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Differential expression of glucagon and glucagon-like peptide 1 receptors in mouse pancreatic alpha and beta cells in two models of alpha cell hyperplasia

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Abstract

Glucose homeostasis is determined by a balance between insulin and glucagon, produced by beta and alpha cells of the pancreas respectively. The levels of circulating hormones is partly determined by the mass of these two endocrine cell types. However, in contrast to β cells, the identity of the signals regulating alpha cell number is not known. Mice with a global deletion of the glucagon receptor ($Gcgr^{-/-}$) and mice with ablation of prohormone convertase 2 (PC2), the enzyme involved in the conversion of proglucagon into mature glucagon, develop alpha cell hyperplasia. These observations and the fact that $Gcgr^{-/-}$ mice exhibit high levels of circulating glucagon-like peptide-1 (GLP-1) suggested that members of the glucagon family of peptides could be directly involved in the regulation of alpha cell number. In this study we sought to determine whether alpha cells express receptors for $Gcgr$ and/or the glucagon-like peptide-1 (GLP1r). We examined the expression of these receptors in islets of $Gcgr^{-/-}$, $PC2^{-/-}$ mice and control littermates, in an alpha ($\alpha TC1/9$) and in a beta ($\beta TC3$) cell line. $Gcgr$ was expressed exclusively by islet beta cells, but not by alpha cells, of the two lines of mice lacking glucagon signaling. Similarly, βTC but not αTC cells, expressed $Gcgr$. The expression of GLP-1r by alpha cells was determined by the genotype and age of the mice. In embryos, GLU+ cells of $Gcgr^{+/+}$ mice express GLP-1r during early development, but not in adults. In contrast, alpha cells of $Gcgr^{-/-}$ mice were GLP-1r+ throughout life, reflecting the immature state of GLU+ cells when $Gcgr$ is deleted. Unlike alpha cells, beta cells of all mice lines examined initiate GLP-1r expression after birth. These results suggest that GLP-1 may affect the maturation of postnatal but not prenatal beta cells. In addition, they also suggest that the incretin could mediate alpha cell proliferation, inducing the development of alpha cell hyperplasia in $Gcgr^{-/-}$ mice.

Keywords

Pancreatic alpha cells; pancreatic beta cells, glucagon receptor; glucagon-like peptide 1 receptor

Introduction

The endocrine cells of the pancreas play a central role in glucose homeostasis. Insulin released from beta cells after a meal promotes the storage of glucose into target organs and its action is

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counterbalanced by glucagon. The regulation of euglycemia is partly determined by the beta cell mass, which is regulated by a balance between the rates of beta cell proliferation and apoptosis (Bonner-Weir, 2000, Scaglia et al., 1997). There is a large body of evidence indicating that the beta cell mass shows considerable plasticity, increasing in only few days in response to hormones (Bonner-Weir, 2000, Bonner-Weir, 2000, Ling et al., 1994, Lopez-Talavera et al., 2004, Nielsen et al., 2001, Sorenson and Brelje, 1997, Stoffers, 2004). Levels of circulating glucagon are also likely to be partly determined by the number of alpha cells. However, in contrast to beta cells, there is scant information regarding the identity of the signals regulating alpha cell number. Analysis of two lines of mice lacking glucagon signaling provided evidence that the absence of glucagon signaling resulted in development of alpha cell hyperplasia. One line is mouse with a deletion in the gene encoding for proprotein convertase-2 (PC2), that fails to convert prosomatostatin and proglucagon to the active form of the hormones (Furuta et al., 1997, Steiner, 1998). Adult PC2 KO mice showed a dramatic hypertrophy and hyperplasia of the alpha, delta, and PP cells without alteration in the normal location of these cells in the periphery of islets (Furuta et al., 1997, Vincent et al., 2003). The growth in the relative alpha cell mass was partly due to a higher rate of alpha cell proliferation in PC2^{-/-} than in PC2^{+/+} mice during the perinatal period (Furuta et al., 1997, Vincent et al., 2003). The administration of glucagon to adult PC2^{-/-} mice led to a reduction in the number of Glu⁺ cells due to increased apoptosis (Webb et al., 2002), suggesting that the lack of glucagon signaling affected both alpha cell proliferation and death.

