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Review

Glucagon receptors

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Abstract. Glucagon is a pancreatic peptide hormone that, as a counterregulatory hormone for insulin, stimulates glucose release by the liver and maintains glucose homeostasis. First decribed as a glucagon binding entity functionally linked to adenylyl cyclase, the glucagon receptor is a member of the family B receptors within the G protein coupled superfamily of seven transmembrane-spanning receptors. During the past decade, considerable progress has been made in the identification of the molecular determinants of the glucagon receptor that are important for ligand binding and signal transduction, in the development of glucagon analogs and of nonpeptide small molecules acting as receptor antagonists, and in the characterization of the mechanisms involved in the regulation of expression of the glucagon receptor gene. In the present review, the current knowledge of glucagon receptor structure, function and expression is described, with emphasis on the metabolic fate of glucagon and on the endocytosis and cell itinerary of both ligand and receptor.

Keywords. Glucagon, glucagon receptor, endocytosis, signal transduction, proteolysis, gene structure, protein expression, ligand binding.

Introduction

Glucagon is a 29-amino acid peptide hormone that is secreted by the islet A cells of the endocrine pancreas. It is synthesized as a 180-amino acid precursor, proglucagon, which also contains two additional glucagon-like sequences at its C terminus, glucagon-like peptide-1 (GLP-1) and GPL-2. Glucagon is the major product of proglucagon processing in pancreatic α cells, while GLP-1 and GPL-2 are produced in intestinal L-cells and in central nervous system. Glucagon secretion is determined by the level of blood glucose, with hypoglycemia and hyperglycemia leading to stimulation and inhibition, respectively. The major action of glucagon is to increase, by stimulating glycogenolysis and gluconeogenesis, glucose production by the liver. This effect, which counterbalances that of insulin, is exerted at multiple levels including gene transcription and modulation of enzyme activity. Glucagon also elicits various effects in extrahepatic tissues, including adipose tissue, kidney, heart, pancreatic β cells, gastrointestinal tract, thyroid and central nervous system.

The glucagon receptor (GR) was first described by Rodbell and his coworkers in 1971 [1] as a glucagon binding entity of liver plasma membranes functionally linked to adenylyl cyclase. These investigators also

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showed that guanine nucleotides affected glucagon binding to membranes [2] and played an obligatory role in the activation of adenylyl cyclase by glucagon [3]. In retrospect, this discovery has been an essential step in the identification of G proteins as membrane components mediating signal transduction by a class of membrane receptors designated as G protein-coupled receptors (GPCRs). Subsequent studies focused on the structural features of glucagon required for receptor binding and action, on the regulation of the GR in animal models and in isolated cells, and on the structural characterization of the receptor protein [4]. Cloning of the GR cDNA in 1993 formally identified this receptor as a seven transmembrane-spanning protein of the GPCR class B and allowed studies on the expression of the GR gene, on the molecular determinants of GR function and on the phenotype of GR knockout mice [4–10].

The aim of this review is to summarize the current knowledge of GR structure, function and cellular expression. Emphasis will be given on the implication of the GR in the organ uptake and endocytosis of glucagon, on the subcellular sites at which glucagon is degraded and on the responsible proteases, and on the ligand-induced endocytosis of the GR. Due to space limitation, studies on glucagon structure-function relationships and GR antagonists will not be covered here, and the reader interested by these topics should consult previous reviews [5, 8, 10].

Identification of the GR

GRs have been identified in liver and other glucagon target tissues as membrane components that bind [^{125}I]iodoglucagon in a time- and temperature-dependent, specific, saturable and reversible manner [4]. Early studies on rat liver plasma membranes showed that glucagon binding displays the same dependence on ligand concentration as glucagon-stimulated adenylyl cyclase [1] and, remarkably, that GTP decreases binding affinity by stimulating the dissociation of bound ligand [2]. In later studies, the interaction of glucagon with its receptor in liver subcellular fractions [11–13] and intact hepatocytes [11, 14, 15] was characterized by analysis of binding data at equilibrium and of association and dissociation kinetics. Using [^{125}I-Tyr^{10}]monoiodoglucagon as ligand, Rojas et al. [12] showed that plasma membranes contain an homogeneous population of receptors with a K_d of 0.8 nM in the absence of GTP and 2.5 nM in the presence of 0.1 mM GTP. Horwitz et al. [15] also described an homogeneous population of receptors in hepatocytes, and presented evidence for a time-, temperature- and GTP-dependent interconversion of two states of the receptor, of low and high affinity, respectively. In contrast, Sonne et al. [14] proposed the existence, in rat hepatocytes, of two classes of non-cooperative glucagon binding sites: high-affinity sites (20 000 sites/cell) with a K_d of 0.7 nM and low-affinity sites (200 000 sites/cell) with a K_d of 13 nM. Canine hepatocytes were also suggested to contain two classes of binding sites, with high-affinity sites accounting for 1 % of total sites [11]. Since a single molecular form of the GR has been identified (see below), receptor heterogeneity may be only apparent and linked to events associated with the glucagon/GR interaction, such as ligand and/or receptor internalization, and/or to experimental conditions, such as radioligand heterogeneity. More recent studies on the binding activity of the recombinant GR have not addressed this issue.

Solubilization and physical characterization of the GR protein

Like most GPCRs of family B, the GR has been difficult to solubilize with retention of ligand binding activity using nondenaturing detergents. However, this has been achieved using Lubrol-PX [16–18], provided that glucagon was allowed to bind to the receptor prior to solubilisation, and CHAPS [18]. Using wheat germ agglutinin-Sepharose as a solid matrix to adsorb receptor-bound ligand, Iyengar and Herberg [19] have shown that the CHAPS-solubilized receptor binds glucagon with a lower apparent affinity (33–70 nM) than the membrane-associated receptor, and unlike the Lubrol-PX-solubilized receptor [17], does not retain GTP sensitivity. Presumably due to instability, it has not been possible to purify the solubilized GR to homogeneity.

The GR has been structurally characterized by reducing SDS-PAGE after affinity labeling by photoactivable glucagon derivatives such as 2-[(2-nitrophenyl)sulfenyl]-Trp^{25}-glucagon [20] and N^{e}-4-azidophenyl-amidino-glucagon [21]. Alternatively, [^{125}I]iodoglucagon has been crosslinked to the receptor using agents such as 1,4-difluoro-2,5-dinitrobenzene and bis(sulfosuccinimidyl)suberate [22, 23], hydroxysucciniminidyl–*p*-azidobenzoate, a photoaffinity crosslinker [18, 19, 24] or UV irradiation [25]. In most of these studies, the covalently labeled receptor was detectable as a single or major protein in the 55–65 kDa range, the abundance of which was decreased by excess unlabeled glucagon and GTP. Treatment by endo-β-*N*-acetylglucosaminidase converted this protein into four components of lower molecular mass, suggesting the presence of four *N*-linked glycans [18]. A 60-kDa protein sensitive to *N*-glycanase and *N*-glycosidase F digestion was also

detectable by Western blotting in liver, kidney and adipose tissue [26], and in transfected cells expressing the recombinant GR [27].

Hydrodynamic studies combining gel filtration and sucrose density gradient centrifugation have allowed determination of the molecular parameters of the covalently labeled and Lubrol-PX solubilized receptor [18]. Based on a calculated molecular weight of 119 000 comparable to that obtained for the functional receptor by target size analysis [28], the GR receptor protein was suggested to be a dimer of the 63-kDa hormone binding subunit. Additional studies based on co-immunoprecipitation of different tagged versions of the receptor or bioluminescence resonance energy transfer between receptors bearing different fluorophores are required to determine whether, as many GPCRs [29], GR undergoes homo- and/or heterodimerization in intact cells, and whether this process is involved in receptor export or signaling.

