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REGULATION OF RAT GLUCAGON RECEPTOR EXPRESSION

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This report describes the isolation of a cDNA for the rat glucagon receptor by using the glucagon-like peptide 1 receptor cDNA as a probe. Northern blot analysis using the cDNA clone showed that the message encoding the receptor is approximately 2.3 kb in size and is expressed only in liver and kidney among seven tissues tested. To study how glucagon receptor expression is regulated _in vivo_, the levels of hepatic glucagon receptor mRNA were measured in diabetic mouse model, db/db and control (db/+) mice. Interestingly, the receptor mRNA levels were similar between diabetic and control mice. In contrast, the number of hepatic glucagon receptors in diabetic mice measured by binding assays was significantly higher than that found in normal mice. These results suggest that the major regulation in hepatic glucagon receptor expression in vivo is at the posttranscriptional level. © 1994 Academic Press, Inc.

Glucagon is a 29 amino acid peptide hormone which plays a major role in regulating hepatic glucose production by stimulating gluconeogenesis and glycogenolysis (1, 2). Biological actions of glucagon are mediated by glucagon binding to a membrane receptor that activates adenylate cyclase via a membrane associated heterotrimetric G protein (3). The glucagon receptor has been extremely difficult to purify, which has impeded the development of antibodies and other reagents needed to investigate its regulation in depth. Thus, very little information is available regarding the molecular details of the interaction between glucagon and its receptor and how this complex activates adenylate cyclase. Importantly, even less information is available on the regulation of the expression of the glucagon receptor gene.

A cDNA for the rat glucagon receptor was recently cloned by an expression cloning strategy (4). The present report describes an alternative strategy which we used to isolate cDNA clones for the rat glucagon receptor. Using this clone, we examined the expression pattern of the glucagon receptor in various tissues and determined whether expression of the glucagon receptor mRNA is modulated under different pathophysiologic conditions.

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MATERIALS AND METHODS

Materials: [Alpha-32P] dCTP (3000Ci/mmol) and Hybond-N nylon (0.45 micron) were purchased from Amersham. 125I-glucagon (1.85 MBq, 2200 Ci/mmole) was purchased from NEN, Du Pont. Nitrocellulose filters (BA 85) were from Schleicher & Schuell. Nonradioisotopic detection kit and restriction endonucleases were purchased from Boehringer Mannheim. Rat liver cDNA libraries were purchased from Clontech (made from adult male Sprague Dawley in lambda GT II) and Stratagene (made from Sprague Dawley, 6 months old male in lambda Zap II). Lambda phage and plasmid DNA were prepared by Qiagen kits. DNA sequencing was done with an Exo-pfu sequencing kit (Stratagene).
Genetically diabetic mice (C57BL/KsJ db/db) and lean control mice were obtained from Jackson Laboratory, Bar Harbor, Maine. Animals were killed by CO_2 inhalation at 10-12 weeks of age and blood and liver were collected. Blood samples were analyzed for plasma glucagon (Peninsula) and glucose concentrations.

Plaque Hybridization: Approximately two million phage plaques (50,000 per plate) were screened with rat glucagon-like peptide-1 (GLP-1) receptor cDNA under low stringency hybridization conditions (30% Formamide, 6 x SSC, 0.1% N-lauroyl sarcosine, 0.2% SDS, 3% blocking reagent). The probe was a gel purified insert fragment of GLP-1 receptor cDNA (5) labelled with digoxigenin dUTP according to the manufacturer's instructions. The filter (Hybond-N nylon) lifts, denaturation, neutralization, hybridization and washings were performed according to manufacturer's instructions except that hybridization was done at 37° C. The washes were performed twice for 20 min each, once at room temperature and the second at 37° C. Filters were then processed according to the Genius System (Boehringer Mannheim) for nonradioactive detection. Positive plaques from the first round of screening were further purified and DNA was prepared by the Qiagen lambda DNA purification procedure.

