reaction (PCR). As a template, cDNA was generated from polyadenylated RNA (5 μg) of the breast cancer cell line SK-BR-3 with reverse transcriptase from avian myeloblastosis virus (Boehringer-Mannheim). The 220-bp PCR product was subcloned into the Bluescript vector with restriction enzyme (Eco RI and Bam HI) cleavage sequences attached to the polymerase chain reaction primers (Stratagene). One clone (P 158) of the 120 sequenced was identified to represent a different PTP and was therefore used as a probe to screen a SK-BR-3 cell, λZAP cDNA library. Sequencing of the longest clone revealed an open reading frame without an upstream termination signal. Full-length cloning was completed by rescreening of the library and by primer extension with an appropriate 5′ oligonucleotide.
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Expression Cloning and Signaling Properties of the Rat Glucagon Receptor

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Glucagon and the glucagon receptor are a primary source of control over blood glucose concentrations and are especially important to studies of diabetes in which the loss of control over blood glucose concentrations clinically defines the disease. A complementary DNA clone for the glucagon receptor was isolated by an expression cloning strategy, and the receptor protein was expressed in several kidney cell lines. The cloned receptor bound glucagon and caused an increase in the intracellular concentration of adenosine 3′, 5′-monophosphate (cAMP). The cloned glucagon receptor also transduced a signal that led to an increased concentration of intracellular calcium. The glucagon receptor is similar to the calcitonin and parathyroid hormone receptors. It can transduce signals leading to the accumulation of two different second messengers, cAMP and calcium.

Glucagon is a 29–amino acid pancreatic hormone that affects the production and distribution of glucose by its target organ, the liver. Glucagon functions to maintain basal concentrations of glucose and is a key hormone in the pathogenesis of diabetes. Glucagon binds to receptors in liver and activates two enzymatic pathways, glycogenolysis and gluconeogenesis, which result in the production of glucose (*1*). It is assumed that these actions of glucagon on the liver result when glucagon binds its hepatic receptor (or receptors) and activates adenylate cyclase, thereby increasing the intracellular concentration of cAMP. However, glucagon may also cause hydrolysis of inositol phospholipids (*2*, *3*) and increases in intracellular calcium concentrations (*4*). Two types of hepatic glucagon receptor have been proposed, and it has been suggested that they signal via two different intracellular messengers (*2*). Despite considerable effort, it has not been possible to isolate a cDNA for the glucagon receptor. We have used an expression cloning strategy to isolate a cDNA that encodes a functional hepatic receptor for glucagon. Like the parathyroid hormone (PTH) and calcitonin (CT) receptors (*5*, *6*), the glucagon receptor, when stimulated, gives rise to increased intracellular concentrations of cAMP and calcium.

We constructed a rat liver cDNA library in a mammalian cell expression vector and tested miniprep DNAs from pools containing 5000 clones by transfection into COS-7 cells (African Green monkey kidney cells) grown on microscope slides (*7*). The transfected cells were analyzed after 72 hours by binding with ^{125}I-labeled glucagon and by emulsion autoradiography. One pool out of the 100 screened reproducibly gave rise to a single cell that bound glucagon per slide. This pool was successively broken down into portions of one-tenth as many clones until a single clone, pLJ4, was isolated.

The pLJ4 plasmid contains a 1.9–kilobase pair insert that encodes a 485–amino acid protein with a predicted molecular size of 54,962 daltons (Fig. 1). The receptor encoded by pLJ4 is related to the CT (*8*), PTH (*5*), secretin (*9*), vasoactive intestinal peptide (VIP) (*10*), and glucagon-like peptide I (GLP I) (*11*) receptors (24, 31, 34, 35, and 42% amino acid identity, respectively). Analysis of hydropathy (*12*) revealed eight clusters of hydrophobic amino acids. The NH_2-terminal cluster does not contain a likely site for signal peptide cleavage (*13*). The remaining hydrophobic clusters correspond to seven transmembrane domains (TMDs), as seen with the other members of the secretin receptor family. Alignment of the rat secretin, rat PTH, porcine CT, and pLJ4 receptor sequences shows that amino acids within and adjacent to the TMDs are conserved (Fig. 1). Just before the sixth TMD the sequence RLAR appears in pLJ4, and a similar sequence motif, KLAK, was shown to be important for the interaction of the β2-adrenergic receptor with the adenylate cyclase–stimulating guanosine triphosphate–binding protein ($G_{s\alpha}$) (*14*). The COOH-terminal segment of pLJ4 shows less similarity to the CT, PTH, and secretin receptors, except for a segment of 16 amino acids adjacent to the seventh TMD. There are four potential N-linked glycosylation sites located in an extended hydrophilic stretch (amino acids 30 to 140) and six cysteine residues conserved among the secretin receptor family in this same segment. This segment may be an extracellular region of the receptor analogous to those predicted for the other receptors in this family.

