Identifying Interactions between Transmembrane Helices from the Adenosine A_{2A} Receptor†

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ABSTRACT: The human adenosine A_{2A} receptor ($A_{2A}R$) is an integral membrane protein and a member of the G-protein-coupled receptor (GPCR) superfamily, characterized by seven transmembrane (TM) helices. Although helix−helix association in the lipid bilayer is known to be an essential step in the folding of GPCRs, the determinants of their structures, folding, and assembly in the cell membrane are poorly understood. Previous studies in our group showed that while peptides corresponding to all seven TM domains of $A_{2A}R$ form stable helical structures in detergent micelles and lipid vesicles, they display significant variability in their helical propensity. This finding suggested to us that some TM domains might need to interact with other domains to properly insert and fold in hydrophobic environments. In this study, we assessed the ability of TM peptides to interact in pairwise combinations. We analyzed peptide interactions in hydrophobic milieus using circular dichroism spectroscopy and Förster resonance energy transfer. We find that specific interactions between TM helices occur, leading to additional helical content, especially in weakly helical TM domains, suggesting that some TM domains need a partner for proper folding in the membrane. The approach developed in this study will enable complete analysis of the TM domain interactions and the modeling of a folding pathway for $A_{2A}R$.

The human adenosine A_{2A} receptor ($A_{2A}R$)[1] is a member of the G-protein-coupled receptor (GPCR) superfamily. GPCRs are integral membrane proteins characterized by seven transmembrane (TM) helices that mediate a plethora of cellular signals across the plasma membrane via coupling to G-proteins. They modulate many physiological processes and are linked to numerous human diseases (*1*−*3*) and, consequently, are the targets of an increasingly large number of drugs (*2*). Despite the importance of this class of receptors, knowledge of the mechanisms that drive their folding and assembly into the membrane is limited. A two-stage model was originally proposed to describe the folding of integral membrane proteins (*4*). In this model, TM domains form independent stable helices in the membrane, which associate to form the active native structure. This model was developed on the basis of thermodynamic arguments, as opposed to a kinetic mechanism for the in vivo or in vitro folding of membrane proteins. This model has yet not been fully tested for complex polytopic integral membrane proteins such as GPCRs. However, recent studies suggested that despite its simplification of the actual process of protein insertion and folding as it occurs in vivo, in which chaperons and translocon complexes play major roles (*5*−*8*), it is relevant to membrane protein folding and can serve to guide experimental analysis of folding mechanisms (*9*, *10*).

In vivo and in vitro studies of membrane proteins and helical membrane peptides have shed more light on the folding processes and mechanisms (*11*−*14*). It is currently envisioned that the folding of the helices in the context of the full-length protein might occur by more complex processes and involve some external constraints or interactions with other helices (*9*, *15*−*17*). In the past few years, refinements to the two-stage model have been proposed (*18*, *19*).

GPCRs, with the notable exception of rhodopsin, are difficult to express and purify (*20*). One approach to circumventing these difficulties and gaining insight into the folding and assembly processes of integral membrane proteins is to study peptides corresponding to the TM domains (*9*, *21*−*26*). A major issue we sought to address is whether TM domains have equivalent properties or whether cofolding of TM domains might be generally required in the assembly of large, multihelix membrane proteins.

We are studying the folding and assembly of the human A_{2A} receptor ($A_{2A}R$), as a representative example of human GPCRs. The adenosine family of receptors belongs to class A of GPCRs (rhodopsin-like), and four members have been identified: A_1, A_{2A}, A_{2B}, and A_3. They are linked to cardioprotective and hypertensive effects during periods of

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[1] Abbreviations: GPCR, G-protein-coupled receptor; TM, transmembrane; $A_{2A}R$, adenosine A_{2A} receptor; SDS, sodium dodecyl sulfate; DMPC, dimyristoylphosphatidylcholine; CD, circular dichroism; FRET, Förster resonance energy transfer; T_m, temperature of the phase transition.

