Glycophorin A Helical Transmembrane Domains Dimerize in Phospholipid Bilayers: A Resonance Energy Transfer Study†

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ABSTRACT: Glycophorin A and its isolated transmembrane region (GpATM) are each known to form sequence-specific dimers in SDS micelles. Whether this behavior accurately reflects behavior in red cell membranes or lipid bilayers, however, has remained unclear. Resonance energy transfer between labeled GpATM peptides has been used to observe dimerization of GpATM in bilayers. Separate populations of GpATM peptides were labeled with 2,6-dansyl chloride as the donor chromophore and dabsyl chloride as the acceptor. Quenching of the 2,6-dansyl chloride by the dabsyl group demonstrated an association of the labeled peptides. The quenching was not affected by increases in the amount of lipid present or by unlabeled heterologous peptides but was decreased by the addition of unlabeled GpATM. GpATM was determined to form dimers by fitting the observed energy transfer for a number of donor to acceptor ratios and fitting to the expected number of donor labeled peptides in an oligomer with an acceptor as a function of oligomer number. The finding that the GpATM peptide forms helical dimers in lipid bilayers supports the idea that GpA is a dimer in the erythrocyte membrane. The resonance energy transfer approach may extend to the study of other oligomeric complexes.

It is increasingly apparent that many, if not most, membrane proteins interact to form oligomers. The detection of such complexes, however, is far from straightforward. In this paper we present a study of the dimerization of the transmembrane helical domain of glycophorin A (GpA)[1] in lipid bilayers using resonance energy transfer. It establishes that the method is useful for this application and that the GpA domain, previously studied in SDS micelles, also forms a dimer in DMPC bilayer membranes. By extension, the observation supports the idea that GpA dimerizes in the erythrocyte membrane.

A number of recent studies have brought to light the importance of noncovalent interactions between transmembrane α-helices in the folding and oligomerization of membrane proteins [see Lemmon and Engelman (1992)]. For example, in the assembly of the T-cell receptor complex, interaction between two of its components, TCRα and CD3δ, is suggested to be mediated by the single transmembrane domain of each of these subunits (Cosson et al., 1991; Manolios et al., 1990). Bacteriorhodopsin can reassemble from three fragments, two of which are single α-helices (Kahn & Engelman, 1992). Transmembrane domain interactions are also proposed to be required for the assembly of the Fcγ receptor of human natural killer cells (Kurosaki & Ravetch, 1989; Lanier et al., 1989). Another possible example of transmembrane helix interaction is the dimerization of p185^{neu*}, where a single Val to Glu substitution in the single transmembrane α-helix of the proto-oncogene may stabilize the interaction (Cao et al., 1992; Sternberg & Gullick, 1990). A single transmembrane α-helix also directs the dimerization of glycophorin A from human erythrocytes (Furthmayr & Marchesi, 1976; Bormann et al., 1989).

Although specific helix–helix interactions do appear to be required for mature protein oligomerization and assembly, in the majority of cases it is not known if the interactions of the transmembrane helices on their own, in isolation from the extramembranous regions, are sufficient to drive associations. An apparent exception is the case of glycophorin A, which forms noncovalent, SDS-resistant dimers, although it has not been shown whether the GpA dimer exists in membranes or is formed upon SDS solubilization. A synthetic peptide, corresponding to the transmembrane helix, is capable of associating with GpA on SDS gels (Bormann et al., 1989). In addition, a chimeric protein containing the transmembrane region of GpA fused to the C terminus of staphylococcal nuclease exhibits all of the dimerization properties of intact GpA in SDS micelles (Lemmon et al., 1992a).

The GpA transmembrane region (GpATM) appears to be a favorable choice for the study of transmembrane helix–helix interactions since the dimer is stable in ionic detergents and requires no extramembranous sequences beyond the end polar regions. Although there is as yet no high-resolution structure of the GpA transmembrane helix dimer, extensive point mutagenesis has identified those residues which are necessary for the dimerization in an SDS micelle (Lemmon et al., 1992b). Analysis of those residues at which conservative substitutions disrupt the dimer leads to the prediction of a tightly packed interface along two parallel helices; interfacial amino acids occur with a periodicity of 3.9 residues per turn of helix. A complementation mutation for a disruptive mutant was found at an adjacent sequence position, which indicates a parallel, as opposed to antiparallel, orientation for the two helices. In addition to the mutagenesis experiments, Treutlein et al. (1992) conducted a series of studies using molecular dynamics to predict and model the interactions. One of the

