

Photo-Cross-Linkers Incorporated into G-Protein-Coupled Receptors in Mammalian Cells: A Ligand Comparison**

Irene Coin, Marilyn H. Perrin, Wylie W. Vale, and Lei Wang*

Although G-protein coupled receptors (GPCRs) are the molecular target of almost half of today's pharmaceuticals, little is known about the molecular basis of the specific interaction with their ligands, owing to the difficulty of obtaining spectroscopic data for such highly flexible and complex systems integrated in the cell membrane. A powerful experimental approach to investigate ligand–receptor interactions in a native environment is the use of photoaffinity cross-linking, which usually involves photo-activatable cross-linkers installed into chemically achievable ligands to establish spatial constraints in the ligand–receptor complex.^[1] However, only a few positions within the ligand can be modified with the photo-cross-linker without affecting binding or signaling behavior.^[2] This limitation prevents comprehensive mapping of the ligand–receptor interaction and prevents comparison of ligands with different pharmacological properties.

Noncanonical amino acids can be genetically incorporated into proteins in live cells through the expansion of the genetic code.^[3] Briefly, an exogenous orthogonal tRNA/aminoacyl-tRNA synthetase (aaRS) pair is introduced into the host cell. The aaRS is engineered to charge a desired unnatural amino acid (Uaa) onto its cognate tRNA, which incorporates the Uaa in response to a unique codon, usually the amber stop codon UAG. Using this approach, photo-activatable amino acids have been introduced into proteins in bacteria, yeast, and mammalian cells,^[4] and a few cross-linking examples have been reported, which are mostly limited either to cytosolic proteins or to validate a known interaction.^[5] Cross-linking with photoactivatable Uaas

genetically incorporated into GPCRs has been achieved in yeast,^[6] and in mammalian cells only with class I GPCRs, which can be overexpressed in high yields and for which high resolution X-ray structures have been determined.^[7]

The corticotropin releasing factor receptor type 1 (CRF-R1, Figure 1) is a class II GPCR which binds endogenous peptide ligands to mediate, among other responses, the organism's response to stress.^[8] Like the other class II GPCRs, CRF-R1 has a large N-terminal domain (NTD, ca. 120 residues), which has been identified as the major binding site of peptide ligands.^[9] Although the isolated NTD has been structurally characterized with NMR and X-ray spectroscopy,^[10] no full-length class II GPCR structure has been solved to date, and information on these receptors is mostly derived from structure–activity relationship (SAR) studies and cross-linking studies with a cross-linker substituting a ligand residue.

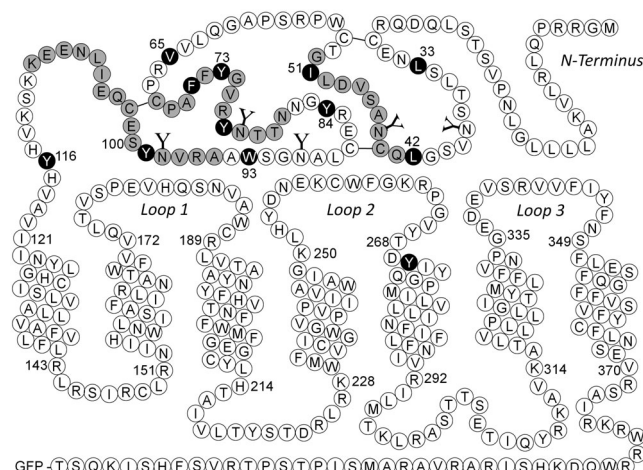


Figure 1. Representation of the rat CRF-R1 fused to GFP. The disulfide arrangement is based on Ref. [9a]. Regions of the NTD involved in ligand binding are in gray.^[9,10] Glycosylation sites are marked by “Y”. Black circles represent residues substituted with Azi.

Herein, we genetically incorporated the photoactivatable amino acid *p*-azido-phenylalanine (Azi) into different positions of CRF-R1 in the native environment of the mammalian cell. By comparing the cross-linking pattern of several peptide ligands, we demonstrate that besides a common interaction site in the hinge region, the ligands come into different proximity with different regions of the receptor.

