Receptor Subtype-specific Docking of Asp$^{6.59}$ with C-terminal Arginine Residues in Y Receptor Ligands*

Received for publication, September 18, 2006, and in revised form, January 4, 2007

Nicole Merten$^1$, Diana Lindner$^1$, Nadine Rabe$^1$, Holger Römpler$^5$, Karin Mörl$^4$, Torsten Schöneberg$^3$, and Annette G. Beck-Sickinger$^2$

From the$^1$Institute of Biochemistry, Faculty of Bioscience, Pharmacy and Psychology, University of Leipzig, Brüderstrasse 34, 04103 Leipzig, Germany and$^3$Institute of Biochemistry, Molecular Biochemistry, Medical Faculty, University of Leipzig, Johannisallee 30, 04103 Leipzig, Germany

Y receptors (YRs) are G protein-coupled receptors whose Y$_1$R, Y$_2$R, and Y$_4$R subtypes preferentially bind neuropeptide Y (NPY) and peptide YY, whereas mammalian Y$_5$Rs show a higher affinity for pancreatic polypeptide (PP). Comparison of YR orthologs and paralogs revealed Asp$^{6.59}$ to be fully conserved throughout all of the YRs reported so far. By replacing this conserved aspartic acid residue with alanine, asparagine, glutamate, and arginine, we now show that this residue plays a crucial role in binding and signal transduction of NPY/PP at all YRs. Sensitivity to distinct replacements is, however, receptor subtype-specific. Next, we performed a complementary mutagenesis approach to identify the contact site of the ligand. Surprisingly, this conserved residue interacts with two different ligand arginine residues by ionic interactions; although in Y$_2$R and Y$_5$R, Arg$^{33}$ is the binding partner of Asp$^{6.59}$, in Y$_1$R and Y$_4$R, Arg$^{35}$ of human PP and NPY interacts with Asp$^{6.59}$. Furthermore, Arg$^{25}$ of PP and NPY is involved in ligand binding only at Y$_2$R and Y$_5$R. This suggests significant differences in the docking of YR ligands between Y$_1$,Y$_2$, and Y$_5$, and Y$_4$ and provides new insights into the molecular binding mode of peptide agonists at GPCRs. Furthermore, the proposed model of a subtype-specific binding mode is in agreement with the evolution of YRs.

Neuropeptide Y (NPY),$^3$ pancreatic polypeptide (PP), and peptide YY (PYY) belong to a conserved peptide hormone family. All members are C-terminally amidated polypeptides consisting of 36 amino acids (1) and are involved in multiple physiological processes, including memory retention (2, 3), feeding behavior (4, 5), control of blood pressure (6, 7), and seizure (8).

This makes them interesting target molecules for therapeutic interventions. Consequently, knowledge of the mechanism of peptide-receptor interaction is of great relevance, and efforts in designing subtype-selective agonists are central. The effects of NPY, PYY, and PP are transmitted by at least five rhodopsin-like GPCRs named Y$_1$ receptor (Y$_1$R), Y$_2$R, Y$_4$R, Y$_5$R, and y$_6$R. The y$_6$R shows a restricted genomic presence, indicated by the lowercase letter, being functionally expressed in rabbits and mice but is truncated in humans and absent in rat (9–11). Y$_1$R, Y$_2$R, and y$_6$R can be combined to a subfamily sharing $\sim$50% overall amino acid identity with each other but only 27–31% with Y$_2$R and Y$_5$R (12, 13). Despite the low sequence similarity between YR subtypes, all bind the same family of peptides.

All of the four most intensely investigated YR subtypes (Y$_1$R, Y$_2$R, Y$_4$R, and Y$_5$R) have their own signature profile of ligand recognition, indicating that the ligand-binding pocket displays subtype-specific differences. In general, Y$_1$R, Y$_2$R, and Y$_4$R preferentially bind NPY and PYY, whereas mammalian Y$_5$R show a higher affinity for PP (14–18). Y$_1$R and Y$_5$R require the complete N terminus of the ligand (15, 19, 20). Y$_2$R accepts peptides with the deletion of the first amino acid (21), and Y$_5$R even binds significantly shorter peptides (NPY (13–36)) or centrally truncated analogs ([Ahx$^5$–24]NPY) with only minor effects on affinity (19, 22). However, the C-terminal pentapeptide of all natural ligands was identified as an essential region for binding to all YRs (23, 24), and these residues of the ligand C terminus seem to represent a core contact domain. Interestingly, studies of analogs containing conformational constraints, when bound at the various YR subtypes, support the concept of structural differences between subtypes in this domain (25–28). However, the two conserved Arg residues at positions 33 and 35 are important contact sites for all YRs.