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Table 1: Sequences of the Peptides Corresponding to TM5 and TM6 Domains of the Adenosine A_{2A} Receptor and Their Variants

amino acid sequence[a]	name
KS**LAIIVGLFALCWLPLHIINCFTFF**CPD	TM6 native sequence
*KKK***LAIIVGLFAL**A**WLPLHIINCFTFF**APD*KK*	TM6
*KKK***LAIIVALFALĀWLPLHIINCFTFF**ĀPD*KK*	G9A
*KKK***LAIIVḠLFALAWLLLHIINCFTFF**APD*KK*	P17L
*KKK***MNYMVYFNFFACV̄LVPLLLMLGVYL***RKKK*	TM5
*KKK(pyrene)***MNYMVYFNFFACVLVPLLLMLGVYL***RKKK*	TM5Pyr

[a] The residues predicted to be part of the TM domains (http://www.gpcr.org/7tm/) are indicated in bold. The Lys residues added at the N- and C-termini are indicated in italics.

stress such as hypoxia and ischemia. $A_{2A}R$ activates adenylate cyclase through coupling to the G_S-proteins, which trigger a cascade of events, including vasodilation. Adenosine receptors are expressed in various tissues, including blood platelets and vessels, heart, lung, and some brain regions. They are important targets in the search for the molecular origins of cardiovascular disease, and numerous biomedical, clinical, and drug discovery efforts are aimed at these receptors (*27*−*29*).

In a recent study, we showed that peptides corresponding to the seven TM domains of $A_{2A}R$ display significant variability in their helical propensity and tendency to insert into or associate with micelles and vesicles (*26*). Four of the peptides (TM3−TM5 and TM7) exhibit very high helical content, near the predicted maximum for their TM segments. TM1 also adopts a relatively high α-helical content. In contrast, two of the peptides, TM2 and TM6, display low α-helicity. Since all peptides were capable of adopting highly helical structures at high concentrations of TFE, we concluded that intrinsic differences in helical propensity exist among these peptides.

Similarly, Hunt et al. reported that the two C-terminal α-helices in bacteriorhodopsin (BR) do not form stable TM helices in their assays. They then proposed that these two helices might interact with one another during insertion into the membrane, potentially forming a stable α-helical hairpin (*30*), suggesting that the folding of BR involves a mechanism somewhat more elaborate than that envisioned by the two-stage model (*31*). Furthermore, Engelman et al. (*18*) suggest that helix interactions can create internal spaces in the membrane that allow subsequent, less regular polypeptide folding. Interesting questions arise from these observations: Do differences in helical propensity reflect steps in the folding pathway? Do particular TM domains need to interact with other domains to become more helical?

Here, we study interactions between synthesized peptides corresponding to the seven TM domains of $A_{2A}R$ to test the hypothesis that interactions between TM domains are required for proper insertion and folding. We first study interactions between pairs of TM peptides by circular dichroism (CD) spectroscopy. We find that some pairs interact productively to increase helical content, suggesting that interaction between adjacent TMs can help helix formation and assembly. We investigate the TM5−TM6 pair in further detail using variants of the TM6 peptide, by CD and fluorescence spectroscopy, and Förster resonance energy transfer (FRET).

While several investigators have studied association of homo-oligomeric TM proteins (*13*, *32*, *33*) and various designed systems, including hairpins (*34*−*36*), we believe that this work represents the first study of interactions between different individual TM peptides. This study also lays the groundwork for more detailed analysis of integral membrane protein helix−helix interactions. Our goal is to study as many helix−helix pairs as possible to propose a model for the folding and assembly of the human adenosine A_{2A} receptor.

MATERIALS AND METHODS

Peptide Design and Synthesis. Sequences of the seven original A_{2A} TM peptides were published previously (*26*). Table 1 shows the amino acid sequences of the additional peptides used in this study. The peptides were designed to correspond to the sixth TM domain (TM6) of the human adenosine A_{2A} receptor as described previously (*26*). Lysine residues were added at the N- and C-termini of the peptides (*26*). The two cysteines at positions 14 and 28 were substituted with alanines to prevent intramolecular disulfide bonds. The cysteine at position 23 was retained, because it is conserved among nearly all adenosine receptors (*37*). Two variants were constructed (G9A and P17L) to modify the peptide helicity. Peptides were synthesized and purchased from SynPep, Co. TM5 and its pyrene-labeled counterpart (TM5Pyr) have been designed and synthesized as previously reported (*38*). All peptides were purified to >95% purity as judged by HPLC. The identity of the purified peptides was confirmed by mass spectrometry. Peptides were stored at −20 °C as solid powders. With the exception of TM5, all individual peptides appear to be monomeric on the basis of PAGE and CD spectroscopy (*26*, *38*).