Effective application of Uaas to study membrane proteins in mammalian cells is often challenging owing to inefficient Uaa incorporation and low protein yields, especially for

[*] Dr. I. Coin, Dr. L. Wang

Jack H. Skirball Center for Chemical Biology and Proteomics
The Salk Institute for Biological Studies
10010 North Torrey Pines Road, La Jolla, CA 92037 (USA)
E-mail: lwang@salk.edu
Homepage: <http://wang.salk.edu/>

Dr. M. H. Perrin, Dr. W. W. Vale
The Clayton Foundation Laboratories for Peptide Biology
The Salk Institute for Biological Studies
10010 North Torrey Pines Road, La Jolla, CA 92037 (USA)

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systems that are not highly expressed. To optimize the Uaa incorporation, we developed a new all-in-one (AIO) construct containing three expression cassettes for the target gene, the orthogonal synthetase gene, and the orthogonal tRNA gene, respectively (Figure 2). A single plasmid obviates the co-transfection of multiple plasmids that often reduces the transfection efficiency and expression level in mammalian cells. The enhanced orthogonal EctRNA^{Tyr}_{CUA}-E2AziRS pair^[5d] was used for site-specific Azi incorporation in response to the UAG codon. We reasoned that a high expression level of the orthogonal tRNA would be beneficial for efficient suppression of the UAG codon, yet excessive tRNA expression would result in uncharged tRNA leading to potential cytotoxicity. To balance the tRNA expression level with the synthetase activity and turnover rate, we tested a series of AIO plasmids bearing an increasing number of tRNA^{Tyr}_{CUA} expression cassettes (Figure 2B). The gene for green fluorescent protein (GFP) bearing a UAG stop codon at a permissive site (Y182, Figure 2B) was used as a reporter to monitor the protein expression level with flow cytometry.^[4b] Indeed, incorporation efficiency of Azi by E2AziRS increased with the number of tRNA^{Tyr}_{CUA} expression cassettes from one to three but started

