Stable interactions between the transmembrane domains of the adenosine A_{2A} receptor

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Abstract

G-protein-coupled receptors (GPCRs) must properly insert and fold in the membrane to adopt a stable native structure and become biologically active. The interactions between transmembrane (TM) helices are believed to play a major role in these processes. Previous studies in our group showed that specific interactions between TM helices occur, leading to an increase in helical content, especially in weakly helical TM domains, suggesting that helix–helix interactions in addition to helix–lipid interactions facilitate helix formation. They also demonstrated that TM peptides interact in a similar fashion in micelles and lipid vesicles, as they exhibit relatively similar thermal stability and α -helicity inserted in SDS micelles to that observed in liposomes. In this study, we perform an analysis of pairwise interactions between peptides corresponding to the seven TM domains of the human A_{2A} receptor (A_{2A}R). We used a combination of Förster resonance energy transfer (FRET) measurement and circular dichroism (CD) spectroscopy to detect and analyze these interactions in detergent micelles. We found that strong and specific interactions occur in only seven of the 28 possible peptide pairs. Furthermore, not all interactions, identified by FRET, lead to a change in helicity. Our results identify stabilizing contacts that are likely related to the stability of the receptor and that are consistent with what is known about the three-dimensional structure and stability of rhodopsin and the β_2 adrenergic receptor.

Keywords: protein structure/folding; membrane proteins; circular dichroism; fluorescence; FRET; GPCR; transmembrane peptides

Membrane receptors are essential mediators of signal transduction between cells and their environments, between compartments within cells, and between different organs. Consequently, proper functioning of these mem-

brane proteins is vital to health, and specific defects are associated with many known human diseases (Shichida and Imai 1998; Gether 2000; Gurrath 2001). G-protein-coupled receptors (GPCRs) are the largest class of membrane proteins in the human genome and regulate and control a wide range of cell processes. Despite recent progress, structural characterization of GPCRs has been particularly challenging.

Difficulties associated with structural and functional characterization of GPCRs arise in part from our limited understanding of the complex mechanisms that underlie their folding, assembly, stability, and conformational flexibility in the membrane (Booth and Curnow 2006; Mackenzie 2006). Interactions between transmembrane (TM) α -helices are believed to play a major role in these processes (Popot and Engelman 1990; Haltia and Freire 1995; White and Wimley 1999). It is therefore appealing to identify specific interactions between secondary structure elements of

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Abbreviations: GPCR, G-protein-coupled receptor; TM, transmembrane; A_{2A}R, adenosine A_{2A} receptor; SDS, sodium dodecyl sulfate; CD, circular dichroism; FRET, Förster resonance energy transfer; PAGE, polyacrylamide gel electrophoresis; DMPC, dimyristoyl phosphatidylcholine; DMPG, dimyristoyl phosphatidylglycerol; D β M, *n*-dodecyl- β -D-maltoside.

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membrane proteins and to seek the structural determinants of such interactions. Moreover, several diseases are associated with mutations that cause destabilization and misfolding of GPCRs (Sung et al. 1991, 1993; Bai et al. 1996; Tanaka et al. 1998; Themmen and Huhtaniemi 2000; Morello and Bichet 2001), thus determining the molecular interactions that stabilize or destabilize GPCRs is fundamental to our understanding of their structure and function.

In the two-stage model, Popot and Engelman hypothesized that, for helical membrane proteins, the “final structure in the transmembrane region results from the accretion of smaller elements (helices), each of which has reached thermodynamic equilibrium with the lipid and aqueous phases before packing” (Popot and Engelman 1990). Experimental support for the two-stage model and the role of helix–helix interactions includes the observation that lateral association of coexpressed protein fragments can regenerate functional polytopic proteins such as bacteriorhodopsin (Huang et al. 1981; London and Khorana 1982; Liao and Khorana 1984; Popot et al. 1986, 1987), the lactose permease (Bibi and Kaback 1990), the α -factor receptor of the yeast *Saccharomyces cerevisiae* (Martin et al. 1999), or the Na^+ – Ca^{2+} exchanger (Ottolia et al. 2001). Moreover, atomic force microscopy data have revealed that upon pulling the C(OOH) terminus of an individual bacteriorhodopsin, the protein is extracted from native purple membrane two helices at a time (Oesterhelt et al. 2000). TM domain interactions are not only a major determinant in the assembly and stability of the native structure of membrane proteins, but also seem to be crucial during the initial folding of helices. For example, it has been proposed that for multispanning membrane proteins, the integration of a TM domain could be facilitated by previously synthesized TM helices (Rapoport et al. 2004). It has also been shown for a double-spanning model protein that a weakly helical TM domain, which is unstable in the lipid phase on its own, is released into bulk lipids by the translocon upon interaction with a previously integrated TM helix (Heinrich and Rapoport 2003). This implies that hydrophobicity is not the only interaction involved in this process and that other factors, such as the amino acid sequence, could also be involved. In other cases, individual TM domains can assume the proper orientation in the translocon but must be paired with another TM helix for efficient integration into the bilayer (van Geest and Lolkema 2000). It has also been suggested that the translocon may be able to accommodate multiple TM helices inside its pore and that they may leave it in pairs or as a group (Johnson and van Waes 1999), although the subsequent determination of the translocon structure does not reveal a space in which it could happen. Taken together, these observations indicate that at least some TM helices need to interact with neighboring helices in the protein to insert properly

and adopt the correct topology in the membrane (Skach and Lingappa 1993; Lin and Addison 1995; Heinrich and Rapoport 2003; Alder and Johnson 2004).