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[1] Abbreviations: DABS, 4-(dimethylamino)azobenzene-4′-sulfonyl; 2,6-DANS, 2-(dimethylamino)naphthalene-6-sulfonyl; DMPC, L-α-dimyristoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; RET, resonance energy transfer; GpA, glycophorin A; GpATM, transmembrane domain of GpA; PMSF, α-toluenesulfonyl fluoride; SN/GpA, staphylococcal nuclease–glycophorin A transmembrane domain fusion protein; SDS, sodium dodecyl sulfate; TEA, triethylamine; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; β-OG, 1-*O*-octyl β-D-glucopyranoside.

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most stable conformations found has the parallel helices aligned with the interface as a right-handed supercoil, approximately 3.9 residues per turn of helix, in striking agreement with the mutagenesis results. Thus, the GpA transmembrane domain dimer is likely to exist as a right-handed coiled coil of helices that is, in SDS, highly sensitive to small changes of packing at the interface.

Although GpA forms a dimer in SDS micelles, its actual oligomerization state in lipid bilayers has not been established. In this paper we use resonance energy transfer (RET) to show that GpATM self-associates as a dimer in DMPC bilayers.

MATERIALS AND METHODS

Preparation of GpATM. GpATM was prepared from a chimeric protein containing the C-terminal regino of GpA (residues 60–131) fused in-frame to the C terminus of staphylococcal nuclease, from which it was released by treatment with trypsin. Construction of the plasmid encoding the chimeric protein (pT7SN/GpA) is described in Lemmon et al. (1992a). This plasmid directs expression of the chimeric protein under the control of the phage T7 promoter (Studier et al., 1990). Large quantities of material are produced by transfection of the plasmid into *Escherichia coli* strain MGT7, which contains the T7 RNA polymerase gene under control of the *lac* promoter.

Conditions for protein expression are given in Lemmon et al. (1992a). Harvested cells were resuspended in a solution of 50 mM Tris (pH 8.0), 5 mM EDTA, and 20 μg/mL PMSF to a total volume of 40 mL/L of culture. The cells, which contain the plasmid pLYS-S, were lysed by three rounds of freeze–thawing, and DNA was hydrolyzed by the addition of Ca^{2+} to 10 mM to activate the nuclease domain on SN/GpA. The lysate was incubated for 30 min on ice, and a pellet containing SN/GpA was then collected by centrifugation at 35 000 rpm (96000*g*) for 30 min at 4 °C in a Beckman Ti45 rotor. Protein was extracted from the insoluble fraction by resuspension with sonication in 1 M NaCl, 50 mM Tris (pH 7.8), 5 mM EDTA, and 20 μg/mL PMSF. The suspension was again centrifuged at 35 000 rpm for 30 min and the extraction repeated. Extracts were dialyzed (300–600 volumes of the extraction buffer at 7 °C) to remove residual nucleotides and nucleic acids. Coomassie-stained SDS gels of the extracts did not reveal bands from contaminating proteins, and the material was proteolyzed without further purification. Protein concentration in the dialyzed extracts was estimated from absorbances, using $\epsilon(280) = 17\,000\ M^{-1}\ cm^{-1}$ (Flanagan et al., 1993).

GpATM, residues 60–100 (101), was produced by reacting the chimeric protein with trypsin (TPCK treated, Sigma) at 1:20 weight ratio for 24–36 h as described in Lemmon et al. (1992a). The acid-precipitated peptides were washed twice with 50 mM sodium acetate (pH 5.2), resuspended in 0.1% NH_4OH, frozen, and lyophilized.

Preparation of Labeled GpATM. 2-(Dimethylamino)-naphthalene-6-sulfonyl chloride (2,6-DANS Cl) and 4-(dimethylamino)azobenzyl-4′-sulfonyl chloride (DABS Cl) were obtained from Molecular Probes (Eugene, OR). Lyophilized peptides were extracted with approximately 5 mL of 50% $CHCl_3$, 50% methanol, and 1% triethylamine (TEA) per milligram of peptide, and the solution was clarified by centrifugation in a JA-20 rotor for 10 min at 10 000 rpm. If the pellet appeared to be a substantial fraction of the original material, the extraction was repeated with half the original volume of organic solvent. Dyes were added to a 40:1 molar excess over peptide as 20 mM solutions in the same solvent. Reactions proceeded at 7 °C with agitation for 24 h in the dark.

