In Vivo Detection of Hetero-association of Glycophorin-A and Its Mutants within the Membrane*

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Protein recognition within the membrane is a crucial process for numerous biological activities. Detection of such interaction is limited because of difficulties that arise from the hydrophobic environment of the membrane. We detected direct hetero-oligomerization of the glycophorin-A (GPA) transmembrane segments in vivo through inhibition of ToxR transcription activator dimer formation. We investigated the amino acids important for hetero-oligomerization within the membrane, using peptide analogs of the transmembrane domain of glycophorin A. The wild type ([WT]GPA) and alanine mutant ([A]GPA) were able to interfere with and inhibit the proper dimerization of the ToxR-GPA transcription factor. Conversely, a second alanine mutant ([A2]GPA), a glycine mutant ([G]GPA), and a scrambled analog ([SC]GPA) were virtually inactive. Binding studies reveal similar membrane partitions for [WT]GPA, [G]GPA, and [SC]GPA, whereas membrane partition of [A]GPA and [A2]GPA are lower. Spectral analysis of fluorescent-labeled analogs revealed a significant blue shift, indicating membrane insertion. Our results suggest that the GXXXG motif, found in homo-oligomerization, is not sufficient for hetero-oligomerization in a biological membrane, whereas an extended motif, LIXXGXXGXXXT, is sufficient. Interfering with hetero-oligomerization within the membrane can be a useful strategy for characterizing such interactions and possibly modulating membrane protein activity.

Experimental Procedures

Peptide Synthesis and Purification—The peptides were synthesized by a standard solid phase method on PAM-resin as described (24, 25). The peptides were cleaved from the resin by HF treatment and purified by reverse phase-HPLC. Purity (>99%) was confirmed by analytical HPLC. The peptide compositions were determined by Platform LCZ electrospray mass spectrometry.

Fluorescent Labeling of Peptides—The boc protecting group was removed from the N terminus of the peptides by incubation with trifluoroacetic acid, whereas all the other reactive amine groups of the attached peptides were kept protected. The acidity was neutralized with 5% (v/v) N,N-diisopropylethylamine (DIEA 5%). The resin-bound peptides were then treated with 4-chloro-7-nitrobenz-2-oxa-1,3-diazole their transmembrane domains. This includes the viral fusion proteins influenza hemagglutinin and hepatitis E1/E2, and the cellular fusion protein synaptobrevin (3, 15, 16). Other examples of biologically important membrane proteins include the M13 major coat protein (17), phospholamban (18), and glycophorin A (GPA) (1). Uncovering the mechanisms that drive these kind of interactions is critical for understanding how these proteins work.

GPA (1), a well documented example of transmembrane homophilic interactions, was previously shown to create α-helical right-handed dimers with a specific homo-association motif (1, 2, 5, 19–21). A part of this motif, GXXXG, was shown to be crucial for the homo-oligomerization process. Furthermore, protein data base studies as well as transmembrane domain bacterial libraries suggest that this motif plays a general role in membrane protein–protein homo-association (19, 22).

We chose GPA as a model protein to investigate hetero-association of different transmembrane segments. GPA has a known homo-oligomerization motif, which was implicated in hetero-oligomerization in vitro (2, 19, 21, 22). Until now, detection of hetero-association between proteins in the membrane was mostly restricted to in vitro methods. In the case of GPA, SDS-polyacrylamide gel electrophoresis experiments suggested hetero-oligomerization of a wild type transmembrane domain linked to staphylococcal nuclease with mutant peptides through the homo-oligomerization motif (19, 23). We devised a scheme to detect direct interaction between hetero-proteins in a biological membrane. We then applied this method to wild type GPA ([WT]GPA) and peptide analogs to study their hetero-association.

Our results suggest the importance of an extended motif for hetero-association of GPA within the membrane of Escherichia coli. This study emphasizes the significance of using such displacement strategies in the investigation of membrane complexes. In turn, these strategies may prove useful in modulation of membrane protein activity.
flouride (NBD-F) (2 eq) in dry dimethylformamide (DMF), leading to the formation of resin-bound N-NBD peptides, respectively. After 1 h, the resins were washed thoroughly with DMF and then with methylene chloride. The labeled peptides were cleaved from the resin and purified as described previously.

