A method for identifying G protein-coupled receptor dimers and their interfaces

Jing Chen[1, 4] # *, Xin Cai[2] #, Maocai Yan[3] #, Zhengwen Wang[1], Zhitong Lv[1], Chunmei Wang[1]

1, Neurobiology Key Laboratory of Jining Medical University, Jining, 272067, China.

2, School of Basic Medicine, Weifang Medical University, Weifang, 261053, China.

3, School of Pharmacy, Jining Medical University, Rizhao, 276826, China.

4, Division of Biomedical Sciences, Warwick Medical School, University of Warwick, Coventry, CV4 7AL, UK. E-mail: Jing.Chen@warwick.ac.uk

# contributed equally to this work.

Highlights

- A combined approach for assessing GPCR dimer interfaces was developed
- The method utilizes a HIV-TAT protein transduction motif, MALDI-TOF-MS, and BRET
- The approach identified Apelin receptor heterodimer interfaces and key dimer residues
- The method is suitable for GPCR homodimers and heterodimers

Abstract

The G protein-coupled receptor (GPCR) dimer interface plays an important role in the formation and stabilization of the dimer. Therefore, identifying the potential receptor-receptor interface is an important part of studying GPCRs. Various strategies have been employed to study the GPCR dimer interface and explore its functional significance, but experimental methods lack robustness and calculations are laborious. Herein, we report a combined

optimized experimental and calculation approach for identifying and structurally characterizing GPCR dimer interfaces, and constructing atomic resolution models. Using a transmembrane domain (TM) peptide containing a human immunodeficiency virus trans-acting transcriptional activator (HIV-TAT) protein transduction motif, matrix-assisted laser desorption tandem time-of-flight mass spectrometry (MALDITOF-MS), and bioluminescence resonance energy transfer (BRET), we successfully identified Apelin receptor (APJ)/Nociceptin receptor 1 (ORL1) and APJ/Vasopressin receptor 2 (V2R) heterodimer interfaces, and two key sites mediating dimerization. This method can identify dimer interfaces of GPCR homodimers and heterodimers.

Keywords: G protein-coupled receptor; dimer; interface; transmembrane domain; mass spectrometry; bioluminescence resonance energy transfer (BRET)

1. Introduction

G protein-coupled receptors (GPCRs) can form dimers in cells, and dimeric molecules can function in different signal transduction pathways from monomers. As we learn more about GPCR dimers, it is becoming increasing apparent that dimerization is important in regulating their functions[1]. Interactions between receptors may result in more stable structural forms, alter G protein coupling of downstream signals through dimer-specific signal transduction, and increase the diversity of GPCR phenotypes[2].

Many technological advancements have been made in determining GPCR structures, and there are now more than 70 crystal structures of individual receptors[3]. However, there remain challenges in understanding and exploiting the structures of GPCRs to support drug design and the pharmaceutical industry. Emerging evidence suggests that interactions between subunits in GPCR dimers involve changes in the conformation of the dimer interface.

In addition to extracellular domains, the transmembrane domain (TM) can also form an interface and is important for the formation of head-to-head interfaces in GPCR dimers. The receptor interface plays an important role in the formation and stabilization of the dimer, and studying the interactions between GPCRs requires the identification of potential receptor-receptor interfaces[4].

Different strategies have been employed to study the dimer interfaces of GPCRs and explore their functional significance [5], including fluorescence/bioluminescence resonance energy transfer (FRET, BRET), cross-linking, co-immunoprecipitation, and computational methods. Many of these approaches use inactive and active receptor models obtained from dimers determined from structural information. However, numerous experiments and calculations are needed to confirm the results.

Herein, we report a simple and practical approach combining experimental and computational methods to identify Apelin receptor (APJ)/Nociceptin receptor 1 (ORL1) and APJ/Vasopressin receptor 2 (V2R) heterodimer interfaces, and some key sites mediating dimerization.