Understanding the folding of membrane proteins can be simplified by considering the insertion process and helix packing in the bilayer separately, as proposed by the two-stage model (Popot and Engelman 1990). Despite its simplification, this model can guide experimental analysis of folding mechanisms (Hunt et al. 1997; Xie et al. 2000; Mackenzie 2006). It suggests that studying peptides corresponding to the individual TM domains is relevant to understanding the overall folding process (Reddy et al. 1994; Deber et al. 1999; Xie et al. 2000; Katragadda et al. 2001; Naider et al. 2001; Lazarova et al. 2004; Compton et al. 2006). Usefully, employing synthetic peptides also circumvents difficulties associated with the expression, purification, and reconstitution of full-length proteins.

In our previous studies, we used synthetic peptides to study the folding and the assembly of GPCRs, and, more specifically, of the human adenosine $\text{A}_{2\text{A}}$ receptor ($\text{A}_{2\text{A}}\text{R}$). While peptides corresponding to each of the seven TM domains of $\text{A}_{2\text{A}}\text{R}$ form independent and stable helical structures in detergent micelles and lipid vesicles, they display significant variability in their helical propensity (Lazarova et al. 2004). Moreover, we found that there is no correlation between hydrophobicity and helical propensity of a single TM domain. While these findings strongly support the first stage of the two-stage folding mechanism, they also raise questions about the role of helix–helix interactions for proper folding and stability of some TM domains in the membrane. Thus, we have chosen to study interactions between TM helices (Thévenin et al. 2005b). Using circular dichroism (CD) spectroscopy and Förster resonance energy transfer (FRET), we found that specific interactions between some TM helices do exist, leading to an increase in helical content, especially in weakly helical TM domains, suggesting that some TM domains need a partner for complete folding in the membrane (Thévenin et al. 2005b). These studies show that we can detect interactions between peptides corresponding to the TM domains of $\text{A}_{2\text{A}}\text{R}$ by mixing them in membrane-mimetic environments and monitoring changes in either helicity or proximity. They also show that each of the seven TM peptides forms a stable and independent α -helical structure in SDS micelles and adopts a higher helical content than in other detergents such as *n*-dodecyl- β -D-maltoside (D β M) and Triton X-100 micelles (Lazarova et al. 2004). Moreover, we have demonstrated that $\text{A}_{2\text{A}}\text{R}$ TM peptides interact in a similar fashion in SDS micelles and DMPC vesicles, as they exhibit relatively similar thermal stability and α -helicity inserted in SDS micelles to that observed in lipid vesicles (Thévenin et al. 2005b). Even though the use of detergents may obscure contributions of protein–lipid or lipid–lipid interactions that would be present in a membrane (Mackenzie

2006), detergent micelles have been proven to be a useful environment in which to study interactions in membrane proteins by various biophysical methods (Cristian et al. 2003; DeGrado et al. 2003; Doura and Fleming 2004; Lear et al. 2004). For instance, there is a remarkably good agreement between the apparent free energy scale obtained by the TOXCAT biological assay (i.e., in lipid bilayer) and the free energy differences from sedimentation equilibrium studies (i.e., in detergent micelles) for point mutants of the glycophorin A, indicating that sequence changes usually affect helix–helix interactions quite similarly in these two very different environments (Duong et al. 2007). More particularly, SDS micelles have also been demonstrated to be a useful milieu (Fisher et al. 1999; Orzáez et al. 2000; Zhou et al. 2000; Fisher et al. 2003), because the disruptive nature of this detergent permits the detection of the strongest helix interactions and because they provide a low amount of turbidity and light scattering (Hoyt and Gierasch 1991; Garavito and Ferguson-Miller 2001). Moreover, the secondary structure of the KcsA potassium channel obtained by nuclear magnetic resonance in SDS micelles (Chill et al. 2006) was found to closely agree with the KcsA crystal structure (Doyle et al. 1998). These findings indicate that the micelle of the anionic SDS detergent is an appropriate membrane-mimetic environment for studying strong and stable interactions between the TM domains of A_{2A}R.

In this study, we perform an exhaustive analysis of interactions between all seven TM helices of A_{2A}R mixed in pairwise combination, with the goal of identifying structural determinants of receptor stability. We found that strong and specific interactions occur in a small subset of peptide pairs. Only seven of the 28 possible pairs have sufficient interaction strength to be observed in SDS micelles. Furthermore, not all pairs detected as interacting pairs by FRET undergo changes in helicity, indicating a variability of the driving and stabilizing forces involved in helix–helix interactions. Overall, our results provide new insights into the stability and folding of the A_{2A} receptor in vitro.

