Interspecies Polymorphisms Confer Constitutive Activity to the Mastomys Cholecystokinin-B/Gastrin Receptor*

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The enteroendocrine hormone, gastrin, exerts trophic effects on the gastric mucosa through the CCK-B/gastrin receptor (CCK-BR). To varying degrees in different species, excess circulating gastrin leads to proliferation of enterochromaffin-like cells and to the development of gastric carcinoid tumors. The African rodent, Marsomys natalensis, is distinguished from other mammals by its propensity toward CCK-BR-mediated growth even in the absence of hypergastrinemia. Here, we report that the Mastomys CCK-BR, when expressed in COS-7 cells, differs from the respective human, canine, and rat receptor homologs by its ability to trigger ligand-independent (i.e., constitutive) inositol phosphate formation. To define the molecular basis of this observation, a series of Mastomys-human chimeric receptors was investigated. Functional characterization of these constructs revealed that a limited segment of the Mastomys CCK-BR, transmembrane domain VI through the C-terminal end, is sufficient to confer constitutive activity to the human protein. Mutagenesis studies within this CCK-BR region defined a combination of three Mastomys amino acids that, when introduced into the human receptor, together conferred a level of ligand-independent signaling comparable with the Mastomys CCK-BR. Complementing prior observations that single point mutations can lead to ligand-independent signaling, our findings suggest that multiple naturally occurring amino acid polymorphisms and/or mutations may together result in an enhanced basal level of receptor activity.

The cholecystokinin-B/gastrin receptor (CCK-BR) is a seven transmembrane domain G protein-coupled receptor that is widely expressed in the gastrointestinal tract and in the central nervous system (1). Stimulation of the CCK-BR by either of the endogenous peptides, CCK-8 or gastrin, triggers phospholipase C-mediated conversion of membrane phospholipids to inositol phosphates and diacylglycerol, which in turn initiates a series of downstream signaling events (1).

In the CNS, the CCK-BR is postulated to modulate anxiety as well as the perception of pain (2, 3). In the stomach, it is well established that activation of this receptor subtype stimulates acid secretion as well as mucosal growth (1). Recent studies in which CCK-BR-deficient mice were generated by targeted gene disruption revealed that absence of the CCK-BR results in thinning of the gastric mucosa and a reduced density of both gastric enterochromaffin-like (ECL) and parietal cells (4, 5). Conversely, receptor overactivity in rodents, induced by hypergastrinemia, leads to gastric mucosal hypertrophy marked by increased synthesis of DNA, RNA, and protein (6). Long-term stimulation of the CCK-BR by elevated circulating gastrin may ultimately result in the development of ECL cell carcinoid tumors (7).

Among mammals, there is a broad spectrum of susceptibility to gastrin-induced proliferation and tumor formation, with the highest sensitivity observed in the African rodent, Mastomys natalensis. During its normal course of aging, Mastomys may develop gastric carcinoid tumors even with normal circulating gastrin levels. In this animal, hypergastrinemia potentiates the enhanced rate of gastric ECL cell tumor development (7). The increased susceptibility of Mastomys to ECL cell growth, even in the absence of elevated circulating gastrin, led us to speculate that this receptor homolog was functionally different from the CCK-BR in other species. There is ample precedent that species-dependent differences in the amino acid sequence of the CCK-BR can lead to alterations in the pharmacologic properties of the receptor. We have previously shown that interspecies polymorphisms markedly alter CCK-BR interactions with synthetic ligands, i.e., a single amino acid difference between the canine and human CCK-BRs results in a reversal of the affinity rank order for non-peptide antagonists (8). In a subsequent study, we demonstrated that single and double amino acid differences between the mouse, human, and dog receptors led to marked alterations in drug-induced inositol phosphate formation, the primary second messenger generated in response to activation of the CCK-BR (9).

In this study, recombinant CCK-BRs from different species were expressed in COS-7 cells and functionally compared. Only the Mastomys receptor had a significant level of ligand-independent (i.e., constitutive) signaling activity. To understand the molecular basis of this finding, we utilized a series of human/Mastomys CCK-BR chimeric receptors to map the limited domain that confers ligand-independent signaling. Point mutations were then introduced within this region of the protein to identify residues that underlie the species-dependent constitutive activity.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs Inc. Iodinated cholecystokinin oc-
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tapeptide I23T CCK-8, specific activity, 2200 Ci/mmol and [3H]myoinositol (specific activity 40–60 Ci/mmol) were obtained from NEN Life Science Products. Unlabeled CCK-8 was purchased from Peninsula Laboratories (Belmont, CA).