Cloning of the GR cDNA, deduced structure of the GR protein and organization of the GR gene

The GR cDNA was cloned first in the rat in 1993 independently by Jelinek et al. [30] and Svoboda et al. [31, 32] by expression cloning and PCR-based cloning, respectively. GR cDNA cloning was achieved shortly thereafter in man [33] and mouse [34], and later in monkey [35], dog [36], amphibians [37, 38] and goldfish [39]. The sequence of rat GR cDNA predics a 485-amino acid protein with a central core consisting of seven membrane-spanning helices connected by alternating extracellular and intracellular loops, an extracellular N-terminal extension and a cytoplasmic C-terminal extension (Fig. 1). The N-terminal extension (amino acids 1–143) contains a putative signal sequence, four potential Asn-linked glycosylation sites, and six Cys that are conserved in family B of GPCRs and, based on studies on other GPCRs of class BB, form three disulfide bonds. An additional conserved disulfide bridge links Cys^{225} and Cys^{295} in extracellular loops e1 and e2, respectively. The C-terminal tail (amino acids 406–485) contains 11 Ser residues that are potential sites of phosphorylation by serine/threonine kinases. The rat GR protein shows some amino acid identity with other receptors of the B family of GPCRs (GLP1, 42%; VIP, 35%; secretin, 34%; PTH, 31%; calcitonin, 24%), with highest identity within and adjacent to the transmembrane domains. A region (FQG-hydr-hydr-VAx-hydr-YCF-xEVQ ; hydr being a hydrophobic and x any amino acid) that is highly conserved in the glucagon/secretin receptor subfamily is present in the seventh transmembrane domain at position 392–409. The mouse, human, monkey and dog GR cDNAs encode proteins of 485, 477, 491 and 495 amino acids, respectively, with a sequence identity with the rat protein of 93%, 82%, 78% and 76%. The amphibian GR cDNAs encode proteins of 492 (*Rana tigrina* [37]), 490 (*Xenopus laevis* [38]) and 489 (*Rana pipiens* [38]) amino acids, which show a 60% identity with the mammalian receptor. Upon transient expression in transfected cells, most mammalian and amphibian recombinant GRs bind glucagon with an apparent K_d of 1–10 nM and display a GTP sensitivity comparable to that of the endogeneous receptor.

The protein coding region of the human [40], mouse [34] and rat [31] GR genes spans at least 4 kb of DNA and contains 13 exons separated by 12 introns (Fig. 2). In the rat gene, the 5'-untranslated domain contains two exons of 133 and 166 nucleotides, separated by an intron of 0.6 kb [41]. The most proximal exon, separated from the first coding exon by an intron of 3.2 kb, is spliced out in liver and less frequently in heart [41]. However, in the mouse [42] and human [43, 44] genes, only a single untranslated exon located 4 kb upstream from the first coding exon has been identified. Furthermore, a single promoter region, located upstream from the most distal untranslated exon, has been defined in the rat GR gene [41], whereas both a proximal and a distal promoter, located upstream from the first coding exon and from the single untranslated exon, respectively, have been described in the mouse [45] and human [44] genes. Structurally, these promoters lack consensus TATA and CAAT boxes, are GC rich and contain several putative Sp1 binding sites, as many promoters of housekeeping genes [46]. Other interesting features include, in the rat promoter (between –545 and –527), a glucose response element consisting of a highly palindromic sequence of 19 nucleotides, centered on two E boxes (CACGTG and CAGCTG) separated by three nucleotides [41]; and in the proximal human promoter (between –1062 and –354), several CRE, C/EBPα and AP-1 elements, known to be involved in cAMP regulation of gene expression [44]. *In situ* hybridization to metaphase chromosome preparations have mapped the human GR gene to chromosome 17q25 [40], but the chromosomal localization of the gene in other species has not been determined.

Tissue distribution of the GR protein and mRNA

The rat GR protein, as identified by *in vitro* (reviewed in [4]) and *in vivo* (Fig. 3) ligand binding, is expressed mainly in liver and kidney and, to a lesser extent, in heart, adipose tissue, spleen, adrenal glands, endocrine pancreas and cerebral cortex. In liver, GRs are

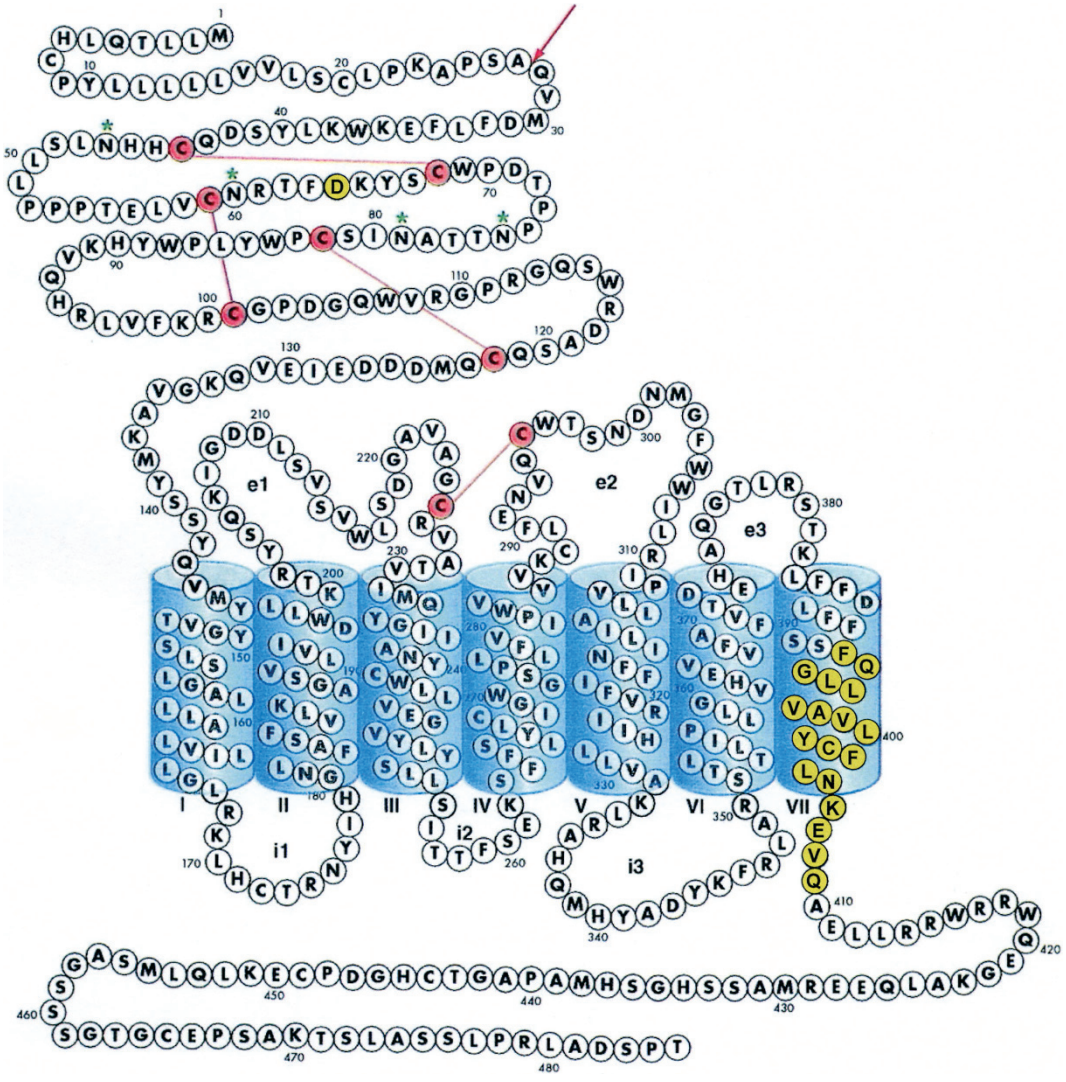

Figure 1. Schematic representation of the primary and secondary structure of rat glucagon receptor (GR). Amino acid residues are depicted in a single-letter code. The extracellular domain (N-terminal tail and extracellular loops e1, e2 and e3) is at the top, and the cytoplasmic domain (C-terminal tail and intracellular loops i1, i2 and i3) at the bottom. The red arrow indicates a putative signal sequence proteolytic site. The four sites of potential *N*-linked glycosylation on the N-terminal tail are labeled with green asterisks. Six Cys in the N terminus, involved in a pattern of three disulfide bonds linking Cys^{44} and Cys^{68}, Cys^{59} and Cys^{101}, and Cys^{82} and Cys^{122}, are shown in red. A disulfide bridge linking extracellular loops e1 and e2 is a conserved feature in all G protein-coupled receptors (GPCRs). The Asp at position 64 and the 392–409 sequence in the seventh transmembrane domain, which are conserved in the members of the glucagon/secretin receptor subfamily, are shown in yellow. Adapted from [8].

