Functional Segregation of the Highly Conserved Basic Motifs within the Third Endoloop of the Human Secretin Receptor

KATHY YUEN-YEE CHAN, RONALD TING-KAI PANG, AND BILLY KWOK-CHONG CHOW
Department of Zoology, University of Hong Kong, Hong Kong, People’s Republic of China

In this study, a mutagenesis-based strategy was employed to assess the roles of two highly conserved motifs (KLR and RLAR) within the third endoloop of the human secretin receptor. Block deletion of KLRT and mutation of Lys323 (R323K) significantly reduced cAMP accumulation, and these mutations did not affect ligand interaction and receptor number significantly reduced cAMP accumulation, and these mutations did not affect ligand interaction and receptor number expression on the cell surface. Thus, the KLRT region at the N terminus of the third endoloop, particularly Lys323, is important for G protein coupling. For the RLAR motif, receptors with substitutions at positions 339 and 342 from Arg to Ala (R339A/342A), Glu (R339,342E), or Ile (R339,342I) as well as block deletion of the RLAR motif were all found to be defective in both secretin-binding and cAMP production. Interestingly, a single mutation at the corresponding positions of Arg339 or Arg342 responded as the wild-type human secretin receptor in all functional assays, indicating that the presence of one Arg at either position within the RLAR motif is sufficient for a normal receptor function. Immunofluorescent staining of these mutant receptors showed that these Arg residues are responsible for surface presentation and/or receptor stability.

THE SECRETIN RECEPTOR has a high affinity for secretin and a relatively low affinity for VIP (1) and belongs to the class II G protein-coupled receptor (GPCR) subfamily. This secretin/VIP receptor subfamily also includes receptors for glucagon, glucagon-like peptide-1 (GLP-1), gastric inhibitory peptide, PTH, pituitary adenylate cyclase activating polypeptide, and GHRH. Generally, the signal transduction mechanism of GPCRs involves ligand-induced changes that affect the conformation of the intracellular surface of the receptors and hence promote the coupling to G proteins (2–6). In the case of the secretin/VIP receptor subfamily, this stimulation process activates adenylate cyclase and eventually leads to the elevation of intracellular cAMP (7–11). Besides cAMP, other intracellular second messengers, such as Ca2+ and inositol phosphates, have also been reported (12–14).

Like other GPCRs, the secretin receptor displays a common structural profile in which seven transmembrane domains are linked by alternative exo- and endoloops. Within the same class, there are many conserved amino acid residues, including six well-conserved cysteine residues in the N-terminal extracellular domain and multiple consensus N-glycosylation sites. Nevertheless, these receptors share only 25–50% of amino acid identity among themselves. When compared with other classes of receptors, such as the rhodopsin/β-adrenergic receptor family, the secretin receptor family is distinct with respect to the primary sequence. Even with this minimal level of sequence homology, comparisons of receptor properties within the family can provide insight into the importance of specific structural domains or motifs. For example, the diversity of the N termini of these receptors in amino acid composition suggests that the N-terminal ectodomain should contribute to specific ligand recognition (15–17). Moreover, sequence homology of the human secretin receptor (hSR) with other class II receptors revealed only one conserved N-linked glycosylation site (position 72) of a total of five putative sites, implicating its functional importance. Mutagenic studies on VPAC-1 (subtype of the VIP 1 receptor/pituitary adenylate cyclase-activating polypeptide) (18), secretin (19), and calcitonin (20) receptors provide solid evidence for this hypothesis.

According to the amino acid sequence alignment of the third endoloop within the secretin receptor subfamily, two highly conserved motifs were identified: the KLR motif at the N terminus and the RLAR motif at the C terminus. It is remarkable that this RLAR (basic-L-L/A/S-basic) motif is present in every member within the class II receptors, and these two basic residues are also found in other families, such as the β2 adrenergic receptor (21) and the lutropin/choriogonadotropin receptor (22) (Fig. 1). The structural conservation of both KLR and RLAR motifs in GPCRs strongly suggests their importance in receptor conformation and/or function. Indeed, from studies of the GPCR superfamily in the past decade, the predicted second and third (IC3) endolopes were believed to contain primary structural and functional determinants for G protein selectivity and interactions (23–25). Studies on m3, acetylcholine and GLP-1 receptors suggested that the G protein activation process involves hydrophobic residues at the N and C termini, respectively (26–29). Basic residue Lys334 within the KLK motif of the GLP-1 receptor was also found to be required for efficient interaction with the G protein.
Role of Basic Motifs in IC3 Loop of hSR

