Structure-activity characterization of glucagon receptor, an example of using HTRF® cAMP assay

Min He, Dehua Yang, Zhiyun Zhang, Caihong Zhou, Xiaoqing Cai, Jia Wang, Yang Feng, Jiejie Deng, Antao Dai and Ming-Wei Wang

The National Center for Drug Screening and the CAS Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences (CAS), 189 Guo Shou Jing Road, Shanghai, China 201203

Abstract

The glucagon receptor (GLR), one of the 15 members of the class B family of G protein-coupled receptors (GPCRs), presents a potential target for treatment of diabetes. Despite a large body of information regarding the ligand binding and signaling characteristics of GPCRs in general, the molecular basis by which GLR selectively recognize and bind its endogenous ligand - glucagon is still largely unknown. To better understand GLR-glucagon interactions, we performed a comprehensive mutagenesis study of GLR at 89 different residue positions. The mutants were expressed in CHO-K1 cells and assessed for expression levels, binding of radioactive glucagon and intracellular cAMP levels. A total of 128 mutants were tested and of which, 51 mutations were found to play important roles in ligand binding and cAMP accumulation. The results are consistent with the three-dimensional model of the glucagon bound GLR structure, based on the recently solved seven-transmembrane domain crystal and the previously reported GLR extracellular domain structures.

Introduction

One of the class B G protein-coupled receptors (GPCRs), glucagon receptor (GLR), is activated by the 29 amino acid hormonal peptide – glucagon and is a drug target for type 2 diabetes. During fasting, the pancreas dispatches glucagon to activate GLR in the liver causing the release of glucose into the blood. The desired anti-diabetic activity for GLR is via an antagonistic drug that reduces blood glucose levels in diabetics.

Unlike class A, all class B GPCRs contain a globular N-terminal extracellular domain (ECD). They are activated by hormonal peptides, which bind to both the ECD and the seven-transmembrane (7TM) domains. Structural details of soluble ECDs, including the ECD of GLR, and their role in selective recognition of peptide hormones' C-termini have been revealed for several class B receptors by X-ray crystallography and NMR studies. However, in the absence of a class B 7TM domain structure, understanding of the ligand-receptor interactions and the mechanism of signal transduction remains very limited. Here, we report an extensive mutagenesis study aimed at better understanding of glucagon interactions and functional features in the context of full-length GLR.

Methods

☐ *Construction of GLR mutants and cell culture transfection*

The cDNA encoding the human GLR was cloned into the expression vector pcDNA3.1/V5-His-TOPO at the Hind III and EcoRI sites. The single and double mutants were constructed by PCR-based site directed mutagenesis. CHO-K1 cells were seeded onto 96-well poly-D-lysine treated cell culture plates at a density of 2.7×10^4 per well. After overnight culture, the cells were transiently transfected with wild-type or mutant GLR DNA using Lipofectamine 2000 transfection reagent.

☐ *Expression level quantitation of constructed GLR in cells by flow cytometry*

☐ *Whole-cell glucagon binding assay*

Cells were harvested 24 h after transfections and incubated with blocking buffer for 2 h at 37° C. For homogeneous binding, the cells were incubated in binding buffer with constant concentration of ^{125}I-glucagon (40 pM) and different concentrations of unlabeled glucagon (0.02 nM ~ 5 μM) at room temperature for 3 h. Cells were washed three times with ice-cold PBS and lysed by 50 μl lysis buffer. The plates were subsequently counted for radioactivity in a scintillation counter using a scintillation cocktail.

☐ *cAMP accumulation assay*

Transfected cells were detached from the culture dish, resuspended in stimulation buffer (F12 medium, 1 mM IBMX), and seeded in 10 μL into a PerkinElmer 384-well plate at a density of 2×10^4 cells per well. After equilibration for 10 min at room temperature, 10μL of stimulation buffer containing different concentrations of glucagons (0 ~ 1 nM) were added. After incubation for 30 min at room temperature, cells were lysed, and the cAMP content was measured using the cAMP dynamic HTRF detection kit (CisBio, France)

