and 73–80, respectively. Removal of domain I (DIA) removes most of the amino acids predicted to form the amphipathic helix, while deletion of domains I and II (D(I+II)Δ) effectively removes the entire hydrophobic domain except for the first five amino acids.

Characterization of the 3AB Domain I Deletion—We analyzed the deletion of domain I in a mutated form of 3AB (DIA), which has a faster electrophoretic mobility than that of wild type 3AB, in the gradient membrane association assay. As shown in Fig. 5, both 3AB-DIA and 3AB-wt behave similarly to cytochrome b5 in that each is found in nearly identical proportions in the high density sucrose fractions when the translations were carried out in the presence of microsomal membranes (lanes 3–8). This result indicated that the 3AB-DIA deletion does not result in a decrease in membrane association, consistent with the previous observations that this region of the hydrophobic domain could tolerate the addition of multiple charged residues without diminishing 3AB membrane association. We cannot, however, rule out the possibility that the mutations in domain I result in reductions of 3AB membrane association that are not detectable by this assay.

One possibility that could explain the presence of 3AB-DIA in the high density sucrose fractions when translated in the presence of membranes (Fig. 5) was that the mutated form of 3AB was able to assemble heteromultimers with the wild type form of 3AB causing 3AB-DIA to appear to be membrane-associated. To test this possibility, 3AB-DIA was translated both in the presence and absence of microsomal membranes and in the presence and absence of 3AB-wt. The results of this analysis are shown in Fig. 6. In this experiment, the in vitro translation reactions were sedimented through a sucrose cushion, and the pellet (membrane fraction) and supernatant (non-membrane fraction) fractions were analyzed. This type of analysis, while less definitive than the gradient assays described above, is much faster for analyzing membrane association under multiple conditions. The presence of 3AB-DIA in lane 4, in which membranes but not 3AB-wt were present, indicated that 3AB-DIA was able to associate with microsomal membranes independently of 3AB-wt. Furthermore, 3AB-DIA did not demonstrate an increase in membrane association when translated in the presence of 3AB-wt (compare lanes 4 and 6). These results strongly suggest that domain I is not required for membrane association in this assay and that 3AB membrane association does not occur via a predicted amphipathic helix within aa 59–73 of the viral protein.

Characterization of Deletions of Domains I and II—Membrane association of 3AB is dramatically different when both domains I and II of the hydrophobic domain are removed. The results of this analysis are shown in Fig. 7. The top panel demonstrates that none of the in vitro translated proteins were found in the bottom half of the gradient in significant amounts when translated in the absence of microsomal membranes, as expected. However, when 3AB-wt and 3AB-D(I+II)Δ were translated along with the model proteins in the presence of membranes, only 3AB-wt and cytochrome b5 were found in the lower half of the gradient (lanes 3–7). 3AB-D(I+II)Δ behaved in a fashion nearly identical to that of the non-membrane protein β-globin, indicating that removal of nearly all of the hydrophobic regions of the hydrophobic domain required for membrane association. Deletions and substitutions within the hydrophobic domain of 3AB that were tested for their effects on membrane association are shown in Fig. 4. Based on restriction sites engineered into the poliovirus cDNA (21, 22) that lie within the nucleotide sequence encoding amino acids 59–81, the hydrophobic domain of 3AB has been divided into two subdomains, domains I and II, corresponding to amino acids 64–72 and 73–80, respectively. Removal of domain I (DIA) removes most of the amino acids predicted to form the amphipathic helix, while deletion of domains I and II (D(I+II)Δ) effectively removes the entire hydrophobic domain except for the first five amino acids.

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bic domain abolished 3AB membrane association. This result is consistent with the results of Datta et al. (29), in which they removed the first 15 amino acids of the hydrophobic domain and saw a partial decrease in membrane association as well as a more diffuse immunofluorescence pattern in transfected cells. Those studies, however, could not discriminate between membrane-associated protein and particulate matter.