**Preparation of Small Unilamellar Vesicles (SUVs)**—Lipid films were prepared at a ratio of phosphatidylethanolamine (PE) : phosphatidylglycerol (PG) (7:3, w/w) as previously described (26). Lipids were resuspended by vigorous vortexing. SUVs were obtained by sonication of the lipid suspensions for 2 min in a water bath-type sonicator.

**In Vivo Detection of Hetero-association of Proteins within the Membrane**—The ToxR transcription activator can be used successfully to assess weak protein interactions within the E. coli membrane. A GPA transmembrane-encoding DNA cassette was previously inserted between the maltose-binding protein and the ToxR transcription activator (2). Transcription activation is mediated by expressing the construct, ToxR-GPA, in the indicator strain FHK12. After transforming FHK12 cells, 1-ml cultures (8 repeats) were grown in the presence of chloramphenicol and 0.1 μM IPTG. After 4 h, plasmid DNA was isolated and introduced into E. coli/BL21(DE3) [pLysS] cells, 1-ml cultures (8 repeats) were grown in the presence of chloramphenicol and 0.1 μM IPTG. Hetero-association was detected using ToxR-GPA grown in the presence of an exogenous peptide. The fluorescence intensity was measured in Miller units (2). Hetero-association was detected by applying non-linear fitting of our results and found the IC50 of [A]GPA to be 2.18 ± 0.01 μM.

**RESULTS**

A series of five peptides corresponding to the transmembrane domain of GPA were synthesized (Table I) using tert-boc strategies. To overcome solubility problems and to deliver the hydrophobic transmembrane segments into the bacterial membrane, a host sequence of two consecutive lysines was used (29). These peptides were tested for their ability to inhibit the formation of a ToxR-GPA transcription activator complex in the inner membrane of E. coli as described in the model depicted in Fig. 1 (2). Briefly, the ToxR-GPA is a transcription factor that activates a lacZ reporter gene as a consequence of dimerization. The dimerization is solely through the transmembrane segment, namely the GPA transmembrane segment (13 amino acids long) (2). Hetero-association of an exogenous peptide with the ToxR-GPA transcription factor will result in a decrease in the lacZ signal.

**Inhibition of the ToxR Transcription Activator Dimer**—ToxR-GPA transcription levels were monitored in the presence of the different exogenous GPA analogs. The [WT]GPA and alanine mutant ([A]GPA) were able to interfere with the proper dimerization of ToxR-GPA, thereby inhibiting reporter gene transcription (Fig. 2). Conversely, glycine ([G]GPA), scrambled ([SC]GPA), and [A2]GPA analogs were virtually inactive. In the [A]GPA peptide, we used the [A]GPA mutant as a backbone, and introduced alanine and glycine mutations. These mutations eliminated the entire “extended motif” and left only the GXXG motif.

Using a simple equilibrium model (Equations 2 and 3), we performed non-linear fitting of our results and found the IC50 of [WT]GPA and [A]GPA to be 2.18 and 6.68 μM, respectively (Table I). A more complex equilibrium is possible, although it is unlikely because the transcription activator homodimer demands a spatial proximity of the two transcription domains for its activity. For example, inhibition of the dimer through complexes such as heterotrimization with the peptide is probably not sufficient to prevent the cytoplasmic domains from dimerization (Fig. 1).

**Weak Membrane Binding Is Not the Reason for Peptide Inactivity**—To eliminate the possibility that the inactivity of [SC]GPA, [G]GPA and [A]GPA were because of poor membrane binding, we compared peptide affinity to bacterial-mimetic membranes (PE/PG 7:3) (26, 30, 31). We found the inactive peptides ([SC]GPA, [G]GPA) and the active [WT]GPA to have similar membrane partitions; both being higher than the

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**TABLE I**

Peptide designations, sequences, and biophysical properties

| Peptide | Sequence | $K_p$ x 10^-4 M^-1 | $\Delta G$ (kcal/mol) | IC50 μM |
|---------|----------|-------------------|----------------------|--------|
| [WT]GPA | KIKLITFLGVMAGVIGT | 15.0 ± 0.4 | -8.60 | 2.18 ± 0.01 |
| [A]GPA | KIKLITFLGVMAGVIAAT | 3.9 ± 0.3 | -7.91 | 6.68 ± 0.45 |
| [A2]GPA | KIKLITFLGVMAGVIAA | 3.6 ± 2.5 | -7.87 | >95 ± 12.0 |
| [SC]GPA | KIKLITFLGVMAGVIAAT | 19.6 ± 8.0 | -8.79 | >200 |
| [G]GPA | KIKLITFLGVMAGVIGT | 23.0 ± 4.0 | -8.88 | >200 |
membrane partition of the active [A]GPA and inactive [A2]GPA (Fig. 3A). The free energy of binding and partition coefficient of all the peptides is within the range reported for known membrane binding peptides (25, 29, 32, 33) (Table I).