predominantly localized in hepatocytes but have also been identified in endothelial and Kupffer cells by autoradiography and biochemical studies [47]. In kidney, GRs have been localized in the distal nephron including the ascending limb of Henle's loop, the distal convoluted tubule and the collecting tubule [48]. In pancreatic rat islets, GRs have been localized by quantitative electron microscopic autoradiography [49, 50], and immunocytochemistry [51] to B cells and a subpopulation of A and D cells, and by ligand binding studies in purified B cells [52]. Although B cells also express GLP-1 receptors, dose-response effects and studies using glucagon and GLP-1 antagonists suggest that, at physiological concentrations, glucagon stimulates insulin release mainly *via* its own receptor [53, 54]. In addition, ablation of the GR gene in mice induces marked alterations in the development and maturation of the three types of islet cells, indicating that the GR associated with these cells is physiologically relevant [55]. In brain, glucagon binding sites have been detected in the olfactory tubercle, hippocampus, anterior pituitary, amygdala, septum, medulla, thalamus, olfactory bulb and hypothalamus [56].

A single GR mRNA transcript of 2.3–2.5 kb is detectable by Northern blotting in poly(A)$^+$-enriched RNA from rat [57] and mouse liver and kidney [58].

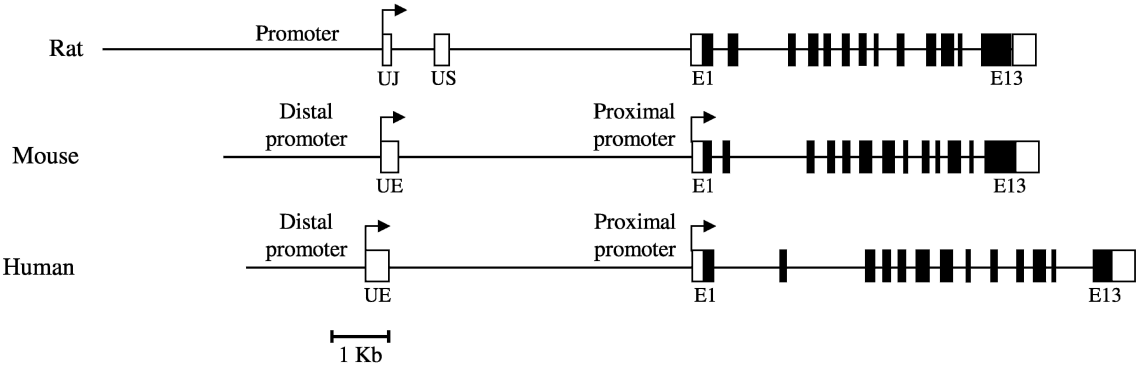

Figure 2. Schematic representation of the organization of the rat, mouse and human GR genes. Open boxes represent untranslated exons and closed boxes coding regions. Arrows indicate transcription start sites. The continuous line represents introns. Adapted from [34, 41, 42, 44].

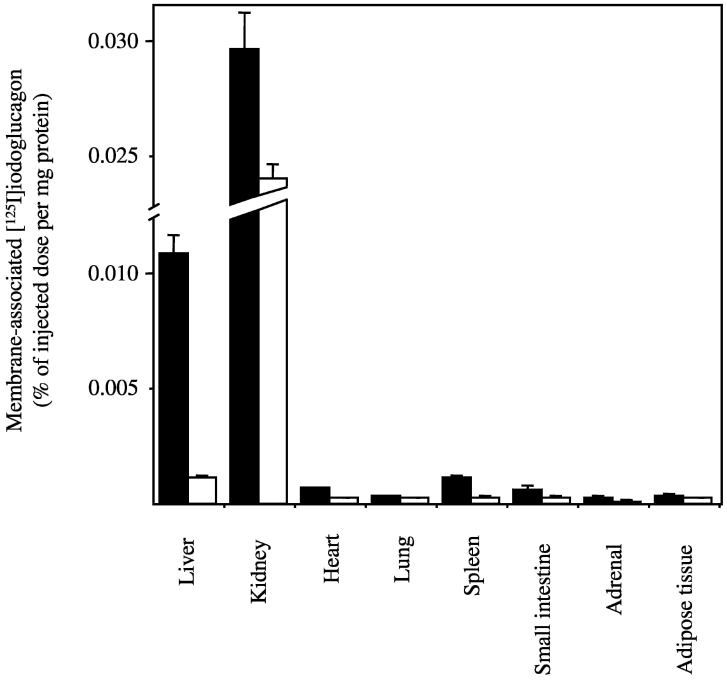

Figure 3. *In vivo* association of injected [^{125}I]iodoglucagon with the membrane fraction of various rat tissues. Male Sprague-Dawley rats (200–240 g body weight) received an i.v. injection (penis vein) of 10–20 pmol [^{125}I]iodoglucagon (about 500 000 cpm/pmol) alone (black bars) or in association with 100 nmol native glucagon (white bars) under ether anesthesia. At 90 s after injection, the indicated tissues were removed and immediately homogenized. A crude membrane fraction was prepared and analyzed for associated radioactivity, with results expressed as % of injected dose per mg protein (mean ± SEM of four to seven determinations on separate rats) (unpublished data). The association of [^{125}I]iodoglucagon with liver membrane as a function of the dose of native glucagon co-injected is shown on Fig. 4.

The relative abundance of GR mRNA in rat [59–61] and mouse [34, 58] tissues has been quantitated by Southern blot analysis of RT-PCR products and nuclease or RNase protection assays. In the rat, GR mRNA distribution somewhat matches GR protein distribution, abundance being highest in liver, intermediate in kidney, heart, spleen, ovary, pancreatic islets and thymus, and low in adrenals, stomach, small intestine, lung and brain. In the mouse, GR mRNA abundance is also highest in liver, followed by kidney, lung and pancreas, and then by adipose tissue and small intestine. *In situ* hybridization studies have shown that hepatic GR mRNA is predominantly expressed in the periportal area, in which the metabolic effects of glucagon also predominantly occur [62].

Signal transduction pathways coupled to the GR

The major consequence of GR activation is the stimulation, *via* the heterotrimeric protein Gs, of adenylyl cyclase activity and resulting elevation of intracellular cAMP concentration. In liver and other tissues that express the GR, the concentrations of glucagon required for half-maximal and maximal effects are in the range of 2–4 nM and 30–100 nM, respectively [4, 63–67]. However, in both liver membranes [68] and hepatocytes [14], as well as in intact liver following glucagon injection (Fig. 4), adenylyl cyclase activity and cAMP production are nonlinearly related to GR occupancy, and half-maximal activation occurs with only about 10 % of the receptors occupied. In addition, in several studies supramaximal concen-