**Role of Domain II in Membrane Association**—Based upon the inability of 3AB-D(1+II)Δ to associate with microsomal membranes while 3AB-DIΔ could, we concluded that the amino acid residues most critical for membrane association were likely to be those in domain II. In order to test this hypothesis, the amino acids in this region were either deleted (3AB-DIIΔ) or substituted with charged residues (3AB-DII-3E). When domain II was deleted from *in vitro* translated 3AB, the mutated poliovirus protein showed a decreased ability to associate with microsomal membranes (Fig. 8). In this experiment, little to no 3AB (wild type or mutant) was seen in the bottom portion of the sucrose gradient when translated in the absence of membranes (*top panel, lanes 1–8*). Furthermore, when these proteins were translated in the presence of microsomal membranes, only 3AB-wt and cytochrome *b₅* could associate with membranes, as indicated by their presence in the bottom half of the gradient (*bottom panel, lanes 3–9*). The amount of 3AB-DIIΔ found in the lower fractions of the gradient following translation in the presence of membranes was similar to that seen when translated in the absence of membranes, indicating that the presence of the hydrophobic amino acids in domain II is crucial for 3AB membrane association.

A logical prediction that stems from the diminished ability of 3AB-DIIΔ to associate with microsomal membranes is that a major determinant for 3AB membrane association is through hydrophobic interactions between the lipid bilayer and the hydrophobic residues present in domain II. Therefore, substitution of charged residues within this region for the highly conserved and most hydrophobic residues (valines 75, 76, and 78) should render this mutant form of 3AB (3AB-DII-3E) deficient in membrane association. When 3AB-DII-3E was examined in the membrane association assay (Fig. 9), membrane association was severely decreased when compared to that of 3AB-wt (*bottom panel*). As was the case for 3AB-DIIΔ (Fig. 8), 3AB-DII-3E behaved essentially like the non-membrane control protein while the wild type form of 3AB behaved like the model integral membrane protein cytochrome *b₅*. These results suggest that maintenance of hydrophobicity within domain II of 3AB is necessary for 3AB to associate *in vitro* with microsomal membranes.

**Strength of 3AB-wt Membrane Association**—Previous stud-
Membrane Association of Poliovirus Protein 3AB

In this study, we have presented evidence that poliovirus protein 3AB behaves in a manner consistent with that of an integral membrane protein. Furthermore, deletion and substitution analysis of in vitro translated 3AB indicates that a crucial domain within 3AB required for membrane association lies within amino acids 73–80 and that introduction of charge into this domain abrogates membrane association. While we have not demonstrated this directly, we speculate that domain II interacts substantially and directly with the hydrophobic core of the lipid bilayer and is able to do so post-translationally. The lines of evidence which support this interpretation of the data are as follows: (a) within the 22-amino acid hydrophobic domain of 3AB, the most hydrophobic (and most “hydrophobically conserved”) region corresponds to that of domain II (aa 73–80), (b) the introduction of negative charge into domain II abrogates membrane association, while the introduction of multiple charged residues into domain I, or its removal entirely, has no measurable effect on 3AB membrane association, and (c) the wild type form of 3AB is extracted from microsomal membranes only when biochemical treatments aimed at disrupting hydrophobic interactions (i.e. nonionic detergent) are used; treatments with 4 M urea, high pH, or high salt have only minimal effects. We recognize that the microsomal membranes used in these experiments contain significant amounts of cellular integral membrane proteins. It is therefore possible that a mechanism for 3AB membrane association is to tightly interact with unidentified cellular integral membrane protein(s) (16). Studies aimed at identifying possible cellular and/or viral protein binding partners of 3AB are ongoing.