Although many efforts have been made to characterize the relevance of the individual amino acids of the ligand, little is known about residues within receptor involved in ligand recognition. For the hY$_1$R only, all extracellularly located Glu and Asp residues have been mutated to Ala to identify possible interaction partners of the Arg residues of the C-terminal pentapeptide of NPY (29–31). In this study, we have now identified one conserved residue Asp$^{6.59}$ (see “Experimental Procedures” for position numbering scheme) and proved its importance in all YRs by replacing it with Ala, Glu, Asn, and Arg. Interestingly, the fine mapping of Asp$^{6.59}$ showed differences between Y$_1$R, Y$_2$R, Y$_4$R, and Y$_5$R, suggesting a subtype-specific binding pocket. Then using a complementary mutagenesis approach to
Subtype-specific Y Receptor Interaction

receptors and ligands, we identified the interaction partner of YR-Asp\textsuperscript{6.59} in the peptide ligands. Surprisingly, a receptor subtype-specific interaction was observed, where Asp\textsuperscript{6.59} of Y\textsubscript{2}R and Y\textsubscript{5}R interacts with Arg\textsuperscript{35} of NPY/PP, and Asp\textsuperscript{6.59} of Y\textsubscript{2}R and Y\textsubscript{5}R contacts Arg\textsuperscript{33} of NPY, suggesting differences in the docking of YR ligands to Y\textsubscript{2}R and Y\textsubscript{5}R. Further receptor subtype-specific interactions have been identified for [Ala\textsuperscript{25}]NPY/hPP and position Asp/Glu\textsuperscript{6.27} in the second extracellular loop (ECL2) of YRs. These data are of great importance not only for YRs. We suggest a binding mode that involves at least two different events and contact sides. Furthermore, we identified one position on top of transmembrane domain (TMD) 6 that might be a general activation residue in many peptide-binding GPCRs, probably representing the second step. Additionally, these results are relevant to the evolution of GPCRs, because the multi-ligand/multi-receptor system has evolved from an ancestor ligand/receptor pair. We speculate that the Asp\textsuperscript{6.59}/Asp\textsuperscript{6.59} interaction may have evolved in the primordial YR first, and the alternate binding of the Asp\textsuperscript{6.59} arose later, after the gene duplication and evolution of Y\textsubscript{2}/Y\textsubscript{5}Rs.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—The following peptides were synthesized by automated solid phase peptide synthesis using the Fmoc-tert-buty (9-fluorenylmethoxy carbonyl-tert-buty) strategy (23): porcine NPY (pNPY), [Ala\textsuperscript{4}pNPY, [Ala\textsuperscript{19}]pNPY, [Ala\textsuperscript{25}]pNPY, [Ala\textsuperscript{26}]pNPY, [Ala\textsuperscript{33}]pNPY, [Ala\textsuperscript{35}]pNPY, human PP (hPP), [Ala\textsuperscript{25}]hPP, [Ala\textsuperscript{26}]hPP, [Ala\textsuperscript{33}]hPP, and [Ala\textsuperscript{35}]hPP. The peptides were purified, and peptide mass was verified by matrix-assisted laser desorption ionization-mass spectrometry. To confirm the correct position of C-terminal Ala residues, bromocyan digest followed by Edman sequencing ([Ala\textsuperscript{25}]hPP and [Ala\textsuperscript{35}]hPP) or tryptic digest combined with matrix-assisted laser desorption ionization mass spectrometry of the fragments ([Ala\textsuperscript{33}]pNPY and [Ala\textsuperscript{35}]pNPY) were performed. CD analysis of Ala substituted peptides were performed (23) and showed no significant differences to wild type pNPY and hPP.

Position Numbering System in YRs—The residues are numbered relative to the most conserved residue contained in the helix, as explained previously (32). On the basis of this scheme, the most conserved residue in TMD 6 is proline, designated with the index number 6.50 and hence referred to throughout as Pro\textsuperscript{6.50}. Asp, located nine residues C-terminal of Pro\textsuperscript{6.50}, is referred to Asp\textsuperscript{6.59} to compare conserved residues in different receptors.

Generation of Mutant YRs—The subcloning of the human Y\textsubscript{2}R and Y\textsubscript{5}R cDNA into the eukaryotic expression plasmid pEGFP-N1 (Clontech, Heidelberg, Germany) was fused to the human Y\textsubscript{2}R and Y\textsubscript{5}R cDNA for fluorescence detection. As previously shown the function of YRs is not altered by C-terminal fusion to fluorescent proteins (33). The correctness of all of the constructs was confirmed by sequencing of the entire coding region.

Cell Culture, Transfection, and Functional Assays—Baby hamster kidney cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum. COS-7 cells (African green monkey kidney cells) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were grown as monolayers at 37 °C in a humidified atmosphere of 5% CO\textsubscript{2} and 95% air.