The second animal model was a line of mice with a global ablation of the glucagon receptor (Gcgr^{-/-}, (Gelling et al., 2003, Parker et al., 2002)). Examination of Gcgr^{-/-} mice (Gelling et al., 2003) indicated the presence of alpha cell hyperplasia in islets, reproducing the phenotype observed in PC2^{-/-} mice. The increase in the non- β cell population in pancreatic islets of Gcgr^{-/-} mice was due primarily to larger numbers of GLU⁺ and SOM⁺ cells and was already evident during late prenatal development in Gcgr^{-/-} fetus (Vuguin et al., 2006). Growth in the number of non- β cells was partly due to increased rates of GLU cell proliferation in islets of embryos and pups (Vuguin et al., 2006).

The identity of the signals inducing an increase in alpha cell number in the absence of glucagon signaling is not known. If alpha cells express Gcgr, it could be argued that the regulation of alpha cell number is determined by an autocrine loop and that this loop becomes dysregulated following ablation of glucagon signaling. It is also possible that glucagon-like peptide (GLP-1), a member of the glucagon family of peptides known to increase beta cell number (Drucker, 2003, Stoffers, 2004), regulates the balance between alpha cell proliferation and apoptosis.

In this study we sought to examine the expression of Gcgr and GLP-1r in alpha cells of PC2^{-/-}, Gcgr^{-/-} mice and control littermates, in a glucagon-producing alpha cell line (α TC1/9) and an insulin-producing beta (β TC3) cell line. The two cell lines have relatively differentiated functions as islet alpha- and beta- cells (Hamaguchi and Leiter, 1990). Our results indicate that Gcgr was expressed by the beta cell line and by beta cells but not by the alpha cell line or by glucagon cells of adult PC2^{+/+} and Gcgr^{+/+} mice. GLP-1r was transiently expressed by alpha cells of Gcgr^{+/+} mice during development and by GLU cells of Gcgr^{-/-}, but not of PC2^{-/-} mice, throughout life. These results suggest that GLP-1 may regulate alpha cell mass in mice lacking Gcgr.

Materials and Methods

Cell lines

Mouse alpha TC1/9 cells (α TC1 clone 9) were obtained from American Type Culture Collection [(ATCC), Manassas, VA]. Cells were grown at 37.0°C in 5% carbon dioxide (CO₂) incubator, propagated in Dulbecco's Modified Eagle's Medium (DMEM) with 4 mM L-

glutamine plus 3.0 g/L glucose (0.016 M), 1.5 g/L sodium bicarbonate (0.018 M), 10% (v/v) heat-inactivated fetal bovine serum; 15 mM HEPES; 0.1 mM non-essential amino acids and 0.02% (w/v) bovine serum albumin. Mouse betaTC (β TC3) cell line was a gift from Dr. Shimon Efrat, University of Tel Aviv, Israel. Cells were propagated in DMEM with 4 mM L-glutamine plus 4.5 g/L glucose (0.025 M), 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate (0.018 M) and 15% (v/v) heat-inactivated fetal bovine serum. Cells were subcultured twice/week.

Animals

The generation of *Gcgr*^{-/-} and of *PC2*^{-/-} mice has been previously reported (Furuta et al., 1997, Gelling et al., 2003). *Gcgr*^{-/-} and *PC2*^{-/-} mice were generously provided by Dr P. Vuguin and M. Charron (Albert Einstein College of Medicine) and by Dr D. Steiner (U. of Chicago) respectively. CD-1 mice were purchased from Charles River (Kingston, NY). All animal protocols were approved by the Institutional Animal Care and Use Committees. Three animals/line were examined/age/experiment.

Islet isolation and Cytospin

Islets were isolated using the intraductal collagenase technique (Shapiro et al., 1996). Islets were collected with a pipette under the microscope and were incubated in a solution of papain following manufacturer's instructions (Worthington, Lakewood, NJ) for one hour at 37°C, dissociated and cytospun on Superfrost plus slides at 1000 rpm for 5 min (Cytospin 3; Shandon, Waltham, MA). Cells were immediately fixed in Zamboni's fixative (2% (w/v) paraformaldehyde and 15% (v/v) picric acid in 0.1 M phosphate buffer) for one hour and processed for in situ hybridization and/or immunostaining.