Results

Table 1 presents the synthetic peptides used in this study. The extent of interactions between two TM peptides was monitored by FRET, using tryptophan (Trp) as a donor and pyrene (Pyr) as an acceptor (Thévenin et al. 2005b). FRET measurements were conducted by measuring the intensity of Trp emission upon mixing of Trp-labeled TM peptide with increasing concentrations of Pyr-labeled peptide in SDS micelles. If the two peptides are close enough for FRET to occur (Förster critical distance for energy transfer between Trp and Pyr is at ~28 Å) (Lakowicz 1999), one should observe a decrease of the

Table 1. Sequences of the peptides corresponding to the seven TM domains of A_{2A}R and their variants

Amino acid sequences ^a	Name
KKKVYITVELAIAVLAILGNVLVCWAKKKK	TM1W
KKKVYITVELAIAVLAILGNVLVCYAKKKK	TM1
KKK(Pyrene)VYITVELAIAVLAILGNVLVCYAKKKK	TM1Pyr
KKKFVVSLLAAADIAVGVLAIPIWAIITIKKKK	TM2W
KKKFVVSLLAAADIAVGVLAIPIYAITIKKKK	TM2
KKK(Pyrene)FVVSLLAAADIAVGVLAIPIYAITIKKKK	TM2Pyr
KKKLFIACFVLVLTQSSIFSLLAIAIDRWKKK	TM3W
KKKLFIACFVLVLTQSSIFSLLAIAIDRYKKK	TM3
KKK(Pyrene)LFIACFVLVLTQSSIFSLLAIAIDRYKKK	TM3Pyr
KKKRAKGHIAICWVLSFAIGLTPMLGWKKKK	TM4W
KKKRAKGHIAICWVLSFAIGLTPMLGYKKKK	TM4
KKK(Pyrene)RAKGHIAICWVLSFAIGLTPMLGYKKKK	TM4Pyr
KKKMNVWMVYFNFFACVLVPLLLMLGVYLRKKK	TM5W
KKKMNVWMVYFNFFACVLVPLLLMLGVYLRKKK	TM5
KKK(Pyrene)MNVWMVYFNFFACVLVPLLLMLGVYLRKKK	TM5Pyr
KKKLAIIIVGLFALAWLPLHIINCFTFFAPDCK	TM6W
KKKLWLMYLAIVLSHTNSVVPFIYAYRIREK	TM7W

^aThe residues predicted to be part of the TM domains (<http://www.gpcr.org/7tm/>) are indicated in bold. The lysines added at the N and C termini are indicated in italics. The residues that differ from the native A_{2A}R sequence are underlined.

donor (Trp) emission at 330 nm. Given seven TM helices, there are 21 possible heterologous pairs and seven possible self-interactions. Of these, 20 heterologous pairs and five self-interactions were examined. A decrease of the Trp fluorescence emission, indicating a productive FRET, was observed for eight pairs. Experimental energy transfer efficiencies (F/F_0) were obtained as described in Materials and Methods. The resulting curves for the eight interacting pairs are presented in Figure 1. In each case, the linear dependence of F/F_0 on the acceptor mole ratio is indicative of heterodimer formation. Importantly, not all pairs showed a measurable change of Trp fluorescence intensity of the donor upon addition of increasing concentrations of the acceptor peptide. Two representative spectra of such pairs are shown in Figure 2. Unlike in interacting pairs, the F/F_0 ratio did not change significantly, indicating that no productive energy transfer occurs between the peptides constituting these pairs. Similar F/F_0 profiles were observed for 22 out of 31 tested pairs. These findings suggest that the sequences of the peptides composing these pairs do not favor heterodimer formation. It is worth mentioning that the data points cover only a limited range of concentration, not allowing determination of the binding constant K_d , since the use of higher peptide concentrations for the titration would lead to an increase of the inner filter effect, which, in turn, could significantly corrupt the experimental data. Nevertheless, these results can be used as a qualitative measure for peptide–peptide interactions.

Figure 1. Summary of energy transfer efficiencies for all peptide pairs presenting productive FRET in SDS micelles. Data were fitted to a linear regression model. The dependence on the acceptor mole ratio is linear indicative of heterodimer formation. Points represent the average of at least three independent experiments; standard errors are given. For specifics regarding each panel, please refer to text.

To investigate whether the interactions between the TM peptides are specific, six pairs were tested with a reversed labeling scheme (TMaW + TMbPyr). A summary of the FRET results for all 31 pairs tested is presented in Table 2. The gray-shaded areas in Table 2 correspond to pairs that have already been tested in the upper part of the table. For instance, the pair TM5Pyr + TM2W corresponds to the pair TM2Pyr + TM5W, which has been tested. In all explored cases, similar F/F_0 values were obtained for TMaPyr + TMbW versus TMbPyr + TMaW. For example, TM2 and TM3 peptides interacted in the same fashion in both cases (Fig. 1B,C; Table 2), while TM1 and TM4 did not display an interaction no matter how they are labeled. These findings support the view that the interactions between peptides are strongly specific.