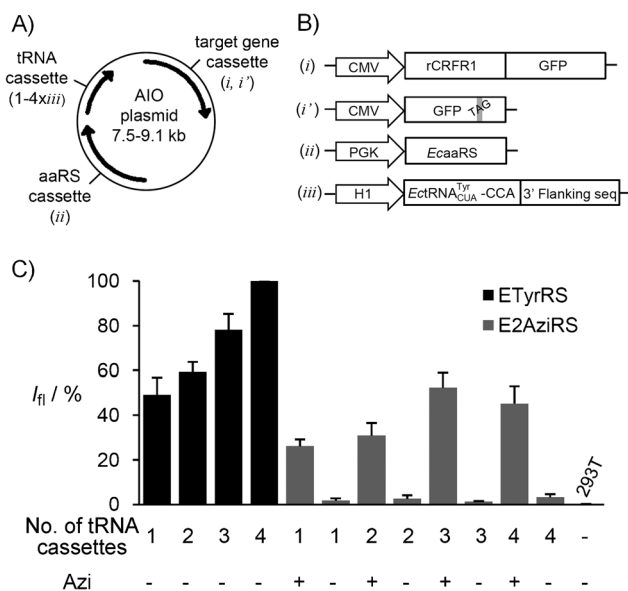


Figure 2. All-in-one (AIO) plasmid for efficient Uaa incorporation in mammalian cells. A) Three independent expression cassettes were built into a unique plasmid. B) Elements included in the cassettes. i) Cassette expressing the wt-CRF-R1-GFP fusion. To incorporate Azi, a TAG stop codon was inserted at the desired position and Azi was added to the cell growth medium. i') GFP-TAG reporter; ii) orthogonal aaRS cassette; iii) monomeric cassette for functionally expressing orthogonal prokaryotic tRNA in mammalian cells.^[4b] This cassette was repeated in tandem (1–4 copies) to increase tRNA expression. C) In vivo fluorescence assay for UAG suppression efficiency. Data represent the average of at least four independent flow cytometry experiments and are normalized to the value obtained with *E. coli* ETyrRS. Total fluorescence intensities I_{fl} : 49.2 ± 7.6 , 59.4 ± 4.4 , 78.2 ± 7.1 , 100 (ETyrRS, 1–4xtRNA), 26.1 ± 3.1 and 1.7 ± 1.0 (E2Azi, 1xtRNA, $\pm$ Azi), 30.9 ± 5.6 and 2.6 ± 1.5 (E2Azi, 2xtRNA, $\pm$ Azi), 52.3 ± 5.7 and 1.2 ± 0.3 (E2Azi, 3xtRNA, $\pm$ Azi), 45.2 ± 7.7 and 3.2 ± 1.4 (E2Azi, 4xtRNA, $\pm$ Azi). The last bar (0.1 ± 0.0) represents non-transfected cells.

to decrease at four, while incorporation of Tyr by the more active enhanced wild-type (wt) TyrRS^[5d] increased from one to four copies of tRNA (Figure 2C).

The AIO plasmid containing three copies of tRNA^{Tyr}_{CUA} was then used to incorporate Azi into the rat CRF-R1. GFP was fused at the C terminus of CRF-R1 (Figure 2B) to allow detection of the receptor's cellular localization without interfering with normal binding and activation.^[11] A series of CRF-R1-GFP mutants bearing Azi at different positions were expressed in 293T cells (Figure 3). We focused on the extracellular NTD and on two regions to which photoactivatable ligands have been reported to cross-link: the hinge between the NTD and the transmembrane domain 1 (TMD1)^[12] and the C-terminal section of extracellular loop 2 (ECL2).^[13]

All receptors mutated at the NTD expressed well and showed well-defined membrane localization (Figure 3A; Supporting Information, Figures S1–S3). Receptor expression was dependent upon the presence of Azi in the cell growth medium. In the absence of the Uaa, only a weak background of GFP fluorescence was observed in the cytosol, but not at the plasma membrane. Likewise, bands attributable to the GFP-receptor fusion were observed in Western blot only for samples treated with Azi, and showed the expected molecular

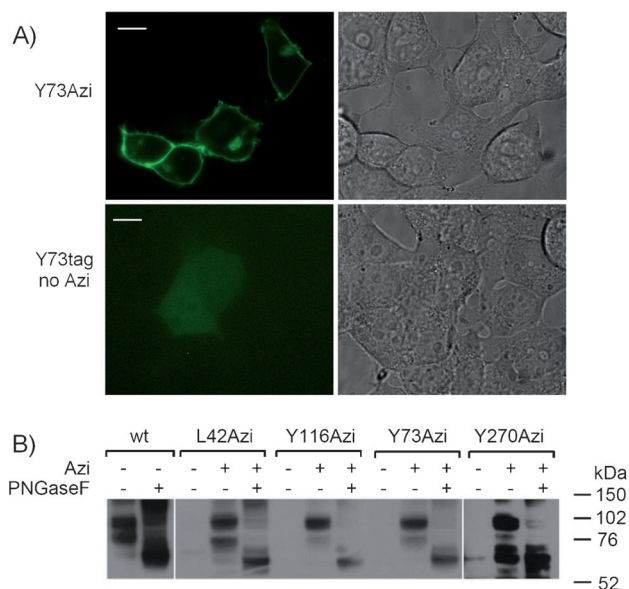


Figure 3. Expression of [Azi]CRF-R1-GFP mutants. A) 293T cells transfected with the AIO plasmid encoding for [Y73TAG]CRF-R1-GFP. Cells were grown in the presence (upper panel) or absence (lower panel) of Azi. Left: confocal fluorescence images, GFP channel (identical settings, scale bars 10 μ m); right: brightfield image of the same areas. Images of other mutants are reported in the Supporting Information. B) Western blot: mouse anti-GFP followed by goat anti-mouse-HRP and ECL detection (see the Supporting Information). Exposure: few seconds, except for the portion of Y270Azi which was exposed for 5 min. The apparent MW of about 100 kDa corresponds to the mature glycosylated CRF-R1-GFP and of about 72 kDa to the high-mannose form. Deglycosylated CRF-R1-GFP migrates at about 62 kDa.^[14] Same volumes of cell lysates were loaded for all samples (10 μ L of 40 μ L cell lysate, about 3.5×10^6 293T cells when harvested), except for the wild-type (wt) sample (0.5 μ L loaded).

