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Visual Detection of Specific, Native Interactions between Soluble and Microbead-Tethered α-Helices from Membrane Proteins†

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ABSTRACT: Using peptides tethered to polymer microbeads, we have developed a technique for measuring the interactions between the transmembrane α-helices of membrane proteins and for screening combinatorial libraries of peptides for members that interact with specific helices from membrane proteins. The method was developed using the well-characterized homodimerization sequence of the membrane-spanning α-helix from the erythrocyte membrane protein glycophorin A (GPA). As a control, we also tested a variant with a dimer-disrupting alteration of a critical glycine residue to leucine. To test for detectable, native interactions between detergent-solubilized and microbead-tethered α-helices, we incubated fluorescent dye-labeled GPA analogues in sodium dodecyl sulfate solution with microbeads that contained covalently attached GPA analogues. When the dye-labeled peptide in solution and the bead-tethered peptide both contained the native glycophorin A sequence, the microbeads readily accumulated the dye through lateral peptide−peptide interactions and were visibly fluorescent under UV light. When either the peptide in solution or the peptide attached to the beads contained the glycine to leucine change, the beads did not accumulate any dye. The usefulness of this method for screening tethered peptide libraries was tested by incubating dye-labeled, native sequence peptides in detergent solution with a few native sequence beads plus an excess of beads containing the variant glycine to leucine sequence. When the dye-labeled peptide in solution was present at a concentration of ≥ 2 μM, the few native sequence beads were visually distinguishable from the others because of their bright fluorescence. Using this model system, we have shown that it is possible to visually detect specific, native interactions between α-helices from membrane proteins using peptides tethered to polymer microbeads. It will thus be possible to use this method to measure the specific lateral interactions that drive the folding and organization of membrane proteins and to screen combinatorial libraries of peptides for members that interact with them.

The recent exponential growth in the use of combinatorial chemistry and high-throughput screening attests to the power of these methods in biomedical research. An important component of this research area is the polymer microbead, first developed for use in solid-phase chemical synthesis (*1*). Polymer microbeads, often made from porous cross-linked polystyrene, are spheres 50−200 μm in diameter that are useful for benchtop-scale combinatorial research because they have densities of 10^5−10^6 beads per gram of dry weight and yet are visible under low-power magnification. One of the

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more powerful high-throughput applications of microbead technology is the screening of microbead-tethered combinatorial peptide libraries synthesized by the split and recombine method (*2*, *3*). In this type of library, each individual bead has 50−200 pmol of the same peptide sequence attached to it but each bead in the library has a different sequence. The value of bead-tethered libraries is that individual beads can be identified and physically separated and the unique peptide sequence on a bead can be determined with standard sequencing methods. Such one-bead/one-compound libraries have been used to screen for specific bimolecular interactions (*4*−*6*) and to screen for enzyme-specific substrate sequences (*7*−*10*). We are developing methods for using microbead-tethered peptides and tethered peptide libraries for the characterization and screening of interactions with the membrane-spanning sequences of membrane proteins. Except for the ToxCat biological screening assay for helix−helix homodimerization (*11*), this is an area that has received little attention to date and yet could be broadly useful in many areas of membrane protein structural biology.

Ideally, one would like to be able to incubate a particular membrane-spanning α-helix with a one-bead/one-compound combinatorial library of helices and then identify those members of the library which specifically interact with the test sequence. For this method to be effective, the following conditions must be met. First, there must be no nonspecific interactions between the necessarily hydrophobic peptides and the matrix of the microbeads. Second, soluble peptide−detergent complexes must equilibrate with the internal matrix of the bead where most of the tethered peptides reside. Third, the interaction of peptides in solution with the microbead-tethered peptides must be readily visible and measurable. Fourth, the helix−helix interactions within the polymer matrix of the bead must faithfully reflect the strength and specificity of native helix−helix interactions in membranes.

To assess whether these necessary conditions can simultaneously be met, we chose as a model system the well-characterized, membrane-spanning α-helix of the erythrocyte membrane protein glycophorin A (GPA).[1] Glycophorin A exists in the erythrocyte plasma membrane as a homodimer which is stabilized by specific surface−surface interactions between the single membrane-spanning α-helices (*12*−*17*). The interactions that drive dimerization have been well characterized; they are mainly nonpolar van der Waals interactions that occur at a right-handed helical cross between residues 75 and 87 of the native sequence. The interacting surface is shown in Figure 1. The glycines at residues 79 and 83 are critical for dimerization and cannot be replaced without inhibiting dimer formation (*15*, *18*, *19*). Importantly for this work, in detergent solution, the glycophorin A helix dimer is stable and is driven by native interactions (*18*, *20*). In these experiments, we use peptides containing the native sequence of the glycophorin A membrane-spanning helix (GPA) and we also use peptides (GPA^{G83L}) in which native dimerization has been disrupted by changing one of the critical glycines, Gly83 in the native sequence, to leucine