Moreover, they further validate the capability of our approach to detect possible interactions between TM domains, and that the pyrene molecule does not perturb these interactions. As we have shown previously, the modifications made to the A_{2A}R TM peptides do not affect their secondary structure (Lazarova et al. 2004).

Moreover, we also showed that all of the peptides except TM5 are monomeric. TM5 appears to be the only peptide to display (1) a ratio of $[\theta]_{222}/[\theta]_{208} > 1$ by CD spectroscopy, (2) a band corresponding to its dimer form by PAGE, and (3) the formation of excimer by fluorescence spectroscopy (Thévenin et al. 2005a). We have also shown by mutagenesis of the full-length A_{2A}R that TM5 may mediate the known dimerization of A_{2A}R (Canals et al. 2004; Thévenin et al. 2005a).

Figure 2. Representative examples of FRET measurement results for noninteracting pairs in SDS micelles. Data were fitted to a linear regression model. The slope of the line is not significantly different from 0, indicating that no heterodimer formation occurs. Points represent the average of at least three independent experiments; standard errors are given.

CD spectroscopy was used to monitor whether the interacting pairs identified by FRET undergo changes in their helicity. CD spectra of peptides incorporated in SDS micelles alone or in pairwise combinations were recorded. The average of single-peptide spectra were calculated to determine their theoretical (noninteracting) spectra, and then compared to their respective experimentally recorded spectra of mixed peptides. The spectra for TM1 + TM7, TM3 + TM4, and TM3 + TM7 are presented in Figure 3. The results for the other pairs have already been reported in our previous studies (Thévenin et al. 2005a,b). The data from the methods used to assess helix–helix interactions are summarized in Table 3.

The comparison between CD and FRET data shows that pairs identified by CD with increased α -helicity (Thévenin et al. 2005b) are also identified as interacting by FRET (TM2 + TM3 and TM5 + TM6). On the other hand, some pairs, composed by peptides exhibiting relatively high helicity on their own (Lazarova et al. 2004), display interaction by FRET but do not exhibit a measurable change in their α -helical content (TM1 + TM7, TM3 + TM4, and TM3 + TM7). Therefore, these data show that helix–helix interactions are not always coupled to an increase in α -helicity. Taken together, these results

show that CD and FRET are complementary and efficient in identifying specific interactions between peptides, and that they correlate very well with each other.

Discussion

The aim of this study was to perform a comprehensive analysis of every pairwise interaction between peptides corresponding to the seven TM domains of the human adenosine A_{2A} receptor, to gain insights on the stability and assembly of the full-length receptor. While FRET is a useful method to measure the strength of TM interaction, it cannot assess changes in helicity induced by these interactions, whereas CD spectroscopy can. Thus, combining the methods gives a more complete view.

The analysis of the FRET data demonstrates that stable contacts in SDS micelles occur only between certain specific peptides (seven of the 28 possible pairs of helices). These results clearly show that these interactions are strong and specific. Among the seven TM domains, TM3 exhibits the highest tendency to interact, forming pairs with four other peptides (TM2, TM4, TM6, and TM7), while TM1, TM2, and TM4 interact only with one other peptide (Table 2). TM6 interacts with three other peptides, and TM5 and

Table 2. Summary of TM helix interactions seen by FRET for the 31 studied peptide pairs mixed in SDS micelles

	TM1W	TM2W	TM3W	TM4W	TM5W	TM6W	TM7W
TM1Pyr	×	×	×	×	×	×	✓
TM2Pyr	×	×	✓	×	×	×	×
TM3Pyr	×	✓	×	✓	×	✓	✓
TM4Pyr	×	×	✓	×	×	×	×
TM5Pyr					✓	✓	×
TM6Pyr						N/D	N/D
TM7Pyr							N/D

Green checkmark (✓): FRET observed. Red cross (×): No FRET observed. N/D: Not Determined (monitoring FRET was not possible for these pairs because the constituting peptides were not available).

Figure 3. CD spectra of noninteracting peptide pairs in SDS micelles. (A) (Filled circles) TM1 (20 μ M) mixed with (open circles) TM7 (20 μ M); (B) (Filled circles) TM3 (20 μ M) mixed with (open circles) TM4 (20 μ M); (C) (Filled circles) TM3 (20 μ M) mixed with (open circles) TM7 (20 μ M). For each figure, (filled triangles) the theoretical spectrum of noninteracting peptides and (open squares) the spectrum of mixed peptides (20 μ M each) are shown. The results for the other pairs have already been reported in our previous studies (Thévenin et al. 2005a,b).