Cloning: *Rat GLP-1 receptor cDNA*: Two primers from the protein coding portion of GLP-1 receptor (sense primer containing ATG: TCCTGAGCGCCCCGCCATGG and antisense primer containing TGA: GGGGCTCAGCTGCAGGA) were synthesized and used to clone a cDNA from a rat islet tumor cell line (RIN) cDNA library by PCR under the standard conditions (1 min at 95°C; 1 min at 55°C; and 1 min at 72°C). The identity of the clone for the rat GLP-1 receptor cDNA was established by restriction mapping and partial sequencing.
Rat glucagon receptor cDNA: Inserts from the positive phage clones were subcloned into pUC 18 or Bluescript vectors for further restriction analysis and nucleotide sequencing.

RNA Analysis: Total RNA was extracted from rat tissues using RNAzol (BIOTECX) solution according to the manufacturer's manual. Poly A+ RNA was purified by oligo(dT)-cellulose chromatography (6). Northern analysis was carried out with 3.5 ug of poly A+ RNA. The RNA was denatured in 70% formamide-6% formaldehyde in MOPS buffer (20 mM morpholinepropanesulfonic acid [MOPS], 5 mM sodium acetate, 1 mM EDTA [pH 7]) at 55° C for 15 min and electrophoresed in a 6% formaldehyde-1.2% agarose gel in MOPS buffer. Conditions for transfer, hybridization and washing were as described previously (7). A rat glucagon receptor cDNA probe that consisted of a 2.1 kb Eco R1 fragment and a mouse ribosomal protein probe, gene 32 (8), were gel purified and labelled with [32P]- dCTP by the oligolabelling kit from Pharmacia.

Glucagon Binding Assays: Glucagon binding to mouse liver membranes was carried out in 96-well plates (0.45 micron Durapore; Millipore Corp.). The liver membranes were prepared according to the procedure by Hubbard et al (9). Approximately 5 ug of membrane protein in 200 µl of 20 mM Tris, 1 mM EDTA, 1 mg/ml BSA and 1 mg/ml bacitracin, pH 7.4, was incubated with 18 fmol of 125I-glucagon in the presence or absence of excess native hormone. After a 60 min incubation at room temperature, the plate was placed on a millipore vacuum apparatus and the membranes captured on the millipore filters. The filters were washed three times with ice-cold buffer and the filter-associated radioactivity determined.

RESULTS AND DISCUSSION

Isolation of cDNA clones

The strategy to isolate cDNA clones for the rat glucagon receptor was based on the assumption that two ligands (i.e. glucagon and glucagon-like peptide 1), closely related both structually and functionally, would have two closely related receptors. Both peptide hormones are similar in size and their amino acid residues are highly conserved (3). Both hormones mediate their biological actions by raising cAMP levels. Importantly, a report by Thorens showed (5) that high concentrations of glucagon can compete with GLP-1 for binding to the GLP-1 receptor. Based on the above information, a rat liver cDNA library was screened with GLP-1 receptor cDNA under hybridization conditions that should detect clones which share more than 50% homology with the probe.

Approximately 2 million plaques were screened and 3 overlapping clones were isolated. Figure 1 shows the alignment of these clones based on restriction mapping and nucleotide sequencing data. The nucleotide sequence of the coding portion of clone 4 (~1.5 kb out of 2.1 kb) is in perfect agreement with the published version (Genbank Accession number M96674).

The prediction that the receptors for glucagon and GLP-1 are closely related proved correct. When the nucleotide and amino acid sequences of several G protein coupled receptors including receptors for secretin, PTH and calcitonin were

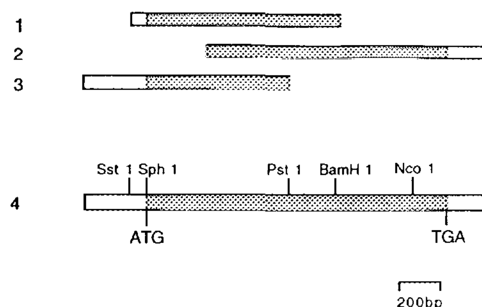

Figure 1. Rat glucagon receptor cDNA clones.

compared, the glucagon receptor has the highest homology to the GLP-1 receptor (67 % overall similarity at the amino acid level, 5), especially in the presumed membrane spanning regions. This is consistent with the finding that glucagon and GLP-1 bind competitively at high concentrations but with different binding affinities to the same receptor. Thus, the physiological effects of each hormone is determined by the binding affinities of its receptor and the tissues in which the receptor is expressed. For example, GLP-1 receptor is expressed primarily in lung, stomach and beta cells of pancreatic islets (5), whereas the glucagon receptor is expressed mainly in liver and kidney (Figure 2).