We expressed pLJ4 transiently in COS-7 cells and established stable baby hamster kidney (BHK) cell lines that express pLJ4. Both types of cells bound ^{125}I-labeled glucagon with similar affinity to that of the receptors in rat liver membranes (*15*). Competition with unlabeled glucagon yielded nearly identical sigmoidal curves for glucagon binding by BHK cell or rat liver membrane preparations (Fig. 2). As indicated by Scatchard analysis, the apparent dissociation constants (K_d's) were 37 nM for the cloned receptor and 38 nM for rat liver membranes. These K_d's correspond closely to published

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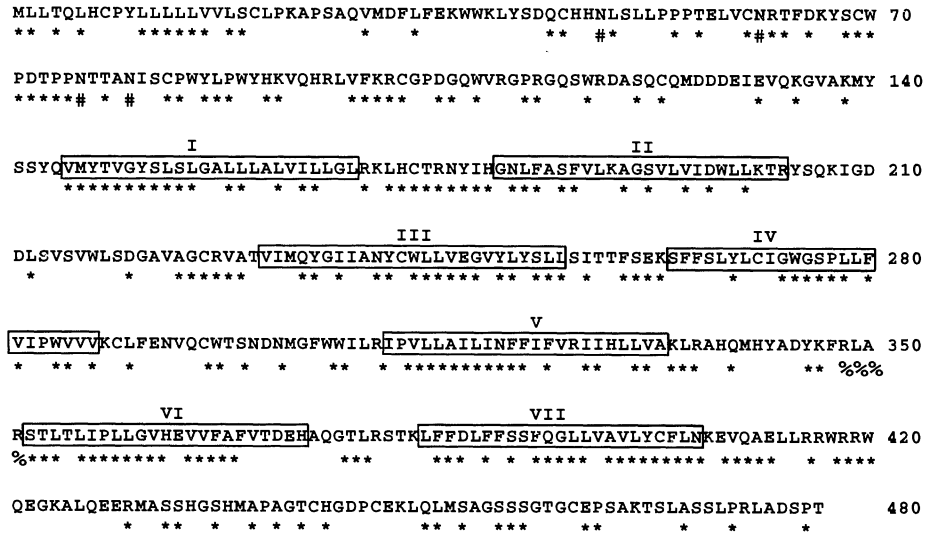

Fig. 1. Structure of the rat liver glucagon receptor. The predicted amino acid sequence of the rat liver glucagon receptor as determined by DNA sequencing of both strands of the 1892–base pair (bp) cDNA present in pLJ4. The GenBank accession number is M96674. The initiator methionine codon corresponds to the only extended open reading frame. Four potential N-linked glycosylation sites are marked (#) and asterisks mark positions where at least one of the three aligned receptors (CT, PTH, and secretin) is identical to the glucagon receptor. Seven putative TMDs (*12*) are boxed, and a sequence motif important for G protein coupling is marked by (%). Polyadenylated RNA was isolated from Sprague-Dawley rat livers and used as a template for cDNA synthesis (*26*). A library containing approximately 1 × 10^6 independent clones was constructed by directional cloning of cDNA larger than 800 bp in a mammalian cell expression plasmid, pZCEP (*27*). COS-7 cells were grown on microscope slides and transfected with miniprep DNA from pools of 5000 bacterial colonies with the use of a DEAE-dextran procedure (*7*). The transfected cells were incubated with 0.5 nM ^{125}I-labeled glucagon (2000 Ci/mmol; Amersham, Arlington Heights, Illinois) for 1 hour at 30°C. The slides were washed three times with phosphate-buffered saline and subjected to emulsion autoradiography (*7*). Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

values (*16*, *17*). The two membrane preparations had a similar capacity to bind glucagon. The maximal amount of glucagon bound (B_{max}) was 78 and 25 pmol per milligram of protein for the cloned receptor and rat liver membranes, respectively. A similar binding experiment with intact COS-7 cells transfected with pLJ4 yielded a K_d of 20 nM, and we estimated that there were 2 × 10^5 receptors present per cell (*18*). The same number of glucagon receptors was estimated for rat hepatocytes (*19*).