2. **Materials and Methods**

Detailed Materials and Methods are provided in Supporting Information 1.

2.1 BRET

For monitoring constitutive APJ and ORL1 interactions, HEK293 cells were transfected with APJ-Rluc or APJ mutant-Rluc and ORL1-Venus or ORL1 mutant-Venus plasmids at a ratio of 1:3. Coelenterazine h (5 μM) was added for BRET measurements using a Tristar LB941 plate reader with Rluc (400–475 nm) and Venus (500–550 nm) filters. To determine the effects of interfering peptides on APJ/ORL1 dimers, HEK293 cells were co-transfected with APJ-Rluc and ORL1-Venus, incubated with TM peptides (TM1 to TM7) or ORL1 (TM3 or

TM5), stimulated with or without agonists, and detected by BRET assay as described previously [6].

2.2 Design and synthesis of TM peptides

TM peptides containing the human immunodeficiency virus trans-acting transcriptional activator (HIV-TAT) protein transduction peptide and GPCR TM primary sequences were prepared (Supplemental Table 1). HIV-TAT (YGRKKRRQRRR) was fused at the N-terminus of even-numbered TMs and at the C-terminus of odd-numbered TMs to obtain the correct orientation of the inserted peptide, since HIV-TAT binds to phosphatidylinositol-(4,5) bisphosphate found on the inner surface of the membrane[7].

2.3 Mass spectrometry (MS)

MS was performed to identify APJ/ORL1 and APJ/V2R dimer interfaces in samples treated with TM peptides. Cells were transfected with APJ, ORL1, or V2R plasmid, incubated for 48 h, and then treated with the indicated HIV-TAT-TM fused peptides (4 μM) for 60 min at 37°C. Cells were stimulated with or without ligand (100 nM) for 15 min as required. Extracted proteins were immunoprecipitated using anti-APJ, anti-ORL1, and anti-V2R antibodies. Protein A/G PLUS agarose beads were incubated with proteins for 2 h, and beads were washed four times with lysis buffer. APJ, ORL1, and V2R complexes were analyzed using a matrix-assisted laser desorption tandem time-of-flight (MALDI-TOF) mass spectrometer as described previously[8]. We also investigated whether the dimeric interface changes during receptor activation. Similar methods were used for measuring the activities of APJ/ORL1 and APJ/V2R heterodimers after stimulating with corresponding peptide.

3. Results and Discussion

We performed calculations to construct atomic resolution models of GPCR dimers. The 3D structures of APJ (PDB ID 5VBL)[9] and opioid-related ORL1 (PDB ID 5DHG) were retrieved from the Protein Data Bank (PDB) database [10]. The 3D model of V2R was built by homology modeling using the homologous GPCR ORL1 (PDB ID 5DHG) as the template. Homology modeling was accomplished using MODELLER 9.20[11,12]. Heterodimer models of APJ/ORL1 and APJ/V2R, in both active and inactive states, were built by manual protein-protein docking followed by molecular dynamics simulations using the drug discovery software package Discovery Studio 2017 (Supplemental Figure 1).

Based on the atomic resolution models of the GPCR dimers, we designed and synthesized TM peptides. The APJ/ORL1 and APL/V2R dimer interfaces in samples treated with TM peptides were identified by MALDI-TOF MS. Figure 1a shows that ORL1+APJ-TM1 and ORL1+APJ-TM2 dimers were detected by MS in the absence of ligand, but ORL1+APJ-TM5 and ORL1+APJ-TM7 dimers were not detected. This indicates that the structural interface of the APJ/ORL1 dimer is composed of TM1 and TM2, and these domains interact in an inactive state (without ligand stimulation; Figure 1a). We then examined the dynamics of this interface to explore how APJ/ORL1 dimers change interface upon activation by Orphanin FQ stimulation. Figure 1b shows that ORL1+APJ-TM5 dimers were detected by MS, but ORL1R+APJ-TM4 and ORL1+APJ-TM6 dimers were not detected. By contrast, upon Apelin-13 stimulation, APJ/ORL1-TM5 dimers were detected by MS, but APJ/ORL1-TM4 and APJ/ORL1-TM6 dimers were not detected in the absence of ligand (Figure 1c). These results suggest that the interface of the active APJ/ORL1 dimer switches from TM1 and TM2 in the absence of ligand (basal or inactive state) to mainly TM5 in the active conformation.