Tissue processing for immunostaining

Three-month-old adult mice were perfused through the heart with a solution of 4% paraformaldehyde in 0.1 M phosphate buffer and postfixed for several hours in the same fixative. Fixed tissues were infiltrated in 30% sucrose, mounted in embedding matrix (Lipshaw Co., Pittsburgh, PA), and 20- μ m cryostat sections were collected on Tissue Tack slides (Polysciences, Warrington, PA). Pregnant females were euthanized at days 12, 15 and 17 of gestation, the embryos dissected and the abdominal region fixed and processed as described above. The day the vaginal plug was found was considered embryonic day 1.

Antibodies

Guinea pig antibody to bovine IN was purchased from Linco Research, Inc. (Eureka, MO). Rabbit antibody to human GLU was purchased from Calbiochem, Inc. (San Diego, CA). Monoclonal antibodies to IN and GLU were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Rabbit antisera to GLP1r and to PC3/1 were generous gifts from Dr J. Habener (Harvard University) and Dr D.F. Steiner (U. of Chicago). Antisera to GLP-1r was also purchased from Novus Biologicals (Littleton, CO). Antibodies were diluted in 0.01M PBS and used at the following dilutions: guinea pig antibody to IN, 1:1000; monoclonal antibody to GLU, 1:6000; rabbit antisera to human GLU, 1:4000 and rabbit antisera to GLP1r, 1:4000 (from Harvard University) and at 1:100 (from Novus). Antisera to PC3/1 was used at 1:2000 dilution. For secondary antibodies, biotinylated goat anti-rabbit or anti-guinea pig IgG and avidin-labeled peroxidase were purchased from Vector Laboratories, Inc. (Burlingame, CA). Alexa fluor 488 antimouse, and antirabbit IgG and Alexa fluor 594 antiguinea pig, antirabbit, and antimouse IgG were purchased from Molecular Probes, Inc. (Eugene, OR).

Immunostaining

These techniques have been previously described (Vincent et al., 2003, Vuguin et al., 2006). In brief, sections and cell cultures were incubated sequentially in an empirically derived optimal

dilution of control serum or primary antibody raised in species X overnight and with a 1:200 dilution of the secondary antibody (in 0.01M PBS). For multiple labeled experiments, antibodies produced in different hosts were used. After completion of the staining procedure, sections were covered with two to three drops of Vectashield Solution (Vector Laboratories, Burlingame, CA). For DAB staining, sections or cell cultures were incubated with a 1:50 dilution of anti- (species x) biotinylated IgG solution in 1% goat serum in PBS for 30 min; and a 1:100 dilution of peroxidase-avidin complex for 30 min (avidin-biotin complex: ABC technique). Following these incubations, the bound peroxidase was visualized by 3,3'-diaminobenzidine (DAB). After the DAB step, sections were dehydrated and mounted with Permount (Fisher Scientific, Fairlawn, NJ).

in situ hybridization

Total RNA from mouse liver was reverse-transcribed and PCR-amplified with primers for mouse *Gcgr* (5'-CAGAAGATTGGCGATGACCT and 5'-CTCGTCAGTCACAAAGGCAA, Accession No. [NM_008101.1](#)). The amplified fragment (502 bp) was cloned into pCR4-TOPO plasmid (Invitrogen, Carlsbad, CA) and verified by sequencing. The recombinant plasmid was propagated in *E. coli*, purified and then linearized using either NotI (for sense) or SpeI (antisense) for 2 hours at 37°C. Digoxigenin-labeled antisense or sense cRNA probe was transcribed in vitro using DIG RNA labeling kit (Roche Applied Sciences, Indianapolis, IN).

In situ hybridization (ISH) was performed as previously described (Braissant et al., 1996). Briefly, frozen tissue sections (10 μ m) were processed for prehybridization. Hybridization was performed at 58°C for 16 hours in buffer containing 0.2 ng/ μ l labeled RNA probe. Then the sections were incubated overnight at 4°C with alkaline phosphatase conjugated antidigoxigenin monoclonal antibody (Roche). For color development, the tissue sections were covered with NBT/BCIP (Roche) for 1h at room temperature. At the end of the reaction, sections were covered with a drop of glycerol and coverslipped. For combined in ISH and immunohistochemistry, tissues were first processed for ISH and then for immunostaining as described above.

Confocal microscopy

Confocal images were obtained using a Radiance 2000 confocal microscope (Bio-Rad, Hercules, CA) attached to a Zeiss Axioskop microscope (Carl Zeiss Inc., Thornwood, NY). Images of 540 $\times$ 540 pixels were obtained and processed using Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA).

RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from islets, cell pellets or tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA), further purified with RNAeasy Kit (Qiagen, Germantown, MD) and treated with recombinant DNase-1. Total RNA was reverse-transcribed using SuperScript III (Invitrogen). The number of cycles was optimized depending on the particular mRNA abundance and chosen to select PCR amplification on the linear portion of the curve to avoid saturation effect. Aliquots were analyzed by electrophoresis. Information about genes is indicated in Table 1.

Real-Time PCR array

Mouse Diabetes RT² Profiler PCR Array and RT² Real-Timer SyBR Green/ROX PCR mix were purchased from SuperArray Bioscience Corporation (Frederick, MD). PCR was performed on ABI Prism 7000 Sequence Detector (Applied Biosystems, Foster City, CA). For data analysis the $\Delta\Delta C_t$ method was used; for each gene fold-changes were calculated as

difference in gene expression between α TC1/9 and β TC. Data were analyzed using web-based PCR data analysis software provided by Superarray website.

Results

Pancreatic alpha cells do not express Gcgr

The specificity of the Gcgr primers was tested on tissues isolated from Gcgr^{+/+} and of Gcgr^{-/-} mice that were reported to express Gcgr (Christophe, 1996, Dunphy et al., 1998, Goldfine et al., 1972, Hansen et al., 1995, Moens et al., 1996). RT-PCR analysis of cDNA prepared from total RNA isolated from kidney of Gcgr^{-/-} mice did not generate a PCR product (Fig. 1A, **lane 1**). In contrast, a 447 bp fragment was amplified from Gcgr^{+/+} kidney (Fig. 1A, **lane 2**). The expression of Gcgr throughout the medulla of the kidney has been previously reported (Marks et al., 2003). In situ hybridization analysis confirmed the presence of *Gcgr* mRNA in kidney medulla of Gcgr^{+/+} mice (Fig. 1D) while kidney from Gcgr^{-/-} mice scored negative (Fig. 1E). No signal was detected in kidney of Gcgr^{+/+} incubated with sense probe (Fig. 1F).

We then examined the expression of Gcgr in endocrine cells. RT-PCR analysis revealed the absence of *Gcgr* mRNA in α TC1/9 (Figure 1C, **lane 1**), while β TC3 cells generated the 447 bp Gcgr fragment (Figure 1C, **lane 2**). Immunohistochemical studies reported the expression of Gcgr by β TC3 cells and it also indicated that the receptor expressed by these cells lacked functional activity (Kieffer et al., 1996). The RT-PCR expression results were confirmed by real-time PCR array, which showed 5.1 fold decrease in α TC vs. β TC (data not shown) suggesting the level of expression of Gcgr in α TC is very low.

Islets isolated from Gcgr^{-/-} and Gcgr^{+/+} mice were used as negative and positive controls respectively. As expected, Gcgr was expressed by islets of Gcgr^{+/+} (Fig. 1B, **lane 2**) but not of Gcgr^{-/-} mice (Fig. 1B, **lane 1**). In agreement with the PCR results, in situ-hybridization experiments demonstrated the expression of *Gcgr* mRNA in β TC3 (Fig. 2E) and the absence of the receptor in α TC1/9 cells (Fig. 2F). Importantly, cytospin preparation of islets of adult CD-1 (Fig. 2A) and PC2^{-/-} mice (not shown) revealed the presence of a population of Gcgr⁺ cells. Cytospins incubated with sense probe did not produce a signal (Fig. 2B). To identify the labeled cells, cytospin preparations of islets were processed for visualization of *Gcgr* mRNA by in situ hybridization and insulin by immunocytochemistry. This analysis revealed that most beta cells expressed the receptor (Fig. 2C) while alpha cells of pancreas did not contain *Gcgr* mRNA (Fig. 2D).

Alpha cells of Gcgr^{-/-} mice express Glp1r

In agreement with previous results (Campos et al., 1994, Schlatter et al., 2007), RT-PCR analysis showed that *Glp1r* mRNA was abundant in β TC3 cells (Fig. 3A, **lane 1**) but it was not detected in α TC1/9 (Fig. 3A, **lane 2**). However, real-time PCR arrays revealed low level of *Glp1r* expression in α TC1/9 cells (Fig. 3B, **lane 1**), which is 13.7 fold lower than in β TC3 (data not shown). To ascertain whether α and β TC cells synthesize the receptor protein, we performed immunohistochemistry with a specific antibody to the GLP-1r. Only a fraction of α TC1/9 cells scored positive (Fig. 3C). In contrast, most β TC3 cells were GLP-1r⁺ (Fig. 3D).