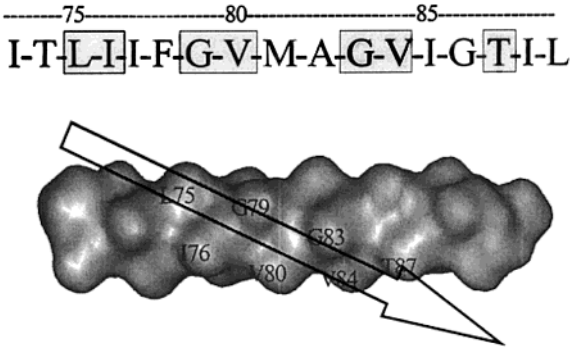

FIGURE 1: Sequence of the transmembrane helix of glycophorin A. The dimerization of the transmembrane helix of glycophorin A is driven by specific interactions occurring at the right-handed crossing (*12*) between residues 75 and 87. Residues that were shown to be important for the interaction (*12*, *14*−*16*, *20*, *38*) are boxed in gray. The surface involved in the right-handed helical crossing of the dimer, shown by the arrow, is apparent when the pattern of these important residues is superimposed on a surface representation of a model α-helix. In this work, we changed the critical glycine at position 83 to leucine to prevent dimerization.

(*15*, *18*). With these model peptides, we will show how the four necessary conditions outlined above can be met and we demonstrate how one can use microbead-tethered peptides or tethered peptide libraries to study or screen the interactions between membrane-spanning α-helices.

MATERIALS AND METHODS

Synthesis. All the peptides were synthesized manually in fritted vessels shaken with a 180° wrist action shaker using standard fast Fmoc chemistry (*21*). See Table 1 for a list of all of the synthesized peptides. Peptides were tethered to either Tentagel S microbeads (*22*) from Advanced Chemtech (Louisville, KY) or Amino PEGA microbeads (*23*) from Novabiochem (San Diego, CA). Peptides to be cleaved and used in solution were synthesized on Fmoc-Ala NovaSyn TGA resin from Novabiochem. A 4-fold excess of Fmoc-amino acids, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), hydroxybenzotriazole (HOBT), and diisopropylethylamine were used for each coupling in dimethylformamide, and a solution of 30% piperidine in dimethylformamide was used for the deprotection of the Fmoc amino group. Couplings were carried out for 30−60 min, and their progress and completion were monitored by ninhydrin colorimetric tests (*24*, *25*). Where indicated by the ninhydrin test, double couplings were performed to achieve completion of the reaction.

After synthesis, a 95:5:2.5:2.5 mixture of trifluoroacetic acid, thioanisole, 1,2-ethanedithiol, and anisole was used to remove the side chain protecting groups and to cleave the synthesized peptide from the NovaSyn TGA resin. For Tentagel S and PEGA beads, the synthesized peptides remained tethered to the beads because they are directly coupled to the primary amino groups of the beads. These beads were repeatedly washed and dried under N_2 with methanol, 2-propanol, and methylene chloride. Washed Tentagel S beads with tethered peptides were stored after thorough drying at low temperatures, while the PEGA beads were stored at −20 °C in methanol because of their fragile nature under dry conditions. Peptides cleaved from NovaSyn TGA resin were dried repeatedly from methanol and 2-propanol and then were lyophilized from acetic acid.

[1] Abbreviations: GPA, residues 73−92 of the membrane-spanning helix of the erythrocyte membrane protein glycophorin A; GPA^{G83L}, variant of GPA in which a glycine is replaced with leucine; PEGA, polyethylene glycol amino; SDS, sodium dodecyl sulfate; UV, ultraviolet.

Table 1: Sequences of the Synthesized Peptides

Free Peptides	Sequence[a]
Arg-GPA	Fluorescent Dye-GRRRR**ITLIIFGVMAGVIGTILLIS**AA-$CONH_2$
Arg-GPA^{G83L}	Fluorescent Dye -GRRRR**ITLIIFGVMALVIGTILLIS**AA-$CONH_2$

Tethered Peptides[b]	Sequence
Arg-GPA	RRRR**ITLIIFGVMAGVIGTILLIS**AA-bead
Lys-GPA	KKKK**ITLIIFGVMAGVIGTILLIS**AA-bead
GPA	**ITLIIFGVMAGVIGTILLIS**AA-bead
Arg-GPA^{G83L}	RRRR**ITLIIFGVMALVIGTILLIS**AA-bead
Lys-GPA^{G83L}	KKKK**ITLIIFGVMALVIGTILLIS**AA-bead
GPA^{G83L}	**ITLIIFGVMALVIGTILLIS**AA-bead