Peptide Concentration. Peptide concentrations were determined using two different approaches: by amino acid analysis (performed at the Purdue University Core Facility PSAL) or by measuring the UV absorbance of the peptides in 6 M guanidine-HCl at 280 nm, using appropriate extinction coefficients for the aromatic tryptophan and tyrosine residues (*39*). Prior to measurements, peptide stock solutions were prepared in an acetonitrile/TFA/water mixture.

Preparation of Vesicles and Micelles. SDS micelles and DMPC vesicles were both prepared in 10 mM Hepes and 10 mM KCl (pH 7) as previously described (*26*). Briefly, the lipid films were prepared by dissolving about 10 mg of lipid in chloroform and methanol (2:1) and then drying them under a stream of N_2. Large unilamellar vesicles were obtained after hydrating in 10 mM Hepes and 10 mM KCl (pH 7) and extruding through two stacked polycarbonate membranes (with a pore size of 100 nm) using a Mini Extruder (Lipofast, Avestin, Ottawa, ON). The concentration of SDS was always well above the critical micelle concentration (*40*).

CD Measurements. Far-UV CD spectra of the peptides were recorded on an Aviv model 215 spectrometer equipped

with a Peltier thermally controlled cuvette holder. All measurements were performed at 25 °C unless noted. Peptide concentrations used in CD experiments were in the range of 5−30 μM. Peptide:lipid molar ratios were 1:100 for all measurements in vesicles. For the peptide association experiments, peptides were mixed at equal concentrations.

CD intensities are expressed as the mean residue molar ellipticity [θ], calculated from the equation

$$[\theta] = \theta_{obs}/10lcn \text{ (in deg cm}^2 \text{ dmol}^{-1}\text{)}$$

where θ_{obs} is the observed ellipticity in millidegrees, l is the optical path length in centimeters, c is the final molar concentration of the peptides, and n is the number of amino acid residues. The percentage helicity was calculated according to the method of Chen et al. (*41*), using an empirical equation for helix length dependence, assuming that the residue ellipticity at 222 nm is exclusively due to α-helix.

$$\text{percentage of } \alpha\text{-helix} = [\theta]_{222}/[\theta]_{222}^{max}(1 - k/n) \text{ (in deg cm}^2 \text{ dmol}^{-1}\text{)}$$

where $[\theta]_{222}$ is the observed mean residue ellipticity at 222 nm, $[\theta]_{222}^{max}$ is the theoretical mean residue ellipticity for a helix of infinite length (−39500 at 222 nm), n is number of the residues, and k is a wavelength-dependent constant (2.57 for 222 nm) (*41*, *42*). Although this a relatively old method and fairly simple compared to more modern techniques, recent studies have shown that this method remains the most reliable for calculating the helicity of transmembrane peptides (*10*, *43*). To minimize effects of scattering and to ensure that the CD spectra were observed from peptides in solution, several precautions were taken as previously described (*26*). The spectra were recorded using a 0.1 cm path length quartz cuvette, from 260 to 190 nm, at a 1 nm step resolution and with an integration time of 3 s.

Interactions between peptide pairs were assessed by comparing the experimentally determined spectra of mixed peptides (experimental spectrum) with the calculated average spectrum of the two individual peptides (theoretical spectrum) (*44*). All manipulations of the spectra were performed after appropriate blank/buffer subtraction. If the experimental spectrum differs from the theoretical spectrum (e.g., an increased apparent helical content), we conclude that the peptides interact.

Förster Resonance Energy Transfer (FRET). Fluorescence measurements were performed on an ISS PC-1 spectrofluorimeter, operating in photon-counting mode, using 10 mm × 10 mm or 2 mm × 10 mm quartz cuvettes at 25 °C unless specified. To minimize light scattering effects, all scans were performed with the emission polarizer oriented at 0° and the excitation polarizer at 90°. Concentrations of the peptides were chosen to prevent inner filter effects. To study the interaction between TM5Pyr and TM6 peptide variants, increasing TM5Pyr concentrations were added to constant TM6 peptide concentrations in the presence of DMPC vesicles. The concentration of the unlabeled TM5 peptide was adjusted to keep the total TM5 concentration (TM5 and TM5Pyr) constant. The samples were excited at 295 nm to monitor Trp emission. The emission spectra were collected from 300 to 600 nm. The experiments with DMPC vesicles were performed at 35 °C, which is well above the T_m of the lipid (23 °C).