In CD-1, Gcgr^{+/+}, PC2^{+/+} and PC2^{-/-} mice, the GLP1r had a differential expression in different islet cell populations. Thus, immunocytochemical analysis of islets of adult CD-1 (Fig. 4A-C), Gcgr^{+/+} (not shown), and PC2^{-/-} (Fig. 4D-F) mice revealed that almost all beta cells were GLP-1r⁺ while no alpha cells expressed the receptor. In contrast, a subset of glucagon cells of both embryonic (Fig. 4J-L) and adult Gcgr^{-/-} mice coexpressed GLP-1r (Fig. 4G-I, Table 2).

The expression of GLP-1r by alpha cells suggests that GLP-1 could be involved in the regulation of alpha cell proliferation through an autocrine loop. However, previous work indicated that GLU⁺ cells of adult *Gcgr*^{-/-} mice, like those of *Gcgr*^{+/+} mice, do not express PC3/1 (Vuguin et al., 2006), the enzyme responsible for the conversion of proglucagon into GLP-1 (Habener, 1998). We confirmed these results (Fig. 5D-F). In addition, analysis of tissues from e18 *Gcgr*^{-/-} embryos, a stage characterized by active alpha cell proliferation (Vuguin et al., 2006), demonstrated that GLU⁺ cells of embryos also lack PC3/1 (Fig. 5A-C). The lack of PC3/1 expression by alpha cells strongly suggests that they do not synthesize GLP-1. Therefore, if GLP-1 plays a role in the regulation of GLU cell proliferation, this role would be through a paracrine rather than autocrine action.

Alpha cells expressing Glp-1r are immature cells

Glucagon cells of adult *Gcgr*^{-/-} mice have been reported to express embryonic traits such as insulin, the transcription factor PDX-1, Glut-2 and nestin (Kedees et al., 2007, Teitelman et al., 1993, Vuguin et al., 2006). These observations raised the possibility that the expression of GLP-1r is an additional embryonic trait of alpha cells of adult *Gcgr*^{-/-} mice. To test this proposition, alpha cells of embryos and adults *Gcgr*^{+/+} and *Gcgr*^{-/-} mice were triple labeled for visualization of glucagon, insulin and GLP-1r. Examination of pancreas of e-12 (not shown) and e15 *Gcgr*^{+/+} embryos indicated that most GLU⁺ cells were GLP1r⁺ (Fig. 6C, Table 2).

GLU⁺GLP-1r⁺ cells of *Gcgr*^{+/+} expressed insulin (Fig. 6D), at e-15 but not at later embryonic stages. In *Gcgr*^{+/+} mice, expression of GLP-1r by alpha cells persisted in the perinatal period (Fig. 6J), but not at later postnatal stages (not shown) or adults (Fig. 4). In contrast, expression of GLP-1r in alpha cells of *Gcgr*^{-/-} mice was not downregulated during the perinatal period. Rather, GLU⁺ cells of *Gcgr*^{-/-} mice retained GLP-1r expression at e-18 (Fig. 4 J-L) and adults (Fig. 4G-I). Alpha cells of adult *Gcgr*^{-/-} mice not only retained the expression of GLP-1r but also stained for insulin (Fig. 6F-G).

Beta cells initiate GLP-1r expression after birth

In contrast to alpha cells, beta cells of e-15 *Gcgr*^{+/+} (Fig. 6A-B) and *Gcgr*^{-/-} mice (not shown) do not contain GLP-1r. The receptor was first expressed by IN⁺ cells after birth (Fig 6 H-I, Table 2). This finding indicates that GLP-1 does not have a direct effect on β cells during development. Taken together, these results indicate that expression of GLP-1r is differentially regulated in alpha and beta cells during development. In *Gcgr*^{+/+} mice, the GLP-1r is expressed by alpha cells during pre and postnatal development but not in adults, while beta cells first express the receptor in the perinatal period. Presumably, the GLP-1r expression in the two islet cell types is regulated by signals related with the initiation of independent feeding.