Peptides were separated from the majority of the unreacted dye and other small contaminants by open-column gel filtration chromatography on a Pharmacia LH-20 resin column running in 50% methanol, 50% $CHCl_3$, and 1% TEA. Fractions were assayed for the presence of peptide by SDS gel electrophoresis (Pharmacia Phastgel system). Approximately 10 μg of SN/GpA was added to aliquots of the fractions which were subsequently dried under vacuum and resuspended in SDS gel sample buffer. Fluorescent peptides were visualized directly by removal of the gel from its plastic backing and illumination by a UVP UV transilluminator viewed through a yellow filter. Unlabeled or nonfluorescent peptides were visualized by Coomassie staining, the presence of peptide being noted principally by the formation of heterodimer with SN/GpA (Lemmon et al., 1992a), although large quantities of peptide bound enough stain to be visible. Pooled fractions of peptide were dried under vacuum and stored at –20 °C.

Substantially pure GpATM was obtained by reversed-phase HPLC. Peptides were dried from $CHCl_3$/methanol and resolubilized in 90% formic acid to approximately 1 mg/mL and filtered through a 0.5-μm-pore filter (Millipore Millex-FH_{13}). Peptides were then chromatographed on a Vydac semipreparative C_4 column with a water/acetonitrile/2-propanol gradient as described in Lemmon et al. (1992a). Fractions containing peptide were pooled and dried in a Speed-Vac under vacuum until only ~10% of the original volume was left. An approximately equal volume of 5% β-OG was added, and the material was then lyophilized. Peptides were resuspended in 50 mM sodium phosphate (pH 8.0) sufficient to make the solution 5% in β-OG.

Dye concentrations were quantitated by absorbance spectroscopy, using the following extinction coefficients: 2,6-DANS, $\epsilon(359) = 5700\ M^{-1}\ cm^{-1}$ (Fung & Stryer, 1978); DABS, $\epsilon(472) = 22\,000\ M^{-1}\ cm^{-1}$, which is the average of the values for labeled amino acids for the chromophore in hydrophilic environment (Lin & Chang, 1975). Labeled peptide concentrations were determined by weighing the dried peptides. Unlabeled peptide concentrations were determined by absorption spectroscopy using $\epsilon(280) = 1400$, from the peptide's lone tyrosine residue.

GpATM Reconstitution and Fluorescence Experiments. The labeled and unlabeled peptides were reconstituted into separate phospholipid vesicle populations by detergent dialysis. GpATM was solubilized in 5% β-OG as described above. Synthetic DMPC in the same buffer was added to a 10:1:: DMPC:GpATM weight ratio. Material was then dialyzed extensively against 50 mM sodium phosphate (pH 8.0). Recovered vesicles were frozen in liquid N_2 and stored at –20 °C.

Vesicles containing DABS and 2,6-DANS GpATM were mixed together and frozen in dry ice/ethanol or liquid N_2. Buffer was added to a final volume of 1.5 mL, giving peptide concentrations of 1–4 μg/mL (0.25–1 μM), and the samples were briefly sonicated in a bath sonicator. A sample of 2,6-DANS GpATM on its own was likewise frozen, brought to the same concentration as in the experimental sample, and sonicated. Concentrations were such that peak absorbances were below 0.05. Prior to fluorescence measurements, vesicles were incubated at 37 °C for at least 1 h. Fluorescence spectra were recorded on an SLM 8000C spectrofluorometer equipped with a double diffraction grating excitation monochromator and a single diffraction grating emission monochromator

operating in ratiometric mode (using 3 mg/mL rhodamine B in ethylene glycol as a quantum counter). The photomultiplier tube for the emission beam was cooled to –20 °C, while the photomultiplier tube for the reference cell was at room temperature. Samples were in a 1 cm × 0.5 cm quartz cuvette, oriented with its long axis perpendicular to the emission measurement path, and maintained at 37 °C. Spectra were corrected for instrument response with correction factors supplied by the vendor.