Radioligand binding studies were performed with [\textsuperscript{3}H]proionylated pNPY ([\textsuperscript{3}H]pNPY; 107 Ci/mmol; Amersham Biosciences) and [\textsuperscript{3}H]proionylated hPP ([\textsuperscript{3}H]hPP) on transiently transfected cells as described previously (34, 35) (for details see supplemental materials). Signal transduction was studied by co-transfection of COS-7 cells with YRs and chimeric G proteins (Go\textsubscript{q41}, Go\textsubscript{q54}, kindly provided by E. Kostenis) and subsequent measuring of the signal of the phospholipase Cβ-pathway (inositol phosphate (IP) accumulation) as described (36, 37). It has been demonstrated that replacement of the four or five C-terminal amino acids of Go\textsubscript{q41} with the corresponding Go\textsubscript{q6} residues (referred to as Go\textsubscript{q41m}) confers the ability to stimulate the phospholipase Cβ pathway onto G\textsubscript{i}-coupled receptors (38, 39). For control, transfection of chimeric G proteins without receptor and only transfection of the receptor and subsequent stimulation experiments were performed and did not show any increased IP accumulation up to 100 µM of the ligand (for details and controls see supplemental Fig. S1).

Immunological and Immunofluorescence Studies—To ligand-independently estimate plasma membrane localization, a cell surface ELISA was performed with wild type and mutant receptors carrying an N-terminal hemagglutinin tag (40) (for details see supplemental materials). Immunofluorescence studies were carried out to determine the subcellular distribution of wild type and mutant receptors. COS-7 cells were transferred into 24-well plates that contained sterilized glass coverslips and transfected. Approximately 48 h later, the cells were fixed with formaldehyde. The cells were treated with an anti-hemagglutinin monoclonal antibody (10 µg of 12CA5/ml) and then incubated with a tetramethylrhodamine isothiocyanate-linked anti-mouse IgG secondary antibody. Fluorescence images were obtained with a confocal laser-scanning microscope (Leica TCS SP2; Heidelberg, Germany).

RESULTS

Identification of Conserved Residues in the Third Extracellular Loop—By comparing the amino acid sequences of ECL3 of human, rat, and mouse YRs (Fig. 1) and further available sequences, the conservation of residue Asp\textsuperscript{6.59} became obvious for YRs, whereas Asp/Glu is not found in any other peptide-binding GPCRs except for the receptors of the neuropeptide FF (NPFF) and pyroglutamylated Arg-Phe-amide peptide (QRFP) family. The general receptor numbering system (32) was used to compare residue positions between receptors. The first digit indicates the transmembrane domain, and the last two digits

7544 JOURNAL OF BIOLOGICAL CHEMISTRY
VOLUME 282 • NUMBER 10 • MARCH 9, 2007
Asp<sup>6.59</sup> is a functional key residue in all YRs — To study whether this conserved aspartate plays an important role in ligand binding, Asp<sup>6.59</sup> was substituted with Ala in Y<sub>R</sub>, Y<sub>R</sub>, Y<sub>R</sub>, and wild type and mutant receptors were transiently expressed and functionally tested. As shown in Table 1, mutation of Asp<sup>6.59</sup> to Ala resulted in a dramatic loss of affinity, and the <i>K<sub>d</sub></i> values could no longer be determined because specific binding was only 2–4% of wild type binding. To further characterize the mutant receptors, IP accumulation assays with chimeric G proteins were performed. IP formation revealed a more than 600-fold reduction in pNPY potency for Y<sub>R</sub>-D6.59A, a 12-fold reduction for Y<sub>R</sub>-D6.59A, and a 99-fold reduction for Y<sub>R</sub>-D6.59A compared with a 461-fold reduction for Y<sub>R</sub>-D6.59A (Table 1). In contrast, mutagenesis of nearby Asp residues in Y<sub>R</sub> and Y<sub>R</sub> to yield Y<sub>R</sub>-D<sup>6.51</sup>A and Y<sub>R</sub>-D<sup>6.62</sup>A showed wild type properties in both binding and signal transduction.