The same method was used to identify the interface in the APJ/V2R dimer. As shown in Figure 1d, upon D-Arg8-Vasopressin treatment, the V2R+APJ-TM5 dimer was detected,

and the V2R+APJ-TM4 dimer was detected without treatment. By contrast, without treatment, V2R TM1 and TM5 did not influence the APJ dimer. When APJ+V2R-TM5 was treated with Apelin-13, the results were similar to those of V2R+APJ-TM5 (both were detected; Figure 1e). These results indicate that TM4 and TM5 are mainly responsible for forming the APJ/V2R dimer interface, but TM1 is not intimately involved. Thus, the APJ/V2R heterodimer switches from a largely TM4-mediated interface in the inactive state to a largely TM5-mediated interface in the active conformation.

BRET was used to evaluate the effect of synthetic peptides corresponding to the TM region on APJ heterodimer formation. BRET ratios were significantly reduced after incubating with HIV-TAT-fused APJ-TM1, TM2, TM4, TM5, and TM6 peptides, but not APJ-TM3 and APJ-TM7 peptides **(**Supplemental Figure 2a**)**. To further confirm the interface, each energy donor-acceptor combination was tested by incubating ORL1-TM3 and ORL1-TM5 with Apelin-13 stimulation, and only the ORL1-TM5 BRET ratio decreased **(**Supplemental Figure 2b**)**. These BRET results are consistent with the MS results (see above).

The importance of TM1, TM2, and TM5 in the formation of the APJ/ORL1 dimer interface was confirmed by constructing point mutations to explore the residues mediating the interactions. TM1, TM2, and TM5 mutant receptors of APJ/ORL1 were generated, including outward-facing and hydrophobic residues. Plasmids encoding Rluc-tagged APJ and its mutants or Venus-tagged ORL1 and its mutants were transfected into HEK-293 cells, and cells were subjected to BRET analysis. Notably, APJ M36$^{1.40}$A, APJ V80$^{2.55}$A, and ORL1 L57$^{1.38}$ A mutants in TM1 and TM2, and APJ L218$^{5.55}$A and ORL1 L299$^{5.52}$A mutants in TM5, exhibited significantly lower BRET signals than native APJ or ORL1, further highlighting the significance of TM1 and TM2 in the formation of the dimer interface in the ligand-free basal (inactive) state (Supplemental Figure 2c). Furthermore, ligand stimulation of APJ 218L$^{5.55}$A and ORL1L 299$^{5.52}$A mutants in TM5 reduced the APJ/ORL1 dimer BRET

signals by nearly 50% (Supplemental Figure 2c). These results demonstrate that the key sites of interaction between APJ/ORL1 dimers are APJ M36$^{1.40}$, APJ V80$^{2.55}$, ORL1 L57$^{1.38}$, APJ 218 L$^{5.55}$, and ORL1 L 299$^{5.52}$.

We demonstrated that the APJ/ORL1 heterodimer has a similar structure to the β1-adrenergic receptor (β1-AR) dimer reported previously[13]. The inactive interface of the APJ/ORL1 dimer spans TM1, TM2, and the C-terminus; the active interface includes TM4, TM5, and two intracellular loops. Thus, there is a significant conformational change in the GPCR protein between inactive and active states, involving a switch between two different interfaces. The same method was applied to identify the interface in the APJ/V2R dimer, and the results showed that the APJ/V2R heterodimer switches from a largely TM4-mediated interface in the basal state to a largely TM5-mediated interface in the active conformation. We also identified five key sites in the APJ/ORL1 heterodimer interface.