A value of 41 Å for $R_0(^2/_3)$ was calculated from fluorescence emission and absorbance spectra of labeled GpATM in DMPC vesicles at 37 °C. The background scattering from the lipids was subtracted from the absorbance spectrum of DABS GpATM. The DABS absorbance peak wavelength had shifted from that obtained in β-OG, indicating an environment change for the chromophore, and the mean value for $\epsilon(430)$ of 29 000 M^{-1} cm^{-1} given by Lin and Chang (1975) for the chromophore in a hydrophobic environment was used (a range of 37–42 Å is obtained using the SEM for the extinction coefficient). A quantum efficiency of 0.35 was determined for the 2,6-DANS chromophore using 5-(dimethylamino)-1-naphthalenesulfonic acid in 0.1 M $NaHCO_3$ as a standard ($\Phi = 0.36$; Chen 1966). The value for the refractive index was taken as 1.4 (Fairclough & Cantor, 1978).

RESULTS

The transmembrane domain of GpA was prepared by reacting SN/GpA131 with trypsin, which has previously been shown to produce approximately equimolar amounts of peptides containing GpA residues 62–100 (GpATM100) and 62–101 (GpATM101) (Lemmon et al., 1992a). Labeling of the peptides with chromophores gave a stoichiometry for the pools of all forms of GpATM of 1.8 mol of DABS and 1.3 mol of 2,6-DANS per mole of GpATM. The sequence of the peptides is shown in Figure 1A. GpATM100 has two possible labeling sites (the amino terminus and Lys-100), while GpATM101 has three (the amino terminus and Lys-100 and -101). The distribution of dyes among the possible sites was not determined. An important control showed that the labeling of the peptides did not interfere with the ability of GpATM to disrupt SN/GpA dimers on SDS gels. Figure 1B shows an SDS gel of SN/GpA on its own and with unlabeled, DABS-labeled and 2,6-DANS-labeled GpATM. Comparable amounts of heterodimer are produced by each of the peptides, showing that the interaction of the transmembrane domains is not significantly altered by the labels.

The fluorescence excitation and emission spectra of 2,6-DANS GpATM in DMPC are compared with the absorbance spectrum of DABS GpATM in DMPC in Figure 2. There is good spectral overlap between the 2,6-DANS emission and DABS absorbance, making this a good donor–acceptor pair. However, DABS in nonfluorescent, so sensitized emission cannot be detected. Rather, RET is detected solely by decreases in 2,6-DANS emission resulting from quenching by DABS.

Specific Dimerization of GpATM in Membranes. A number of different salt concentrations and sonication procedures were tested to determine the optimum conditions to produce RET. The best results were found with measuring the fluorescence of the vesicles in 1 M NaCl following (>5 min) bath sonication. Measurements of the fused vesicles containing 2,6-DANS and DABS GpATM showed a decrease in 2,6-DANS fluorescence which was not produced by merely freezing the 2,6-DANS vesicles on their own or by fusing the 2,6-DANS vesicles with unlabeled GpATM. Experiments

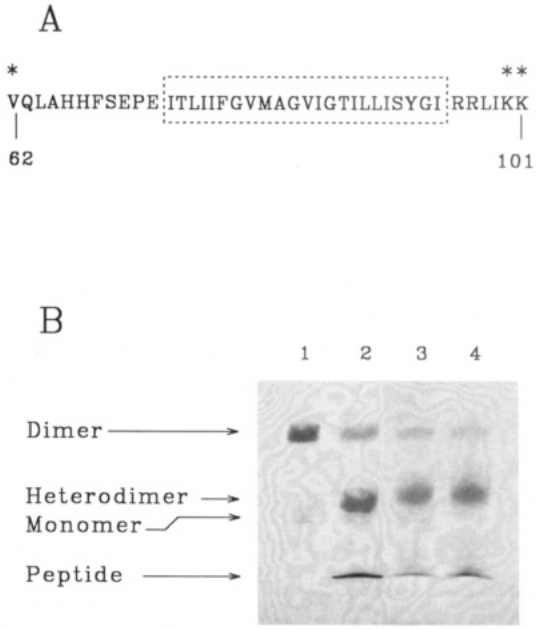

FIGURE 1: (A) Sequence of GpATM indicating potentially labeled positions (*). The putative transmembrane region is boxed. (B) 12.5% SDS gel (Pharmacia Phastgel) of SN/GpA showing competition with labeled and unlabeled peptides. Each lane contains 4 μL of a 10 μM solution of SN/GpA. Lane 1 contains SN/GpA without GpATM; lanes 2, 3, and 4 contain SN/GpA and a 15-fold molar excess of unlabeled GpATM, DABS-labeled GpATM, and 2,6-DANS-labeled GpATM, respectively. The positions for SN/GpA dimer and monomer, SN/GpA-GpATM heterodimer, and GpATM are indicated. The labeled and unlabeled peptides appear equivalent in forming heterodimers.