weight (MW) shift (30–40 kDa) upon deglycosylation (Figure 3B).^[14] By comparison of signal intensities, we estimated the expression level of these mutants to lie in the range of 1/20 to 1/50 of the wt CRF-R1-GFP expression. Lower expression levels were observed for Azi incorporation in the ECL2 region (Figure 3; Supporting Information, Figure S3, Y270Azi). Decreasing suppression yields for UAG codons placed toward the 3'-end of the gene have been reported also for rhodopsin^[15] and might be an intrinsic characteristic of the suppression technique when applied to membrane proteins. The same yield decrease occurred when ETyrRS substituted for E2AziRS in the AIO plasmid to incorporate the native residue Tyr. This excludes the possibility that the phenomenon is related to intolerance of the chosen positions toward Azi substitution.

To test if the mutant receptors bind CRF-R1 ligands, we estimated the apparent inhibitory binding constants (K_i) for the antagonist astressin (Ast)^[16] (Table 1). In most cases the

Table 1: Inhibitory binding constants (K_i) of the wild-type and [Azi]CRF-R1-GFP receptor mutants derived from competitive displacement of ¹²⁵I-[D-Tyr⁰]Ast by increasing concentrations of Ast.^[a]

Receptor	K_i [nM]	Receptor	K_i [nM]
wt	0.87 (0.85–0.88) ^[b]	Y77Azi	3.6 (3.1–4.2)
L33Azi	0.77 (0.63–0.94)	Y84Azi	1.1 (0.91–1.4)
L42Azi	0.78 (0.61–0.98)	W93Azi	— ^[c]
I51Azi	3.8 (2.6–5.5)	Y99Azi	1.6 (1.2–2.0)
V65Azi	0.95 (0.64–1.4)	Y116Azi	1.2 (0.68–2.3)
Y71Azi	0.5 (0.21–1.2)	Y270Azi	2.0 (1.8–2.3)
Y73Azi	0.87 (0.80–0.93) ^[b]		

[a] The logarithmic means are shown for at least three measurements from different binding experiments performed in triplicate. Binding was measured on cell membrane preparations from transiently transfected 293T cells. [b] 95 % confidence interval. [c] Low affinity, not determined.

introduction of Azi did not significantly affect binding of Ast. The mutant receptors showed affinities comparable to that of the wt ($K_i \approx 1$ nM), with the maximal difference of a factor 4 in the case of I51Azi and Y77Azi ($K_i \approx 4$ nM). The only exception is the receptor mutated at Trp93 (W93Azi), a residue involved in a Trp–Trp structural motif stabilizing the NTD folding.^[10] Although this mutant was correctly targeted to the cell membrane (Supporting Information, Figure S3), its specific binding to Ast* was too low to measure the K_i accurately.

Cross-linking experiments were carried out on intact 293T cells transfected with the AIO plasmids of receptor mutants. Cells were irradiated in the presence of different ligands, and cell lysates analyzed with SDS-PAGE. Peptide ligands were radiolabeled with ¹²⁵I to allow autoradiographic detection of the cross-linked products (radioactive species are indicated by an asterisk).