Construction of mutants and transfection

A c-Myc epitope tagged human secretin receptor (hSR-cMyc) at the C terminus was constructed previously and was found to be functionally similar to the wild-type (WT) receptor (19). This construct was subcloned into the plasmid vector pALTER-1 and was used as a template for site-directed mutagenesis (Promega Corp., Madison, WI). Mutations were verified by DNA sequencing with a T7 DNA sequencing kit (Amersham Pharmacia Biotech, Arlington Heights, IL). The WT hSR-cMyc and the mutant receptor cDNAs were subcloned into the expression vector pRc-CMV (Stratagene, La Jolla, CA) for transfection into Chinese hamster ovary (CHO) cells. Approximately 0.18 × 10⁶ cells were seeded onto the 6-well plates (Costar, San Diego, CA) 48 h before transfection and cultured in minimum essential medium supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Life Technologies, Inc., CA). The cells were transiently transfected with 2 μg of various constructs using Lipofectamine (Life Technologies, Inc.) according to the manufacturer’s protocol.

Competitive binding analysis

Human secretin was labeled with [125I]-sodium by the method described by Chan and Chey (31), and the labeled peptide was used for binding assays essentially according to the protocol described previously (19). The transiently transfected cells were washed twice with assay buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.2% BSA at 5% sucrose, 1% IBSA) before being cultured for an additional 48 h. The cells were incubated with 100,000 cpm of [125I]-secretin and various concentrations (10⁻⁸ to 10⁻³ M) of unlabeled peptide in a final volume of 200 μl for 1 h at 22°C. After incubation, the cells were washed out with ice-cold binding buffer (assay buffer with 0.1 mM PMSE, 0.1% BSA) and lysed with 1 mM NaOH. The cell-associated radioactivity was measured by a γ-counter. Specific binding measured in the presence of excess human secretin (1 μM) was determined for the WT and the mutant receptors. The Kᵦ binding capacity and Bmax values were calculated by the homologous competitive binding approach using the PRISM version 2.0 computer software (GraphPad Software, Inc., CA).

Measurement of intracellular cAMP

To monitor the transfection efficiency, CHO cells were cotransfected with the receptor construct (1 μg/well) and the pCMV-SPORT-gal plasmid (0.25 μg/well) (Life Technologies, Inc.) using Lipofectamine as described previously. After transfection, the cells were cultured for 48 h before peptide stimulation (10⁻⁴ to 10⁻⁷ M). Thereafter, the intracellular cAMP content of the stimulated cells was measured using a CAMP RIA kit (NEN Life Science Products Inc., Boston, MA) according to the manufacturer’s protocol. The basal and maximal cAMP levels were on average, 3.5 and 25.5 pmol/well, respectively.

Northern blot analysis

Permanently transfected CHO cells were grown on 150-mm tissue culture dishes 48 h before initiation of experiments. Total RNAs of the mutant and WT receptors were harvested with Solution D (4 mM guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl). Ten micrograms of total RNA was size fractionated by electrophoresis in a denaturing formaldehyde gel and followed by blots to UV cross-linking onto a Hybond N⁺ membrane (Amersham Pharmacia Biotech). Fifty nanograms of the full-length hSR cDNA was radiolabeled with 10 μCi [32P]-deoxyribonucleoside triphosphate (Amersham Pharmacia Biotech) by the RadPrime DNA labeling kit (Life Technologies, Inc.). After 18 h hybridization in the Rapid Hyb buffer (Amersham Pharmacia Biotech) at 65°C, the blot was washed out with 2 × saline sodium citrate (SSC), 1 × SSC, and then 0.1 × SSC for 15 min each at 65°C.

Materials and Methods

Reagents

Synthetic human secretin was purchased from Calbiochem (La Jolla, CA). General chemicals and reagents were from Sigma (St. Louis, MO). Restriction enzymes, DNA sequencing, and tissue culture supplies were obtained from Life Technologies, Inc. (Gaithersburg, MD). Anti-c-Myc mouse monoclonal antibody (clone9E10) was obtained from Roche Molecular Biochemicals (Indianapolis, IN). The secondary antibody Cy-2 labeled rabbit antimouse IgG was obtained from Pierce Chemical Co. (Rockford, IL). Saponin and IBMX were purchased from United States Biochemical Corp. (Cleveland, OH) and RBI (Natick, MA), respectively.
C. The membrane was exposed to BioMax film (Eastman Kodak Co., Rochester, NY).