**TABLE 1**

Functional characterization of wild type and mutant Y receptors

| Construct | Radioligand binding (<i>K<sub>d</sub></i>)<sup>a</sup> | IP assay<sup>b</sup> | ELISA (cell surface expression)<sup>c</sup> |
|-----------|-----------------|-----------------|-----------------|
|            |                  | <i>F<sub>max</sub></i> | EC<sub>50</sub> | EC<sub>50</sub>/EC<sub>50 WT</sub> | % wild type |
| Y<sub>R</sub> | 2.0 ± 0.4        | 12.1 ± 4.7      | 3.0 ± 0.1      | 1.0 | 100 |
| Y<sub>R</sub>-D6.59A | ND<sup>d</sup> | 11.2 ± 19.9 | 1.384 ± 496 | 461 | 135 ± 31 |
| Y<sub>R</sub>-D6.59E | ND<sup>d</sup> | 10.3 ± 4.22 | 6.5 ± 1.4 | 2.2 | 111 ± 15 |
| Y<sub>R</sub>-D6.59N | ND<sup>d</sup> | 15.6 ± 2.43 | 404 ± 39 | 135 | 156 ± 18 |
| Y<sub>R</sub>-D6.59R | ND<sup>d</sup> | 1.4 ± 0.5 | ND<sup>d</sup> | 110 ± 5 |
| Y<sub>R</sub> | 0.2 ± 0.1 | 1.4 ± 0.93 | 9.7 ± 4.8 | 1.0 | 100 |
| Y<sub>R</sub>-D6.59A | ND<sup>d</sup> | 2.9 ± 0.2 | >6,000 | >600 | 135 ± 30 |
| Y<sub>R</sub>-D6.59E | ND<sup>d</sup> | 4.5 ± 1.1 | 199 ± 97 | 20 | 83 ± 13 |
| Y<sub>R</sub>-D6.59N | 1.4 ± 0.6 | 3.7 ± 0.3 | 1,694 ± 484 | 175 | 89 ± 19 |
| Y<sub>R</sub>-D6.59R | ND<sup>d</sup> | 1.0 ± 0.1 | ND<sup>d</sup> | 102 ± 17 |
| Y<sub>R</sub>-D6.61A | 0.25 ± 0.01 | 4.3 ± 1.2 | 15.3 ± 1.3 | 1.6 | 61 ± 9 |
| Y<sub>R</sub> | 4.0 ± 0.1 | 8.4 ± 0.1 | 10.5 ± 0.8 | 1.0 | 100 |
| Y<sub>R</sub>-D6.59A | ND<sup>d</sup> | 9.0 ± 0.7 | 134 ± 15 | 12 | 257 ± 7.7 |
| Y<sub>R</sub>-D6.59E | 6.9 ± 1.6 | 10.2 ± 2.1 | 21.7 ± 11.8 | 2.1 | 163 ± 11 |
| Y<sub>R</sub>-D6.59N | ND<sup>d</sup> | 9.3 ± 1.5 | 214 ± 29 | 20 | 208 ± 54 |
| Y<sub>R</sub>-D6.59R | ND<sup>d</sup> | 6.4 ± 0.3 | >6,000 | >600 | 214 ± 29 |
| Y<sub>R</sub> | 21.9 ± 0.7 | 2.9 ± 0.1 | 13.1 ± 4.0 | 1.0 | 100 |
| Y<sub>R</sub>-D6.59A | ND<sup>d</sup> | 2.6 ± 0.2 | 1303 ± 381 | 99 | 113 ± 9 |
| Y<sub>R</sub>-D6.59E | 2.6 ± 0.4 | 2.8 ± 0.5 | 14.5 ± 0.6 | 1.1 | 99 ± 7 |
| Y<sub>R</sub>-D6.59N | ND<sup>d</sup> | 2.6 ± 0.4 | 890 ± 105 | 68 | 100 ± 15 |
| Y<sub>R</sub>-D6.59R | ND<sup>d</sup> | 0.7 ± 0.2 | ND<sup>d</sup> | 98 ± 12 |
| Y<sub>R</sub>-D6.62A | 1.6 ± 0.4 | 2.6 ± 0.1 | 22.3 ± 2.9 | 1.7 | 106 ± 5 |

<sup>a</sup> <i>K<sub>d</sub></i> values were determined in saturation experiments using [<sup>3</sup>H]pNPY for Y<sub>R</sub>, Y<sub>R</sub>, Y<sub>R</sub>, and [<sup>3</sup>H]pPP for Y<sub>R</sub>.<br><sup>b</sup> <i>F<sub>max</sub></i> and EC<sub>50</sub> values were obtained from concentration-response curves. The <i>F<sub>max</sub></i> values are presented as fold over basal IP levels of the corresponding wild type YRs (Y<sub>R</sub>, 2237 ± 397 cpm/well; Y<sub>R</sub>, 670 ± 97 cpm/well; Y<sub>R</sub>, 4809 ± 1329 cpm/well; and Y<sub>R</sub>, 1252 ± 140 cpm/well) at 10 μM peptide (asterisks indicate 100% peptide).<br><sup>c</sup> Cell surface expression levels were measured by an indirect cellular ELISA. Specific optical density readings (optical density value of hemagglutinin-tagged construct minus OD value of green fluorescent protein- or yellow fluorescent protein-transfected cells) are given as percentages of wild type hemagglutinin-tagged YR. The nonspecific OD<sub>492 nm</sub> values were 0.279 ± 0.143 (green fluorescent protein) and 0.228 ± 0.062 (yellow fluorescent protein), and the OD<sub>492 nm</sub> values of the hemagglutinin-tagged wild types were 0.686 ± 0.236 (Y<sub>R</sub>) and 0.455 ± 0.080 (Y<sub>R</sub>). For the measurement of the hemagglutinin-tagged Y<sub>R</sub> and Y<sub>R</sub> wild types (OD<sub>492 nm</sub> = 0.501 ± 0.036; Y<sub>R</sub>; 0.703 ± 0.100) concentration of peroxidase-labeled streptavidin conjugate was increased, which also resulted in higher nonspecific OD<sub>492 nm</sub> values (OD<sub>492 nm</sub>, yellow fluorescent protein: 0.310 ± 0.096; green fluorescent protein: 0.419 ± 0.038). The ELISA data are given as means ± S.D. of two to five independent experiments, each carried out in quadruplicate.<br><sup>d</sup> ND, not determinable with sufficient accuracy.
Subtype-specific Y Receptor Interaction