Construction of Chimeric Receptor cDNAs—The Mastomys CCK-BR cDNA (generously provided by Dr. T. Chiba) was subcloned into the expression vector pcdNA I (Invitrogen) and sequenced. Two differences in translated amino acid sequence from the originally published version (10) were noted: 31GPGLASANQAA27 (old) versus 31GPGLPRPGPRQA27 (revised) and 325C (old) versus 325R (revised). Of note, the 325PR revision results in the introduction of an additional amino acid, thus increasing the numbering by 1 of residues C-terminal to position 324. The revised Mastomys cDNA has been submitted to GenBank. Naturally occurring PatI sites, in the same relative position of the human (11) and the Mastomys (10) CCK-BR (corresponding to human cDNA nucleotides 607–612), were used to generate the PatI chimeras. Oligonucleotide-directed mutagenesis was used to introduce XhoI and MluI sites (corresponding to human cDNA nucleotides 910–915 and 986–992) into the respective receptor cDNAs from each species (12). The XhoI and MluI chimeric receptor cDNAs were constructed by replacing segments of the human CCK-BR cDNA with the corresponding Mastomys sequence. Mutant receptors, in which 1–3 amino acids were replaced, were generated using oligonucleotide-directed mutagenesis (12). All chimeric and mutant receptor cDNA constructs were confirmed using the Applied Biosystems 373A DNA sequencer.

Cell Culture and Transfection—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and gentamicin (100 μg/ml). The cells were maintained at 37 °C in a 5% CO2 atmosphere. Utilizing the DEAE-dextran method (13), cells (106/10-cm dish) were transiently transfected with 5 μg of either the expression vector, pcdNA I, or the relevant CCK-BR cDNA construct. In control experiments (see Fig. 4), the amount of transfected DNA was adjusted (1–5 μg/10-cm dish) to obtain comparable receptor expression levels of the human and Mastomys CCK-BR.

Radioligand Binding Experiments—24 h after transfection, cells were trypsinized and divided into 24-well plates (5 × 103 cells/well). The following day, competition binding experiments were performed in Hank’s balanced salt solution supplemented with 10% fetal calf serum and gentamicin (100 μg/ml). The cells were maintained at 37 °C in a 5% CO2 atmosphere. Utilizing the DEAE-dextran method (13), cells (106/10-cm dish) were transiently transfected with 5 μg of either the expression vector, pcdNA I, or the relevant CCK-BR cDNA construct. In control experiments (see Fig. 4), the amount of transfected DNA was adjusted (1–5 μg/10-cm dish) to obtain comparable receptor expression levels of the human and Mastomys CCK-BR.

Measurement of Inositol Phosphate Formation—Transfected COS-7 cells were cultured overnight in serum-free medium containing 3 μCi of [3H]myoinositol/ml. Ligand-independent and agonist (0.3 μM CCK-8)-stimulated [3H]inositol phosphate production were assessed at 37 °C in the presence of 10 mM LiCl. After incubation of the cells for either 0.5 or 1 h (see text), inositol metabolites were extracted with methanol/chloroform, and the upper phase was analyzed for inositol phosphates by strong anion exchange chromatography. The production of inositol phosphates was expressed as a fraction of the total cellular tritium content. To determine the receptor-mediated component of IP production, all measurements were corrected by the amount of IP formation in COS-7 cells expressing pcDNA I (the expression vector) alone.

RESULTS

Second messenger signaling by COS-7 cells expressing the human (11, 14, 15), canine (13), rat (16), or Mastomys (10) CCK-B receptors was assessed in the presence and in the absence of cholecystokinin octapeptide (CCK-8). With agonist stimulation, each receptor led to comparable levels of IP production (Fig. 1A). In contrast, only the Mastomys protein triggered ligand-independent IP formation that was significantly higher than the level observed in cells transfected with the expression vector, pcDNA I (Fig. 1B).