Tissue specific expression

Since the liver is a major target organ for glucagon action, it should show preferential expression of the glucagon receptor gene. A profile of tissue expression of glucagon receptor mRNA using our cDNA clone is illustrated in Figure 2. The Northern blot showed a single 2.3 kb band that hybridizes to the glucagon receptor cDNA probe. Among seven tissues tested, this band was found only in liver and kidney. The amount of glucagon receptor mRNA in the kidney is estimated to be approximately one third of that in liver. Recently, Gillin and Sands (10) suggested that glucagon might be an important physiological regulator in the proximal tubule. The physiological role of glucagon pertaining to carbohydrate metabolism in kidney needs to be examined in detail. In our preliminary studies small amounts of high affinity glucagon binding activity was detected in kidney plasma membrane preparations, consistent with the Northern analysis.

Regulation of glucagon receptor expression

Previous studies have shown that the rates of basal hepatic glucose output are elevated in Type II diabetes and that this elevation is correlated with the increased

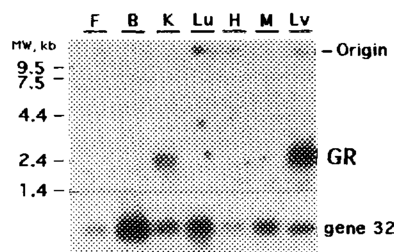

Figure 2. Expression of rat glucagon receptor mRNA. Poly A+ RNA (3.5 μg) samples from rat tissues (F, fat; B, brain; K, kidney; Lu, lung; H, heart; M, muscle; Lv, liver) were analyzed according to the protocol described in Methods. The probe used was the insert fragment of clone 4 (see Figure 1) and the filter was reprobed with a control probe, a ribosomal protein gene 32, to ensure that all lanes contained approximately equivalent amounts of RNA.

hepatic response to glucagon (1, 11). This is consistent with the findings in rodent models for Type II diabetes, such as db/db mice (12).

The availability of glucagon receptor cDNA clones enabled us to directly investigate whether or not mRNA expression is affected by different pathophysiological states. For these studies, the levels of glucagon receptor mRNA were compared in livers of genetically diabetic (db/db) and control (db/+) mice by Northern blot analysis. Animals were killed at 10-12 weeks when the blood profile showed a marked hyperglycemia in the db/db animals, i.e. a mean blood glucose concentration of approximately 660 mg/dl and a mean glucagon level of 880 pg/ml (Table 1). Liver tissues collected from four mice from each group were divided into two parts, one half for RNA extraction and the remaining half for the plasma membrane preparation. The results of Northern analysis using these samples are shown in Figure 3. Interestingly, we did not observe any significant changes in hepatic glucagon receptor mRNA levels between the diabetic and control mice, either in the liver nor in the kidney (see Figure 3a and b). However, glucagon binding studies using liver membranes from individual mice showed that the diabetic mice have much more 125I-glucagon binding activity than that of control animals (data not shown). To obtain enough material for Scatchard analysis of the glucagon binding activity, the liver membrane samples for each group were combined. We estimate from Figure 3c that the number of functional glucagon receptors present in the liver membranes of db/db mice is ~3 times higher than that of control animals. Our efforts to correlate the binding activities in the kidney plasma membranes with RNA analysis were unsuccessful due to the low level of glucagon binding in the kidney plasma membrane preparations.

Nonetheless these results suggest that glucagon receptor expression in the liver (likely in the kidney as well) is modulated at a step after mRNA formation. This is the first study performed to investigate the regulation of the glucagon receptor expression in vivo. We speculate that the rate of turnover of the glucagon receptor or covalent modifications that alter its binding activity likely provide the major

Table 1. Plasma glucose and glucagon levels in db/db and db/+ mice

Animals*	Glucose (mg/dl)	Glucagon (pg/ml)
db/db , mean (+/- SD)	660 (89)	880(324)
db/+ , mean (+/-SD)	230 (2)	282 (33)

* Four animals were used in each group.