**FIGURE 2. Functional characterization of wild type and Asp<sup>6.59</sup> mutant YRs.** COS-7 cells expressing wild type YRs (wt) or the corresponding Asp<sup>6.59</sup> mutants and a chimeric G protein were incubated with increasing concentrations of pNPY (Y<sub>1</sub>R, Y<sub>5</sub>R, and Y<sub>7</sub>R) or hPP (Y<sub>2</sub>R). IP accumulation assays were performed as described under “Experimental Procedures”. Data of two independent experiments each performed in duplicate are shown and expressed as fold over wt basal IP level (Y<sub>1</sub>R, 2237 ± 397 cpm/well; Y<sub>2</sub>R, 670 ± 97 cpm/well; Y<sub>4</sub>R, 4809 ± 1329 cpm/well; and Y<sub>5</sub>R, 1252 ± 140 cpm/well).

To test the importance of the negative charge at position 6.59, we set out to identify the basic counterpart of the receptor Asp<sup>6.59</sup> within NPY/PP hormones by a complementary mutagenesis approach. As already suggested by an Ala scan and binding studies (23) Lys<sup>5</sup>, Arg<sup>19</sup>, and His<sup>26</sup> in NPY do not significantly influence agonist potency and efficacy at Y<sub>2</sub>R when compared with wild type NPY (data not shown). However, [Ala<sup>25</sup>]pNPY resulted in a 10-fold increase, and [Ala<sup>33</sup>]pNPY and [Ala<sup>35</sup>]pNPY resulted in a more than 100-fold increase in EC<sub>50</sub> values with Y<sub>2</sub>R expressing cells (Table 2). To dissect which of the two most important Arg residues (Arg<sup>33</sup> and Arg<sup>35</sup>) in NPY participated in a postulated electrostatic interaction with Asp<sup>6.59</sup>, the two pNPY-Ala peptides were functionally tested on COS-7 cells transfected with Y<sub>2</sub>R-D6.59A. In such a complementary mutagenesis approach, one can assume that the decreased potency of a mutant receptor, in which an interacting residue was substituted, remains unchanged when tested with wild type and mutant peptide. In other words, in the mutant receptor, one partner of a peptide-receptor bond has already been disrupted, and the mutation of the counterpart in the peptide should have no further impact on this interaction. Thus, [Ala<sup>33</sup>]pNPY and [Ala<sup>35</sup>]pNPY were tested on Y<sub>2</sub>R-D6.59A. Interestingly, concentration-response curves to [Ala<sup>35</sup>]pNPY were further right-shifted compared with those to pNPY for cells expressing the mutant receptor (Fig. 3). This indicates that Arg<sup>35</sup> in pNPY contacts residues in Y<sub>2</sub>R that are different from Asp<sup>6.59</sup>. In contrast, the potency of [Ala<sup>33</sup>]pNPY remained unchanged at this mutant receptor, implying that Arg<sup>33</sup> of pNPY and Asp<sup>6.59</sup> in Y<sub>2</sub>R form an electrostatic bond.

**Interaction of Asp<sup>6.59</sup> with Arg<sup>33</sup> and Arg<sup>35</sup> Depends on the YR Subtype**—To test whether Arg<sup>33</sup> of NPY interacts with Asp<sup>6.59</sup> not only in Y<sub>2</sub>R, we extended the two-sided mutagenesis approach to all other human YRs. As seen with the Y<sub>2</sub>R, replacement of Lys<sup>5</sup>, Arg<sup>19</sup>, and His<sup>26</sup> in NPY and Arg<sup>35</sup> in PP by Ala changed neither affinity nor activity at all other YRs. [Ala<sup>25</sup>]NPY/PP had no effect on Y<sub>1</sub>R or Y<sub>4</sub>R, respectively, but the NPY analog led to an approximately 10-fold loss of activity in Y<sub>2</sub>R (Table 2), whereas [Ala<sup>33</sup>]pNPY/hPP and [Ala<sup>35</sup>]pNPY/hPP significantly reduced binding at all wild type receptors. Functional assays were used to determine the potencies of
[Ala\textsuperscript{33}]pNPY and [Ala\textsuperscript{35}]pNPY (for Y1R and Y2R) or [Ala\textsuperscript{33}]hPP and [Ala\textsuperscript{35}]hPP (for Y3R). As with Y2R, the potency of [Ala\textsuperscript{33}]pNPY was not significantly different at Y2R-D6.59A transfected cells compared with pNPY, whereas the potency of [Ala\textsuperscript{35}]pNPY was further reduced at the mutant receptor (Fig. 3 and Table 2). This indicates that Asp6.59 in Y5R also forms a salt bridge with Arg33 of peptide ligands.