Increased expression of a constitutively active receptor will result in a parallel increase in both agonist-induced and ligand-independent second messenger signaling (17). Consistent with this expectation, a positive correlation was observed between CCK-8-stimulated and ligand-independent signaling in COS-7 cells expressing the Mastomys CCK-BR (Fig. 2). In contrast, signaling by the human CCK-BR in the absence of ligand was always close to zero regardless of the level of CCK-8-induced IP formation. These observations confirm that the Mastomys CCK-BR is constitutively active, whereas the human homolog is not.

A comparison of IP production after 0.5 and 1 h provided additional support that only the Mastomys CCK-BR is constitutively active. As shown in Fig. 2A, a time-dependent increase in basal IP production is observed in cells expressing the human CCK-BR, whereas this is not the case in cells expressing the human receptor. In a control, the maximal level of CCK-8-induced signaling was also compared at 0.5- and 1-h incubation times. Consistent with expectation, agonist-induced IP production by either the Mastomys or the human CCK-BR increased to a similar extent independent of the respective basal level of signaling (Fig. 2B).

It has been recently established that constitutive activity of
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**Fig. 2.** Ligand-independent IP production in cells expressing the *Mastomys* CCK-BR increases as a function of the CCK-8-induced maximum. [3H]Inositol phosphate production in the presence of 0.3 μM CCK-8 (x axis) and in the absence of ligand (y axis) are compared in COS-7 cells expressing either the *Mastomys* (black squares) or the human (open circles) CCK-BRs. IP production is expressed as a fraction of the total tritium incorporated after an overnight exposure of transfected cells to tritiated myoinositol. Data represent the means ± S.E. of 10 independent experiments (n.s., non-significant). Asterisk denotes a significant increase (0.5 h versus 1 h) in IP production (p < 0.01). A, basal IP production in COS-7 cells expressing the *Mastomys* CCK-BR significantly increases between 0.5 and 1 h. In contrast, in cells expressing the human CCK-BR, no significant increase (n.s.) in IP production is observed. B, when stimulated with a saturating concentration of CCK-8 (0.3 μM), cells expressing either the *Mastomys* or the human CCK-BR show a similar increase in IP production. Of note, after either 0.5 or 1 h, ligand-independent IP production expressed as a fraction of the CCK-8-induced maximum [basal IP production/CCK-8-stimulated IP production] × 100] approximates 13% for the *Mastomys* CCK-BR versus 0% for the human CCK-BR.

**Fig. 3.** A time-dependent increase in basal signaling suggests that the *Mastomys* CCK-BR is constitutively active. [3H]Inositol phosphate production was assessed in COS-7 cells expressing either the *Mastomys* or the human CCK-BR. IP production (y axis) is expressed as a fraction of the total tritium incorporated after an overnight exposure of transfected cells to tritiated myoinositol. Data represent the means ± S.E. of ≥10 independent experiments. Asterisk denotes a significant increase (0.5 h versus 1 h) in IP production (p < 0.01). A, basal IP production in COS-7 cells expressing the *Mastomys* CCK-BR, no significant increase (n.s.) in IP production is observed. B, when stimulated with a saturating concentration of CCK-8 (0.3 μM), cells expressing either the *Mastomys* or the human CCK-BR show a similar increase in IP production. Of note, after either 0.5 or 1 h, ligand-independent IP production expressed as a fraction of the CCK-8-induced maximum [basal IP production/CCK-8-stimulated IP production] × 100] approximates 13% for the *Mastomys* CCK-BR versus 0% for the human CCK-BR.

G protein-coupled receptors can be attenuated by a novel class of ligands, inverse agonists (18, 19). The availability of such a compound for the CCK-BR, L-740,093 (R enantiomer), enabled us to provide additional confirmation of constitutive signaling by the *Mastomys* CCK-BR. L-740,093 R attenuated ligand-independent IP production by the *Mastomys* receptor but had no effect on the basal activity of the human CCK-BR (Fig. 4A). Half-maximal inhibition of ligand-independent IP production by L-740,093 R in cells expressing the *Mastomys* receptor was achieved at a concentration of 1.73 nM (Fig. 4B).