One interesting question that remains to be answered is how can such a small hydrophobic domain facilitate such a tight interaction with the microsomal membranes. Removal of domain I leaves only 13 amino acids of the hydrophobic region, which, by itself, is unlikely to contain enough hydrophobic character to anchor 3AB into the membrane in a manner consistent with that of an integral membrane protein. At least 20 amino acids are required to span a lipid bilayer in an a-helical structure (34), more amino acids than remain in the hydrophobic domain of 3AB-DI. Recently a characterization of synaptobrevin, a member of a class of proteins that utilize COOH-terminal membrane anchors, revealed that a minimum of 12 consecutive hydrophobic residues were required for post-translational membrane insertion in a manner resistant to pH 11.5 (35, 36). This mechanism of synaptobrevin insertion into membranes, while post-translational, is both ATP- and protein-dependent and results in a complete spanning of the membrane by the COOH-terminal anchor. Given that 1) 3AB requires a carboxyl-terminal hydrophobic sequence for membrane association, 2) 3AB can associate with microsomal membranes post-translationally, and 3) 3AB behaves biochemically like an integral membrane protein, it is conceivable that 3AB uses a

Tractus, indicating that these agents had slight effects on the membrane association of these proteins. Similarly, treatment with high salt up to 1.5 M NaCl also had little or no effect on 3AB-wt and cytochrome b5 membrane association (lanes 10–13). Taken together, these results suggest that electrostatic interactions are not the primary 3AB membrane association determinant. Our data do not rule out stabilizing or orientation functions for the charged residues that flank the hydrophobic domain. In contrast, treatment with a nonionic detergent that should disrupt hydrophobic interactions abolished both 3AB-wt and cytochrome b5 membrane association (lanes 8 and 9). This latter result is consistent with a model in which the hydrophobic residues in domain II of 3AB are the crucial residues necessary to allow 3AB to interact with the lipid environment.

**DISCUSSION**

In this study, we have presented evidence that poliovirus protein 3AB behaves in a manner consistent with that of an integral membrane protein. Furthermore, deletion and substitution analysis of in vitro translated 3AB indicates that a crucial domain within 3AB required for membrane association lies within amino acids 73–80 and that introduction of charge into this domain abrogates membrane association. While we have not demonstrated this directly, we speculate that domain II interacts substantially and directly with the hydrophobic core of the lipid bilayer and is able to do so post-translationally. The lines of evidence which support this interpretation of the data are as follows: (a) within the 22-amino acid hydrophobic domain of 3AB, the most hydrophobic (and most “hydrophobically conserved”) region corresponds to that of domain II (aa 73–80), (b) the introduction of negative charge into domain II abrogates membrane association, while the introduction of multiple charged residues into domain I, or its removal entirely, has no measurable effect on 3AB membrane association, and (c) the wild type form of 3AB is extracted from microsomal membranes only when biochemical treatments aimed at disrupting hydrophobic interactions (i.e. nonionic detergent) are used; treatments with 4 M urea, high pH, or high salt have only minimal effects. We recognize that the microsomal membranes used in these experiments contain significant amounts of cellular integral membrane proteins. It is therefore possible that a mechanism for 3AB membrane association is to tightly interact with unidentified cellular integral membrane protein(s) (16). Studies aimed at identifying possible cellular and/or viral protein binding partners of 3AB are ongoing.

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**FIG. 6.** Effect of the wild type form of 3AB on the membrane association of 3AB-DI. Three 25-µl in vitro translation reactions carried out using mRNAs coding for the mutated form of 3AB (3AB-DIΔ), cytochrome b5, and b-globin. In one reaction, an additional mRNA coding for the wild type form of 3AB was included (lanes 6). Following translation, each reaction was centrifuged at 50,000 x g through a 20-µl 15% w/v sucrose cushion (sucrose was 1 x RSB) for 30 min. The corresponding pellet and supernatant fractions of each sample were harvested and immunoprecipitated as described under “Materials and Methods.” The samples were resolved by electrophoresis on 15% polyacrylamide-SDS gels.

1. 2. 3. 4. 5. 6. 7.