Further Evidence for Different Subtype-selective Ligand-binding Pockets in YR—To generate further evidence for a different ligand-binding pocket in Y2R versus Y1R, the analogs [Ala\textsuperscript{25}NPy/PP were studied in more detail. Indeed, only at Y2R and Y5R was a 10-fold loss in potency found, whereas Y1/4Rs showed wild type properties to these agonists (Fig. 4A). Thus, we mutated all relevant acidic residues within all four receptors and Table 2), rejecting the hypothesis that Arg25 of the peptide forms an ionotropic interaction with an acidic residue of ECL2 of Y2R and Y5R. Similar results were seen with the [Ala\textsuperscript{33}]NPY and [Ala\textsuperscript{35}]NPY peptide analogs at Y3R-Glu5.24Ala and Y5R-Glu5.27Ala. Therefore, it is more likely that either π-cation interactions form the second contact, or more than one Glu/Asp residue is involved. Accordingly, the complementary mutagenesis approach revealed that none of the three Arg residues of the ligand acts as the interacting partner, so a Y2/3R-selective intramolecular salt bridge can be postulated. This further supports the concept of a different binding pocket and again is nicely in agreement with evolution in which Glu\textsuperscript{5.27} of the Y3R is not conserved, and Val is present at this position in the rat Y3R.

**DISCUSSION**

Identification of receptor residues that are involved in ligand binding is a fundamental step in elucidating and understanding the principles of ligand-receptor interactions. The only known detailed structure of a GPCR bound to its ligand has been reported for rhodopsin (41), in which the characterized seven-transmembrane receptor is in its inactive state. Significant structural rearrangements, however, are required to obtain active GPCRs. Much effort has been made for many small ligand GPCRs, but little is known so far for peptide GPCRs (43–45). In peptide GPCRs one of the most challenging problems is the identification of the binding mode of agonists (42).

To identify receptor residues that are important for agonist binding of the whole YR family, we applied the approach of screening for residues conserved within the receptor family by comparing all of the available sequences for conserved positions. With this approach, Asp\textsuperscript{6.59} was found to be the only YR-specific acidic residue in the ECLs that is conserved throughout all subtypes and species for which receptor sequences have been reported so far. This is in agreement with earlier reports suggesting that Asp\textsuperscript{6.59} of YR at the junction of TMD6 and ECL3 plays an important role in agonist (29, 31) and antagonist (30) binding at YR. Interestingly, in other peptide-binding GPCRs, position 6.59, in combination with position 6.58, is also frequently a residue conserved within a family. Thus, receptors binding orexin contain a conserved Lys\textsuperscript{6.58}/Lys\textsuperscript{6.59}, cholecystokinin receptors Arg\textsuperscript{6.58}/Ala\textsuperscript{6.59}, endothelin receptors Lys\textsuperscript{6.58}/Lys\textsuperscript{6.59}/Leu\textsuperscript{6.59}, bradykinin receptors Asp\textsuperscript{6.58}/Phe\textsuperscript{6.59}/Thr\textsuperscript{6.59}, angiotensin receptors Asp\textsuperscript{6.58}/Val\textsuperscript{6.59}, and many more receptors show conserved trifunctional residues at position 6.58 and/or 6.59 that qualify for ligand binding. A further indication of a very distinct interaction at this position can be concluded because nearby Asp residues in the Y3R and Y5R (Asp\textsuperscript{6.61} and Asp\textsuperscript{6.62}) do not contribute to ligand binding. Position...
tions 6.58/6.59 are on top of TMD6; many studies in rhodopsin and other GPCRs suggest that this region of TMD6 and the neighboring ECL are important in receptor-specific agonist binding (46–49). Aspartate residues can participate in several types of interactions, and their role can be characterized by replacing Asp with, for example, Glu and Asn. In the gonadotropin-releasing hormone receptor, Asp$^{6.59}$ has been reported to interact with His$^{7}$ of the ligand by hydrogen bonds (50), whereas Porter and Perez (51) described Asp$^{125}$ of $\alpha_{1b}$-adrenergic receptor to form a salt bridge between the ligand and the receptor. To identify the mode of action of the conserved Asp$^{6.59}$ in YR, we replaced the Asp$^{6.59}$ of all YRs with Ala, Glu, and the Ala analogs (Fig. 3). This approach allowed us to investigate the effects of mutating Asp$^{6.59}$ in the YR subtypes.
Asn, and Arg. In all YRs, replacement of Asp with Glu is tolerated, whereas the replacement of Asp with Ala, Asn, and Arg leads to a significant loss in affinity and receptor activation. This is in agreement with a major ionic component of the ligand-receptor interaction. However, small subtype specific differences were observed; thus, in Y1R the conservative mutation of Asp6.59 to glutamate (Y1R-D6.59E) revealed an anomalous loss of binding but wild type-like affinity in functional assay. This can be explained by different k_{off}/k_{on} constants. As shown for the orexin A receptor, high k_{off} values lead to receptors for which binding is very difficult to measure (52), but activity can be studied easily. This might be the case for this mutant as well, because of the size of the binding pocket caused by the Asp-Glu mutation. In addition to the ionic component, the slight decrease in affinity of Y1/2R-D6.59A compared with Y1/2R-D6.59N suggests the additional involvement of some polar ligand-receptor interactions at Y1R-Asp6.59 and Y2R-Asp6.59 that can be still fulfilled by Asn but not by Ala.