[a] Sequences of the synthesized peptides. Free peptides have amidated C-termini. The native sequence of the membrane-spanning helix of glycophorin A (GPA) is shown in bold and corresponds to residues 73−92 of the native sequence. The glycine that was changed to leucine in the control peptides (Gly83 in the native sequence) is double underlined. Two alanines were added at the C-terminus to increase helicity and to move the tethered peptides farther away from the bead polymer. The Arg and Lys sequences have four cationic residues on their N-termini. Two fluorescent dyes, fluorescein and Texas Red, were used for labeling the N-termini of the peptides in solution. [b] Each tethered peptide variant was synthesized on Tentagel beads and also on PEGA beads. See the text for details.

Mass Spectrometry and Peptide Sequencing. Mass spectrometry of the crude free peptides, Arg-GPA and Arg-GPA^{G83L} (see Table 1), by the MALDI-TOF technique was carried out on a Voyager-DE mass spectrometer from Applied Biosystems (Foster City, CA). Almost no deletion peptides were detected in the crude peptides. The predominant peaks in the Arg-GPA and Arg-GPA^{G83L} samples were both within the experimental uncertainty (0.4 mass unit) of the expected values of 2869.4 for Arg-GPA and 2925.5 for Arg-GPA^{G83L}. The sequences of the bead-tethered peptides were confirmed by direct amino acid sequencing.

Purification of the Free Peptide. The free peptides (Table 1) were designed with multiple cationic residues on their N-terminus to increase solubility and manageability (*26*); therefore, peptides were purified by cation exchange HPLC using PolyCAT A resin (*27*) (Western Analytical Products, Inc., Murietta, CA). Peptide was loaded on a 5 cm × 0.5 cm PolyCAT A column using a 70% (v/v) 10 mM ammonium acetate solution (pH 5.5) with 20% 2-propanol and 10% methanol. Hydrophobic impurities were eluted from the column by pulsing with 2-propanol and methanol, and the peptide was eluted with the loading solvent with additional 15% acetic acid.

Labeling with Fluorescent Dyes. Purified, lyophilized peptides were labeled on their N-terminal amino groups with the fluorescent dye fluorescein or Texas Red. The succinimidyl esters of these dyes (Molecular Probes, Eugene, OR) were used in excess over peptide to ensure the complete labeling of the terminal amino group. The labeled peptides were repurified by cation exchange HPLC as described above. Monitoring was carried out at the absorption maximum for the dyes. Retention times of the labeled peptides were reduced by several minutes due to the loss of the terminal charge.

Reconstitution into SDS Micelles. Purified labeled peptides were reconstituted into SDS micelles by dissolving them in a 2 mM SDS solution and then lyophilizing the solution to dryness (*28*). The dried powder was redissolved into the original volume of water to obtain stable solutions of peptides in 2 mM SDS detergent.

Fluorescence Quenching. Fluorescence was measured on an SLM Aminco 8100 fluorometer at room temperature. The level of quenching of fluorescein-labeled GPA analogues by Texas Red-labeled GPA analogues was measured in SDS solutions to assess the ability of these peptides to dimerize. Because of the overlap in the emission spectrum of fluorescein and the broad excitation spectrum of Texas Red, Texas Red was expected to quench fluorescein fluorescence (*29*) when the two are in proximity, as they will be in the native parallel GPA homodimer. In the quenching experiments, we measured the intensity of fluorescein-labeled peptides (GPA or GPA^{G83L}) in the presence and absence of Texas Red-labeled peptides. The total peptide concentration was equal to 1.5 μM, and the fluorescein intensity was corrected for dilution in the samples that contained both dyes. In the absence of Texas Red-labeled peptides, the fluorescein intensity was directly proportional to its concentration; therefore, the quenching experiment is not detecting any nonlinear self-quenching or aggregation phenomena.