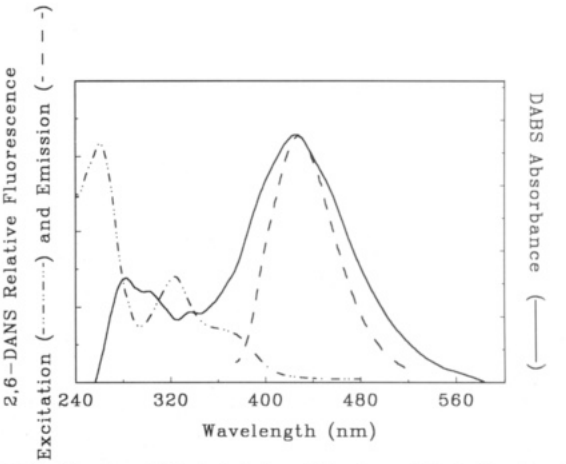

FIGURE 2: Spectra of the labeled peptides in vesicles indicating the spectral overlap between 2,6-DANS emission and DABS absorbance: (-·-·-) 2,6-DANS absorbance spectrum; (- - -) 2,6-DANS emission spectrum; (—)DABS absorbance spectrum. 2,6-DANS spectra are from fluorescence emission and excitation spectra; DABS spectrum is an absorbance spectrum. A baseline corresponding to the light scattering from the vesicles has been subtracted from the DABS spectrum.

were performed to determine whether the RET produced by this system is due to a specific self-association of GpATM. The RET signal was constant over a 10-fold increase in the amount of DMPC in the system (from 10:1 to 100:1 w/w, approximately 60:1 to 600:1 mole ratio DMPC:GpATM), which indicates that the observed signal is not simply the result of nearby but noninteracting peptides. In addition, the RET signal was also resistant to competition from heterologous transmembrane peptides when DMPC vesicles containing these peptides were fused to the GpATM vesicles. The peptides used corresponded to the transmembrane regions of the

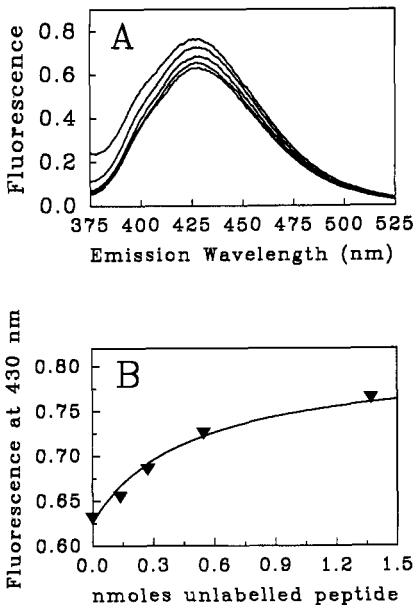

FIGURE 3: (A) Spectra of a constant amount of labeled peptides (0.25 nmol of 2,6-DANS and 0.30 nmol of DABS GpATM) in DMPC vesicles with 0.0, 0.14, 0.27, 0.55, and 1.4 nmol of unlabeled GpATM added. Lipid to peptide ratios were held constant at 10:1 (w/w) DMPC:GpATM for all peptides (labeled and unlabeled). Vesicles are in 1.5 mL of 1 M NaCl with 50 mM sodium phosphate (pH 8.0) at 37 °C. (B) Peak emission of the spectra in (A) plotted against the amount of unlabeled peptides added (▼), showing competition by unlabeled peptide for dimer formation with labeled peptides. Curve is the best fit to the data with eq 10.

epidermal growth factor receptor (EGF-TM), proto-*neu* oncogene (*neu*-TM), and HER-2 (HER-2-TM) and were the same as those used by Lemmon et al. (1992a) to test disruption of SN/GpA dimers on SDS gels. The overall amino acid composition and charge organization of these peptides are quite similar to those of GpATM. The absence of competition demonstrates that the observed RET is due to an interaction between the peptides that is determined by the exact sequence of GpATM, rather than by a general tendency of any transmembrane peptide to associate.