To finally elucidate the residue within the ligand that interacts with Asp6.59, a complementary mutagenesis approach was taken. In Y2R and Y5R, the conserved Arg33 residue of the ligand NPY is suggested to be the interacting partner of Asp6.59, whereas in Y1R and Y4R, the conserved Arg35 of NPY and hPP form the ionic contact. The fact that the conserved Asp6.59 of YRs can interact either with Arg33 or Arg35 of the peptide provides unexpected insights into the mode of peptide-receptor interaction. The choice of which of the two ligand Arg residues contacts the receptor Asp6.59 must be guided by the receptor itself, because the peptide ligand is identical for Y1R and Y2R, for example. Structural analyses of NPY and PP revealed that Arg33 and Arg35 are located at the very C terminus of an α-helix (53, 54). This free accessibility of both Arg residues would allow for an alternative contact with Asp6.59. However, the observed receptor-subtype specificity in the formation of a salt bridge is not compatible with such a model. It instead suggests different scenarios in which the formation of an Arg-Asp interaction is secondary to an initial, subtype-specific contact between the peptide ligand and the receptor.

FIGURE 4. Identification of YR subtype-selective peptide-receptor interactions. Further evidence for a subtype-selective binding is supported by the receptor subtype-selective recognition of Arg25 of the ligand (A) and the receptor subtype-selective sensitivity of Asp/Glu5.27 of YRs (B). COS-7 cells were transfected with chimeric G protein and wild type (wt) or Asp/Glu5.27Ala constructs. Concentration-response curves were determined in IP assays using increasing concentrations of pNPY, hPP, [Ala25]pNPY, or [Ala25]hPP. Fold stimulation over basal IP levels are given as the means ± S.E. of two independent assays, each performed in duplicate.
Subtype-specific Y Receptor Interaction

FIGURE 5. Proposed subtype-specific interaction between NPY/PP and YRs. Two conserved Arg residues, Arg43 and Arg45, at the C-terminus of peptides of the NPY/PP family participate in the formation of high affinity interactions to YRs. Because Arg residue interaction with Asp6.59 in YRs is strictly subtype-dependent, this interaction must be guided by further receptor-ligand contacts. Arg45 may be a candidate for such interaction because its relative positioning within the α-helical part of the ligand is spatially conjunct with the two Arg residues, and consistently, it participates in subtype-specific binding. A possible candidate for the steric hindrance might be a subtype-selective salt bridge in ECL2 at position 5.27. TM, transmembrane.

One scenario might be that a first interaction site differs between the Y1R/Y4R and the Y2R/Y5R subfamilies and subsequently also causes a difference in the relative orientation of the peptide toward Asp6.59. We identified Arg33 as a candidate that shows high subtype-specific binding properties. Interestingly, NMR studies demonstrate that Arg25 is located at the same face of the C-terminal α-helix as is Arg43 and Arg45, which qualifies this residue to serve as a first contact point in Y2R (Fig. 5). Then because of the inflexibility of Arg43 within the α-helix, this important position can interact with Asp6.59 (proximity model). This is further supported by the importance of the α-helix of the ligand for Y1R binding (26). A second reasonable model suggests that the first interaction site is identical in all YRs but also different from the interaction of Asp6.59. In this model, one or more subtype-specific residues would constrain the peptide and restrict Asp6.59 to interaction with only one of the Arg residues (hindrance model). A hydrophobic face that has been discussed previously for Y1R might serve as a first and common binding site (54). However, hydrophobic interactions work only at much shorter range than do ionic interactions, making such scenario not very likely. In a third model, the ligand nonspecifically binds first to the membrane and then enters the receptor (55), probably between TMD5 and 6 (56). The overall size of the binding pocket will then determine how deep the ligand can insert into the receptor. In the case of the Y2R/Y5R subfamily, residues might block the entrance and just allow the formation of the activating contact of Asp6.59 via Arg33, whereas in the case of the Y1R/Y4R receptor, different docking might occur. One candidate for such a subtype-selective sensitivity position is Asp6.59 in ECL2, which might form a salt bridge, preventing the ligand from getting any deeper into the binding pocket. In any case, a receptor subtype-specific event must occur first, and then the ionic contact of Asp6.59 can be formed in a second step. As a fourth possibility, Y2R/Y5R ligand binding can be postulated to affect Gq coupling in a different and selective manner. Ongoing work now seeks to identify further subtype-specific interaction partners, which subsequently will help to further elucidate the binding mechanism.