Confocal laser scanning microscopy

CHO cells were cultured on 12-mm glass coverslips and transiently transfected with the WT or mutant receptor cDNA as described above. After 48 h, the transfected cells were fixed with 0.5% paraformaldehyde for 10 min at 4°C and were permeabilized by washing twice with PBS-saponin (0.1% saponin wt/vol in PBS). The cells were incubated for 20 h at 4°C with the mouse anti-c-Myc monoclonal antibody (9E10) at the final dilution of 1:250. Then the cells were incubated with Cy-2-labeled rabbit antimouse IgG for 1 h at 22°C. The cells were washed with PBS-saponin and excited with a laser at 488 nm. Images of cells expressing WT or mutant receptors were obtained using a confocal laser microscope (MRC 600, Bio-Rad Laboratories, Inc., Richmond, CA).

Statistics

All values were expressed as the mean ± SE of at least six independent observations. Statistical analyses were performed using one-way ANOVA, and the differences between WT and mutant receptors were considered significant when P < 0.01.

Results

Effects of mutation within the central region of the hSR

The third endoloop of class II GPCRs can be divided into three regions according to the amino acid sequence alignment study (Fig. 1): the N terminus KLR sequence, the intervening region, and the C-terminus RLAR/K motif. The intervening sequence between the two consensus motifs exhibits a high level of sequence diversity. Block deletion (IC3–2, QETR, IC3–3, GNEV, and IC3–4, SHYK) or single (R330I) substitution mutations within this central region did not alter the function of the receptor as indicated by binding studies and cAMP assays (Table 1). It is likely that the intervening sequence between the two basic motifs contains no structural determinant and is not directly involved in the process of G protein activation.

Effects of deletion or amino acid substitution within the KLRT at the N terminus of the third endoloop on receptor function

To determine its functional role, the KLR motif at the N terminus of the IC3 loop was deleted (mutant IC3–1, block deletion of KLRT). In addition, the basic residue Lys323 was mutated to isoleucine (K323I) to study the function of this highly conserved residue within this motif on G protein activation. The WT, mutant IC3–1, and K323I receptors were transiently expressed in CHO cells, and their biological activities were tested by 125I-secretin-specific binding as well as secretin-stimulated cAMP production. There were no significant differences in binding affinities and maximal binding capacities of either mutant when compared with the WT receptor (Fig. 2A and Table 2). However, both IC3–1 and K323I mutants showed significant decreases in maximal cAMP responses (41% and 50% reduction, respectively, P < 0.01) (Fig. 2B) as well as increases in EC50 values (9- and 6-fold, respectively). These data indicate that the KLR motif is neither directly nor indirectly involved in ligand interaction. On the other hand, this motif is important for the process of G protein coupling, and hence the block deletion mutant IC3–1, after binding to secretin, is unable to activate adenylate cyclase to produce cAMP. The observation that Ile substitution of Lys at position 323 resulted in a mutant with similar binding and G protein coupling properties as the block deletion mutant strongly indicated that the residue Lys323 is of paramount importance to the function of this motif.

<p>| TABLE 1. Biological activities of the WT and mutants: R330I, IC3–2 (QETR), IC3–3 (GNEV), and IC3–4 (SHYK) in the transfected CHO cells |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Mean EC50 ± SE (10–10 M)</th>
<th>Max. cAMP res. (% WT)</th>
<th>Kd ± SE (nM)</th>
<th>Bmax ± SE (cpm × 103/106 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.3 ± 0.2</td>
<td>100 ± 3</td>
<td>14.1 ± 1.7</td>
<td>33.6 ± 1.9</td>
</tr>
<tr>
<td>R330I</td>
<td>0.3 ± 0.2</td>
<td>98 ± 2</td>
<td>10.7 ± 0.5</td>
<td>25.6 ± 0.4</td>
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<tr>
<td>IC3–2</td>
<td>0.2 ± 0.1</td>
<td>116 ± 1</td>
<td>13.8 ± 0.2</td>
<td>34.0 ± 0.7</td>
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<tr>
<td>IC3–3</td>
<td>0.2 ± 0.1</td>
<td>125 ± 6</td>
<td>7.4 ± 0.7</td>
<td>40.0 ± 2.3</td>
</tr>
<tr>
<td>IC3–4</td>
<td>0.2 ± 0.2</td>
<td>110 ± 1</td>
<td>89.0 ± 8.4a</td>
<td>7.3 ± 1.0a</td>
</tr>
</tbody>
</table>

P < 0.01 Statistically significant change (n ≥ 3).