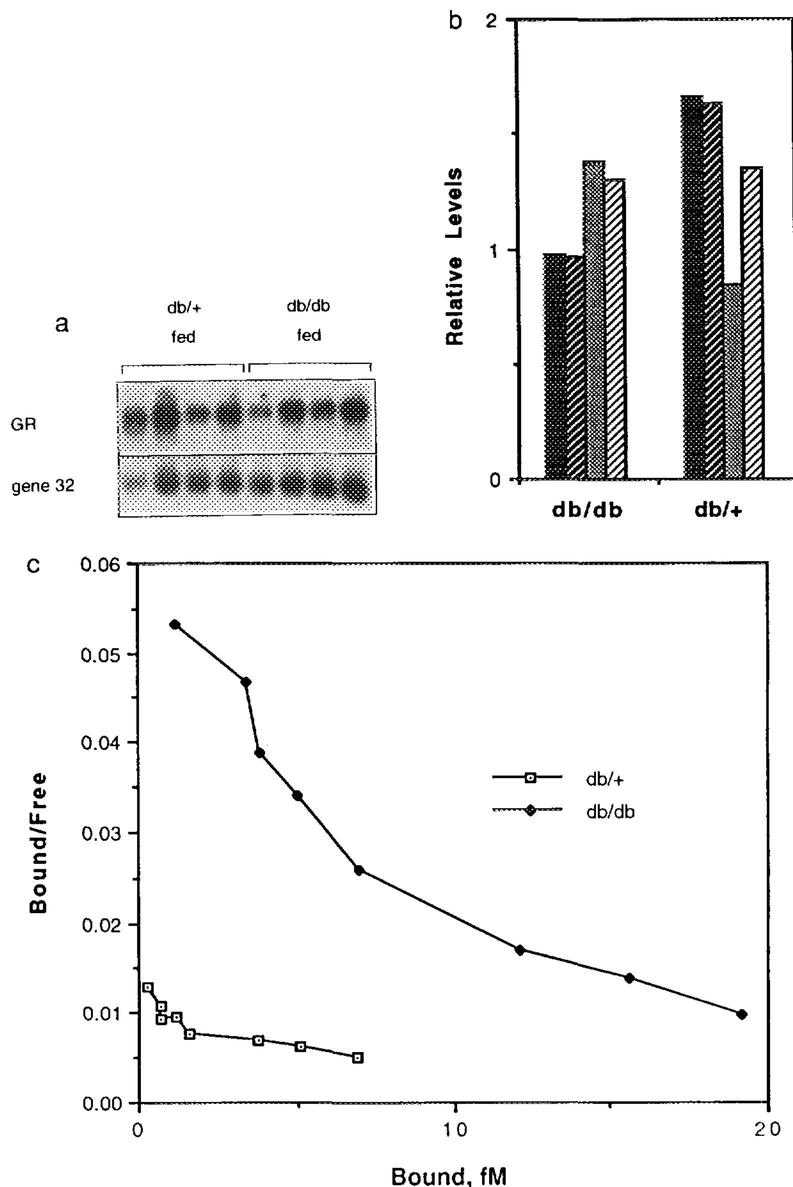

Figure 3. Glucagon receptor expression in vivo. a. Northern analysis of the hepatic glucagon receptor mRNA. b. Relative levels of the glucagon receptor mRNA in a. Intensity of glucagon receptor mRNA was scanned using an image scanner and was normalized according to the intensity of a control probe, gene 32. c. Scatchard analysis of 125I-glucagon binding data from studies of liver membranes isolated from db/db and db/+ mice. These measurements were done in duplicates using four animals in each group in two independent sets of experiments.

control for determining its steady state level rather than the rate of gene expression. In order to understand the precise mechanism(s) that regulate the glucagon receptor levels, specific antibodies are currently being prepared.

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