Sedimentation Equilibrium Analytical Ultracentrifugation. Sedimentation equilibrium experiments were performed at 25 °C using a Beckman Coulter ProteomeLab XL-1 analytical ultracentrifuge with six-sector cells. The samples were centrifuged for 20 h at each speed to achieve equilibrium. Data obtained from absorbance at 280 nm were analyzed and fitted using SEDPHAT (www.analyticalultracentrifugation.com) (*45*). TM5 and TM6 peptides were mixed in equal amounts in 100 mM phosphate buffer (pH 7) containing 10 mM dodecylphosphocholine, density matched using D_2O (*46*). For each global fit, nine data sets were collected, consisting of three different peptide concentrations (5, 10, and 15 μM) analyzed at three different speeds (40 000, 45 000, and 48 000 rpm).

RESULTS AND DISCUSSION

Assessment of Peptide Association by CD Spectroscopy. As we previously reported, the peptides corresponding to the seven TM domains of $A_{2A}R$ have different α-helical contents when inserted into micelles and vesicles (*26*). For instance, TM2 and TM6 peptides display lower helicity than the five others. This result suggested to us that some TM helices might need to interact with other domains to properly fold in hydrophobic environments.

We first used far-UV CD spectroscopy to study interactions between TM helices in membrane-mimetic environments. CD spectra of peptides incorporated in SDS micelles or in DMPC vesicles alone or in pair combination were recorded. The average of single-peptide (20 μM) spectra was calculated to determine their theoretical (noninteracting) spectra, and then compared to their respective experimentally recorded spectra of mixed peptides. Representative examples of interacting and noninteracting peptides are presented in panels A and B of Figure 1, respectively. In Figure 1A, the TM2 + TM3 experimental spectrum shows a net increase in helicity in comparison with the theoretical spectrum, indicating a productive interaction between TM2 and TM3. In contrast, for TM3 and TM6, the experimental and theoretical spectra are very similar (Figure 1B), indicating either that TM3 and TM6 do not interact or that interaction between these peptides does not lead to an increase in helicity.

Data for peptide pairs in SDS micelles are summarized in Figure 2. Of the pairs tested, TM2 and TM3, TM5 and TM6, and TM6 and TM7 exhibit notable increases in helicity, indicating that these pairs interact productively. These change are probably due to helix formation in the less helical TM domain peptides (TM2 and TM6), since TM3−TM5 and TM7 are almost 100% helical in their TM regions (*26*).

These interactions appear to be specific, rather than just aggregation of hydrophobic peptides. For instance, TM6, which is one of the least helical peptides, undergoes changes only when mixed with TM5 or TM7, not with TM3. Similarly, TM2 shows productive interactions with TM3, but not with TM1. We observe the same trend when peptides are mixed in DMPC vesicles. For instance, Figure 3 shows clearly that TM5 and TM6 interact productively in DMPC vesicles. We chose to use the TM5 and TM6 pair as a representative model and as a starting point for further investigation.