- Cyt. b5
- b-globin
- 3AB wt
- 3AB DIΔ

26 kD
12 kD

- Membranes
- +
- M P S P P S

This result was consistent with that seen in Fig. 5 and likely reflects a limiting amount of microsomal membranes added to each in vitro translation. When parallel reactions were treated with either 4.0 M urea or adjusted to pH 11.0 (lanes 4 and 5 and lanes 6 and 7, respectively), cytochrome b5 and 3AB-wt were only partially ex-
similar post-translational mechanism to insert into membranes. However, a complete spanning of the membrane by the hydrophobic domain of 3AB is not predicted. If 3AB contains a transmembrane helix, this would predict the existence of two membrane-spanning domains since the NH₂ and COOH termini of 3AB should be on the same side of the membrane to be recognized by the viral proteinase and mediate potential RNA binding functions. Protein structures that would require fewer amino acids to span the lipid bilayer consist of β-sheets and random coils but, unless in the form of a β-barrel, these are energetically unfavorable in a lipid environment (34). Therefore, we speculate that the positively charged residues flanking the hydrophobic domain (Arg-54, Arg-58, and Lys-81 denoted by asterisks (*) in Fig. 1) contribute to membrane binding via strong electrostatic interactions with the negatively charged phospholipid head groups. The role of electrostatic interactions is consistent with the partial membrane extraction by the 4 M urea and the high pH treatments shown in Fig. 10.

It is probable that domain I contributes to membrane association of the wild type form of 3AB, but its deletion is a tolerable one under the conditions tested. In the studies with 3AB DIA, membrane binding only under physiological conditions was examined. This deleted form of 3AB may be more susceptible to biochemical treatments such as high pH or high ionic strength, results that would suggest the existence of additional contributions to membrane binding by flanking electrostatic interactions. In addition, it is possible that determinants for membrane association lie outside of the hydrophobic domain. Theoretical predictions of membrane interactive domains have been previously contradicted by experimental data (37). Our current structural model for how 3AB associates with biological membranes is that the hydrophobic domain forms an α-helical insertion sequence minimally consisting of the amino acids present in domain II (and the first five residues at the
beginning of the hydrophobic domain), and this sequence further utilizes the flanking arginine and lysine residues as stabilizing forces.

Our studies are consistent with the hypothesis that poliovirus protein 3AB associates tightly with biological membranes in a manner that would allow it to serve as a lipophilic anchor for the poliovirus RNA replication complex (9). A potential biological advantage for the use of such a membrane-protein interaction would be to limit diffusion of protein and RNA replication components to two dimensions, resulting in increased local concentrations (22). There are a number of recent reports identifying multiple 3AB interactions with components (both RNA and protein) of the RNA replication machinery (38–42) that would substantiate a role for 3AB as the anchor for the replication complex and a primary component of the RNA replication process. 3AB association with membranes may also cause a conformational change in 3AB structure that is necessary for recognition by the viral encoded proteinase (43) and possibly other functions of 3AB. One intriguing observation with respect to the amino acid composition of the 3AB hydrophobic domain (especially domain II) is the high degree of conservation of \( \beta \)-branched hydrophobic amino acids (V and I). These \( \beta \)-branched amino acids are hydrophobic residues that destabilize \( \alpha \)-helices and promote \( \beta \)-sheet formation in globular proteins. However, when these \( \beta \)-branched amino acids are placed in a membrane environment, they can be readily accommodated into \( \alpha \)-helices, suggesting an environment-dependent modulation of protein conformation (44, 45). Given the multiple functions proposed for 3AB combined with the regulated asymmetric synthesis of viral RNA within the replication reactions, a conformational switch dictated by degrees of 3AB membrane association is indeed an attractive one. We are currently studying the role of membrane association on the multiple functions of 3AB and how this protein may exert its effects within the poliovirus RNA replication complex. Likewise, we are examining determinants of membrane association in addition to those contained within domain II of poliovirus protein 3AB.

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