We first demonstrated that photo-cross-linking to CRF-R1 mutants containing Azi takes place only with ligands specifically bound to the receptor (Figure 4). 293T cells

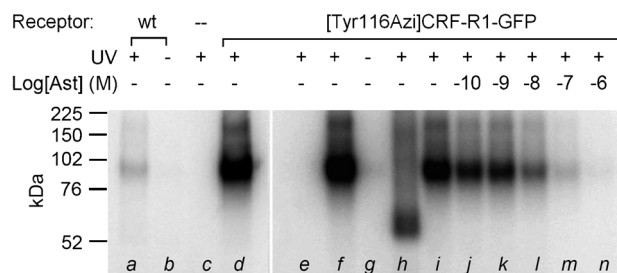


Figure 4. Specific cross-linking of Ast* to [Y116Azi]CRF-R1-GFP. Autoradiography of cell lysates resolved by SDS-PAGE is shown. Samples were normalized for constant cell numbers for each lane. Before irradiation (365 nm, 16 W, ca. 1 cm distance, 10 min), samples were incubated for ca. 2 h with a)–d) 1 nM Ast*; e) 1 nM Ucn 3*; f) 1 nM Ast*/1 μ M Ucn 3; g)–n) 1 nM Ast* and Ast as indicated. Sample (h) was deglycosylated with PNGaseF. Lane (c) represents non-transfected cells.

expressing Y116Azi mutant were incubated either with about 1 nM Ast* or Ucn 3*, a CRF-R2 specific ligand that does not bind CRF-R1.^[17] Ast* but not Ucn 3* gave a radioactive band at the MW expected for the cross-linked complex. The radioactive signal decreased gradually when increasing amounts of unlabeled Ast were added during incubation, but did not change by adding a 1000-fold concentration of unlabeled Ucn 3. A faint radioactive band was detectable in the lane of the wt receptor irradiated in the presence of Ast*, which was probably due to minimal direct cross-linking not mediated by Azi.^[18] However, based on the relative expression level, the amount of wt receptor loaded on this gel was at least 20 times higher than that of the mutants.

Differential cross-linking was tested in parallel on four [Azi]CRF-R1 mutants (Figure 5) with four radiolabeled

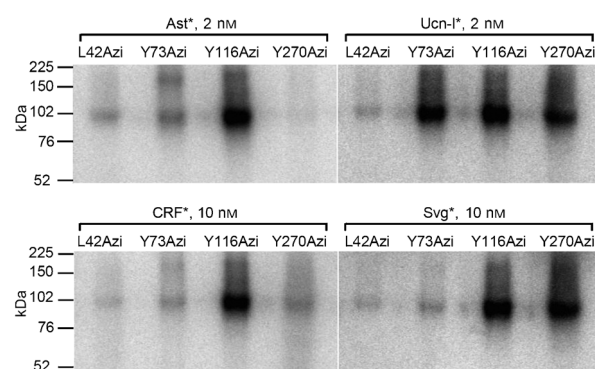


Figure 5. Differential cross-linking of radiolabeled ligands to [Azi]CRF-R1-GFP mutants. Autoradiography of cell lysates resolved by SDS-PAGE is shown. Identical aliquots of the same transfected cell batches were treated in parallel with the ligands. CRF* and Svg* were applied at higher concentrations, because they showed lower total binding to CRF-R1 than did Ast* and Ucn 1*. The amounts of receptor are Y270Azi $\ll$ Y73Azi $\approx$ Y116Azi $<$ L42Azi, as determined by a Western blot on an aliquot of the same samples.

ligands: the antagonist Ast*, and the agonists urocortin 1 (Ucn 1*), CRF*, and sauvagine (Svg*). Azi was placed at positions in the receptor proposed to be in different proximities to the ligands. L42 belongs to a portion of the NTD not involved in ligand binding and was chosen as negative control, while Y73 is located in a loop domain predicted to come into close proximity to the bound ligand^[10] (Figure 1). Y116 and Y270 are close to cross-linking sites identified using photoactivatable derivatives of Svg^[12] and Ucn 1.^[13] To ensure that the mutant receptors correctly interact with the peptide agonists, we measured the stimulated intracellular accumulation of cyclic AMP (Supporting Information). All receptors were active with each of the three agonists in the concentration range used for cross-linking.