TM7 interact with two partners. Importantly, not all interacting pairs identified by FRET reveal an increase in helicity. CD measurements showed that two of the least α -helical peptides, TM2 and TM6 (Lazarova et al. 2004; Thévenin et al. 2005b), are involved in the only three interacting pairs displaying an increase in helicity (TM2 + TM3, TM3 + TM6, and TM6 + TM7). Since SDS micelles preserve only the stronger helix interactions (Fleming and Engelman 2001), these observations identify key points of stability for the folding and function of this GPCR.

In some cases, the helix–helix interaction appears to increase the extent of helix formation. Consider helix 6: As previously shown, the TM6 peptide forms an independent helical structure, but its helicity strongly depends on the environment. Indeed, this peptide folds into an α -helical structure in SDS micelles and DMPC vesicles, but forms β -sheet aggregates in *n*-dodecyl- β -D-maltoside (D β M) and Triton X-100 micelles (Lazarova et al. 2004). The same study also reported that most of the peptides corresponding to the TM domains of A_{2A}R exhibit higher α -helicity in anionic SDS than in neutral D β M micelles or even liposomes, even though SDS and D β M share similar hydrophobic properties (i.e., carrying the same C₁₂ hydrophobic tail). More dramatically, TM2 is disordered in zwitterionic DMPC vesicles, but α -helical in negatively charged DMPC/DMPG vesicles (Lazarova et al. 2004). Our results show that both lipid–peptide contacts and peptide–peptide contacts help to give a proper environment for the folding of some of the TM helices of A_{2A}R. They also provide strong experimental evidence that in addition to the hydrophobic potential, the electrostatic field may contribute to the initiation of the helix formation and folding.

The role of α -helicity in helix–helix interactions

We have previously proposed that a change in α -helicity plays a role in the interaction between the TM5 and TM6 domains of A_{2A}R (Thévenin et al. 2005a). CD measurements showed that the TM6 peptide, which displays a low α -helical content, could not fold and insert properly in detergent micelles and lipid bilayers by itself. However, it exhibits a higher helicity in the presence of TM5, indicating that this particular TM domain needs a partner with a higher helicity, such as TM5, to properly fold and insert in the membrane. This is in agreement with the fact that, in the present experiments, pre-mixing of TM5 and TM6 peptides is necessary prior to the addition of the surfactant. Altogether, these findings suggest that interactions between some TM peptides might occur before their partition in the membrane, as it has been proposed for the translocon machinery (Mothes et al. 1997; Johnson and van Waes 1999; van Geest and Lolkema 2000).

Table 3. Comparison of TM helix pairwise interactions seen by FRET, circular dichroism, fluorescence, and PAGE

	TM1	TM2	TM3	TM4	TM5	TM6	TM7
TM1	CD: ✗ ^b Excimer: ✗ ^b PAGE: ✗	FRET: ✗ ^a CD: ✗	FRET: ✗	FRET: ✗	FRET: ✗	FRET: ✗	FRET: ✓ CD: ✗
TM2		CD: ✗ ^b Excimer: ✗ ^b PAGE: ✗	FRET: ✓ ^a CD: ✓	FRET: ✗	FRET: ✗	FRET: ✗	FRET: ✗
TM3			CD: ✗ ^b Excimer: ✗ ^b PAGE: ✗	FRET: ✓ CD: ✗	FRET: ✗	FRET: ✓ ^a CD: ✗	FRET: ✓ CD: ✗
TM4				CD: ✗ ^b Excimer: ✗ ^b PAGE: ✗	FRET: ✗ ^a CD: ✗	FRET: ✗	FRET: ✗
TM5					^b FRET: ✓ ^b CD: ✓ ^b Excimer: ✓ ^b PAGE: ✓	FRET: ✓ ^a CD: ✓	FRET: ✗
TM6						^b CD: ✗ ^b PAGE: ✗	^a CD: ✓
TM7							^b CD: ✗ ^b PAGE: ✗

Green check mark (✓): Interaction observed. Red cross (✗): No interaction observed. ^{a,b} Data reported in our previous studies (Thévenin et al. 2005a; Thévenin et al. 2005b).

Similarly to TM6, TM2, which also shows a low α -helical content, may need the support of the neighboring TM3 domain for its proper folding and insertion in the membrane. The qualitative comparison of these data shows that, while TM5 + TM6 (Fig. 1H) displays a steep quenching curve, the curve for TM2 + TM3 (Fig. 1B,C) is nearly saturated in the same concentration range, suggesting a stronger interaction between TM2 and TM3 peptides. Furthermore, these cases suggest that the helix–helix interaction energy is favorable enough to overcome the unfavorable TM2 and TM6 helix formation energy. On the other hand, in the case of the TM3 + TM6 pair, which also combines a highly helical peptide (TM3) and a low helical peptide (TM6), the interaction does not result in an increase of helicity. It is important to point out that TM3 and TM6 are not adjacent in the sequence of A_{2A}R and are, therefore, not expected to assist each other during the initial helix formation and insertion. Moreover, the other interacting pairs (TM1 + TM7, TM3 + TM4, and TM3 + TM7), which are composed of the most helical TM domains of A_{2A}R (Lazarova et al. 2004), were identified as interacting by FRET, but do not display an increase of helicity by CD spectroscopy (Table 3). However, we would only expect an increase in helicity for the less helical peptides. It is also possible in these cases that the intermolecular dimerization surface is already present in the α -helical monomers. The possible role of these interactions in the stability of the full-length A_{2A}R is discussed below. Taken together, these observations imply that the α -helical content plays a role in TM domain interaction. However, interactions not resulting in a change of helicity suggest that the association of TM domains in the full-length receptor is likely to be a complex process, mediated by a variety of forces.