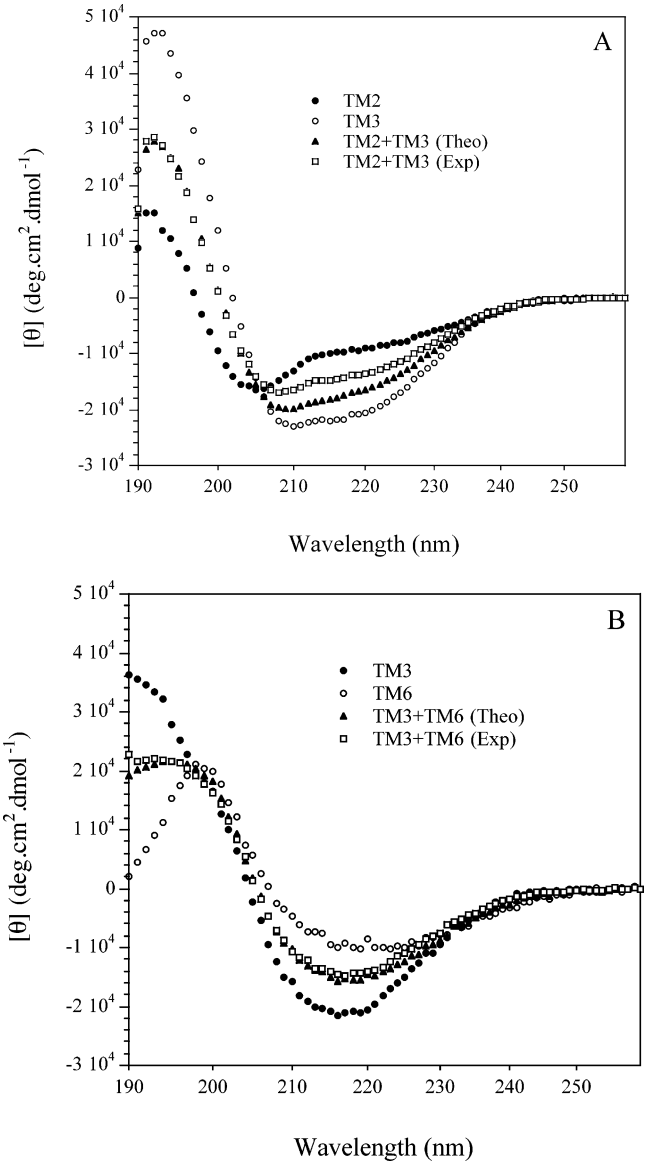

FIGURE 1: Representative CD spectra of interacting and noninteracting peptide pairs in SDS micelles. (A) TM2 (20 μM) (●) mixed with TM3 (20 μM) (○): theoretical spectrum of noninteracting peptides (▲) and spectrum of mixed peptides (20 μM each) (□). (B) TM3 (20 μM) (●) mixed with TM6 (20 μM) (○): theoretical spectrum of noninteracting peptides (▲) and spectrum of mixed peptides (20 μM each) (□).

Secondary Structure Determination of TM6 Peptide Variants. The fact that two of the least helical peptides, TM2 and TM6, are involved in the interacting pairs suggests that α-helical content might play a critical role in peptide interactions. To characterize this effect in further detail, we studied the interaction between TM5 and two TM6 analogues, G9A (where glyince at position 9 has been substituted with alanine) and P17L (where proline at position 17 has been changed to leucine). These changes were made in an attempt to increase peptide helicity; glycine is known for its high flexibility, and proline can be a helix breaker (*47*, *48*).

We first characterized the secondary structure of these variants by CD spectroscopy. Both peptide variants form independent and stable α-helices and show a net increase in their helical content in DMPC vesicles in comparison with TM6 (Figure 4). Similar results were obtained in SDS micelles. The G9A and P17L variants also interact productively with TM5 when incorporated into DMPC vesicles, but induce less change in the CD spectrum than TM6, as shown in Figure 5. The TM5 and TM6 pair undergoes the most change (~70% increase in relative helicity) in comparison with the two other variants. Since we presume that the observed change in the percentage increase in helicity is mainly due to a change in TM6 helicity, it makes sense that the least helical peptides undergo the largest change. As expected, TM6, which is the least helical of the three TM6 variants, shows the most change when mixed with TM5. On the other hand, the helical content of G9A and P17L, which are already more helical than TM6, does not seem to change much. These results then appear to correlate well with the helicity of the TM6 peptides analogues.

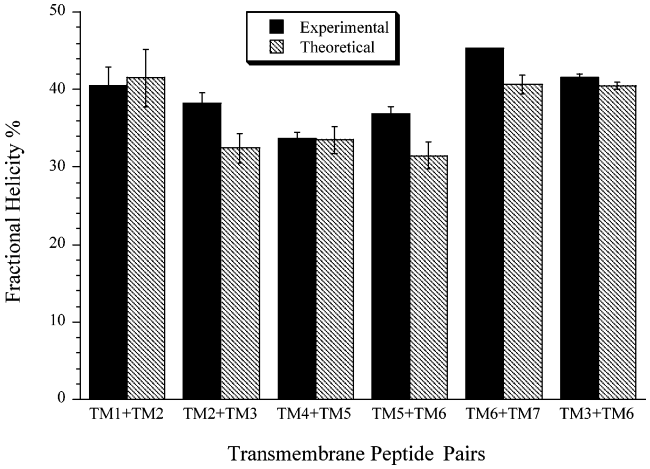

FIGURE 2: Comparison between theoretical and experimental fractional helicities for peptide pair combinations in the presence of SDS micelles. Bars represent the average of three independent experiments; standard errors are given.