<p>| TABLE 2. Biological activities of the WT hSR and mutants constructed within the third endoloop in the transfected CHO cells |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Mean EC50 ± SE (10–10 M)</th>
<th>Max. cAMP res. (% hSR wt)</th>
<th>Kd ± SE (nM)</th>
<th>Bmax ± SE (cpm × 103/106 cells)</th>
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<tr>
<td>WT hSR</td>
<td>0.3 ± 0.2</td>
<td>100 ± 3</td>
<td>14.1 ± 1.7</td>
<td>33.6 ± 1.9</td>
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<tr>
<td>IC3–1</td>
<td>2.6 ± 0.8a</td>
<td>59 ± 6a</td>
<td>12.6 ± 0.9</td>
<td>32.4 ± 0.4</td>
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<tr>
<td>K323I</td>
<td>1.7 ± 0.6a</td>
<td>50 ± 2a</td>
<td>3.8 ± 0.3a</td>
<td>23.6 ± 0.3</td>
</tr>
<tr>
<td>R339A</td>
<td>0.5 ± 0.2</td>
<td>82 ± 5</td>
<td>48.7 ± 5.6</td>
<td>25.4 ± 1</td>
</tr>
<tr>
<td>R342A</td>
<td>0.6 ± 0.2</td>
<td>85 ± 5</td>
<td>32.5 ± 3.3</td>
<td>25.7 ± 5.3</td>
</tr>
<tr>
<td>R339,342A</td>
<td>4.9 ± 1.4a</td>
<td>41 ± 6a</td>
<td>302.4 ± 11a</td>
<td>12.0 ± 1.5a</td>
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<tr>
<td>R339I</td>
<td>0.4 ± 0.2</td>
<td>88 ± 4</td>
<td>26.8 ± 4.9</td>
<td>31.4 ± 4.3</td>
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<tr>
<td>R342I</td>
<td>1.7 ± 1.2</td>
<td>128 ± 9</td>
<td>34.8 ± 5.7</td>
<td>29.3 ± 0.5</td>
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<tr>
<td>R339,342I</td>
<td>9.7 ± 0.5a</td>
<td>35 ± 5a</td>
<td>39.1 ± 1.2</td>
<td>14.3 ± 0.7a</td>
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<tr>
<td>R339,342E</td>
<td>11.1 ± 0.1a</td>
<td>15 ± 3a</td>
<td>274.0 ± 9.9a</td>
<td>7.5 ± 2.1a</td>
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<tr>
<td>IC3–5</td>
<td>46.0 ± 0.1a</td>
<td>24 ± 1a</td>
<td>873.0 ± 5.4a</td>
<td>4.7 ± 0.2a</td>
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P < 0.01 Statistically significant change (n ≥ 3).
Effect of deletion or amino acid substitution within the RLAR motif at the C terminus of the third endoloop on receptor function

To determine the function of the conserved RLAR motif, a series of mutant receptors with block deletion, and double- and single-substitution were generated and transiently expressed in CHO cells. The binding properties of these receptors were analyzed (Table 2). Mutant IC3–5 (block deletion of RLAR) and double mutants (R339, 342A, R339, 342E, and R339, 342I) have greatly reduced binding capacities (86 ± 1%, 64 ± 4%, 78 ± 6%, and 57 ± 2% reductions, respectively) and also binding affinities when compared with the WT receptor (Figs. 3A, 4A, and 5A). The Kd values of the above mutants were found to be 3- to 60-fold greater than that of the WT receptor. However, the competitive binding assay revealed that the single mutants (R339A, R342A, R339I, and R342I) re-

A, Binding Analysis

![Graphs showing binding analysis](image)

FIG. 2. Comparisons of the WT with a block deletion mutant IC3–1 (KLRT deleted) and a single mutant, R323I, within the KLR motif at the N terminus of IC3 loop on receptor functions (A, competitive binding analysis and B, dose-dependent cAMP assay). The intracellular cAMP accumulation and secretin binding in response to increasing secretin concentrations from 10^{-12} to 10^{-7} M and 10^{-12} to 10^{-6} M, respectively, were measured and expressed as a percentage of the maximal response of the WT receptor. These results are representatives of at least two independent measurements, each of which was performed in triplicate.