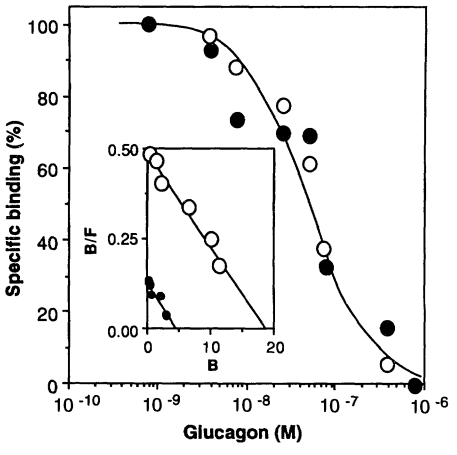

Fig. 2. Binding of ^{125}I-labeled glucagon to its receptor. Displacement of ^{125}I-labeled glucagon by unlabeled glucagon from rat liver membranes (open circles) and from membranes of BHK cells expressing pLJ4 (filled circles). The inset shows Scatchard plots of each curve, where B is bound glucagon (nanomolar) and F is free glucagon. Radioligand assays contained 10 μg of BHK cell membrane or 146 μg of rat liver cell membrane. Each point is the mean of triplicate determinations. The experiment was done three times with essentially the same results.

To test the specificity of the cloned receptor for ^{125}I-labeled glucagon, we attempted to compete for receptor binding with an excess of several related peptide hormones (*15*). Only native glucagon and *des*-His^1 [Glu^9] glucagon amide (an analog of glucagon with His^1 removed and position 9 substituted by Glu) blocked binding of ^{125}I-labeled glucagon. Secretin, which is very similar to glucagon (15 of 29 amino acids are identical), reduced ^{125}I-labeled glucagon binding by only 20% when present at 2000 times the concentration of glucagon. A similar excess of secretin did not significantly reduce the binding of ^{125}I-labeled glucagon to rat liver membranes (*20*). The more distantly related ligands, CT and PTH, reduced glucagon binding by 17 and 0%, respectively.

In COS-7 cells transfected with pLJ4 that were stimulated with glucagon, the amount of cAMP was approximately five times that in untreated cells or in cells transfected with a control plasmid (Fig. 3). The median effective concentration (EC_{50}) for stimulation of cAMP concentrations was 0.7 nM for the cloned receptor and corresponded closely to the EC_{50} of 1 nM observed for glucagon receptors in rat hepatocytes (*19*). The EC_{50} of 0.7 nM for the cAMP response was lower than the apparent K_d of 37 nM (Fig. 2). This difference may be explained if the EC_{50} reflects a small fraction of the expressed receptors that are coupled to guanosine triphosphate–binding proteins (G proteins) and that are sufficient to trigger a cAMP response. A similar discrepancy between the apparent K_d and the EC_{50} was observed for the cloned PTH receptor expressed in COS-7 cells (*5*). In pLJ4-transfected cells, VIP, secretin, PTH, GLP1, and CT all failed to increase the amount of cAMP when added at concentrations 10 to 100 times their published K_d's (*18*). These results indicate that pLJ4 encodes a receptor that is similar to the glucagon receptor in rat

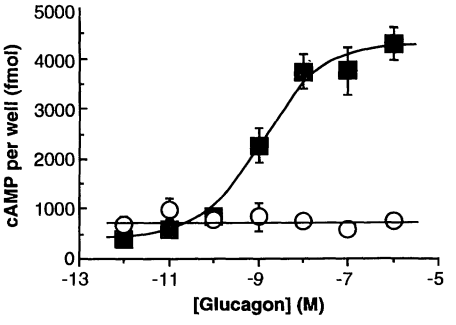

Fig. 3. Accumulation of cAMP in COS-7 cells transfected with pLJ4 and stimulated with glucagon. Increasing concentrations of glucagon were used to stimulate COS-7 cells expressing pLJ4 (squares) or control COS-7 cells expressing the human β-2 adrenergic receptor (circles). Each point was done in triplicate; error bars indicate the range of values around the mean. Similar results were obtained in BHK cells transfected with pLJ4 (*18*). COS-7 cells were plated at 5 × 10^4 cells per well in a 24-well plate and transfected with 1 μg of plasmid DNA per well (*7*). After growth for 72 hours the cells were washed, and binding media (*15*) containing 10 μM 3-isobutyl-1-methyl-xanthine (IBMX) and glucagon (or related peptides) was added. Cells were incubated for 10 min at 37°C; the cultures were then stopped by the addition of 800 μl of boiled water, and the supernatant was saved for assay. The supernatants were assayed in duplicate with a cAMP scintillation proximity assay system (Amersham) (acetylation protocol supplied).