In conclusion, based on the sequence and structural characteristics of GPCRs, we generated atomic resolution models of APJ heterodimer interfaces using a combined approach involving TM peptides, MALDI-TOF-MS, and BRET. The method is suitable for identifying the dimer interfaces of both GPCR heterodimers and homodimers [14]. In future work, we will develop additional techniques that are more sensitive and accurate for examining GPCR dimerization and interfaces in cells.

Supporting Information

Supporting Information 1.

Supplemental Figure 1 and Figure 2.

Supplemental Table 1.

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Author Contributions

JC designed the study, and wrote and revised the manuscript; ZW W, ZT L, and CM W conducted most of the experiments; QX, XC, and JC wrote the manuscript; XC and MC Y analyzed and interpreted the data; all authors reviewed and approved the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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Figure legends

Figure 1. Identification of the interaction interface of APJ/ORL1 and APJ/V2R heterodimers using interfering peptides and MS.

(a) Analysis of the interface between ORL1/APJ heterodimers (without ligand stimulation) using interface peptides and MS. ORL1+APJ-TM1 and ORL1+APJ-TM2 dimers were detected (TM1 MW = 4067.95, TM2 MW = 4054.84), but ORL1+APJ-TM5 and ORL1+APJ-TM7 dimers were not detected. APJ MW = 42.66 kDa, ORL1 MW = 40.5 kDa.

(b) Analysis of the interface between ORL1/APJ heterodimers (with Orphanin FQ stimulation) using interface peptides and MS. ORL1+APJ-TM5 dimers (TM5 MW = 4085.9) were detected by MS, but ORL1R+APJ-TM4 and ORL1+APJ-TM6 dimers were not detected.

(c) Analysis of the interface between APJ/ORL1 heterodimers using ORL1-TM5 and MS. With Apelin-13 stimulation, the APJ/ORL1-TM5 dimer (TM5 MW = 4109) was detected by MS, but the APJ/ORL1-TM5 dimer was not detected in the absence of Apelin-13.

(d) Analysis of the interface between APJ/V2R heterodimers (with deamino-Cys1 and D-Arg8-Vasopressin stimulation) using interface peptides and MS. The V2R+APJ-TM5 dimer (TM5 MW = 4085.9) was detected, but the V2R+APJ-TM4 dimer (MW = 3824.75) was not detected. Without ligand treatment, the V2R+TM4 dimer was detected, but the V2R+APJ-TM5 dimer was not detected.

(e) Without ligand treatment, V2R TM1 (TM1 MW = 3981) and TM5 (TM5 MW = 3794) do not influence the APJ dimer. When the APJ+V2R-TM5 dimer was treated with Apelin-13, the results were similar to those of V2R+APJ-TM5 (both dimers were detected; V2R-Myc MW = 41.46 kDa).

Author Contributions

JC designed the study, and wrote and revised the manuscript; ZW W, ZT L, and CM W conducted most of the experiments; QX, XC, and JC wrote the manuscript; XC and MC Y analyzed and interpreted the data; all authors reviewed and approved the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Highlights