Relating TM peptide–peptide interaction to the folding and stability of the full-length A_{2A} receptor

Even though we recognize the fact that loops contribute to thermodynamic stability and that they are often required for the correct folding and function of integral membrane proteins (Kahn et al. 1992; Marti 1998; Kim et al. 2001; Ulmschneider et al. 2005), we believe that their absence in our model system is not detrimental to our aim of study. Indeed, as it has been shown extensively for bacteriorhodopsin (Liao and Khorana 1984; Popot et al. 1987; Luneberg et al. 1998; Marti 1998) and other membrane proteins (Kobilka et al. 1988; Stühmer et al. 1989; Bibi and Kaback 1990; Berkower and Michaelis 1991; Tang et al. 1991; Zen et al. 1994; Schöneberg et al. 1995), TM helices can often find their way to the final compact state in the absence of loops. Moreover, the specificity of helix–helix packing does not depend on the connecting links (White and Wimley 1999). In the case of the full-length A_{2A}R, the loop connecting TM1 to TM2 is short (Fig. 4A), but no interaction is observed between these domains. On the other hand, TM5 and TM6 domains, which are connected by a fairly long and flexible loop, interact in our assay without the help of a loop. We consider that the loops are not absolutely required for bringing the helices together or holding them together correctly, as it has been suggested in other studies (Kahn et al. 1992; Marti 1998).

The fact that we identify helix–helix interactions in SDS micelles implies that these interactions represent the strongest helix–helix contacts. It is also consistent with the observation that SDS-denatured bacteriorhodopsin, which retains ~60% of its native α -helical structure, gains helicity in distinct kinetic phases (Booth et al. 1997).

Figure 4. (A) Schematic representation of the human adenosine A_{2A}R. (B) Top view of the organization of the membrane-embedded helices of A_{2A}R obtained by homology modeling based on the X-ray crystal structure of bovine rhodopsin (Palczewski et al. 2000). (Circles) Helices; (arrows) observed interactions between helices.

Booth and colleagues hypothesized from experiments with bacteriorhodopsin and GPCRs (Riley et al. 1997; Marti 1998; Booth and Curran 1999; Booth et al. 2001; Rader et al. 2004; Klein-Seetharaman 2005) that membrane proteins can form a partially structured “folding core” composed by pre-existing helices that are stable in SDS (Booth and Curnow 2006). This core will act as a nucleation site upon which remaining helices can be strengthened, ultimately leading to the assembly of the native structure. In the case of A_{2A}R, we believe that TM2, TM3, TM4, and TM6 could form such a core. The fact that TM3 also interacts with TM6 and TM7, which are thought to be close to each other in the three-dimensional model of A_{2A}R based on the X-ray crystal structure of bovine rhodopsin (Fig. 4B; Palczewski et al. 2000), supports this hypothesis. Thus, the interactions within TM3 + TM6, TM3 + TM7, and, more importantly, TM1 + TM7 are likely to take place during the last stages of helix packing, stabilizing the final native structure of A_{2A}R.

Furthermore, the postulated core for A_{2A}R is also in good correlation with the rigid core, identified for bovine rhodopsin by dynamic force spectroscopy (Sapra et al. 2008) and by computational studies (Rader et al. 2004; Tastan et al. 2007), which includes elements from α -helices II, III, IV, and V. It is further strengthened by the recently reported crystal structure of the human β 2-adrenergic receptor (β 2AR) (Cherezov et al. 2007; Rasmussen et al. 2007), which suggests that structurally conserved helices provide a common core present throughout the class A GPCRs (Cherezov et al. 2007). It would be of great interest to confirm whether this helix

bundle might form by studying three or more peptides at a time and determining whether they facilitate and stabilize the assembly. In addition, the interaction observed between TM3 and TM6 is in accord with the X-ray structures of both bovine rhodopsin and β 2AR and with the role of these two helices in G-protein coupling. Indeed, in the inactive state of rhodopsin, helix III, and helix VI interact via a network of hydrogen bonds and charge interactions referred to as the “ionic lock” (Ballesteros et al. 2001). These interactions maintain bovine rhodopsin in an inactive conformation. The ionic lock residues are highly conserved in the rhodopsin family members and seem to have a similar role in β 2AR (Scheer et al. 2000; Ballesteros et al. 2001). Since these residues are also found in A_{2A}R, it is very likely that a similar interaction between TM3 and TM6 is involved in the G-protein-coupling mechanism.