B, cAMP Assay

![Graphs showing cAMP assay](image)

FIG. 3. Effect of alanine substitutions within the RLAR motif at the C terminus of IC3 loop on receptor functions (A, competitive binding analysis and B, dose-dependent cAMP assay). Both WT and three mutants, including a double mutant, R339,342A, and two single mutants, R339A and R342A, were expressed in CHO cells. The intracellular cAMP accumulation and secretin binding in response to increasing secretin concentrations from 10^{-13} to 10^{-7} M and 10^{-12} to 10^{-6} M, respectively, were measured and expressed as a percentage of the maximal response of the WT receptor. These results are representatives of three independent measurements, each of which was performed in triplicate.
tained their abilities to bind $^{125}$I-secretin as indicated in their maximal binding capacities and affinities (Table 2). The abilities of the mutant receptors to functionally couple to Gs protein were monitored by measuring the intracellular cAMP contents upon secretin stimulation. Similar to the binding assays, cAMP responses of the block deletion mutant IC3–5 and double substitution mutants, $R^{339,342I}$ and $R^{339I}$, were expressed in CHO cells. The intracellular cAMP accumulation and secretin binding in response to increasing secretin concentrations from $10^{-13}$ to $10^{-7}$ M and $10^{-12}$ to $10^{-6}$ M, respectively, were measured and expressed as a percentage of the maximal response of the WT receptor. These results are representatives of three independent measurements, each of which was performed in triplicate.

maximal cAMP responses and EC50 values of the single substitution mutants, $R^{339A}$, $R^{342A}$, $R^{339I}$, and $R^{342I}$, did not differ from those of the WT receptor ($P < 0.01$, Table 2).

The functional impotence of IC3–5 and $R^{339,342A/I/E}$ is due to the lack of receptor presentation to the cell surface

The competitive binding and cAMP data suggested that deletion of RLAR (IC3–5) or substitution mutation of both arginines within this motif abolished ligand binding and hence cAMP production. However, the data obtained from
those single mutant (R339A, R342A, R339I, and R342I) transfected cells were found to be indistinguishable from that of the WT receptor, which implies that the presence of one arginine residue at either position, 339 or 342, is sufficient to confer full receptor function. To find out whether these basic residues are important for conformation/maturation or cell surface presentation, we carried out an immunofluorescent study. The c-Myc-tagged WT or mutant receptors were used, and their presence in the cytoplasm and on the surface of the transfected cells was detected by the anti-c-Myc antibody. Our data clearly showed that the WT receptors were located throughout the cell surface and cytoplasm. In contrast, double-substitution (R339,342A, R339,342E, and R339,342I) and block deletion (IC3-5) mutant receptors were hardly detected in the cytoplasm and plasma membrane (Fig. 6). Interestingly, single-substitution mutants at position 339 or 342 were present in the cytoplasm and cell surface. The lack of immunofluorescent signals in the IC3–5, R339, 342A, R339, 342E, and R339, 342I mutants could be due to the instability of either the mRNA or protein. To verify this hypothesis, Northern blot was performed. It was found that the mRNA expression levels of the WT receptor, single- and double-substitution, and block deletion mutants were similar (Fig. 7). These data implied that double and deletion mutant receptors were expressed normally but failed to be presented to the cell surface. It seems that one arginine residue within the RLAR motif is already sufficient for cell surface presentation or/and receptor stability of the hSR.

Discussion

Among different G protein-coupled receptor families, the third endoloop is believed to be involved in G protein coupling because it presents logical contact points for various G
proteins (32). Within the third endoloop, the function of the conserved basic motif at the N-terminal region as the site for G protein interaction has been a focus of interest, and the results obtained are controversial. Evidence supporting this notion can be found in studies of the GLP-1, rhodopsin, and β2-adrenergic and muscarinic receptors (30, 32–34). Instead, other reports have suggested that the hydrophobic residues at the junction of the fifth transmembrane domain of GLP-1 receptor (29) and those at the end of the third endoloop of β-adrenergic receptor (35) are involved in G protein coupling. To address this issue, in this study, several mutant receptors were constructed within this region and their activities were tested. We found that the ligand-binding characteristics of the mutants (KLR deletion and K325I) were indistinguishable from the WT receptor, and their abilities to activate cAMP production were much reduced. Hence, our data confirmed that the KLR motif, particularly the lysine residue, contains the G protein activation determinants in the hSR.