Discussion

To date, it is not known if pancreatic alpha cells function as direct metabolic sensors, or whether they only respond to signals from other islet cells. A recent study reports that glucagon modulates its own secretion by binding to the glucagon receptor, which was detected by physiological assays and by PCR analysis on FACS isolated rat alpha cells (Ma et al., 2005, Moens et al., 1996). This observation, however, differs from previous reports indicating the absence of *Gcgr* expression by alpha cells (Gromada et al., 2007). The development of alpha cell hyperplasia in mice lacking glucagon signaling again raised the possibility of *Gcgr* expression by glucagon cells. It was hypothesized that the increase in the number of GLU⁺ cells was due to a disruption of an autocrine loop (Vuguin et al., 2006). Our results, however, demonstrate the absence of *Gcgr* expression in α TC1/9 by RT-PCR and real-time PCR array and in alpha cells of islets by in situ-hybridization. The reasons for the discrepancy between

the different studies is not known and they may be related to species differences (mouse vs. rat) and/or to different analytical techniques used.

The expression of the Glp1r in alpha cells has also been a controversial issue. *Glp1r* mRNA and protein expression was reported in a small subset of alpha cells from rat islets and in alpha cell lines (Heller et al., 1997). In addition, GLP-1 was found to have direct effects on isolated alpha cells (Gromada et al., 1998). However, other reports indicated that only beta cells express Glp1r (Moens et al., 1996, Tornehave et al., 2008). Our findings indicated that GLP-1r was expressed by glucagon cells of adult *Gcgr*^{-/-} mice but not by alpha cells of adult CD-1, *Gcgr*^{+/+}, *PC2*^{+/+} or *PC2*^{-/-} mice.

The high levels of circulating GLP-1 in *Gcgr*^{-/-} mice may induce an increase in the rate of proliferation of the GLU^+ , which express the GLP-1r throughout life. However, this activity would not be mediated by an autocrine loop because Glu^+ cells of *Gcgr*^{-/-} mice do not express PC3/1 in the perinatal period, when the replicating activity of the cells increase, or in adults (Vuguin et al., 2006). These observations raise the question of the source of the high levels of circulating GLP-1 in adult *Gcgr*^{-/-} mice. Recent evidence suggests that the most likely source is the L cells, the enteroendocrine cells responsible for GLP-1 synthesis, which increase in number in mice lacking *Gcgr* (Grigoryan et al., 2007). Of interest would be to test whether the pharmacological or genetic inhibition of GLP-1 activity in *Gcgr*^{-/-} mice affects the development of alpha cell hyperplasia. Observations in *PC2*^{-/-} mice suggest that the incretin has no role. *PC2*^{-/-} mice have normal levels of GLP-1, and, while its islets contain a thick mantle of alpha cells, these cells do not express GLP-1r. Alternatively, it is possible that different signals regulate alpha cell number in islets of *Gcgr*^{-/-} and *PC2*^{-/-} mice.

It can also be proposed that alpha cell number is regulated by insulin, which plays a key role in modulating glucagon secretion (Asplin et al., 1981, Maruyama et al., 1984). Since insulin has an inhibitory effect on glucagon, decreased levels of insulin could result in a dysregulation and increased proliferation of alpha cells. While circulating insulin levels are normal in *Gcgr*^{-/-} mice (Gelling et al., 2003), the level of insulin mRNA and of genes that regulate insulin function is low (Vuguin et al., 2006), suggesting a decrease in the amount of intra-islet insulin. However, specific ablation of the insulin receptor in alpha cells (Kitamura et al., 2004) or a reduction of insulin levels due to a mutation in *PC3/1*, the main convertase involved in the conversion of proinsulin to insulin, did not result in alpha cell hyperplasia (Zhu et al., 2002, Zhu et al., 2002).