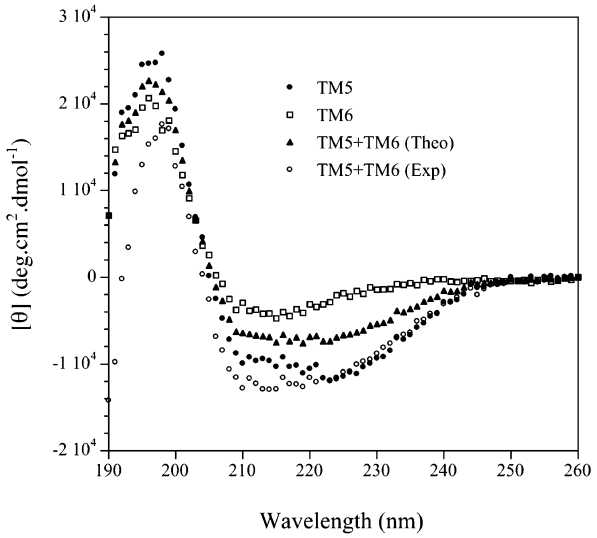

FIGURE 3: Representative CD spectra of TM5 mixed with TM6 in DMPC vesicles. TM5 (20 μM) (●) mixed with TM6 (20 μM) (○): theoretical spectrum of noninteracting peptides (▲) and spectrum of mixed peptides (20 μM each) (□).

Förster Resonance Energy Transfer (FRET). We sought to confirm the conclusions drawn from the CD spectroscopy experiments. We also sought to develop a method for measuring peptide associations even when the interactions do not result in an increase in helicity (e.g., between fully helical peptides). We used Förster resonance energy transfer,

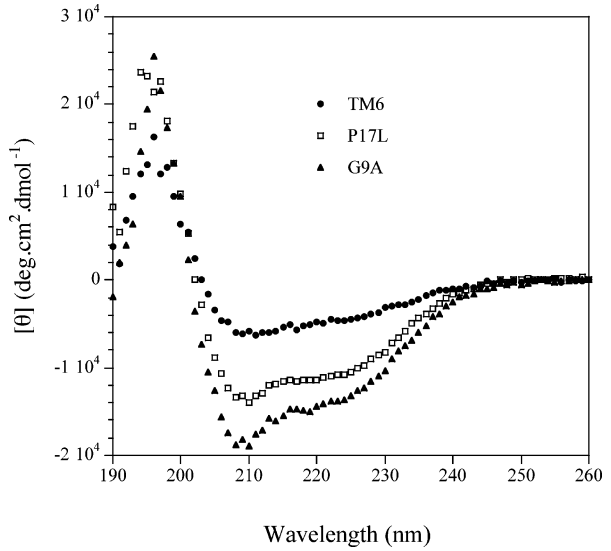

FIGURE 4: CD spectra of TM6 peptides variants (20 μM) in DMPC vesicles at 30 °C: TM6 (□), G9A (●), and P17L (▲).

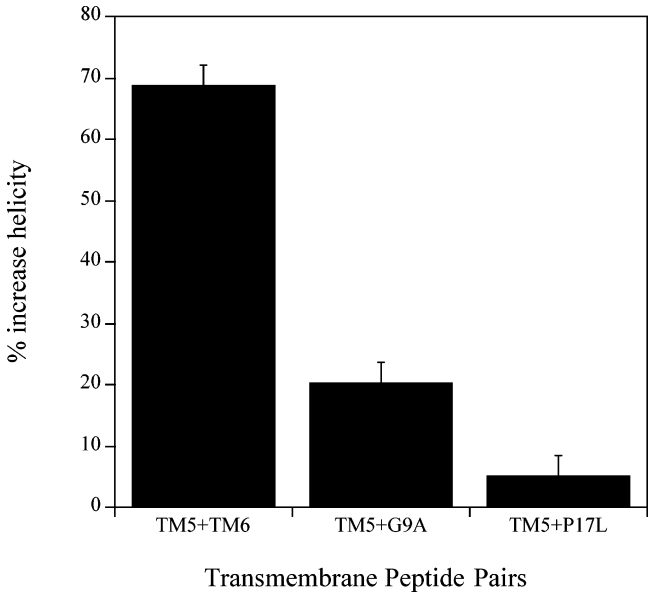

FIGURE 5: Fractional helicity increase for TM5 (20 μM) in combination with TM6, G9A, or P17L (20 μM each) in DMPC vesicles at 30 °C. Bars represent the average from three independent measurements; standard errors are given.