Interactions between Bead-Tethered and Free Peptides. To screen for interactions between detergent-solubilized and bead-tethered peptides, the beads must be incubated with peptide solutions and the peptide in solution must equilibrate with the bead interior. To prepare microbeads with tethered peptides for incubation, they were first swelled in a 2 mM SDS solution for ∼4 h in sealed silanized glass vials. The swelled beads were then settled to the bottom of the vial by centrifugation for 5 min at 5000 rpm. The supernatant SDS solution was completely removed, and the freshly reconstituted Texas Red-labeled peptide in an SDS solution was added to the vials. The tightly sealed vials were held at ∼40 °C overnight in a water bath. After overnight equilibration, the beads were prepared for examination to determine if any accumulation of the Texas Red dye could be detected. For examination, the beads were settled by centrifugation and the Texas Red-labeled peptide solution was siphoned off with a glass capillary. Beads were then washed three times with a cold 2 mM SDS solution to remove any excess labeled peptide solution. At this point, the beads were examined under long-wave (nominal, 365 nm) UV illumination and

compared to control beads to determine if there is a visible accumulation of Texas Red on the bead. For photography, the beads were removed from the vials using a glass capillary and air-dried on a glass cover slip that had been painted black.

RESULTS AND DISCUSSION

Design of the Model System. The membrane-spanning helix of glycophorin A was chosen to test for interactions between α-helices in a detergent solution and α-helices tethered to polymer microbeads. The homodimerization of the glycophorin A transmembrane helix is strong, and specific and native interactions drive dimerization in detergent solutions, such as sodium dodecyl sulfate (SDS), as well as in membranes (*12*−*16*). SDS was chosen as the detergent we would use in these experiments because it drives dimerization of glycophorin A and also because it been shown to promote native interactions between membrane proteins other than glycophorin (*30*−*33*). Thus, SDS probably has widespread applicability to membrane protein systems. All of the peptides synthesized for this work are listed in Table 1. The part of the GPA sequence used in these studies, residues 73−92 of the native sequence, encompasses the interaction surface (*12*) between residues 75 and 87, as shown in Figure 1. As a control for nonspecific or non-native interactions, we also synthesized homologous peptides in which the critical Gly83 was replaced with Leu (GPA^{G83L}). In several previous studies, this change was shown to prevent GPA dimerization in SDS micelles (*15*, *18*). The three-dimensional structure of the dimer offers an explanation for this observation; in the dimer, the Gly83 C_α atom is less than one carbon diameter from the side chain of Val84, leaving insufficient space for any Gly83 C_α constituent larger than a proton (*12*, *13*). For the free peptides, four arginine residues were added to the N-terminus to increase manageability (*26*), and a glycine was added to the N-terminus as a spacer to improve the labeling of the N-terminal amino group. The two free peptides used in this study are termed Arg-GPA and Arg-GPA^{G83L} (see Table 1). Two C-terminal alanine residues were added to all peptides to act as spacers from the polymer matrix of the microbeads and to promote helicity (*34*). For the bead-tethered peptides, we synthesized peptides ending in the native isoleucine 73, and we synthesized peptides that were terminated with four additional positively charged residues, either Arg or Lys. This design strategy was used to minimize the nonspecific self-association of the peptides on the microbeads to improve their accessibility to the aqueous detergent solution.

Selection of the Dye. Our primary objective in this work was to show that we could achieve specific, native, visually detectable interactions between dye-labeled peptides in detergent solution and peptides tethered to polymer microbeads. After testing a number of fluorescent dyes, we chose Texas Red for labeling the peptides in solution because its bright red fluorescence is highly visible when excited with standard, hand-held, long-wave UV light sources. This property permits simple benchtop screening of bead libraries using a low-power microscope. Also, even at very faint intensities, the red color of Texas Red can be confidently distinguished from the intrinsic yellowish or bluish color of the microbeads and from the bluish color of the reflected long-wave UV light. In an experiment in which we directly labeled Tentagel S beads with varying amounts of Texas Red, we found that we could visually distinguish labeled from unlabeled beads when the degree of Texas Red labeling was ∼1% of the bead's active amino groups.

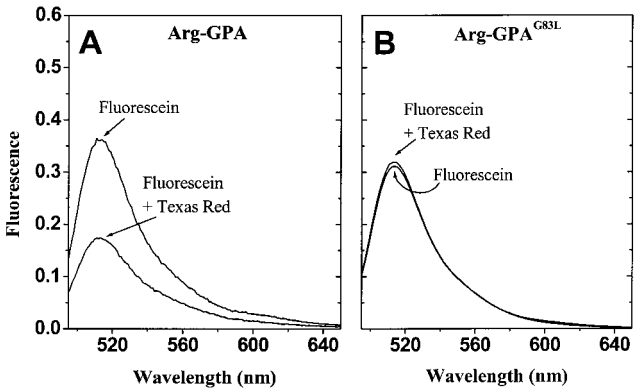

FIGURE 2: Fluorescence quenching in GPA homologue peptides. Fluorescence spectra of fluorescein-labeled peptides and an equimolar mixture of fluorescein- and Texas Red-labeled peptides: (A) Dye-labeled Arg-GPA and (B) dye-labeled Arg-GPA^{G83L}. In all cases, the total peptide concentration was 1.5 μM and the SDS concentration was 2 mM. The spectra in each panel are for fluorescein emission excited at 480 nm. The fluorescein emission spectra measured in the equimolar mixtures were corrected for dilution. Fluorescein-labeled Arg-GPA is strongly quenched by Texas Red-labeled Arg-GPA (A), indicating a close, parallel association. There is no quenching, and therefore no parallel association, between the dye-labeled Arg-GPA^{G83L} peptides (B).