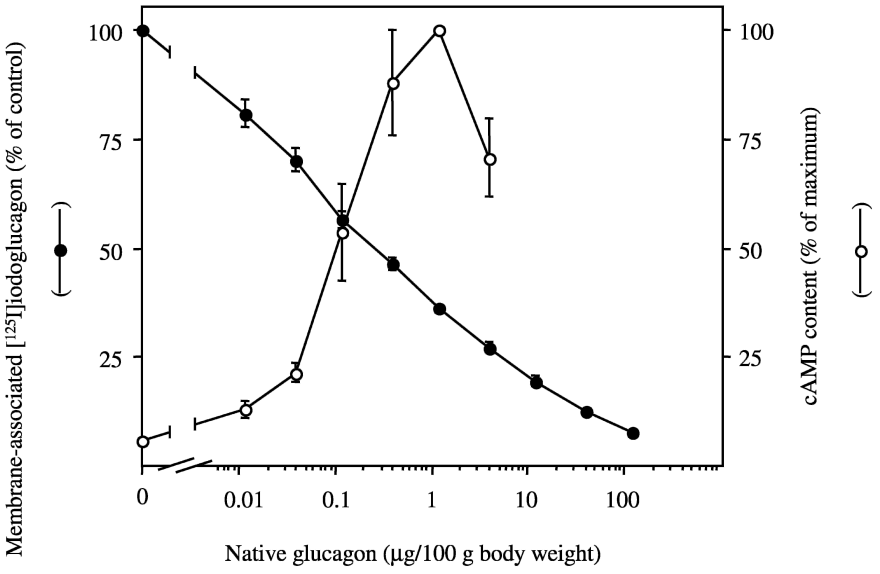

Figure 4. Dose-dependence of the *in vivo* effect of native glucagon on the membrane association of injected [^{125}I]iodoglucagon and cAMP production in rat liver. Two groups of male Sprague-Dawley rats (200–240 g body weight) received an i. v. injection (penis vein) of native glucagon at the indicated dose. In one group, 10–20 pmol [^{125}I]iodoglucagon (about 500 000 cpm/pmol) was co-injected with native glucagon and 3 min after injection, the association of radioactivity with a crude liver membrane fraction was measured (closed symbols). In a second group, the liver content of cAMP was determined at the same time using a specific radioimmunoassay (open symbols). Results (mean ± SEM, five to seven determinations for membrane association of [^{125}I]iodoglucagon and three determinations for cAMP content) are expressed as the percentage of control values (no native glucagon co-injected). In control rats, the association of [^{125}I]iodoglucagon with liver membranes was 0.0108±0.0009 % of total injected counts and liver cAMP content was 0.49±0.03 nmol/g tissue (mean ± SEM of six determinations) (unpublished data).

trations of glucagon have been shown to induce an inhibition of adenylyl cyclase activity and cAMP production [69], an effect also observed in intact rat liver *in vivo* (Fig. 4). In transfected cells expressing the recombinant GR [27, 30, 31, 33, 35, 37, 57, 70–73], stimulation of adenylyl cyclase activity by glucagon is also concentration dependent, but concentrations of glucagon required for half-maximal effect (0.1–3 nM) are somewhat lower than with the endogeneous receptor. Studies by Rodbell and his coworkers [4] on the adenylyl cyclase system in liver plasma membranes have shown that GTP and Mg^{2+} are essential requirements for activation by glucagon.

A series of observations (reviewed in [74]) have established that the activation of adenylyl cyclase by glucagon is mediated by the heterotrimeric protein Gs. As with other adenylyl cyclase systems coupled to Gs (reviewed in [75]), this process involves the following sequence of events: (1) an exchange of GDP for GTP on the α subunit of the Gs protein; (2) a conformational change in GTP-bound Gsα, which induces a dissociation of Gsα from the βγ complex and allows the α subunit to interact with adenylyl cyclase; (3) the hydrolysis of Gsα-bound GTP to GDP by the intrinsic GTPase associated with Gsα, which terminates activation; and (4) the reassociation of Gsα-GDP to the βγ complex, which allows a new cycle of activation. Although both the long (Gsα-L) and short (Gsα-S) splice variants of Gsα mediate adenylyl cyclase activation by the GR, studies using GR-Gsα-S and GR-Gsα-L fusion proteins have shown that the former binds glucagon with the same affinity as wild-type GR, whereas the latter shows a tenfold higher affinity [76]. In the presence of GTPγS, GR-Gsα-L reverts to a low-affinity conformation, suggesting that the coupling of GR to Gsα-L may account for GTP-sensitive high-affinity binding.

Based on target size analysis of the liver adenylyl cyclase system [28], Rodbell [77] has proposed that, in the basal state, the GR and Gs protein are linked together as multimeric or oligomeric structures, and that a concerted activation of the receptor by ligand and GTP causes disaggregation of these structures. Consistent with this model, glucagon treatment of liver membranes was shown, in hydrodynamic studies, to decrease the size of the Gs protein in subsequently prepared detergent extracts [78]. However, this line of research has received little further attention over the last decade, and current views on the physical organization of GPCRs and associated G proteins favor a model in which a GPCR dimer binds a single α/β/γ heterotrimer [79].

As with other GPCRs of class B, GR activation may also lead to activation of the phospholipase C inositol phosphate pathway and mobilization of intracellular Ca^{2+}. Thus, in perfused liver and hepatocytes, gluca-

gon increases both Ca^{2+} influx and Ca^{2+} release from intracellular stores, with a half-maximal effect at 1 nM [80–83]. However, it is still unclear whether this response is a cAMP-mediated effect or the result of a separate signaling pathway. In some studies, the glucagon-induced Ca^{2+} mobilization was mimicked by cAMP analogs, suggesting that it may be mediated by cAMP [84, 85]. However, in other studies, effects of glucagon on Ca^{2+} mobilization were shown to be cAMP independent [86, 87], and in two instances glucagon induced an increase in the concentration of inositol trisphosphate in rat hepatocytes [83, 87]. In more recent studies, glucagon has been found to stimulate Ca^{2+} mobilization in transfected cell lines expressing the human [72, 88] and rat [30, 71] recombinant GRs. In one of these studies, glucagon-induced Ca^{2+} mobilization was not mimicked but potentiated by cAMP, was sensitive to pertussis toxin and was accompanied by a modest increase in inositol trisphosphate [88]. Based on these results, glucagon was suggested to activate a G protein-coupled pathway leading to stimulation of phospholipase C and release of Ca^{2+} from intracellular stores, but the G protein involved was not identified.

Structural determinants of the GR involved in cell surface expression, ligand binding and signal transduction

The minimal structural determinants of the GR involved in expression and function have been characterized with the use of various mutants expressed in CHO, COS-7 or HEK293 cells. These have included single point mutants [27, 73, 89], deletion and truncation mutants [70, 72, 90] and hybrid glucagon/dopamine [71], glucagon/GLP-1 [91–94] and glucagon/secretin [92] receptors. In addition, the ligand-binding domain of the endogenous GR has been probed using antibodies against synthetic peptides corresponding to different extramembrane segments of the receptor [95].

Proper maturation and cell surface insertion of the rat GR as judged by immunofluorescence staining and endo-H sensitivity of the receptor protein requires all seven transmembrane segments, the distal region of the first intracellular loop (tripeptide Asn^{175}-Tyr^{176}-Ile^{177}) and the proximal region of the C-terminal tail, but does not require an intact N-terminal tail [70–72, 92]. In addition, unlike for other membrane glycoproteins, N-linked glycosylation is not required for the receptor to reach the cell surface [70].

High-affinity glucagon binding to GRs expressed at the cell surface requires specific segments of the extracellular N-terminal tail (Asp^{64}, sequences 103–117 and 126–137), of the first extracellular loop (Arg^{202}, sequence 206–219) and of the third, fourth and sixth transmembrane domains [27, 91, 92]. Consistent with mutagenesis studies, antibodies directed against the 126–137 and 206–219 extracellular epitopes inhibit glucagon binding to the liver endogeneous receptor, whereas an antibody against the intracellular C-terminal sequence 468–485 does not affect binding [95]. Based on these observations, Unson [8] has proposed a model whereby glucagon interacts first *via* its hydrophobic C-terminal region with the N-terminal tail of the GR and subsequently *via* its N-terminal half with a predominantly ionic pocket of the first extracellular loop. Studies using hybrid glucagon/GLP-1 receptors have identified three distinct epitopes in the GR core domain that determine specificity of the GR for the glucagon N terminus [94].