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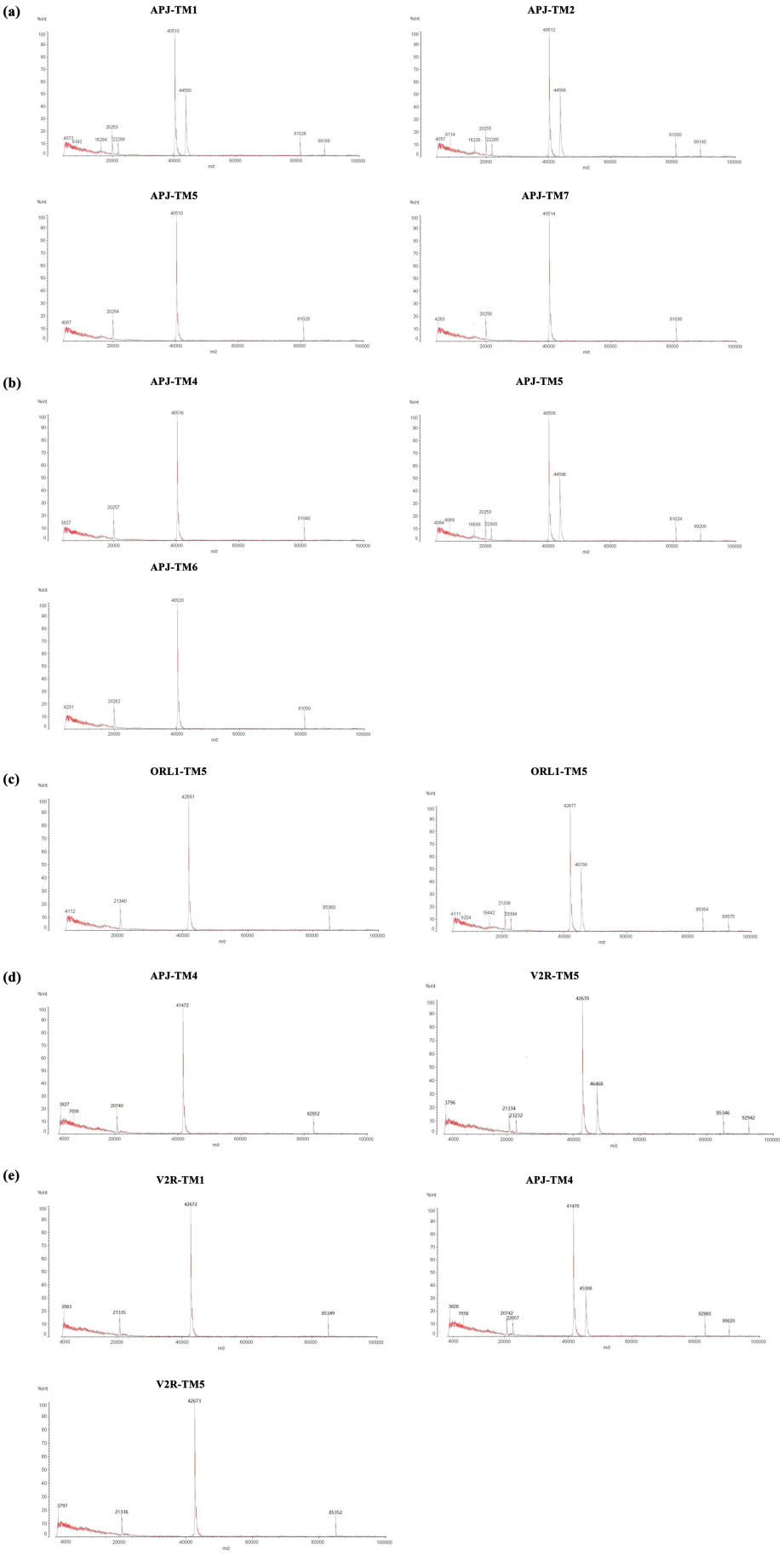
(a)
APJ-TM1
40510
44580
20253
81026
89168
APJ-TM2
40512
44566
20255
81030
89130
APJ-TM5
40510
20254
81028
APJ-TM7
40514
20256
81036
(b)
APJ-TM4
40516
20257
81040
APJ-TM5
40508
44596
20253
81024
89200
APJ-TM6
40520
20262
81050
(c)
ORL1-TM5
42681
21340
85360
ORL1-TM5
42677
46788
21338
85354
93575
(d)
APJ-TM4
41472
20740
82952
V2R-TM5
42670
46468
21334
85346
92942
(e)
V2R-TM1
42672
21335
85349
APJ-TM4
41476
45306
20742
82980
90620
V2R-TM5
42673
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85352

Figure 1