Selection of the Microbeads. To assess the importance of the physical and chemical properties of the microbeads, we tested two very different types of beads. First we used Tentagel S NH_2 beads which are ∼130 μm diameter beads made of cross-linked polystyrene with attached polyethylene glycol. The active amino group is on the end of a long (50−60-ethylene unit) polyethylene glycol chain linked to the polystyrene backbone. These beads are very stable, uniform, and physically robust, but they are also relatively hydrophobic. Second, we also tested amino PEGA beads (*23*), which are made from acrylamide-cross-linked polyethylene glycol. The PEGA beads are very hydrophilic and have much higher porosity and accessibility to large soluble molecules. However, they are also physically less robust than Tentagel beads. PEGA beads must be handled more carefully and cannot be stored in dry form at any time.

Specific Dimerization in Detergent Solution. Before we investigated the association between microbead-tethered and detergent-solubilized peptides, we first had to confirm that our GPA analogues were capable of nativelike dimerization in SDS solution. Circular dichroism spectroscopy showed that both free peptides, Arg-GPA and Arg-GPA^{G83L}, are soluble and fully helical in a 2 mM SDS solution (not shown), and we found that both GPA analogues, prepared in a 2 mM SDS solution as described above, remained stable and in solution for at least several weeks. To characterize the homodimerization potential of Arg-GPA and Arg-GPA^{G83L} in solution, we adapted the fluorescence quenching procedure of Adair and Engelman (*35*) by using fluorescein-labeled peptides as fluorescence donors and Texas Red-labeled peptides as quenchers. Because of the overlap between fluorescein emission and Texas Red absorption, we expected Texas Red to quench fluorescein when the two are in proximity. In the quenching experiments (Figure 2), we

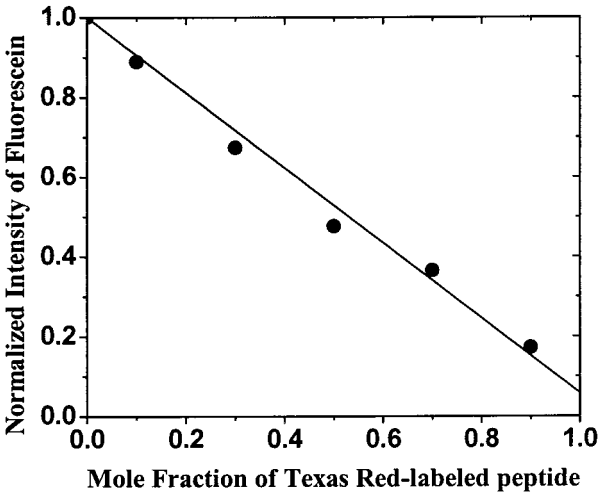

FIGURE 3: Fluorescence quenching between Arg-GPA peptides. For each point, we mixed various fractions of fluorescein-labeled Arg-GPA and Texas Red-labeled Arg-GPA in 2 mM SDS and measured the fluorescence of the fluorescein after overnight equilibration at 40 °C. The total Arg-GPA concentration (fluorescein + Texas Red) was maintained at 1.5 μM. Each measured intensity was corrected for fluorescein dilution. The linearity of the plot supports the idea that the associated peptides are dimeric (*35*). All mixtures containing Arg-GPA^{G83L} peptides gave a normalized intensity of ~1.0 in this experiment, as shown in Figure 2.

show the intensity of fluorescein-labeled peptides in the presence and absence of Texas Red-labeled peptides. The total peptide concentration was 1.5 μM. Complete equilibration required an overnight incubation at 40 °C. The fluorescence emission of fluorescein-labeled Arg-GPA is strongly quenched by an equimolar amount of Texas Red-labeled Arg-GPA (Figure 2A). The strong quenching of fluorescein by Texas Red indicates a close, parallel association between the peptides. As expected, we observed no quenching when the free dyes were mixed in solution. Most importantly, when an equimolar mixture of fluorescein and Texas Red-labeled Arg-GPA^{G83L} was equilibrated in 2 mM SDS, there was no quenching of fluorescein (Figure 2B), demonstrating that there is no nativelike, parallel association between the G83L peptides. Furthermore, no quenching of fluorescein fluorescence was observed in hetero mixtures of dye-labeled Arg-GPA and Arg-GPA^{G83L} peptides. These results indicate that a parallel association of Arg-GPA peptides occurs in detergent solution and that this association is absent in G83L peptides.