exploiting the properties of two fluorescent groups: tryptophan and pyrene. As shown in Table 1 and described in Materials and Methods, in addition to the "wild-type" TM5 peptide, a variant (TM5Pyr) labeled with a pyrene on the third N-terminal Lys was synthesized (*38*). Since TM6 contains a Trp residue at position 15, we can measure the extent of energy transfer; the fluorescence emission spectrum of Trp overlaps very well with the excitation spectrum of pyrene, leading to an excellent energy transfer between these two groups. The Förster critical distance for energy transfer between Trp and pyrene is 28 Å (*49*), enabling us to monitor helix−helix association. In these experiments, we followed Trp fluorescence emission upon mixing of TM6 variants with increasing concentrations of TM5Pyr in DMPC vesicles. When exciting Trp at 295 nm, we observe a clear decrease in Trp fluorescence emission at 330 nm when TM5Pyr is added to TM6 (Figure 6), indicating a productive energy transfer between Trp and pyrene. This finding confirms our

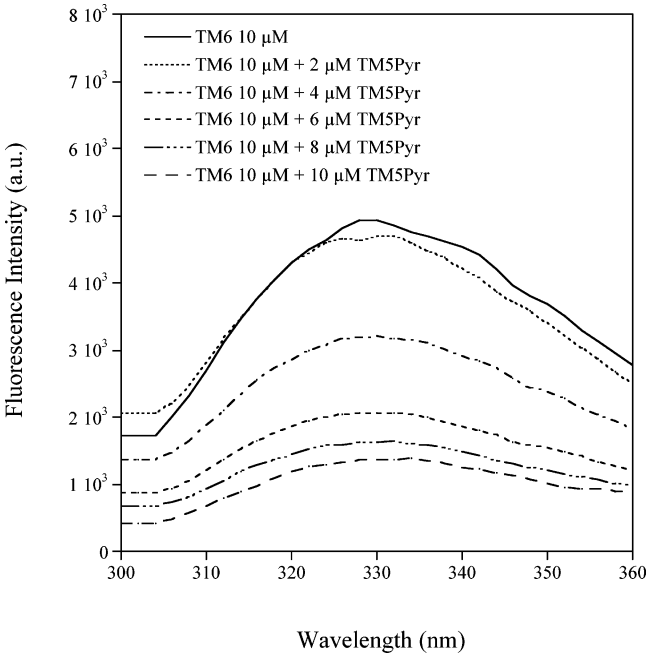

FIGURE 6: TM5−TM6 interaction monitored by FRET. TM6 tryptophan fluorescence emission spectra (300−360 nm) upon excitation at 295 nm in the presence or absence of TM5Pyr. The TM6 peptide concentration was kept constant at 10 μM. The total absorbance in all cases was <0.1 at the excitation wavelength to prevent inner filter effects. The spectra presented in Figure 6 and the spectra used to generate the data in Figure 7 have been baseline subtracted, and were normalized to the pyrene monomer emission peak at 380 nm.

conclusion that TM5 and TM6 interact. Again, this effect is specific: other TM peptides do not exhibit energy transfer with TM5. Moreover, on the basis of fluorescence spectroscopy, gel electrophoresis, analytical ultracentrifugation, and the concentration dependence of the CD signal, there was no appreciable homodimerization of either TM5 or TM6 at the concentrations used in these experiments.

We have not yet determined unambiguously whether the peptide dimers are oriented antiparallel, as would be expected in the native receptor. FTIR experiments performed in DMPC vesicles do show that both peptides are oriented perpendicular to the plane of the membrane (T. Lazarova, personal communication).

Similar results were obtained with G9A and P17L and are summarized in Figure 7. These data clearly indicate that the TM5 peptide interacts productively but differently with each of the TM6 variants. They also correlate very well with the results observed in the CD spectroscopy experiments. Indeed, FRET signals in Figure 7 follow the same trend as the α-helicity presented in Figure 4; the more helical TM6 is, the better the interaction with TM5 seems to be. This finding supports the view that the TM5−TM6 interaction is specific, and not simply a consequence of the hydrophobic nature of these peptides.