The marked difference in ligand-independent second messenger signaling by the *Mastomys* and human CCK-BR homologs was observed despite 92% amino acid identity between the two proteins. In an attempt to define a minimal domain of the *Mastomys* receptor, which conferred ligand-independent activity, a series of chimeric receptors was constructed with varying N-terminal portions of human sequence linked to complementary C-terminal portions of the *Mastomys* protein (PstI, XhoI, and *Mlu*I chimeras, see Fig. 5). When functionally assessed in COS-7 cells, each of these chimeric receptors had a level of constitutive activity approaching that of the *Mastomys* wild-type CCK-BR.

Constitutive activity of the *Mlu*I chimera suggested that transfer of a small segment of the *Mastomys* receptor was sufficient to confer constitutive activity to the human protein. Sequence comparison between the *Mastomys* and human receptors within this limited domain, which spans transmembrane domain VI through the C terminus (see Fig. 5), revealed four candidate amino acids that could potentially confer constitutive signaling activity (Fig. 6A). Many previously reported examples of constitutively active G protein-coupled receptors are the result of single amino acid substitutions (20–22). Based on this precedent, a series of four mutant human CCK-BRs was generated. In each receptor, one of the four human amino acids was replaced with the corresponding *Mastomys* residue. None of the single point mutations in the human CCK-BR was sufficient to significantly increase ligand-independent signaling relative to the human wild-type value (data not shown). The absence of ligand-independent signaling with single amino acid substitutions suggests that a combination of several *Mastomys* residues is required to confer constitutive signaling activity.

To determine in which of the four candidate *Mastomys* amino acids contributed to constitutive activity, an alternative strategy was utilized. With the cDNA encoding the human/*Mastomys* *Mlu*I chimera as a template for mutagenesis, each residue unique to the *Mastomys* receptor (Leu544, Ile353, Asp407, Pro423) was sequentially replaced with its corresponding human homolog (Val544, Val353, Glu407, Ala419). A second series of four mutant human receptors was thus generated, each including a
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**Fig. 4.** Ligand-independent signaling by the *Mastomys* CCK-BR is inhibited by the inverse agonist L-740,093. COS-7 cells were transfected with either the *Mastomys* or the human wild-type CCK-BR. Receptor numbers were determined by homologous 125I-CCK-8 radioligand competition experiments. The amounts of cDNA transfected were adjusted to obtain comparable expression levels. A, [3H]inositol phosphate production was measured in the absence of ligand (basal) or in the presence of the inverse agonist, L-740,093. IP production (y axis) is expressed as a percentage of the CCK-8-induced maximum. Half maximal inhibition of IP production (as a fraction of the total tritium incorporated) was as follows (mean ± S.E., n = 10): *Mastomys* wild-type CCK-BR, 0.097 ± 0.010; *Mastomys* wild-type CCK-BR, 0.127 ± 0.012; *Pst I* chimera, 0.126 ± 0.024; *Xho I* chimera, 0.148 ± 0.028; *Mlu I* chimera, 0.139 ± 0.024.

In contrast, triple mutant 4 maintained constitutive activity comparable with the level of the *Mastomys* protein, despite substitution of Pro423 by an alanine residue. The latter finding suggests that the residue at position 423 played no appreciable role in conferring constitutive activity to the *Mastomys* CCK-BR.

**DISCUSSION**

Comparison of the recombinant canine, rat, *Mastomys*, and human CCK-BRs expressed in COS-7 cells revealed that only the *Mastomys* receptor triggered ligand-independent IP formation. Constitutive signaling activity of the *Mastomys* protein was confirmed by three additional observations: (i) the level of ligand-independent signaling correlated with respective levels of CCK-8-induced signaling, (ii) ligand-independent [3H]inositol phosphate formation increased with extended incubation time, and (iii) ligand-independent signaling could be inhibited in a concentration-dependent manner by the inverse agonist, L-740,093. Of note, none of these criteria applied to the human CCK-BR, which was tested in parallel. Together, these findings establish that the *Mastomys* receptor is constitutively active as a consequence of interspecies variability in the CCK-BR.

Since the *Mastomys* cDNA utilized in this study was isolated from an enterochromaffin-like cell carcinoïd (10), it was important to consider whether the corresponding protein resulted from a somatic mutation in the tumor. The possibility is unlikely based on sequence identity between the cDNA used in our experiments and CCK-BR cDNAs isolated from a number of normal *Mastomys* tissues, including isolated gastric parietal cells and cerebral cortical tissue (7).