We further explored the association between Arg-GPA molecules in detergent solution by measuring the dependence of quenching on composition. The results are shown in Figure 3. In this experiment, the relative fraction of Arg-GPA labeled with each of the two dyes was varied and the total peptide concentration was held constant at 1.5 μM. The emission intensity of the fluorescein was corrected for dilution. Quenching was observed to be a linear function of the mole fraction of quencher over the entire composition range. Adair and Engelman (*35*) showed that only dimerization would give rise to a linear function in this experiment and that higher-order oligomerization would give curved quenching plots. When the same experiment was repeated with Arg-GPA^{G83L} peptides, or hetero mixtures of Arg-GPA and Arg-GPA^{G83L}, there was no measurable quenching of fluorescein fluorescence, even in the presence of a large

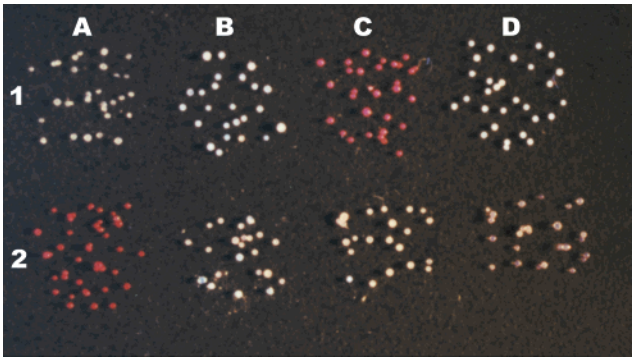

FIGURE 4: Tentagel S beads photographed under simultaneous visible and UV illumination. The beads in sections 1A and 2A have not been incubated with a peptide solution. Those in section 1A are unmanipulated, while those in section 2A have been directly labeled with Texas Red on their active amino groups. The beads in columns B−D have all been incubated with 20 μM Texas Red-labeled peptides in 2 mM SDS as follows: section 1B, Arg-GPA in solution, with no tethered peptide on the beads (only amino group acetylation); section 2B, Arg-GPA^{G83L} in solution, with no tethered peptide on the beads (only amino group acetylation); section 1C, Arg-GPA in solution, with GPA tethered to the beads; section 2C, Arg-GPA in solution, with GPA^{G83L} tethered to the beads; section 1D, Arg-GPA^{G83L} in solution, with GPA tethered to the beads; and section 2D, Arg-GPA^{G83L} in solution, with GPA^{G83L} tethered to the beads. Each bead is approximately 130 μm in diameter.

excess of the Texas Red-labeled peptide. We conclude from these fluorescence quenching experiments that the Arg-GPA molecules form native, parallel α-helical dimers in 2 mM SDS, and that this dimerization is completely prevented by a single G83L change.

Taken together, the CD and fluorescence results demonstrate that the peptides we have synthesized (Table 1) are stably soluble and fully helical and undergo specific, native dimerization in 2 mM SDS. These are important observations, in part, because 2 mM is less than the reported CMC of 5-10 mM for SDS in water (*36*, *37*). However, in experiments at various SDS concentrations, above and below the reported CMC, we found no difference in the interactions, solubility, or stability of the peptides. Presumably, even below the CMC, the SDS molecules associate with the hydrophobic peptides and stabilize them in water in their α-helical configuration. Only at very high SDS concentrations of ~70 mM did we observe any change at all. Specifically, high SDS concentrations caused a decrease in the level of interactions between soluble and bead-tethered GPA peptides, presumably due to the dilution of the peptides in the detergent. To maximize the effective peptide concentration, most experiments were performed at 2 mM SDS.