Confirming the site-specificity of this new cross-linking approach, none of the ligands was cross-linked by L42Azi, but all were cross-linked by Y116Azi. The general cross-linking by Y116Azi indicates the hinge region between the NTD and TMD1 to be an important interaction domain not only for Svg,^[12] but also for other CRF-R1 ligands. The identification of an interaction site common to different ligands adds important structural information to the mapping of the receptor binding pocket because no spectroscopic information is available for this hinge region.

Differentiation between ligands was observed at the NTD and at the C-terminal juxtamembrane section of ECL 2. The antagonist Ast* did not cross-link to Y270Azi. In contrast, both agonists Ucn 1* and Svg* gave an intense cross-linking band with the Y270Azi mutant. These results are consistent with the current binding model,^[19] proposing that agonists interact not only with the NTD but also with the juxtamembrane region to activate the receptor, while some peptide antagonists such as astressin interact mainly with the NTD. However, it is interesting to observe that the cross-linking of the agonist CRF* to Y270Azi was much lower compared to that of Ucn 1* and Svg*. This observation may suggest that CRF exerts its activity by coming into closer proximity to a different region of the extracellular loop domains.

A strong radioactive band was visible for cross-linking of Y73Azi to Ucn 1*. This is the first time that cross-linking has been observed at the glycosylated NTD of a CRF receptor, and is a direct proof that in a native environment, also, the NTD of CRF-R1 is involved in ligand binding. This is in line with data on other class II GPCRs.^[20] In addition, the lack of cross-linking of Svg* and CRF* to Y73Azi is consistent with the observation that CRF binds the isolated NTD with low affinity ($K_i \approx 600$ nM) and that Svg does not bind to the NTD at all.^[9a,b]

However, there is strong evidence in the literature that the CRF-R1 NTD contains major binding determinants not only for Ucn 1, but also exclusively for Ast.^[9a,21] Moreover, X-ray and NMR data for CRF-R1 and other class II GPCR NTDs indicate multiple interactions between ligands and receptor NTDs.^[22] Nevertheless, beside the Y73Azi-Ucn 1* cross-linking, we did not observe any other intense cross-linking with any ligand to the mutant receptors in which Azi replaced L33, L42, I51, V65, F71, Y73, Y77, Y84, or Y99 in the NTD. Although the positions, based on spectroscopic and SAR data, should lie in regions both involved and not involved in

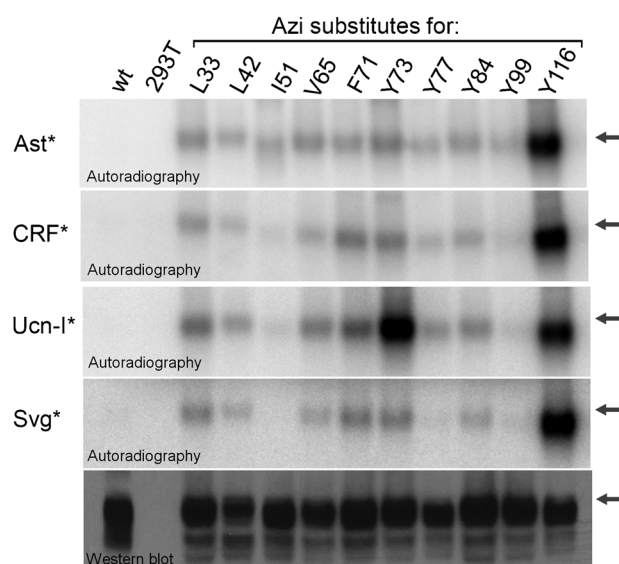


Figure 6. Cross-linking at the NTD. Analysis of cell lysates resolved by SDS-PAGE. The arrows indicate a molecular mass of 102 kDa. Cell aliquots from the same transfected 6 cm dish were incubated with the indicated ligands followed by irradiation (Ast* and Ucn 1* ca. 2 nM, CRF* and Svg* ca. 10 nM). For experimental details, see the Supporting Information.

ligand binding, all these mutants gave weak radioactive cross-linked bands, compared to the signals obtained with Y116Azi (Figure 6). After normalization to mutant expression levels, the intensities of the Y73Azi- and Y116Azi-Ucn 1* cross-linked bands were comparable, but the intensity of Y73Azi-Ast* band was about 1/10 that of the Y116Azi-Ast* band (Supporting Information, Figure S6).