It has also been suggested (Furuta et al., 1997) that the increase in the non- β cell population is caused by the mild hypoglycemia characteristic of the mutant strains. Central and peripheral glucoreceptors detect circulating blood glucose levels and relay this information to the nervous system via central autonomic circuits that regulate the release of glucagon and of other glucogenic signals (Brunnicardi et al., 1995, Satin and Kinard, 1998). According to this hypothesis, a decrease in circulating blood sugar levels would signal, *via* islet innervation, an augmentation in the number of glucagon cells and glucagon secretion, which would lead to a rise in gluconeogenesis and a rise in the blood sugar levels in the mild hypoglycemic mutant mice. However, expression of a transgene comprised of the insulin I promoter linked to the coding region of the *Gcgr* gene in *Gcgr*^{-/-} (RIP-*Gcgr*) mice led to restoration of *Gcgr* expression in beta cells (Vuguin et al., 2005). Importantly, Rip-*Gcgr* mice were normoglycemic and displayed alpha cell hyperplasia (Vuguin et al., 2005). Another possibility is that GABA, produced and released by the beta cells (Bailey et al., 2007, Brogren et al., 1983, Wendt et al., 2004), plays a role in the regulation of alpha cell proliferation. This possibility remains to be tested.

In addition to its effects on alpha cell number, ablation of the glucagon receptor appears to stall alpha cell maturation. Since therapies involving the inhibition of Gcgr are currently being developed for treatment of type II diabetes, it will be important to determine the nature of the mechanisms involved in glucagon cell maturation and proliferation and whether the process of differentiation is affected by the pharmacological ablation of the receptor.

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Figure 1.

Expression of glucagon receptor (Gcgr) mRNA in kidney (A) of Gcgr^{-/-} (lane 1) and Gcgr^{+/+} (lane 2) mice, in islets (B) from Gcgr^{-/-} (lane 1) and Gcgr^{+/+} (lane 2) and in α TC1/9 (lane 1) and β TC3 (lane 2) cell lines (C) by RT-PCR. PCR products were resolved on 2% agarose gels and stained with ethidium bromide. The bands represent the only products observed. No products were detected from RNA samples that were not reverse transcribed (not shown). Glucagon receptor (447 bp amplicon) was not detected in α TC1/9 and Gcgr^{-/-} islets and kidney. All RT-PCR experiments included the co-amplification of a housekeeping gene (*18sRNA* or equivalent) from the same RNA sample and yielded a PCR amplicon of the expected size. D- Gcgr mRNAs were localized by in situ hybridization in kidney of Gcgr^{+/+} mice using a Dig-labelled mouse Gcgr antisense riboprobe; blue precipitates indicate the presence of Gcgr mRNA. E- kidney of Gcgr^{-/-} mouse incubated with antisense probe. F- Kidney of Gcgr^{+/+} mice incubated with sense probe. Note the lack of precipitate in E and F. Bars in D and E= 80 μ m .

Figure 2.

Localization of Gcgr mRNA by in situ hybridization (ISH). **A,B** - dispersed islet cells of Gcgr +/- mice obtained by cytopsin incubated with either antisense probe (**A**) or sense probe (**B**), bar= 20um. **C**: dispersed islet cells processed for visualization of Gcgr mRNA (ISH, purple) and insulin (immunostaining, brown), bar=15um. Note that most cells are IN⁺ Gcgr⁺. **D** - Islet cells processed for visualization of Gcgr mRNA and glucagon, bar= 15um. Note that alpha cells (brown) do not express Gcgr mRNA (blue). **E**- In situ hybridization of Gcgr mRNA in β TC3 cells. Most β TC cells express Gcgr mRNA bar: 20um. **F**- α TC cells processed for visualization of Gcgr mRNA documents the lack of ISH product. Cultures were processed for hematoxylin staining to facilitate visualization.

Figure 3.

A- Expression of Glp1r in β (lane 1) and α (lane 2) TC cells by RT-PCR. Note that no band was detected in α TC cells. Arrows indicate the band corresponding to the GLP-1r (523bp) and 18sRNA (68bp). B- Analysis of array products. After a real-time PCR array run, a standard melting curve program was performed and dissociation curve of each gene was checked for generation of a gene-specific single peak (not shown) without co-amplification of primer dimers or non-specific secondary products. Amplicons were separated on 2% agarose gel; each gel lane contained only one band of the predicted size, this analysis demonstrated that GLP1-r (lane 1) is expressed in αTC1/9 in a very low level (average CT: 27.7) compared to e.g. Gpd1 (Glycerol-3-phosphate dehydrogenase 1, average CT: 25.6, lane 2), Gsk3b (Glycogen synthase

kinase 3 beta, average CT: 23.6, lane 3) or Hmox1 (Heme oxygenase 1, average CT: 23.5, lane 4). C- D, Immunolocalization of the GLP1-r in α TC and β TC cells respectively. Brown precipitate (DAB) indicates the presence of the receptor. C illustrates the presence of GLP1r⁺ and GLP1r⁻ in α TC cells. bar: 20um .