The ability of the ligand-occupied GR to stimulate adenylyl cyclase activity and elevate intracellular Ca^{2+} concentration requires intracellular loops i2 and i3, consistent with recent models for the mechanism of activation of G proteins by class I GPCRs [71]. Presumably, the conformational changes in the receptor protein induced by ligand binding enable specific regions of loops i2 and i3 to interact with G proteins. However, neither the N-terminal tail nor the majority of the C-terminal tail are required for adenylyl cyclase activation [92]. Deletion of the sequence 252–259 within intracellular loop i2 renders the ligand binding activity of the GR insensitive to Gpp(NH)p and divalent cations, suggesting uncoupling of this protein from Gs [90]. Replacements of His^{178} by Arg and Thr^{352} by Ala in the rat GR result in constitutively active receptor mutants [73].

Regulation of expression of the GR in target cells

In rodents, conditions leading to a sustained elevation of plasma glucagon concentration such as glucagon treatment, fasting, liver regeneration induced by partial hepatectomy and, at least in some studies [96, 97], streptozotocin-induced diabetes, are generally associated with a decrease in GR number in hepatocytes and liver membranes, suggesting that glucagon is negative regulator of the expression of the GR protein in liver (reviewed in [4]). Conversely, liver GR number has been shown to progressively increase in rats during the first month of life in the face of decreasing plasma glucagon [98]. Importantly, *in vitro* addition of glucagon to freshly prepared rat hepatocytes [99] and primary cultures of rat [100] and chicken [101] hepatocytes causes a decrease in GR number, indicating a direct effect of the hormone on the expression of the receptor protein. In chicken hep-

atocytes, an early and transitory increase in low-affinity GRs uncoupled from Gs (insensitive to guanine nucleotides) accompanies the decrease in high-affinity GRs [101].

Subcellular fractionation studies have shown that, whereas an acute and transitory hyperglucagonemia does not affect total cellular GR number in liver [23], long-lasting hyperglucagonemias often do so, suggesting a reduced synthesis and/or an increased degradation of the receptor protein. In chicken hepatocytes, the glucagon-induced decrease in GR number is partially reversible in the absence of new protein synthesis, suggesting that receptors lost from the cell surface are largely stored within the cell rather than degraded [101].

In addition to inducing a decrease in GR number, exposure of rat [100] and chicken [101] hepatocytes to glucagon results in a time-dependent decrease in the responsiveness of adenylyl cyclase to glucagon and to other activators, which has been referred to as homologous and heterologous desensitization, respectively. Comparable studies on freshly isolated rat hepatocytes have shown that the desensitization of glucagon-stimulated adenylyl cyclase precedes GR down-regulation, is cAMP and Ca^{2+} independent, is mimicked by hormones that stimulate inositol phospholipid metabolism, and is blocked by pertussis toxin by a process that does not involve the Gi protein [102, 103]. Based on effects of protein kinase C (PKC) inhibitors and alkaline phosphatase treatment, a PKC-induced phosphorylation reaction has been implicated [103]. Studies on primary cultures of chicken hepatocytes have attributed the heterologous desensitization to both a decrease in Gs activity [104] and a protein kinase A-mediated phosphorylation of the adenylyl cyclase catalytic component [105]. Another important mechanism which temporally limits the increase in cAMP concentration induced by glucagon is the ability of this hormone to activate, *via* cAMP and PKA, cAMP phosphodiesterase 3B [106].

Within the past decade, the cloning of GR gene has made possible studies on the developmental, metabolic and hormonal regulation of expression of the GR gene *in vivo* [57, 58, 107] and in cultured cells [107–109]. In mice, liver GR mRNA level was shown to progressively increase from the first day of postnatal life to adulthood [107]. In rats rendered hyperglycemic by a glucose infusion or streptozotocin treatment, liver GR mRNA level was increased [107], suggesting that circulating blood glucose, or rather than glucose itself, the glucose flux in liver, is a positive regulator of expression of the GR gene [107]. In rats bearing glucagon-secreting tumors, liver GR mRNA level is decreased, suggesting a negative, pretranslational effect of circulating glucagon on the expression of the GR gene [110]. Other *in vivo* regulators of the expression of the GR gene include thyroid status, which modulates GR mRNA level differentially in liver, brown adipose tissue and white adipose tissue [111], and cold exposure, which down-regulates GR mRNA level in brown adipose tissue in a sympathetic nervous system-dependent manner [112].

In primary cultured rat hepatocytes, glucose, cAMP and glucagon (*via* cAMP) have been identified as major regulators of GR mRNA level [107, 108]. A 24-h exposure of hepatocytes to glucose induces a concentration-dependent increase in GR mRNA, as do fructose, mannose and the gluconeogenic substrates glycerol and dihydroxyacetone. In contrast, neither L-glucose, which is not metabolized, nor glucosamine affect GR mRNA. A 24-h exposure of hepatocytes to drugs which elevate cellular cAMP level, such as IBMX and forskolin, cause a two- to four-fold decrease in GR mRNA level, which is acompanied by a marked reduction in the number of glucagon binding sites [108]. Furthermore, a 24-h exposure to glucagon dose-dependently decreases hepatocyte GR mRNA level with about 30% decrease at 0.1 nM and 70% decrease at 100 nM [108]. In parallel studies on cultured pancreatic islet cells, glucose was shown to up-regulate, and cAMP to down-regulate, GR mRNA expression, and glucocorticoids were identified as negative regulators of expression [109].

Studies in which partial sequences of the rat GR gene promoter have been fused to a reporter gene and expressed in insulin-producing cells have mapped glucose regulation to the palindromic glucose response element identified at position –545 to –527 [41]. Indeed, deletion or mutation of this sequence abolishes glucose regulation. In subsequent studies, only the most 5'E box of the glucose response element was found to be crucial for the glucose transcriptional effect, and USF1/USF2 transcription factors were shown to be part of the DNA binding proteins involved [113]. In addition, an accessory factor binding site was identified in the DNA region just upstream from the glucose response element [113]. Comparable studies, in which partial sequences of the human GR gene promoter have been fused to luciferase and expressed in HepG2 hepatoma cells, have shown that the activity of the proximal promoter, but not that of the distal promoter, is inhibited by cAMP [44]. In these studies, peroxisome proliferator-activated receptor γ coactivator 1α was also found to abolish cAMP-induced down-regulation of GR mRNA.

Table 1. Glucagon-degrading activities associated with plasma serum and glucagon-target cells.

	In vitro studies	*In vivo* studies	Glucagon affinity (K_m; μM)	Inhibitors	Class of protease	Optimal pH	Main subcellular localization	Main site of activity	Ref.
Dipeptidyl peptidase IV	Yes	Yes	3	Ile-Thia	Serine	7	Cell surface	Blood	[114, 115]
N-arginine dibasic convertase	Yes	No	nd	EDTA	Metallo	7	Cytoplasm (microtubular structures, secretory granules)	Pancreatic α-cells	[125]
Glucagon-degrading aminopeptidase	Yes	Yes	nd	nd	nd	7	Plasma membrane	Liver	[119, 122]
Receptor-linked glucagonase	Yes	No	40–130	PMSF	Serine	7	Plasma membrane	Liver	[22, 120]
Endosomal neutral glucagonase	Yes	Yes	≈ 0.5	PMSF	Serine	7	Endosome	Liver	[140]
Cathepsin D	Yes	Yes	nd	Pepstatin A	Aspartic acid	4	Lysosome (endosome)	Liver	[139]
Cathepsin B	Yes	Yes	nd	E64	Cysteine	4, 7	Lysosome (endosome	Liver	[139]
Insulin-degrading enzyme	Yes	No	6	EDTA	Metallo	7	Cytoplasm, peroxisome	Liver	[145]

The major biochemical characteristics of well-characterized glucagonase activities are presented. The identity of glucagon-degrading aminopeptidase, receptor-linked glucagonase and endosomal neutral glucagonase has not yet been defined. The site of activity of each proteolytic activity represents the main locus of interaction between glucagon and the proteolytic enzyme. The *in vivo* studies have been performed in rat (cathepsin D, cathepsin B and dipeptidyl peptidase IV), or in man and dog (glucagon-degrading aminopeptidase). nd, not defined.