The observation that species-dependent polymorphisms can result in constitutive activation of a G protein-coupled receptor...
Fig. 6. A combination of multiple amino acids confers constitutive activity to the Mastomys CCK-BR. A, sequence comparison between the human and Mastomys CCK-BRs revealed four candidate amino acids that could potentially confer constitutive signaling to the MluI chimeric receptor. These amino acids are highlighted (●) in the cartoon of the CCK-BR; their position in the human receptor is noted. N, amino terminus; C, carboxyl terminus. B, substitution of three human amino acids with the corresponding Mastomys residues (human mutant 4) confers constitutive activity to the human CCK-BR. The four amino acids in the MluI chimera, which are unique to the Mastomys receptor (Asp344, Ile353, Gln407, Pro423), were sequentially substituted with the corresponding human residues (human triple mutants 1–4: human CCK-BR with three amino acid alterations. The observation that multiple naturally occurring receptor variants within a given species rather than a species-dependent phenomenon as observed with the Mastomys CCK-BR.

The vast majority of naturally occurring constitutively active receptors reported in the literature can be attributed to single amino acid mutations. These point mutations have been identified in extracellular (25), transmembrane domain (21, 26–28), and intracytoplasmic segments (20, 22, 29, 30) of the corresponding G protein-coupled receptors. In contrast to this precedent, it is of note that three amino acids act in concert to confer constitutive activity to the Mastomys CCK-BR. Two of these residues (Leu344, Ile353) are located in transmembrane domain VI of the Mastomys CCK-BR, consistent with reports indicating a role of TMD VI in receptor activation. Studies with the photoreceptor rhodopsin, another member of the seven transmembrane domain receptor superfamily, suggest that movement of TMD VI is required for G protein activation (31). In addition, rotation of TMD VI has been demonstrated with either ligand or mutation-induced activation of the β2 adrenergic receptor (32, 33). Complementing these prior observations, the occurrence of activating polymorphisms in TMD VI of the CCK-BR further illustrates that this portion of the protein may be an important regulator of receptor-mediated signaling.

The third activating polymorphism in the Mastomys CCK-BR (Asp407 versus Glu407 in the human receptor) is found in the carboxyl terminus. This region of G protein-coupled receptors has been hypothesized to play a role in constraining the protein through palmitoylated cysteine residues that are believed to be anchored in the cell membrane. Truncations of the carboxyl terminus can result in constitutive activity as shown for the PGE3 receptor (24) and the thyrotropin-releasing hormone receptor (34). Alternatively, deletion or truncation of the carboxyl terminus may leave the signaling properties of a G protein-coupled receptor unchanged (35). The variable consequences of carboxyl-terminal truncations may indicate that this domain may act in concert with other regions of the receptor to define the basal level of signaling. Consistent with this possibility, our results suggest that a combination of transmembrane domain VI and carboxyl terminus amino acids together confer constitutive activity to the Mastomys CCK-BR.

Several naturally occurring constitutively active G protein-coupled receptors, each the result of single amino acid mutations, cause alterations of physiologic function both in animals as well as in humans. Constitutively active melanocyte-stimulating hormone receptor isoforms result in darkening of fur and skin color in mice (26). Activating mutations in the luteinizing hormone receptor lead to precocious puberty (21), whereas constitutively active parathyroid hormone receptors result in Jansen-type metaphyseal chondrodysplasia (29). Constitutively active mutants of the thyroid-stimulating hormone receptor have been identified in patients with thyroid adenomas (20).

In contrast to constitutively active receptors that arise from single point mutations (discussed above), ligand-independent signaling by the Mastomys CCK-BR results from a triple amino acid alteration. The observation that multiple naturally occurring residue changes can lead to constitutive receptor activity introduces a new consideration in the search for receptor abnormalities underlying physiologic alterations. In the case of the Mastomys CCK-BR, the in vivo consequences of receptor overactivity remain to be determined. Based on precedent with constitutively active G protein-coupled receptors resulting in tumor formation (20, 36), it is intriguing to speculate that the ligand-independent signaling by the Mastomys CCK-BR may in part explain the increased susceptibility of this species to the
development of ECL cell hyperplasia as well as ECL cell-derived carcinoid tumors.

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