With respect to the primary sequences of the central region of the third endoloop, members of the class II GPCR subfamily are not conserved. This observation suggested that this region is responsible for receptor-specific functions, or it may be functionally unimportant. In our studies, block-deletion mutations within this central region had no adverse effects on ligand-binding and peptide-stimulated cAMP production (Table 1). These data clearly indicate that the intervening sequences between two basic motifs of the IC3 loop do not contain any structural determinant for receptor function including G protein coupling. These data are consistent with the results of the human M1 muscarinic receptor (36). In contrast, deletion of the threonine, aspartic acid, and isoleucine (TDI) motif within the central region of the third endoloop of the GLP-1 receptor led to a significant reduction in receptor expression (30). The corresponding mutant in the hSR is IC3-3 with the glycine, asparagine, glutamic acid, valine (GNEV) motif deleted, and this mutant is functionally similar to the WT receptor (Table 1).

As shown in Fig. 1, the basic RLAR motif is conserved among human (9), rabbit (37), rat (38), and other receptors from the secretin/VIP/glucagon family. A similar motif with two basic amino acids is also present in classes I and IV receptor families, and receptors from classes III and V contain at least one basic residue at the C termini. Based on studies of β3 and β2 adrenergic and M1-acetylcholine receptors, this C-terminal region of the third endoloop appeared to be essential for Gs, Gi, or G0 protein coupling (34, 39–40). Interestingly, the corresponding KERK motif in the third endoloop of M1-acetylcholine receptor is well conserved, but only the second lysine reside at position 439 was found to have functional importance (36, 41–42). To examine whether this highly conserved basic motif is also a G protein activation site in the secretin/VIP/GLP-1 receptor family, a number of mutants were constructed and studied in this report. Contrary to previous findings, we found that an alanine substitution of either one of the arginines within the RLAR motif had no effect on ligand interaction and cAMP production (Fig. 3). Similar results were obtained when replacing one of these arginines with isoleucine, which has a more bulky hydrophobic side chain (Fig. 4). These data indicated that the loss of one basic residue (Arg339 or Arg342) had no effect on the function of the hSR. This observation is consistent with previous studies; mutation of one of the basic residues at the C-terminal end of the third endoloop had no adverse effects on receptor function in the rat lutropin/CG receptor (43, 44). These studies suggested that the activation of Gs does not involve hydrophilic residues at the C terminal, which is similar to our findings.

The RLAR block deletion rendered the receptor totally defective, and this result is inconsistent with mutants containing a single arginine substitution. For this reason, mutants with both arginine residues (Arg339 and Arg342) replaced with alanine, glutamic acid, or isoleucine were constructed. These mutant receptors were found defective in ligand binding and hence were unable to accumulate intracellular cAMP upon peptide stimulation (Figs. 3–5). It seems that the presence of one arginine residue within the RLAR motif is already sufficient to guarantee proper function of the receptor. The question of why both positively charged residues were conserved in every member of the secretin receptor family during evolution remains unanswered. A possible explanation is that if one arginine is mutated within the RLAR motif, the other positively charged residue may act as a backup to keep the receptor functioning.

There are several possible explanations for the defective secretin-binding capabilities observed in some of the block deletion and double mutant: 1) the RLAR motif is the structural/conformational determinant for receptor function; 2) the motif is responsible for channeling the receptor to the cell surface; and 3) mutation affects the stability of the transcript or the protein. To determine the function of this positively charged motif, laser confocal microscopy coupled to immunofluorescent staining was employed. The WT receptors as well as the single mutants (R339A, R342A, R339I, and R342I)
were detected on the plasma membrane and within the cytosol (Fig. 6). However, the signals of the double mutants (R339, 342E and R339, 342I) and block deletion mutant (IC3–5) were much lower and were almost undetectable on the cell surface. These drastic reductions in receptor expression are not the results of the variations of mRNA expression as shown in Fig. 7. Our data suggested that these defective receptors were properly transcribed but were unable to follow the normal processing and cell surface presentation pathway.

In summary, the positively charged motifs within the IC3 loop of the hSR play different functional roles. The N-terminal KLR motif, particularly the lysine residue, is responsible for the process of G protein coupling. On the other hand, the arginine residues of the RLAR motif at the C-terminal contain the structural/conformational determinants for receptor maturation and stability. The functional importance of these motifs are reflected by the conservation of the positively charged residues in these motifs in the secretin/glucagon receptor family as well as other classes of receptor, and this observation suggests more general and universal functions of these motifs in all GPCRs.

Acknowledgments

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Address all correspondence and requests for reprints to: Dr. Billy K. C. Chow, Department of Zoology, University of Hong Kong, Pok-falum Road, Hong Kong, SAR, PRC. E-mail: bkc@hku.hk.

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