Blood and organ metabolism of circulating glucagon

There have been reports that glucagon degradation begins in blood or plasma but, compared to organ degradation, the level of blood proteolysis is low [114]. The blood compartment is positioned both temporally and physically in the pathway between the secreting tissue (α-pancreatic cells) and target tissue (hepatic parenchyma). Selective glucagon degradation at this locus may be an additional mechanism further regulating plasma level of glucagon with subsequent regulation of glucagon action after glucagon binding to its cellular receptor. Blood may also represent a site of production of bioactive glucagon peptides with changes in the biological activity of metabolites which may serve functions (adenylyl cyclase activation and Ca^{2+}-Mg^{2+}-ATPase inhibition) different from that of the mother hormone [114, 115]. Studies indicate that the circulating serine protease dipeptidyl-peptidase IV (DPPIV) is a primary enzyme for glucagon degradation in blood (Table 1). Inhibition of DPPIV by isoleucyl-thiazolidine (Ile-Thia), a specific inhibitor of DPPIV, completely blocked serum-mediated glucagon degradation [114]. Glucagon is hydrolyzed by DPPIV to produce glucagon-(3–29) and glucagon-(5–29); in human serum, [pyroglutamyl(pGlu)^3]glucagon-(3–29) is formed from glucagon-(3–29), and this prevents further hydrolysis of glucagon by DPPIV [115]. DPPIV hydrolysis of glucagon yields low-affinity agonists of the GR in cAMP stimulation assays and competition binding experiments, [pyroglutamyl(pGlu)^3]glucagon-(3–29) being the most potent partial agonist. As the majority of the DPPIV activity in the blood circulation is membrane bound (endothelial DPPIV), the majority of the conversion of glucagon to its truncated form *in vivo* likely occurs at the blood vessel wall rather than in the plasma matrix [116]. The role of DPPIV degradation in glucagon metabolism has also been studied using N-terminally modified glucagon analogs, *i.e.* [D-Ser^2]-glucagon, [(P)-Ser^2]-glucagon, [Gly^2]-glucagon and [D-Gln^3]-glucagon, with the objective of generating DPPIV-resistant glucagon analogs. *In vitro* studies have shown that [D-Ser^2]-glucagon has the greatest affinity for DPPIV but was completely resistant to DPPIV degradation. [Gly^2]-glucagon has a K_d similar to that of [D-Ser^2]-glucagon but is degraded at a slightly slower rate than wild-type glucagon by purified DPPIV. Using an *in vivo* assay system, the [D-Ser^2] substitution is the only analog that possesses enhanced ability to increase circulating glucose levels relative to wild-type glucagon. The greater potency *in vivo* can be attributed to the lack of degradation by DPPIV, as the *in vitro* potency was found to be moderately reduced [115]. Other N-terminally modified glucagon analogs were not suitable to demonstrate the contribution of DPPIV to the degradation of glucagon.

The metabolic clearance rate (MCR) of exogenous glucagon is 36±5 ml kg^{-1} min^{-1} in rat and 12.6±0.8 ml kg^{-1} min^{-1} in dog, with respective half-life of 1.9±0.1 and 5.5±0.5 min [117, 118]. The tissues responsible for

the clearance of glucagon from the circulation are somewhat controversial; however, consensus exists that the liver and kidneys play a dominant role in the MCR and degradation of glucagon. The liver is thus an important site for glucagon removal and accounts for 34.7±2.4 % of the MCR in the dog. Hepatic glucagon degradation occurs by more than one mechanism, one requiring receptor binding (saturable process) and the other(s) not (nonsaturable mechanism) [117]. Acute bilateral nephrectomy reduces the MCR of glucagon by 34 % in the rat, supporting an important role of the kidney in glucagon metabolism [118]. Studies by several groups indicated that after glomerular filtration in the kidney, glucagon is hydrolyzed by apical surface of the renal proximal tubules. Relevant to this finding is the presence of high concentration of DPPIV, a glucagon-degrading enzyme, in the renal tubular brush border [116]. As expected, injection of [^{125}I]iodoglucagon to rats is followed by the rapid association of this peptide with liver and kidney membranes, and to a much lesser extent with heart, lung, spleen, adipose tissue, small intestine and adrenal membranes (Fig. 3). Co-injection of excess native glucagon inhibits by 90 % the association of [^{125}I]iodoglucagon with liver membranes, suggesting that this process is predominantly receptor mediated. In contrast, native glucagon inhibits the association of [^{125}I]iodoglucagon to membranes by only 30 % in kidney and 30–60 % in other tissues, indicating that membrane association at these sites is only partially receptor mediated (Fig. 3).

A large number of studies have proposed the plasma membrane as a major physiological locus for glucagon degradation. At least three neutral proteolytic activities have been proposed to operate at the hepatic cell surface (Table 1). Incubation of rat or canine hepatocytes (or derived plasma membranes) with mono[^{125}I]iodoglucagon isomers resulted in the identification of three degradation products that arose from proteolytic cleavages at the peptide bonds Gln^3-Gly^4 [glucagon-(4–29)], Phe^6-Thr^7 [glucagon-(7–29)] and Tyr^{13}-Leu^{14} [glucagon-(1–13)] [119, 120]. Cleavage at these bonds does not reflect the specificity of any known proteases. Nevertheless, deletion of three and six amino acids from the N terminus of glucagon [glucagon-(4–29) and -(7–29)] could involve the action of tripeptidyl aminopeptidase that has been isolated from a microsomal extract of rat liver [121]. This neutral extralysosomal protease could act sequentially to effect cleavage at peptide bonds Gln^3-Gly^4 and Phe^6-Thr^7. Glucagon-(4–29) release into the cell incubation medium was unaffected by various acidotropic agents (chloroquine, dansyl cadaverine and procaine), indicating that hormone processing probably occurred on the hepatocyte surface (Table 1) [122]. However, the nonspecific protease inhibitor bacitracin partially inhibited the conversion of glucagon to lower molecular weight peptides resulting from proteolytic processing in the C-terminal region of the hormone [122]. Evidence suggests that glucagon-(4–29) is produced *in vivo*. Thus, a peptide having the size and immunological properties expected of glucagon-(4–29) was demonstrated in the plasma of both man and dog. Furthermore, the concentration of glucagon-(4–29) in the posthepatic circulation exceeded that in the portal circulation. In contrast, the absence of a gradient for glucagon and glucagon-(4–29) across the canine kidney has suggested that the liver is the major (if not the sole) site for the production of this hormone fragment [122]. A GR-linked protease in plasma membranes appears to be responsible for cleavages at internal and C-terminal regions of the glucagon molecule and for the generation of two fragments, glucagon-(1–13) and -(14–29), the former remaining tightly membrane bound (Table 1) [22, 120]. This is compatible with the observation of Balage et al. [123] that about 20 % of [^{125}I]iodoglucagon bound to hepatic plasma membranes was of decreased apparent molecular weight as assessed by gel filtration. Formation of this fragment was inhibited by phenylmethylsulfonyl fluoride (PMSF), but not aprotinin, soybean trypsin inhibitor, bacitracin or *N*-ethylmaleimide, indicating that the relevant enzymatic activity resulted from a serine protease [22, 120]. Also, the production of glucagon-(1–13) fragment is inhibited by the addition of glucagon to incubations, but is unaffected by the addition of the glucagon homologs secretin and vasoactive intestinal peptide or by the addition of arginine vasopressin, suggesting that the responsible proteolytic activity is apparently glucagon specific [22, 120]. Formation of glucagon-(1–13) fragment also depended on the prior association of the hormone with high-affinity glucagon binding sites, its production being sensitive to GTP, which is known, *via* interaction with GTP-binding proteins, to promote dissociation of glucagon from its receptor [22, 120]. The partially purified enzyme appeared to have a different molecular size than endoprotease 24.11 and insulin-degrading enzyme (IDE), showed a broad neutral pH optimum (pH 7–9) and was sensitive to the presence of salt. Several minor degradation products generated by this partially purified protease have been identified, including glucagon-(1–10), -(14–25) and -(23–29) [22, 120].