Evolutionary and structural comparison studies suggest that Y1R and Y4R form a distinct subfamily of YRs, and Y2R and Y5R orthologs showed closer structural relation to each other than to members of the Y1R/Y4R subfamily (13). This is in good agreement with the identified differences in the binding mode of the ligand. Further comparison of GPCRs revealed that only receptors that bind the RF-amide peptides also contain Asp6.59. RF-amide peptides such as neuropeptide FF, AF, and QRFP share a common C-terminal Pro-Gln-Arg-Phe-NH2 motif. It can be speculated that this Arg has the same function as Arg25 of NPY and hPP and interacts with the conserved Asp6.59 of the NPF-family, NPFF1, NPFF2, and QRFP receptor. According to this, the evolutionarily older binding mode would be the interaction between Arg33 and Asp6.59, and later, a second mode of binding characterized by the Arg33/Asp6.59 interaction evolved.

Acknowledgments—We thank Undine Dietrich for assistance at the confocal microscope, Doris Haines for peptide synthesis, and William F. Colmers and Jürgen Wess for carefully reading the manuscript and helpful discussion.

REFERENCES

1. Cerda-Reverter, J. M., and Larhammar, D. (2000) Biochem. Cell Biol. 78, 371–392
2. Cleary, J., Semotuk, M., and Levine, A. S. (1994) Brain Res. 653, 210–214
3. Nakajima, M., Inui, A., Teranishi, A., Miura, M., Hirose, Y., Okita, M., Himori, N., Baba, S., and Kasuga, M. (1994) J. Pharmacol. Exp. Ther. 268, 1010–1014
4. Pau, M. Y., Pau, K. Y., and Spies, H. G. (1988) Physiol. Behav. 44, 797–802
5. Lecklin, A., Lundell, I., Paananen, L., Wikberg, J. E., Mannisto, P. T., and Larhammar, D. (2002) Br. J. Pharmacol. 135, 2029–2037
6. Capurro, D., and Huidobro-Toro, J. P. (1999) Eur. J. Pharmacol. 376, 251–255
7. Abrahamsson, C. (2000) J. Cardiovasc. Pharmacol. 36, 451–458
8. El Bahh, B., Balosso, S., Hamilton, T., Herzog, H., Beck-Sickinger, A. G., Sperk, G., Gehlert, D. R., Vezzani, A., and Colmers, W. F. (2005) Eur. J. Neurosci. 22, 1417–1430
9. Gregor, P., Feng, Y., DeCarr, L. B., Cornfield, L. J., and McCaleb, M. L. (1996) J. Biol. Chem. 271, 27776–27781
10. Matsumoto, M., Nomura, T., Momose, K., Ikeda, Y., Konou, D., Akiko, H., Togami, J., Kimura, Y., Okada, M., and Yamaguchi, T. (1996) J. Biol. Chem. 271, 27217–27220
11. Weinberg, D. H., Sirinathsinghji, D. J., Tan, C. P., Shiao, L. L., Morin, N., Rigby, M. R., Heavens, R. H., Rapoport, D. R., Bayne, M. L., Cascieri, M. A., Strader, C. D., Linemeyer, D. L., and MacNeil, D. J. (1996) J. Biol. Chem. 271, 16435–16438
12. Larhammar, D., Wraith, A., Berglund, M. M., Holmberg, S. K., and Lundell, I. (2001) Peptides 22, 295–307
13. Larhammar, D., and Salaneck, E. (2004) Neuropeptides 38, 141–151
14. Larhammar, D., Blomqvist, A. G., Yee, F., Jazin, E., Yoo, H., and Wahlestedt, C. (1992) J. Biol. Chem. 267, 10935–10938
15. Bard, J. A., Walker, M. W., Branchek, T. A., and Weinshank, R. L. (1995) J. Biol. Chem. 270, 26762–26765
16. Gerald, C., Walker, M. W., Yass, P. J., He, C., Branchek, T. A., and Weinshank, R. L. (1995) J. Biol. Chem. 270, 26758–26761
17. Berglund, M. M., Lundell, I., Eriksson, H., Soll, R., Beck-Sickinger, A. G., and Larhammar, D. (2001) Peptides 22, 351–356
18. Lundell, I., Boswell, T., and Larhammar, D. (2002) J. Mol. Endocrinol. 28, 225–235
19. Beck-Sickinger, A. G., and Jung, G. (1995) Biopolymers 37, 123–142
20. Eriksson, H., Berglund, M. M., Holmberg, S. K., Kahl, U., Gehlert, D. R., and Larhammar, D. (1998) Regul. Pept. 75–76, 29–37
21. Gerald, C., Walker, M. W., Criscione, L., Gutfsohn, E. L., Batzila-Hartmann, C,