Lack of strong cross-linking at various sites of the NTD suggests an intriguing possibility that in the native receptor–ligand complex the ligands are not tightly bound to the receptor NTD. However, in addition to proximity, the photochemical properties of cross-linker may also play a role in cross-linking efficiency.^[2b,23] Aryl azides cross-link through a photogenerated nitrene intermediate, which reacts with X–H bonds (X: C, N, S, O) and is easily quenched in aqueous environment. A low cross-linking yield could result from the dynamic binding equilibrium at the well solvated extracellular receptor NTD, in agreement with the structural flexibility observed in the NMR spectroscopy studies on the isolated NTD.^[10a] Alternatively, it is also possible that the ligand binds to the native full-length receptor in a pocket different from that identified in the non-glycosylated isolated NTD. We will further test these possibilities using a benzophenone-based cross-linker, which offers specificity for C–H bonds and is less susceptible to quenching.^[2b]

In conclusion, by establishing a robust expression system that incorporates photoactivatable amino acids at specific positions into class II GPCRs, we were able to compare for the first time the behavior of different ligands at the same receptor in the native context of mammalian cells. By examining cross-linking patterns at distinct binding domains, we have gained new insights in the map of the ligand–receptor interaction. This new method has enormous potential for

addressing fundamental biological questions, such as whether and in what manner agonists showing different Gs/Gi activation bind to different regions of the receptor, as well as many other biochemical aspects related to the functioning and interaction networks of receptors and membrane proteins.

Experimental Section

Methods and experimental details for the construction of the AIO plasmid, culture of cells, FACS measurements, imaging, cell membrane preparation, binding assay, cAMP assay, Western blot and photo-cross-linking experiments are described in the Supporting Information, together with images of the other CRF-R1-GFP mutants, all binding curves, cAMP assay data, and other experiments.