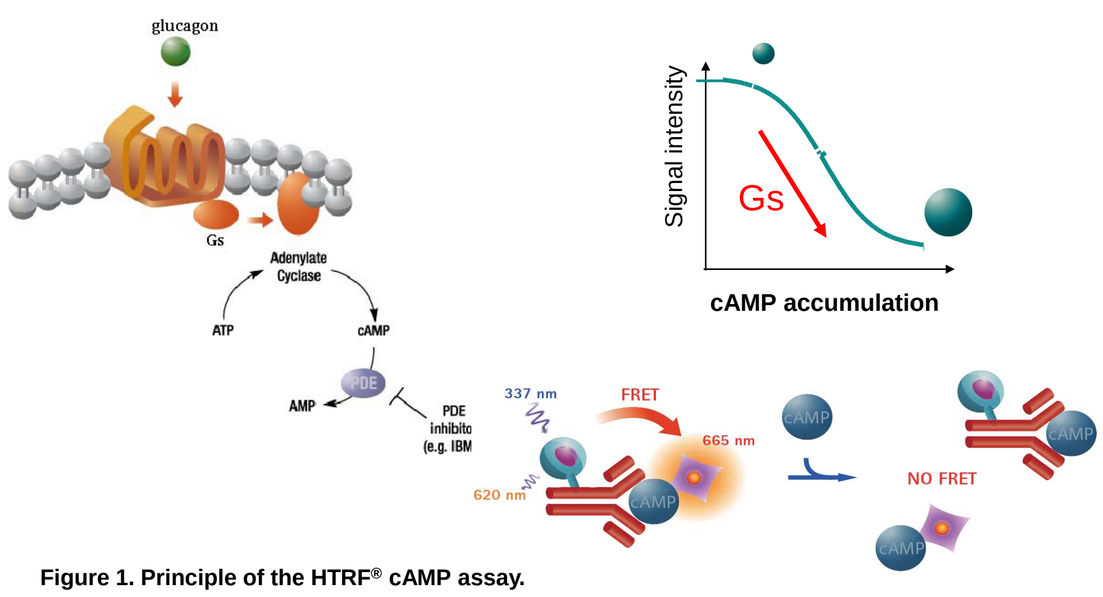

Figure 1. Principle of the HTRF® cAMP assay.

Results

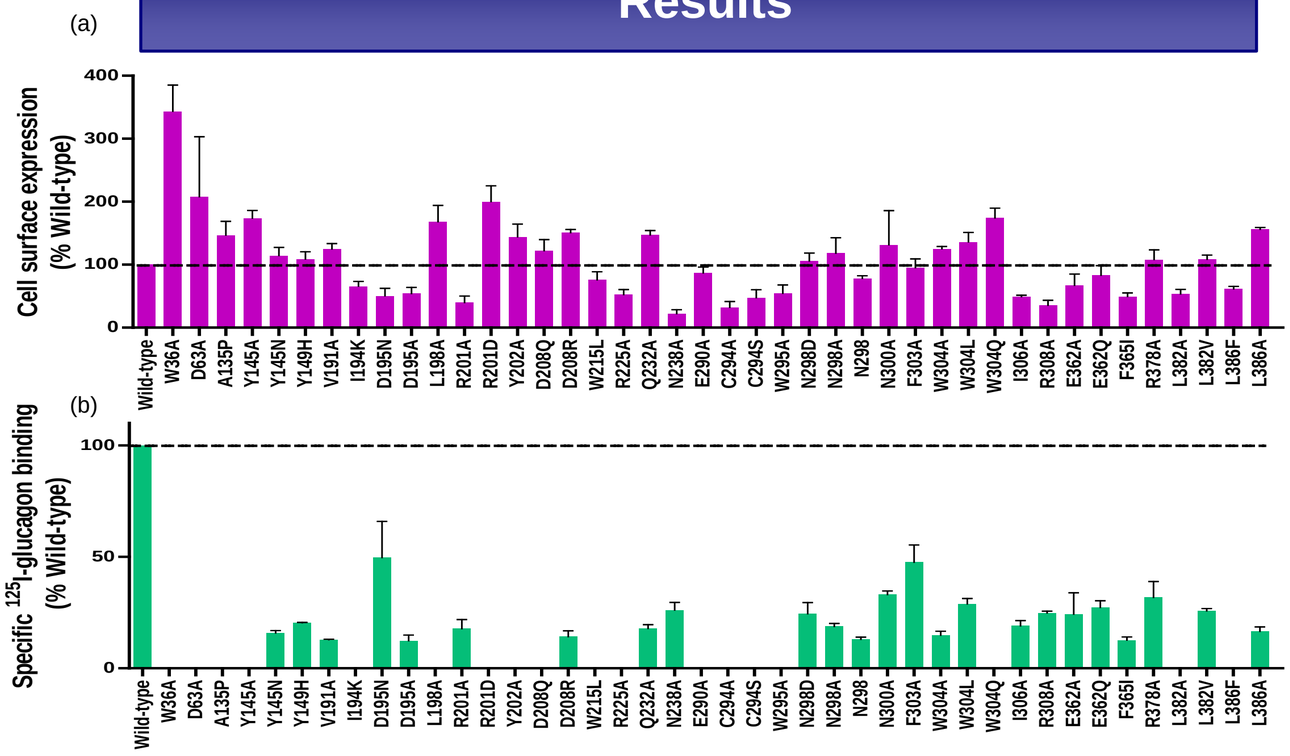

Figure 2. Cell surface expression profiles of human GLR mutants with significantly reduced specific ^{125}I-glucagon binding. Cell surface expression profiles of each of the human GLR mutants are compared with wild type transiently transfected into CHO-K1 cells as determined through flow cytometry (a) and by specific ^{125}I-glucagon (b).