Competition experiments conducted with unlabeled GpATM derived from SN/GpATM are shown in Figure 3. All of the peptides were at 10:1 lipid:protein weight ratio, so the concentration of GpATM (all forms) did not change during the experiment. In contrast to the heterologous peptides, addition of unlabeled GpATM decreases RET. Fluorescence approaches, but does not reach, the unquenched level. Unfortunately, the amount of unlabeled material that may be added is limited by light scattering artifacts produced by the large amount of lipid, and an additional spectrum from this experimental series (3.7 nmol of unlabeled peptide added) is not included due to the fact that the light scattering signal provided a large background obscuring the fluorescence at 430 nm. The data for the unlabeled GpATM competition experiment have been fitted to a curve by assuming the unlabeled peptides are competing to separate donors and acceptors of labeled dimers. The derivation of the dependency of fluorescence on unlabeled peptide concentration used to fit the curve in Figure 3B is given in the Appendix. Agreement between the model and the experimental data is quite good; R^2 for the curve fit was 0.96.

The number of peptides associating in an oligomer was determined by analyzing the dependence of RET on the relative amount of labeled peptides present. As derived in the Appendix, the amount of quenching will be proportional to

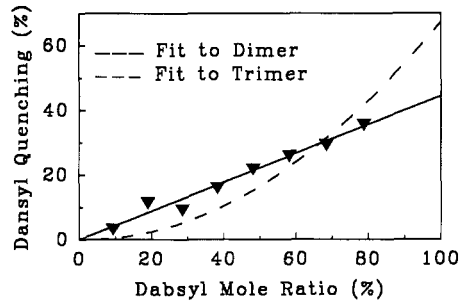

FIGURE 4: Plot of the amount of quenching of a constant amount of 2,6-DANS GpATM with various amounts of DABS GpATM added (▼), showing that GpATM forms a dimer in DMPC bilayers. Spectra were recorded from DMPC vesicles in 1 M NaCl, 50 mM sodium phosphate (pH 8.0) at 37 °C. DMPC:GpATM weight ratios were constant 10:1 for all forms of GpATM. Lines are the best fit to the data with eq 9 given $n = 2$ (dimer) (—) and $n = 3$ (trimer) (- - -).

$1 - (P_{2,6\text{-DANS}})^{n-1}$, where $P_{2,6\text{-DANS}}$ is the mole ratio of 2,6-DANS-labeled peptide and n is the oligomer number. The results for an experiment with various mole ratios of 2,6-DANS GpATM and DABS GpATM are shown in Figure 4. A constant amount of 2,6-DANS GpATM in DMPC vesicles has various amounts of DABS GpATM added by vesicle fusion. The weight ratio of peptide to lipid is kept constant at 1:10. The data are displayed as the quenching at 430 nm as a function of P_{DABS} ($=1 - P_{2,6\text{-DANS}}$). Data have been fit with a least-squares method using eq 9 to a line for dimer (quenching = aP_{DABS}) and for trimer [quenching = $a(P^2_{\text{DABS}} - 2P_{\text{DABS}})$]. The fit is far better for a dimer than a trimer: R^2 was 0.97 for the fit to a dimer and 0.68 for the fit to a trimer. The dimer line in Figure 4 extrapolates to a maximum of 44.4% (i.e., the molar fluorescence of a quenched donor is 0.44 times the unquenched fluorescence).

DISCUSSION AND CONCLUSION

The transmembrane region of glycophorin A had previously been known to form sequence-specific dimers in SDS micelles; it was not known, however, whether this behavior accurately reflected the behavior of GpATM in lipid bilayers, which more closely represent the native environment in red cell membranes. The results for oligomerization and sequence specificity obtained with 2,6-DANS- and DABS-labeled GpATM in DMPC vesicles reproduce the behavior seen on SDS gels. The data from this study show that in DMPC bilayers residues 62–100(101) of GpA form a dimer that is sensitive to the correct amino acid sequence but insensitive to increases in lipid to protein ratios. When the data from the unlabelled GpATM competition experiment are plotted in the form of quenching vs P_{DABS}, the data fall on the same line as the data from the mole ratio mixing experiment (not shown). This clearly indicates that both the unlabeled and 2,6-DANS-labeled molecules have the same propensity to form a dimer with the DABS-labeled molecules, one of the assumptions which predicted this behavior.