In light of these results, we believe that FRET, which monitors peptides' proximity, is a more versatile measure of peptide interactions, as opposed to circular dichroism, which requires a change in helicity. However, we have shown that these two methods correlate very well with each other and are valuable tools for characterizing peptide interactions in membrane-mimetic environments.

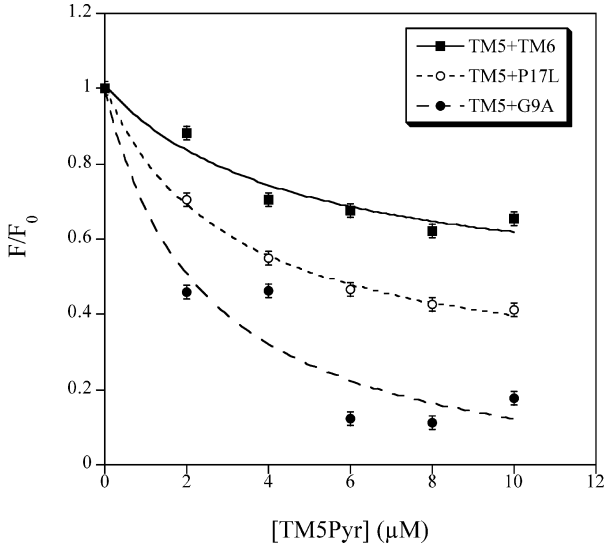

FIGURE 7: Summary of interaction of TM5 with TM6 (■), G9A (●), and P17L (○) monitored by FRET. F/F_0 represents the ratio between the maximum fluorescence at 330 nm of TM6 and its variants in combination with TM5Pyr (F) and the maximum tryptophan fluorescence at 330 nm of TM6 or its variants alone (F_0). Lines correspond to the best fit of a single-species binding model. Standard errors are given.

In a recent study, we showed that among the $A_{2A}R$ peptides the TM5 peptide has the unique property of forming dimers in SDS micelles and in DMPC vesicles, at concentrations above ~10 μM (*38*). We were concerned that TM5 dimers could interfere with the interaction between TM5 and TM6. Therefore, in the TM5−TM6 association experiments, we kept TM5 concentrations under 10 μM to ensure that TM5 remains monomeric.

Finally, to rule out trimer or higher-order oligomer formation, we performed sedimentation equilibrium analytical ultracentrifugation experiments using the TM5−TM6 mixture. The data fit well to a dimeric species, consistent with our model (data not shown). Since TM5 does not dimerize under these conditions (*38*), we conclude that the TM5−TM6 interaction yields discrete dimers.

Structural Implications. It is interesting to consider these results in the context of the structure of the full-length receptor. Assuming that A_{2A} receptor shares the overall topology of rhodopsin, we would expect some helix−helix interactions between all sequential pairs of helices, as well as those that are adjacent in the final structure (e.g., TM3 and TM6). The fact that only some combinations lead to increased helicity (e.g., TM2−TM3 but not TM1−TM2) suggests that our experiments may report on the subset of interactions that occur during the folding process, which lead to increases in helicity, rather than the larger set that are present in the final folded state. Alternatively, the interactions we do observe may just represent the strongest helix−helix contacts. A complete analysis of all pairwise interactions, including CD, FRET, and analytical ultracentrifugation, will allow a more detailed analysis of these features.

CONCLUSION

The aims of this study were to test the hypothesis that interactions between TM domains are required for proper insertion and folding and to lay the groundwork and develop the necessary tools for a complete analysis of the interactions between TM domains of $A_{2A}R$. To achieve these goals, we identified interactions between synthetic peptides corresponding to the seven TM domains of $A_{2A}R$ mixed in pair combinations. The interactions are peptide- and sequence-specific, are limited to dimers, and depend of the structures of the partners. Overall, the results show that specific interactions between some TM helices occur, leading to helix formation, especially in weaker helical TM domain peptides. Thus, some TM domains (e.g., TM6 and TM2) may need partners for proper folding in the membrane. Folding of polytopic integral membrane proteins such as GPCRs is likely to involve complex mechanisms, analogous to those of soluble proteins, in which long-range interactions are needed for establishing secondary and tertiary structures.

This study also shows that we can robustly monitor interactions between peptides, by monitoring changes in either helicity (using CD) or proximity (using FRET). Relating pairwise interactions of the seven TM domains to the folding pathway and structure of the adenosine receptor is the subject of our current investigations.

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