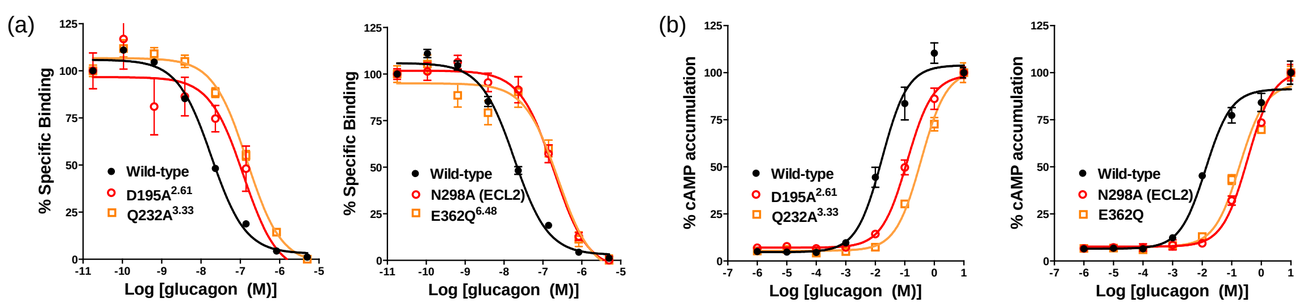

Figure 3. Representative binding and cAMP accumulation curves of GLR mutants with glucagon. Data are expressed as a percentage of specific ^{125}I-glucagon binding in the absence of unlabeled peptide (a) or as a percentage of the highest glucagon stimulated cAMP accumulation (b).

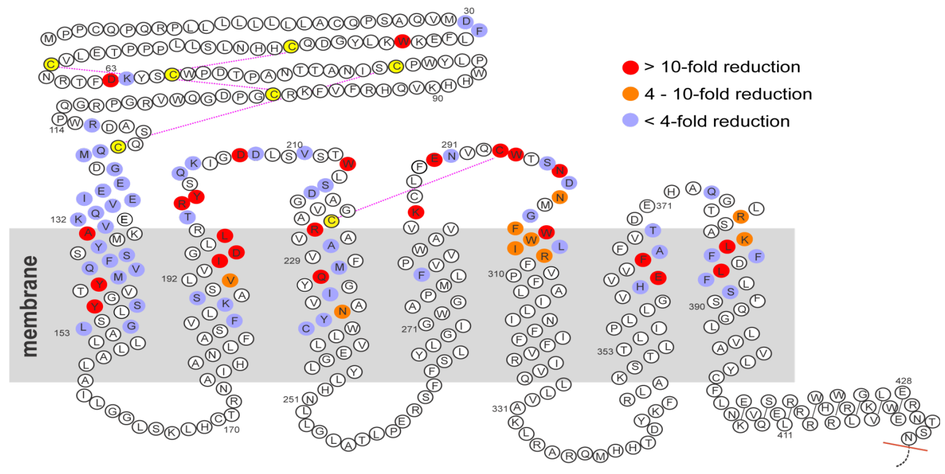

Figure 4. Effects of mutation studies plotted onto GLR snake plot. Mutagenesis study of GLR binding to glucagon mapped onto GLR snake plot. Residues that show < 4-fold, 4 to10-fold, and > 10-fold changes of IC_{50} and EC_{50} values for glucagon binding and cAMP accumulation are colored purple, orange, and red, respectively.

Conclusions

The results of these GLR mutation studies illustrate that the residues that play an important role in glucagon binding line a binding site that covers parts of ECL1, ECL2, ECL3, and helices I, II, III, V, VI, and VII. Most importantly, the binding site extends deep into the 7TM cavity.

Reference

1. Koth, C.M. *et al*. Molecular basis for negative regulation of the glucagon receptor. *Proc Natl Acad Sci USA* 109, 14393-14398 (2012).
2. Unson, C.G. *et al*. Roles of specific extracellular domains of the glucagon receptor in ligand binding and signaling. *Biochemistry* 41, 11795-11803 (2002).
3. Roberts, D.J., Vertongen, P. & Waelbroeck, M. Analysis of the glucagon receptor first extracellular loop by the substituted cysteine accessibility method. *Peptides* 32, 1593-1599 (2011).
4. Cascieri, M.A. *et al*. Characterization of a novel, non-peptidyl antagonist of the human glucagon receptor. *J Biol Chem* 274, 8694-8697 (1999).
5. Prevost, M. *et al*. Mutational and cysteine scanning analysis of the glucagon receptor N-terminal domain. *J Biol Chem* 285, 30951-30958 (2010).