Moreover, we have previously demonstrated that TM5 is the only domain to homodimerize and that it may be involved in the dimerization of A_{2A}R (Thévenin et al. 2005a). We have also found that the TM5 homodimers are stable to temperature and chemical denaturation, indicating that strong interactions are involved. These interactions may contribute to the further stabilization of the receptor by the formation of dimers.

Finally, the interactions we observed might also occur as steps in the folding process. As discussed above, our results are consistent with the progressive emergence of the TM domains and with what is known for the translocon machinery. For example, TM3 and TM5 could assist TM2 and TM6, respectively, to fold and insert in

the membrane, as Heinrich and Rapoport (2003) have previously suggested. Alternatively, TM5 could wait inside the pore of the translocon for TM6 to be properly folded before being released into the bulk lipids. In both cases, the interactions help the weaker helix to properly fold and insert. Moreover, the identification of an interaction between TM1 and TM7 confirms its expected role in ultimately closing the seven-helix bundle and stabilizing the native structure of the receptor.

Overall, our results show that specific and strong interactions between some pairs of TM helices occur, leading in some cases to the formation of additional helical content, especially in less helical TM domain peptides. In addition, assuming that the A_{2A} receptor shares the overall topology of rhodopsin and the β_2 adrenoreceptor, 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 TM pair combinations lead to productive association implies that the interactions detected by our method are sequence specific and depend on the structures of the partners. Our data likely correspond to the subset of interactions that represent the strongest helix–helix contacts. These interactions may guide both the folding pathway and the stabilization of the final A_{2A} receptor structure.

Conclusion

We have identified a set of interactions between TM domains of the human adenosine A_{2A} receptor that are likely related to the stability and folding of the receptor. This study represents the first analysis of a comprehensive set of pairwise interactions for TM domains of a GPCR *in vitro*. By doing so, we gained insights in understanding the nature and the role of these interactions in the complex mechanisms of GPCR stability and assembly. Interactions that are stable enough to persist in a detergent micelle and in the absence of linking loops should dominate the formation of the folded, functional molecule. It would be of great interest to obtain more detailed thermodynamic data and to identify the residues involved in these interactions. Indeed, disrupting one of the stabilizing intramolecular interactions may favor either a more active conformation or denaturation of the receptor. Ultimately, it may also lead to the discovery of a variant with decreased conformational flexibility and increased protein stability, which would make a better candidate for crystallization than the structurally dynamic wild-type protein. It would also be essential to determine whether similar patterns of interactions take place in other GPCRs and integral membrane proteins. The ability to identify crucial interhelical contacts will be a valuable tool in the comprehension of the stability, assembly, and function of these important and challenging proteins.

Materials and Methods

Peptide design and synthesis

Table 1 shows the amino acid sequence of every peptide used in this study. The peptides were designed to correspond to the seven TM domains of the human adenosine A_{2A} receptor as described previously (Lazarova et al. 2004). Lysine residues were added at the N and C termini of the peptides, an approach used frequently in studies of hydrophobic peptides (Deber et al. 1999; Xie et al. 2000). The added lysines increase solubility and minimize aggregation, thus facilitating the chemical synthesis and purification of the desired peptide without significantly altering its properties in detergent micelles or lipid vesicles (Wang and Deber 2000; de Planque et al. 2001; Ding et al. 2001, 2002; Zhou et al. 2001). Additionally, in order to monitor peptide interaction and association, several peptide variants were constructed. We substituted Y for W in the peptide (TMxW) when necessary, and labeled the peptide with a pyrene-butyric acid derivative on the third N-terminal lysine (TMxPyr). These changes do not alter the secondary structure of the peptides; peptides with or without these modifications display equivalent CD spectra (Lazarova et al. 2004). Peptides were synthesized by SynPep or Anaspec. 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 powders.

Peptide concentration

Peptide concentrations were determined by amino acid analysis (performed at Purdue University Core Facility PSAL) or by measuring UV absorbance of the peptides in 6 M Guanidine-HCl at 280 nm, using appropriate extinction coefficients for the aromatic tryptophan (W) and tyrosine (Y) residues (Brandts and Kaplan 1973). Prior to measurements, peptide stock solutions (~500 μ M) were prepared in acetonitrile/water (1:1). Whenever necessary, a minuscule amount of TFA (<0.1%) was added in order to dissolve the peptide.