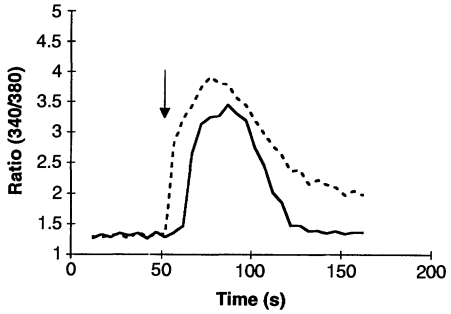

Fig. 4. Effect of glucagon on $[Ca^{2+}]_i$ in BHK cells expressing pLJ4. The ratio of emissions from excitation at two wavelengths (340 and 380 nm) is plotted versus time. The arrow indicates the time of addition of glucagon (100 nM final concentration). Representative traces for two different cells are shown. This experiment was repeated three times; at each time 6 to 40% of the cells in a field responded similarly. Similar responses were obtained with COS-7 cells expressing pLJ4 (*23*).

liver membrane preparations (*16*, *19*).

Glucagon causes an increase in the concentration of intracellular calcium ($[Ca^{2+}]_i$) (*4*, *21*) and small increases in the amount of inositol phosphates in hepatocytes (*2*, *3*). We monitored $[Ca^{2+}]_i$ in BHK cells transfected with pLJ4 with the fluorescent probe fura-2 (*22*). The addition of glucagon (100 nM) to BHK cells expressing the glucagon receptor caused a rapid increase in $[Ca^{2+}]_i$ (Fig. 4). Control cells expressing the β-adrenergic receptor showed no change in $[Ca^{2+}]_i$. Glucagon concentrations as low as 2 nM stimulated a similar increase in $[Ca^{2+}]_i$ (*23*). Glucagon (200 nM) also caused a rapid increase in $[Ca^{2+}]_i$ in COS-7 cells transfected with pLJ4, whereas COS-7 cells transfected with our vector, pZCEP, did not respond (*23*). These results indicate that activation of the cloned glucagon receptor leads to a rapid increase in $[Ca^{2+}]_i$, which is characteristic of receptors signaling via inositol phospholipid metabolites (*24*). Concentrations of intracellular Ca^{2+} and cAMP are both increased by signals from the related PTH (*5*) and CT receptors (*6*). It is possible that the alternate signaling pathways used by the glucagon receptor derive from its interaction with different G proteins, as previously suggested (*3*).

We have not detected a second glucagon receptor by expression cloning after screening 5×10^5 clones from our rat liver library, nor have we detected a different glucagon receptor by hybridization with pLJ4 as a probe. Thus, it is likely that only one receptor is responsible for both types of signaling observed in hepatocytes. We have also identified a human homolog (83% amino acid identity) of the rat hepatic receptor from human pancreatic islet cell cDNA (*25*). This suggests a role for the liver-derived receptor or a very similar receptor in the pancreatic islets as well.

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28. We thank T. W. Whitmore (ZymoGenetics) for advice and assistance.

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Ultrastructural Evidence for Hair Cell Regeneration in the Mammalian Inner Ear

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It has long been thought that hair cell loss from the inner ears of mammals is irreversible. This report presents scanning electron micrographs and thin sections of the utricles from the inner ears of guinea pigs that show that, after hair cell loss caused by treatment with the aminoglycoside gentamicin, hair cells reappeared. Four weeks after the end of treatment, a large number of cells with immature hair bundles in multiple stages of development could be identified in the utricle. Thin sections showed that lost type 1 hair cells were replaced by cells with a morphology similar to that of type 2 hair cells. These results indicate an unexpected capacity for hair cell regeneration in vivo in the mature mammalian inner ear.

The inner ears of vertebrates contain the organs for hearing and balance. Their mechanoreceptive auditory and vestibular epithelia are formed of mosaics of sensory "hair" and supporting cells. Hair cells can be damaged and lost after exposure to noxious agents including noise and ototoxic drugs such as aminoglycoside antibiotics. In cold-blooded animals, hair cells are continuously produced throughout life and damaged cells can be replaced (*1*). In birds,

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