Labeling of the ϵ-amino group of Lys-100 or -101 and the α-amino group of Val-62 with fairly large substitutents thus does not interfere with dimerization in bilayers, in keeping with the properties seen in SDS gels. This result was expected for Val-62, where the label occupies the position of residue 61. Additional amino acids at the amino terminus do not disrupt dimerization in SDS, and this position is thought to be far enough away from the residues important in dimerization that additional noninteracting substitutents do not interfere; steric hindrance of dimerization from nuclease in chimeric

constructs only appears to begin at residue 69 (Lemmon et al., 1992a). The DABS label appears to be in nonaqueous environments; the absorbance spectra on labeled peptides shifts from a maximum of 470 nm in β-OG solubilized peptides to 430 nm in DMPC vesicles, indicating a removal of the chromophore from contact with water (Lin & Chang, 1975).

The extrapolation of the line in Figure 4 to P_{DABS} = 100% gives a value for maximum quenching of 44%. This might seem an unexpectedly small value. For this donor–acceptor pair under experimental conditions, $R_0(^2/_3)$ = 40.5 Å; thus, for uniform labeling and random chromophore orientation, an interchromophore distance of a little over 40 Å would produce the observed result. This distance is larger than that expected for chromophores on the ϵ-amino groups of lysines on the outside surfaces of a dimer of α-helices, which are not likely to be more than 30 Å apart (20 Å for the helix diameters and an additional 4–5 Å for the lysine chains). The N terminus extends 8–10 residues beyond the transmembrane region, and these residues might not in fact be in a helical conformation. Thus, labels at the N terminus might be much farther than 20 Å apart. Labeling is not uniform (not every site is occupied), and many labeled donor–acceptor pairs might be farther apart (a 2,6-DANS at the amino terminus and the DABS on the lysines would be 40 residues apart, approximately 60 Å, if the peptides were helical throughout). In addition, some of the peptides might not be labeled at all. As demonstrated in the Appendix, the presence of unlabeled peptides would not change the behavior in mole ratio mixing experiments but would decrease the total amount of quenching seen.

The RET methods described herein provide a useful method for monitoring helix–helix interactions in bilayers and will, in the future, allow more accurate analysis of the effects of single amino acid substitutions on the stability of GpATM interactions. The finding that the transmembrane region of GpA dimerizes in a lipid bilayer supports the idea that GpA exists as a dimer in the erythrocyte membrane. In addition, these methods may be applicable in other systems where helix–helix interactions are disrupted by ionic or other detergents and could be used to document oligomeric interactions more generally.

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APPENDIX

The number of subunits in the GpATM oligomer was determined by deriving the dependence of the number of quenched 2,6-DANS peptides (and hence the net fluorescence) on the ratio of 2,6-DANS to DABS peptides in the membranes. The determination of oligomer number does not depend on uniformity of labeling and is unaffected by the presence of unlabeled peptides. The method is similar to that developed by Milligan and Koshland (1988) for identifying the oligomeric state of the aspartate chemoreceptor by determining the dependency of a number of cysteine-cross-linked proteins as a function of relative amounts of cysteine-containing and cysteine-less proteins in the solution. The measured fluorescence, F, is given by

$$F = f_D(N_D - NQ) + f_Q N_Q \quad (1)$$

where f_D is the molar fluorescence of unquenched donor, f_Q is the molar fluorescence of quenched donor, N_D gives the total moles of donor, and N_Q gives the moles of quenched donor. f_Q is the average value for the entire population and depends on the actual quenching of the different labeling isomers and their distribution in the population of donor and acceptor oligomers. Specifically, if F_{11} is the molar fluorescence from a dimer with donor labeling isomer 1 and acceptor labeling isomer 1 and F_{12} is the fluorescence from donor isomer 1 and acceptor isomer 2, then the total fluorescence measured will be $F = N_1F_{11} + N_2F_{12} + ... + N_nF_{mn} + N_{f1}F_1 + N_{f2}F_2 + ... + N_{fn}F_n$, where $N_1...N_n$ give the number of donor–acceptor pairs for each isomer and N_{f1} etc. give the number of each donor isomer uncomplexed with acceptor. The model does make the assumption that the number of donor–acceptor pairs formed is a function only of the concentration of the different forms in the membrane (a simple probability function). Thus, in a situation with half as many donor–acceptor pairs being formed there will be half as many F_{11}, F_{12}, etc. and twice as many of the corresponding unquenched donor isomers, so the net quenching when there are half as many donor–acceptor pairs formed will be half as much. More generally, f_Q will be a constant and is treated so here. In this notation the fluorescence of the donor in the absence of acceptor (F_0) is $f_D N_D$. The value for quenching is defined as $1 - F/F_0$ so

$$\text{quenching} \equiv 1 - \frac{F}{F_0} = \left(1 - \frac{F_Q}{F_D}\right)\frac{N_Q}{N_D} \quad (2)$$