Plasma membranes contain a third glucagon-degrading system, which processes glucagon to its bioactive fragment glucagon-(19–29) or miniglucagon [124, 125]. The miniglucagon fragment has its own bio-

logical activity and is a modulator of the action of glucagon, the mother molecule. At picomolar concentrations, miniglucagon inhibits the hepatic plasma membrane Ca^{2+} pump without interfering with the adenylyl cyclase activity [126]. At the same concentration, glucagon-(19–29) displays a negative inotropic effect on myocyte contraction and, at nanomolar concentrations, its potentiates the positive inotropic effect of glucagon [127]. At concentrations ranging from 0.01 to 1000 pM, miniglucagon dose-dependently inhibits by 80–100 % the insulin release triggered by glucose, glucagon, glucagon-like peptide-1-(7–36) amide (tGLP-1) or glibenclamide in the MIN6 cell line (which displays characteristics that compare well with that of normal β cells), but not that induces by carbachol [128]. The miniglucagon action on insulin release is probably mediated by closing voltage-dependent Ca^{2+} channels linked to pathway involving a pertussis toxin-sensitive G protein. These cellular studies have been confirmed using the rat isolated-perfused pancreas, which shows that picomolar concentrations of miniglucagon inhibit glucose-induced and tGLP-1-potentiated insulin secretion [129]. The miniglucagon fragment appears to be produced either from the circulating glucagon at the surface of target cells or inside the islets of Langerhans. A membrane proteolytic activity that cleaves glucagon and produces glucagon-(19–29) has been isolated from rat liver plasma membranes [124]. It is inhibited by the sulfhydryl-blocking reagent *p*-chloromercuribenzoate (pCMB) and the metal-chelating reagent *o*-phenanthroline, and activated by the thiol compound 2-mercaptoethanol (maximal activation 5 mM) or dithiothreitol (maximal activation 0.06 mM). Other protease inhibitors E64 and PMSF had no effect on the endopeptidase activity [124]. Another miniglucagon-producing activity that might be related to the rat liver enzyme has been characterized in culture media from pancreatic mouse αTC1.6 cell line [125]. Evaluation of the effect of various protease inhibitors on the miniglucagon-producing activity released from the αTC1.6 cells showed that miniglucagon production is reduced by the metal chelator 1,10-phenanthroline or EDTA, and the thiol-blocking reagent pCMB [125], three protease inhibitors reported to inhibit the rat liver enzyme [124]. In addition, zinc or cobalt, two cations known to control the catalytic activity of metalloendoproteases, dose-dependently inhibited the production of miniglucagon by the αTC1.6 cells, whereas calcium, necessary for prohormone convertase activity, had no effect [125]. Furthermore, after inhibition of miniglucagon production by the metal chelator 1,10-phenanthroline, the glucagonase activity was restored by addition of zinc or cobalt, consistent with the involvment of a Zn^{2+}/Co^{2+}-endoprotease activity [125]. In addition, the aminopeptidase inhibitors amastatin and bestatin inhibited the miniglucagon production, suggesting the implication of an aminopeptidase activity in the proteolytic process. In view of this sensitivity towards protease inhibitors and cations, the glucagon-degrading activity isolated from culture media has been proposed to be a set of two metalloproteases, namely *N*-arginine dibasic convertase (NRDc) (Table 1) and aminopeptidase B (Ap-B). Glucagon might be sequentially processed by NRDc at the level of the Arg^{17}-Arg^{18} basic site followed by trimming of the remaining Arg^{18} residue by Ap-B, leading to the final (19–29)-miniglucagon product. Recombinant NRDc and Ap-B are able to process glucagon into miniglucagon *in vitro*. Using confocal microscopy, a granular immunostaining of both enzymes has been observed in the αTC1.6 cells as well as in native rat α-cells from islets of Langerhans [125]. Since a colocalization of miniglucagon with glucagon has also been demonstrated in mature secretory granules of α-cells using confocal and electron microscopy analysis [129], NRDc and Ap-B may well be responsible for the production of miniglucagon at the pancreatic locus. However, the proteolytic activity at the surface of the target cells that appears to produce miniglucagon from the circulating glucagon remains to be firmly defined.

Endocytosis and intracellular proteolysis of glucagon

The subcellular localization of [^{125}I]iodoglucagon taken up by liver cells has been assessed morphologically and biochemically. When freshly isolated hepatocytes are incubated with [^{125}I]iodoglucagon and studied morphologically by quantitative electron microscope autoradiography, the radiolabeled hormone localizes to the plasma membrane of the cell at early times (10 min), and after a brief delay, is internalized and found to associate preferentially with lysosome-like structures [130]. Thus, upon interaction with isolated hepatocytes, glucagon undergoes receptor-mediated endocytosis and follows the same intracellular pathway as insulin and epidermal growth factor (EGF), although quantitative differences in receptor mobility and rate of internalization have been observed [131]. [^{125}I]Iodoglucagon binds initially and preferentially to the microvillous surface of the hepatocyte and undergoes moderate redistribution to the nonvillous surface of the cell, whereas the degree of lateral mobility of [^{125}I]iodoinsulin and [^{125}I]iodo-EGF is much pronounced. Comparably, [^{125}I]iodoglucagon is internalized to a much lower extent than [^{125}I]iodoinsulin and [^{125}I]iodoEGF [131]. Binding and endocytosis of [^{125}I]iodoglucagon or colloidal gold-

labeled glucagon to nonparenchymal cells (mainly endothelial and Kupffer cells) isolated from mouse liver was also examined by quantitative electron microscope autoradiography [47]. Both cells internalize glucagon, but, whereas the hormone is internalized into coated vesicles of endothelial cells *via* coated pits and transported to endosomal and lysosomal structures, glucagon bound to the smooth plasma membranes of Kupffer cells is internalized into cytoplasmic tubular structures *via* clathrin-independent plasma membrane invaginations [47]. Finally, using the same morphological techniques, specific binding of [^{125}I]iodoglucagon has been also demonstrated on neonatal rat islet cells [50]. Cell surface labeling was followed by internalization of the radioligand into endocytic vesicles of islet A cells (homologous cells), and islet B and D cells (heterologous cells). After initial internalization the further intracellular progression of the endocytosed [^{125}I]iodoglucagon occurs freely in heterologous cells (transfer from endosomal to lysosomal structures) but poorly in homologous cells [50].

Biochemical studies with isolated hepatocytes have shown that, with time, cell-associated [^{125}I]iodoglucagon becomes less dissociable by acid treatment, confirming internalization of cell surface ligand [132]. Internalization is inhibited by low temperature, phenylarsine oxide, and by blocking receptor binding, consistent with receptor-mediated endocytosis. Approximately 30 % of the total cell-associated hormone is internalized after 30 min of incubation at 37°C [132].

Subcellular fractionation of rat liver confirmed and extended these studies. When injected periphally, radiolabeled or immunoreactive native glucagon is taken up in part into the liver and associates first (2–10 min) with plasma membrane fractions, and then (10–24 min) accumulates in nonlysosomal low-density endocytic fractions [133]. By the application of the diaminobenzidine shift protocol of Courtoy et al. [134] it was conclusively demonstrated that these vesicular structures were non-Golgi [133]. Contrasting with the high revovery of internalized [^{125}I]iodoglucagon in endocytic vesicles, little of this hormone was found in acid phosphatase-containing lysosomal structures, with maximal lysosomal association observed at 20 min post injection [133]. The moderate lysosomal association of [^{125}I]iodoglucagon was next confirmed *in vivo* using a cell-free assay for the transfer of endocytosed ligand-receptor complexes to lysosomes in hepatic tissue [135]. In this *in vitro* system containing *in vivo* endocytosed [^{125}I]iodoglucagon, the time course of the endo-lysosomal transfer (observed 10–20 min after endosome-lysosome interaction) is consistent with the time taken for glucagon to appear in lysosomes in whole liver (>20 min; [133]). The endo-lysosomal transfer of internalized [^{125}I]iodoglucagon is temperature dependent (optimal at 37°C), energy dependent (it requires ATP and an ATP-regenerating system), requires the addition of cytosol and is not accompanied by an important release of low molecular weight degradation products [135].