Dimerization with Microbead-Tethered Peptides. The quenching experiments showed that the GPA peptides in Table 1 undergo specific, nativelike dimerization in an SDS solution. Can we observe the same selectivity and specificity in the interactions between peptides in SDS solution and peptides tethered to polymer microbeads? In Figure 4, we show the results of an experiment that was designed to answer this question and to test for the four necessary conditions for screening interactions between tethered and free membrane-spanning helices: (1) no nonspecific interactions with the beads, (2) proper equilibration with the bead interior, (3) visual detectability of the interaction, and (4) nativelike specificity. The two sections of column A contain control Tentagel S beads that had not been exposed to the

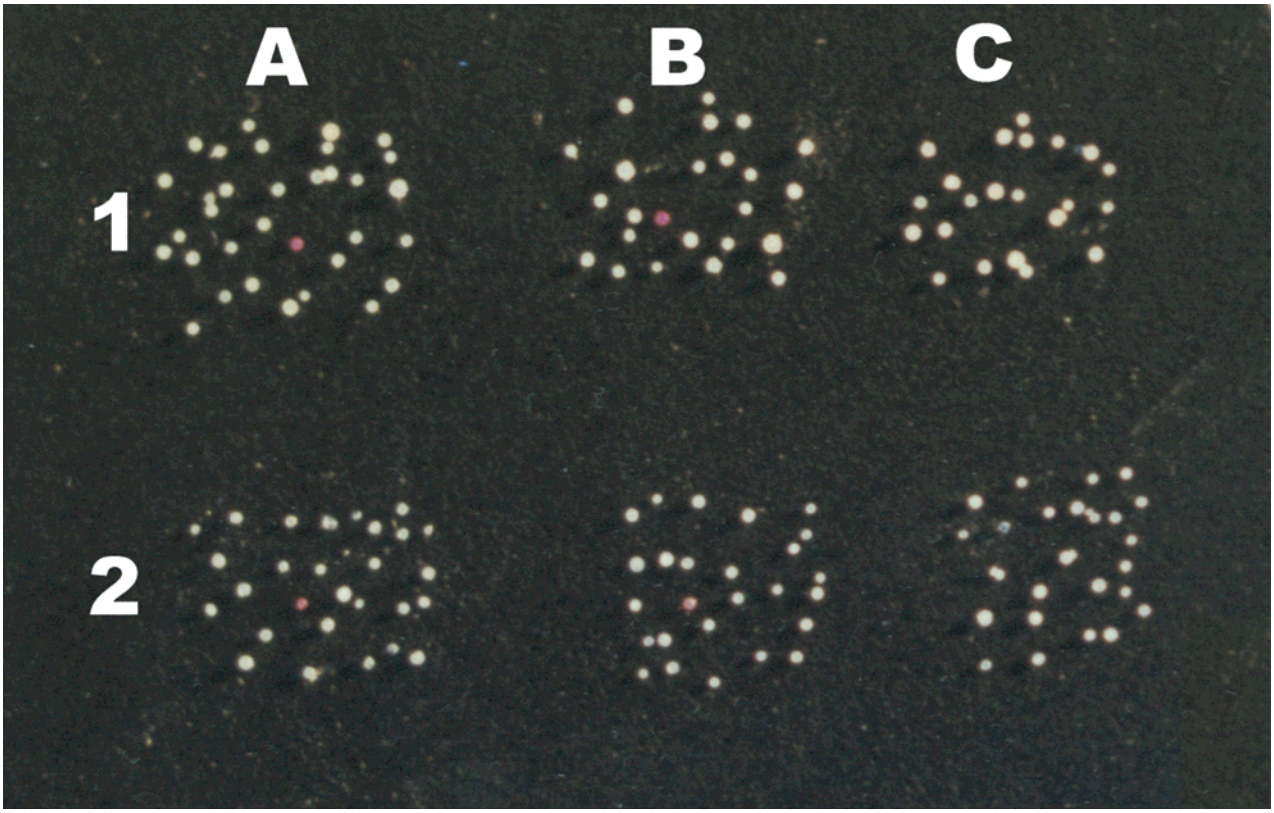

FIGURE 5: Visibility of individual positive beads in a high-throughput screening assay. The photograph was taken under simultaneous visible and UV illumination. Each cluster of beads in this photograph contains ~30 beads that have tethered GPA^{G83L} and a single bead with a tethered native sequence GPA (see Table 1): row 1, Tentagel S beads; and row 2, PEGA beads. These beads were incubated overnight together in the same vial with various concentrations of Texas Red-labeled Arg-GPA in 2 mM SDS: sections 1A and 2A, 20 μM Texas Red-labeled Arg-GPA; sections 1B and 2B, 2 μM Texas Red-labeled Arg-GPA; and sections 1A and 2A, 0.5 μM Texas Red-labeled Arg-GPA. In the leftmost two columns, the single GPA beads are readily distinguishable from the excess of GPA^{G83L} beads by their fluorescence. Each bead is approximately 130 μM in diameter.