Figure 4.

Alpha cells of *Gcgr*^{-/-} mice express GLP-1r. A-C immunohistochemical visualization of GLP1-r (**A**) and glucagon (**B**) in an islet of CD-1 mouse. Note that glucagon cells do not express GLP-1r ; **C**=**A**+**B**. bar= 40um. **D-F**- immunostaining for Glp1-r (**D**) and glucagon (**E**) in an islet from an adult *PC2*^{-/-} mouse. Note that the islet does not contain GLP-1r+ GLU+ cells. **F**=**D**+**E**. Bar= 40um. **G-I**- immunostaining for Glp1-r (**G**) and glucagon (**H**) in a *Gcgr*^{-/-} islet. **I**=**G**+**H**. The islet contains GLP-1r+ GLU+ cells (yellow). Arrowhead indicates a representative cell coexpressing glucagon and Glp-1r, bar= 30um. J-O. Photomicrograph illustrates an islet of e-18 *Gcgr*^{-/-} mouse immunostained for GLP1-r (**J**, green) and glucagon (**K**, red). A subset of alpha cells express the receptor. Bar=30um.

Figure 5.

Alpha cells of *Gcgr*^{-/-} mice do not express proprotein convertase 3/1. Immunolocalization of PC3/1 (green) and glucagon (red) in islets of e-18 (A-C) and adult (D-F) *Gcgr* mice. This figure illustrates that glucagon cells do not express the proprotein convertase, suggesting that they do not synthesize GLP-1. Bar: 40 μ m.

Figure 6.

Beta cells initiate GLP1-r expression in the perinatal period. Figures illustrate the presence of insulin (blue), and/or Glp-1r (green) and/or glucagon (red). **A-C-** Section of pancreas of e-15 *Gcgr*^{+/+} embryos immunostained for IN, GLU and GLP-1r (**A**), IN+ GLP-1r (**B**) or GLU +GLP1r (**C**). Note that GLP-1r is expressed by most GLU⁺ cells but not by IN⁺ cells. Bar= 30um. **D-** At e-15, some GLU⁺ cells coexpress IN and GLP-1r. Bar= 15um. Cell indicated with an arrow in **D**, and illustrated in high magnification in **E**, is IN⁺ GLP-1r⁺ Glu⁺. Bar in E= 4um. **F-** Islets of adult *Gcgr*^{-/-} mice contain IN⁺GLP-1r⁺GLU⁺ cells. Cell indicated with an arrow, illustrated in higher magnification in **G** is IN⁺GLP-1r⁺GLU⁺. Bar= 5um. **H-J-** Islet of postnatal day 2 *Gcgr*^{+/+} mice. Note that GLP-1r (green) is expressed by beta cells (blue, **I**) and by alpha cells (red, **J**). **H=I+J**. Bar= 20um.-

Table 1

Gene	Accession No.	Primers sequence	Product size
Glucagon receptor (<i>Gcgr</i>)	NM_008101.2	5'-ACA TCA TTC ACC TTC TTG TGG-3' and 5'-GCC AGC GAG GTC TCC ATA G-3'	447 bp
Glucagon like peptide 1 receptor (<i>Glp1r</i>)	NM_021332	5'-TGA ACC TGT TTG CAT CCT TCA-3' and 5'-ACT TGG CAA GCC TGC ATT TGA-3'	523 bp
18S ribosomal protein (<i>18srRNA</i>)	BK000964	5'-AGT CCC TGC CCT TTG TAC ACA-3' and 5'-GAT CCG AGG GCC TCA CTA AAC-3'	68 bp
Beta actin (<i>Actb</i>)	NM_007393.3	5'-TGG CAC CAC ACC TTC TAC AAT GAG C-3' and 5'-GCA CAG CTT CTC CTT AAT GTC ACG C-3'	387 bp

Table 2

	Differential expression of GLP1r			
	Alpha cells		Beta cells	
	Gcgr+/+	Gcgr-/-	Gcgr+/+	Gcgr-/-
e-15	+	+	-	-
P2	+	+	+	ND
adult	-	+	+	+