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- [1] V. I. Pham, P. M. Sexton, *J. Pept. Sci.* **2004**, *10*, 179.
- [2] a) M. Beyermann, N. Heinrich, K. Fechner, J. Furkert, W. Zhang, O. Kraetke, M. Bienert, H. Berger, *Br. J. Pharmacol.* **2007**, *151*, 851; b) D. Fillion, G. Lemieux, L. L. Basambombo, P. Lavigne, G. Guillemette, R. Leduc, E. Escher, *J. Med. Chem.* **2010**, *53*, 2063.
- [3] Q. Wang, A. R. Parrish, L. Wang, *Chem. Biol.* **2009**, *16*, 323.
- [4] a) C. C. Liu, P. G. Schultz, *Annu. Rev. Biochem.* **2010**, *79*, 413; b) W. Y. Wang, J. K. Takimoto, G. V. Louie, T. J. Baiga, J. P. Noel, K. F. Lee, P. A. Slesinger, L. Wang, *Nat. Neurosci.* **2007**, *10*, 1063.
- [5] a) N. Hino, Y. Okazaki, T. Kobayashi, A. Hayashi, K. Sakamoto, S. Yokoyama, *Nat. Methods* **2005**, *2*, 201; b) H. Mori, K. Ito, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 16159; c) C. Y. Majmudar, L. W. Lee, J. K. Lancia, A. Nwokoye, Q. Wang, A. M. Wands, L. Wang, A. K. Mapp, *J. Am. Chem. Soc.* **2009**, *131*, 14240; d) J. K. Takimoto, K. L. Adams, Z. Xiang, L. Wang, *Mol. Biosyst.* **2009**, *5*, 931.
- [6] L. Y. Huang, G. Umanah, M. Hauser, C. Son, B. Arshava, F. Naider, J. M. Becker, *Biochemistry* **2008**, *47*, 5638.
- [7] a) S. X. Ye, C. Kohrer, T. Huber, M. Kazmi, P. Sachdev, E. C. Y. Yan, A. Bhagat, U. L. RajBhandary, T. P. Sakmar, *J. Biol. Chem.* **2008**, *283*, 1525; b) A. Grunbeck, T. Huber, P. Sachdev, T. P. Sakmar, *Biochemistry* **2011**, *50*, 3411.
- [8] M. H. Perrin, W. W. Vale, *Ann. N. Y. Acad. Sci.* **1999**, *885*, 312.
- [9] a) M. H. Perrin, W. H. Fischer, K. S. Kunitake, A. G. Craig, S. C. Koerber, L. A. Cervini, J. E. Rivier, J. C. Groppe, J. Greenwald, S. Moller Nielsen, W. W. Vale, *J. Biol. Chem.* **2001**, *276*, 31528; b) J. Klose, K. Fechner, M. Beyermann, E. Krause, N. Wendt, M. Bienert, R. Rudolph, S. Rothemund, *Biochemistry* **2005**, *44*, 1614; c) S. Wille, S. Sydow, M. R. Palchaudhuri, J. Spiess, F. M. Dautzenberg, *J. Neurochem.* **1999**, *72*, 388; d) I. Q. Assil, L. J. Qi, M. Arai, M. Shomali, A. B. Abou-Samra, *Biochemistry* **2001**, *40*, 1187.
- [10] a) C. R. Grace, M. H. Perrin, J. Gulyas, J. E. Rivier, W. W. Vale, R. Riek, *J. Biol. Chem.* **2010**, *285*, 38580; b) A. A. Pioszak, N. R. Parker, K. Suino-Powell, H. E. Xu, *J. Biol. Chem.* **2008**, *283*, 32900.
- [11] O. Kraetke, B. Wiesner, J. Eichhorst, J. Furkert, M. Bienert, M. Beyermann, *J. Recept. Signal Transduction Res.* **2005**, *25*, 251.
- [12] I. Assil-Kishawi, T. A. Samra, D. F. Mierke, A. B. Abou-Samra, *J. Biol. Chem.* **2008**, *283*, 35644.
- [13] O. Kraetke, B. Holeran, H. Berger, E. Escher, M. Bienert, M. Beyermann, *Biochemistry* **2005**, *44*, 15569.
- [14] M. Alken, C. Rutz, R. Kochl, U. Donalies, M. Oueslati, J. Furkert, D. Wietfeld, R. Hermosilla, A. Scholz, M. Beyermann, W. Rosenthal, R. Schulein, *Biochem. J.* **2005**, *390*, 455.
- [15] S. Ye, T. Huber, R. Vogel, T. P. Sakmar, *Nat. Chem. Biol.* **2009**, *5*, 397.
- [16] J. Gulyas, C. Rivier, M. Perrin, S. C. Koerber, S. Sutton, A. Corrigan, S. L. Lahrichi, A. G. Craig, W. Vale, J. Rivier, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 10575.
- [17] K. Lewis, C. Li, M. H. Perrin, A. Blount, K. Kunitake, C. Donaldson, J. Vaughan, T. M. Reyes, J. Gulyas, W. Fischer, L. Bilezikjian, J. Rivier, P. E. Sawchenko, W. W. Vale, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 7570.
- [18] a) M. Suchanek, A. Radzikowska, C. Thiele, *Nat. Methods* **2005**, *2*, 261; b) M. Thibonnier, *J. Biol. Chem.* **1987**, *262*, 10960.
- [19] S. R. Hoare, *Drug Discovery Today* **2005**, *10*, 417.
- [20] L. J. Miller, M. Dong, K. G. Harikumar, F. Gao, *Biochem. Soc. Trans.* **2007**, *35*, 709.
- [21] M. H. Perrin, S. Sutton, D. L. Bain, W. T. Berggren, W. W. Vale, *Endocrinology* **1998**, *139*, 566.
- [22] C. Parthier, S. Reedtz-Runge, R. Rudolph, M. T. Stubbs, *Trends Biochem. Sci.* **2009**, *34*, 303.
- [23] J. J. Tate, J. Persinger, B. Bartholomew, *Nucleic Acids Res.* **1998**, *26*, 1421.