Preparation of peptide pairs

To study the interactions between TM domains, the peptides were mixed in pairwise combinations in sodium dodecyl sulfate (SDS) micelles. First, peptide stock solutions (2–10 μ L) were mixed at desired final concentrations. Subsequently, 20 mM SDS in 10 mM HEPES and 10 mM KCl buffer, pH 7, was added to the peptide combination and briefly vortexed (final volume = 400 μ L). Each mixture was equilibrated for 30 min at room temperature prior to measurements (Fisher et al. 1999). This sample preparation was used in both CD and fluorescence spectroscopy measurements. The concentration of SDS was always well above the critical micelle concentration, which was found to be 1.6 mM by analino-naphthalene sulfonic acid (ANS) fluorescence. A graph of the fluorescence intensity as a function of detergent concentration exhibits a discontinuity at the detergent concentration where micelles form, corresponding to the CMC (data not shown). The pH was maintained at pH 7, and the final amount of TFA was negligible (<0.025%).

Fluorescence spectroscopy measurements

Fluorescence measurements were performed on an ISS PC-1 spectrofluorimeter, operating in photon-counting mode, using

10 × 10-mm or 2 × 10-mm quartz cuvettes at 25°C unless specified otherwise. 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 with an OD₂₈₀ lower than 0.2 to prevent inner filter effects. The appropriate blanks were subtracted from the recorded spectra in every experiment. The reported spectra are the average of three or more independent measurements, which were performed using peptides from different stock solutions.

Förster resonance energy transfer (FRET) measurements

FRET measurements were performed using two kinds of peptides: TMaPyr and TMbW (where a and b refer to different TM domains, with a and b = 1, 2, 3, 4, 5, 6, or 7). TMaPyr peptides are labeled with a pyrene, which acts as the acceptor fluorophore, and TMbW peptides include a Trp residue in their sequences serving as the donor fluorophore (Table 1). The experiments were carried out by adding increasing concentrations of TMaPyr to constant TMbW peptide concentrations in the presence of SDS micelles. The concentration of unlabeled TMa peptide was adjusted to keep the total peptide concentration (TMa + TMaPyr) constant (Thévenin et al. 2005a,b). When Trp emission was monitored, the samples were excited at 290 nm, and the emission spectra were taken from 300 to 500 nm. For monitoring pyrene emission, the excitation wavelength was 345 nm, and the emission spectra were scanned from 360 to 550 nm. The TMbW peptide concentration was kept constant at 10 μM. The total absorbance in all cases was <0.2 at the excitation wavelength to avoid inner filter effects. Experimental energy transfer efficiencies (F/F_0) were obtained by calculating the ratio between the intensity of fluorescence at 330 nm of the Trp-labeled peptide in combination with the pyrene-labeled peptide (F) and the maximum tryptophan fluorescence at 330 nm of the Trp-labeled peptide alone (F_0). Data were fitted to a linear regression model. The P -value, residuals, and runs test were calculated to determine whether the slope is significantly different from 0, and whether the data differ significantly from a straight line (Prism 4.0 for Macintosh). F/F_0 depends linearly on the acceptor mole fraction only when the oligomer is a dimer. Given seven TM helices, there are 21 possible heterologous pairs and seven possible self-interactions. With the exception of the three TM6 + TM7 pairs, for which TM6Pyr and TM6Y peptides were not available, every other possible pairwise combination was tested. To determine whether the interactions between the TM peptides are specific, six pairs were tested with a reversed labeling scheme (TMaW + TMbPyr).

Circular dichroism spectroscopy measurements

Far-UV CD spectra of the peptides were recorded on an Aviv model 215 spectrometer equipped with a Peltier thermal-controlled cuvette holder. All measurements were performed at 25°C and with peptide concentrations equal to 20 μM. For the peptide association experiments, peptides were mixed at equal concentrations.

CD intensities are expressed in Mean Residue Molar ellipticity [θ] calculated from the equation:

$$[\theta] = \frac{\theta_{obs}}{10 \cdot l \cdot c \cdot n} \text{ (in degrees cm}^2 \cdot \text{dmol}^{-1}\text{)}$$

where θ_{obs} is the observed ellipticity in millidegrees, l is the optical pathlength in centimeters, c is the final molar concentration of the peptides, and n is the number of amino acid residues. To minimize the effects of scattering and to ensure that the CD spectra were observed from peptides in solution, several precautions were taken as previously described (Lazarova et al. 2004). Samples were measured in a 0.1-cm pathlength quartz cuvette. Raw data were acquired from 260 nm to 190 nm at 1-nm intervals using a 2-s averaging time, and at least two scans were averaged for each sample. The reported spectra were averaged from at least three independent experiments.

To assess interactions between peptide pairs, the experimentally determined spectra of mixed peptides (experimental spectrum) were compared with the calculated average spectrum of the two individual peptides (theoretical spectrum) (Kliger and Shai 2000; Thévenin et al. 2005b). All manipulations of the spectra were performed after appropriate blank/buffer subtractions. If the experimental spectrum statistically differs from the theoretical spectrum (e.g., an increased apparent helical content), it is concluded that the peptides interact. We used the paired t -test with 95% confidence intervals using a two-tail P -value (Prism 4.0 for Macintosh) to evaluate whether or not the experimental and theoretical spectra were statistically different. The spectra are not significantly different if a large P -value (>0.05) is observed.

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