peptide in solution. The beads in section 1A were unmanipulated and serve as a negative control, while the beads in section 2A had been fully and directly labeled on their free amino groups with Texas Red. These beads serve as a positive control. The beads in columns B−D were incubated with 20 μM Texas Red-labeled peptide in a 2 mM SDS solution and contain, respectively, no tethered peptide (only amino group acetylation), tethered GPA, and tethered GPA^{G83L}. These were incubated with Texas Red-labeled Arg-GPA (row 1) or with Texas Red-labeled Arg-GPA^{G83L} (row 2). Only the beads in section 1C accumulated the dye-labeled peptide from solution. Importantly, this is the only combination in Figure 4 in which both the bead-tethered peptide *and* the peptide in solution contained the native GPA sequence. Nonspecific interactions with the resin are ruled out by the inability of any other beads to accumulate the dye-labeled peptides, including those with closely related peptide sequences. Proper equilibration with the bead interior, after overnight equilibration at 40 °C, is shown by the observation that the beads in section 1C are nearly as bright as the fully labeled beads in section 2A and that the accumulated dye-labeled peptide is not washed away by several washing steps with a cold SDS solution. We also showed that the association is fully reversible by incubating these beads overnight at ~40 °C with a large excess of an SDS solution and observing that they were no longer fluorescent. The visibility and detectability of the native GPA−GPA interaction are demonstrated by the brightness of the beads in section 1C compared to the other samples. The Texas Red label accumulated by these beads is readily visible to the naked eye under UV light, and the beads are distinctly red even under normal room lights. We estimate that we can visually detect an accumulation of peptide of as little as 2% of the amount in the beads in section 1C; thus, the sensitivity of the assay is very high. Finally, the interactions are shown to be highly specific, native interactions by the observation that the accumulation of the dye-labeled peptide on the beads was completely prevented when either the tethered sequence or the sequence in solution contained the single G83L substitution.

We repeated the experiment depicted in Figure 4 with other Tentagel S beads containing the Arg- and Lys-GPA and Arg- and Lys-GPA^{G83L} sequences shown in Table 1. We also tested the same sequences tethered to the more hydrophilic PEGA beads. In all cases, the results were the same. When the tethered and soluble peptides both contained the native GPA sequence, the beads accumulated the dye-labeled peptides from solution, but when either sequence, or both, contained the G83L substitution, the beads did not accumulate any detectable dye. Thus, we conclude that neither the polymer matrix of the bead nor the terminus of the tethered peptides has a significant effect on the ability of peptides in solution to interact with peptides tethered to polymer microbeads. We also tested the effect of using different concentrations of an SDS solution while maintaining the Texas Red-labeled peptide at 20 μM. The interaction is

relatively insensitive to SDS concentration in the regime between 2 and 20 mM. At a very high SDS concentration of ~70 mM, the specificity of the interactions was maintained; however, the visible accumulation of the Texas Red-labeled GPA on GPA beads was noticeably smaller.

Usefulness as a Screening Assay. Ultimately, this assay is being developed to screen combinatorial peptide libraries for members that have specific lateral interactions with particular helices in detergent solution. As a test of such a high-throughput screen, we mixed a few GPA beads of known numbers with an excess of GPA^{G83L} beads and incubated the bead mixtures with 20, 2, and 0.5 μM Texas Red-labeled Arg-GPA in an SDS solution. For the 20 and 2 μM peptide solutions, the few GPA beads were readily distinguishable from the excess of GPA^{G83L} beads under UV light because the GPA beads readily accumulated the Texas Red-labeled Arg-GPA from solution and the GPA^{G83L} beads did not. However, at the lowest concentration that was tested, 0.5 μM Texas Red-labeled Arg-GPA, the GPA beads were difficult to distinguish from the GPA^{G83L} beads as shown in Figure 5. To show the contrast between the positive and negative beads in this screen, we present in Figure 5 a photograph of ~30 GPA^{G83L} beads from each sample of Tentagel and PEGA beads, along with one of the fluorescent GPA beads from the same sample. This experiment demonstrates that it is possible to clearly and unambiguously distinguish a single bead-tethered peptide sequence with which a labeled peptide interacts from a large excess of very similar but noninteracting sequences. This ability is necessary and sufficient for screening combinatorial peptide libraries for members that interact with a specific membrane-spanning α-helix.

Conclusions. We have shown that we can visually observe highly specific, native interactions between peptides in detergent solution and peptides tethered to polymer microbeads. Importantly, we also showed that we can use this method to select positive, interacting sequences from a large excess of very similar but noninteracting sequences. Taken together, these features fulfill all the requirements for high-throughput screening of combinatorial libraries of potential membrane-spanning α-helices. Screening can be done either visually, for qualitative selection of interacting species, or by digital imaging for quantitative measurements. These techniques thus offer a very powerful, new approach that can be applied to diverse problems in the engineering, design, and characterization of membrane proteins.

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