For a donor molecule to be quenched it must be in the same oligomer with at least one acceptor. With a random donor and acceptor content in the oligomers the number of oligomers with any donor and acceptor concentration may be found with the binomial probability function. The total number of quenched donors is thus given by

$$N_Q = N_T \sum_{k=1}^{n-1} k\binom{n}{k} P_D^k P_A^{n-k} \quad (3)$$

where n is the number of units in the oligomer, N_T is the number of oligomers, P_D is the mole ratio of donor, and P_A is the mole ratio of acceptor. In eq 3, k counts the number of donors, and the sum includes all oligomers except $k = 0$ (oligomers with acceptors only) and $k = n$ (oligomers with donors only). Combining eq 3 with the expression for quenching in eq 2 gives

$$1 - \frac{F}{F_0} = \left(1 - \frac{f_Q}{f_D}\right)^{\frac{N_T \sum_{k=1}^{n-1} k\binom{n}{k} P_D^k P_A^{n-k}}{N_D}} \quad (4)$$

Equation 4 may be simplified by using the expression for the total number of donors as the fraction of the total number of peptides

$$N_D = nN_T P_D \quad (5)$$

and the expression using the binomial probability function to count the total number of donor labeled peptides:

$$N_D = N_T \sum_{k=1}^{n} k\binom{n}{k} P_D^k P_A^{n-k} \quad (6)$$

Here, k again counts the number of donor labeled peptides and runs from 1 to n to include all oligomers with any donor. Separating the final ($k = n$) term from the sum in eq 6 and

setting it equal to the expression for N_D in eq 5 gives

$$\left(N_T\sum_{k=1}^{n-1}k\binom{n}{k}P_D^nP_A^{n-k}\right) + N_TnP_D^n = nP_DN_T \quad (7)$$

Rearranging eq 7 and substituting it into the sum in the numerator of eq 4 and further substituting the value for N_D in eq 5 into the denominator of eq 4 gives

$$1 - \frac{F}{F_0} = \left(1 - \frac{f_Q}{f_D}\right)\frac{nN_TP_D - nN_TP_D^n}{nN_TP_D} \quad (8)$$

which simplifies to

$$1 - \frac{F}{F_0} = \left(1 - \frac{f_Q}{f_D}\right)(1 - P_D^{n-1}) \quad (9)$$

It should be stressed that this treatment makes a number of assumptions: principally, that labeling does not influence oligomerization in any way and that the interactions are truly random, that all of the peptides are oligomerizing, and that the population of labeled peptides in any vesicle is large enough to ensure that the labeled peptide distribution accurately reflects the distribution in the total population.

The analysis for unlabeled peptide competition is very similar. In this case both the unlabeled and donor peptides are combined in the same terms since they have the same quenching ability, i.e., a donor will not be quenched if it is in an oligomer with another donor or with an unlabeled peptide. The equations may be recast for RET quenching with an unlabeled population by substituting N_{DU}, the total moles of unlabeled and donor peptide, for N_D, and likewise P_{DU} for P_D, and setting N_Q equal to the number of unlabeled or donor labeled molecules in an oligomer with at least one acceptor. To account for the decreased molar fluorescences that result from combining the unlabeled and donor labeled peptide populations, the molar fluorescences for quenched and unquenched populations of peptides, f_D' and f_Q', become f_DN_D/N_{DU} and f_QN_D/N_{DU}, respectively. The line in Figure 3B was fit to the data by rearranging eq 9

$$F = F_0 - \left(\frac{f_Q'}{f_D'}\right)(1 - P_{DU}^{n-1}) \quad (10)$$

and setting $n = 2$, so $(1 - P_{DU})^{n-1} = P_{DABS}$. Since the total number of 2,6-DANS- and DABS-labeled peptides is fixed, for this experiment P_{DABS} is expressed as a function of the amount of unlabeled peptide $0.38/(0.63 + N_U)$, where 0.38 and 0.63 are the amounts in nanomoles of DABS- and DABS+DANS-labeled peptides.

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