**DISCUSSION**

This study sets a precedent for the detection of direct in vivo-specific hetero-oligimerization. We found that the homo-oligimerization motif (GXXXG) (19) is not enough for hetero-association within bacterial membranes, although an extended motif (LIYXGXXXXGXXXT) (19, 21) is essential for hetero-association. In fact, [A]GPA retains wild type oligimerization capability despite multiple alanine mutations, suggesting that the extended motif alone is enough for hetero-association. Moreover, when comparing the partition coefficients of the [WT]GPA to that of [A]GPA one can see that [A]GPA has about four times weaker binding, and consequently the IC50 is around three times higher (Table I). This indicates that the specificity of [A]GPA is very similar to that of [WT]GPA. In contrast, the [A2]GPA peptide, containing only a GXXXG motif, had very low affinity toward the wild type transmembrane domain. This implies a crucial role for the extended homo-oligimerization motif. Moreover, the [G]GPA peptide, having mutations in the extended homo-oligimerization motif, completely loses its affinity toward the wild type transmembrane domain. This reduction in its membrane binding affinity. A possible explanation for the loss of hetero-assembly is that the glycine mutations interfere with the helical packing. Statistical analysis has previously shown that β-branched amino acids in positions I and I + 4 are favorable for packing two transmembrane domains, especially when next to the GXXXG motif (19, 22). Mutating two such pairs may have lowered the packing energy of the oligomer.

It is worth noting that GXXXG is the most common motif found in oligomeric transmembrane domains (19, 22). By creating a second GXXXG motif in [G]GPA, situated on a different helical face than the original, we allowed further freedom. However, this motif did not contribute to oligimerization. The comparison of the extended motif on the [A]GPA peptide with the GXXXG motif on the [A2]GPA peptide further confirms the importance of the extended motif for hetero-oligimerization.

Polarity is the major characteristic implicated in determining the specificity of membrane interactions (37). Additionally, hydrophobic amino acids and especially β-branched amino acid...
pairs are also characteristic of transmembrane segments. The 
\( \beta \)-branched amino acid pairs were previously suggested to be 
structurally important for helical packing (19, 22). We have 
challenged these characteristics by introducing multiple alanine 
mutations in hydrophobic and \( \beta \)-branched positions, leaving 
the polar amino acids alone. It is logical to assume that 
these drastic structural changes are bound to interfere with the 
helical packing. Still, the specificity of [A]GPA remains similar 
to that of the wild type, suggesting that in this case the polar 
interactions play a larger role than the structure and helical 
packing.

In contrast, [G]GPA lost its ability to hetero-associate 
with the wild type transmembrane domain, despite the 
presence of the polar amino acids, supporting the notion that hy-
drophobic interactions are sufficient for assembly. Other hints 
to this end can be found in the literature where all-hydrophobic 
transmembrane segments were shown to specifically interact 
with each other in a biological membrane (38).

Protein-protein recognition and cross-talk in the membrane 
remains a largely uncharted field. Nevertheless, structural 
data of membrane proteins is scarce (39). The method pre-
sent in this paper can directly detect the interaction between two 
different transmembrane domains provided that one of the 
helices can also homo-oligomerize. Thus, this method can ad-
vance structural and functional research of a broad range of 
such membrane proteins. In the case of the T cell receptor, for 
example, exogenous addition of a peptide corresponding to the 
transmembrane segment is known to modulate the activity of 
the receptor (40). Our method can clarify whether this is due 
to direct displacement of the corresponding helix from the recep-
tor complex. Characterization of the mechanism by which the 
transmembrane domain affects receptor activity is highly im-
portant both in terms of the basic understanding of receptor 
mechanics and from a therapeutic perspective. Our results 
indicate that interfering with hetero-oligomerization within 
the membrane can be a useful strategy to characterize such 
interactions, as well as to modulate membrane protein activity.

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