With earlier observations that trichloroacetic acid-soluble radioactive products of [^{125}I]iodoglucagon were found in hepatic endosomes [133], the hypothesis that liver endocytic vesicles represent a major site of degradation of internalized glucagon was elaborated. This was supported by subsequent studies of chloroquine injection into rats, which was accompanied by a net increase in the glucagon content within isolated endosomal vesicles [133]. These findings indicated that, as with insulin [136], an acidic pH was required for the optimal degradation of glucagon in the endosomal compartment. Radiosequence analysis of glucagon-degradation products extracted from rat hepatic endosomes following the injection of [^{125}I]iodoglucagon revealed three cleavage sites in the glucagon sequence, *i.e.*, Ser^{2}-Gln^{3}, Thr^{5}-Phe^{6} and Phe^{6}-Thr^{7} bonds, and an undefined cleavage located C-terminal to Tyr^{13} [133]. The endosomal proteolysis of glucagon was then analyzed by two approaches, the first using intact cell-free endosomes and the second using an endosomal lysate. The first approach employed a cell-free system in which isolated intact endosomes were shown to degrade previously internalized glucagon [137]. Although detectable at pH 7, the degradation of glucagon in hepatic endosomes was maximal at pH 4 and functionally linked to ATP-dependent endosomal acidification. Thus, ATP stimulated endosomal glucagonase activity at neutral pH by promoting endosomal acidification as judged by acridine orange uptake [137]. The ability of ATP to stimulate glucagon degradation in endosomes did not occur if the isotonic medium was depleted of Cl^- (as expected from the electrogenic properties of the vacuolar proton pump [138]) or supplemented with weak bases, proton ionophore or inhibitor of the vacuolar type H^+-ATPase. The data also indicated that inhibiting the dissociation of glucagon from its receptor in endosomes reduced glucagon degradation [137]. Hence, free intraluminal glucagon was concluded to be the physiological substrate for endosomal glucagonases.

A reverse-phase HPLC assay based on measurement of the initial degradation step of native glucagon by purified endosomal lysates, with the subsequent identification of the early glucagon intermediates, allowed for the rigorous screening of endosomal glucagonase activities [139, 140]. This methodology

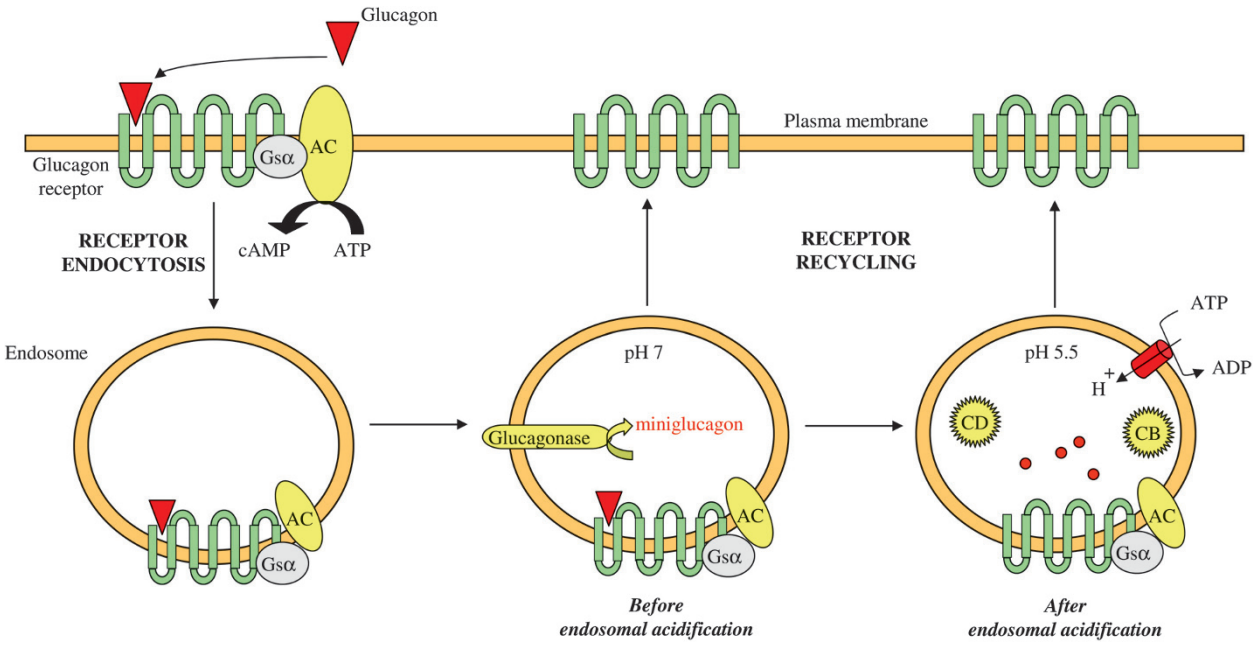

Figure 5. Internalization and endosomal fate of glucagon-GR complex in rat hepatocytes. Following binding of glucagon to its cell surface receptor, the occupied GR is rapidly internalized and Ser phosphorylated both at the plasma membrane and within endosomes [151]. Coincident with glucagon-GR complex endocytosis, both the 45- and 47-kDa Gsα proteins are massively internalized. An endosomal membrane serine glucagonase (glucagonase) transforms glucagon to glucagon-(19–29) or miniglucagon at neutral pH. Then, endosomal acidity maintained by an ATP-dependent proton pump facilitates glucagon dissociation and subsequent degradation of free intraluminal hormone mediated by aspartic acid protease cathepsin D (CD) and cysteine protease cathepsin B (CB). Afterwards, the intracellular GR is recycled to the cell surface to associate again with the transducing system at the plasma membrane level. The role of lysosomal apparatus in the subsequent post-endosomal fate of glucagon, GR and GR-transducing system is not illustrated. AC, adenylyl cyclase; GR, glucagon receptor.

revealed that internalized glucagon is rapidly proteolyzed within hepatic endosomes before (at neutral pH [140]) and after ATP-dependent endosomal acidification (at acidic pH [139, 140]) (Fig. 5). Evaluation of the effect of various protease inhibitors on the neutral glucagon-degrading activity showed that glucagon proteolysis was strongly inhibited by the serine protease inhibitor PMSF and the peptide antibiotic bacitracin, whereas metal-chelating agents such as EDTA, EGTA and 1,10-phenanthroline had no significant effect. The endosomal neutral glucagonase is membrane associated, distinct from miniglucagon endopeptidase (MGE) and furin [125], displays a 170-kDa molecular mass, contains *N*-linked oligosaccharides and generates the bioactive miniglucagon peptide among 13 degradation products (Table 1) [140]. However, it remains unclear as to how the endosomal glucagon-(19–29) fragment would come into contact with the plasma membrane Ca^{2+}-Mg^{2+}-adenosine triphosphatase. In an acute hyperglucagonemic state, as internalized endosomal GRs are recycled back to the cell surface with changes in glucagon-binding activity in plasma membrane completely reversed in less than 2 h [23], a recycling pathway may also apply to the endosome-associated miniglucagon [140]. In support of this, using circular dichroism and fluorescence methods, it has been shown that the Ca^{2+}-binding capacity of glucagon is maintained in the glucagon-(19–29) fragment, and that both peptides display some ability to penetrate the lipid bilayer in a hydrophobic environment with the bound Ca^{2+} ion [141]. Thus, potential translocation of the bioactive glucagon-(19–29) fragment across the endosomal membrane may be conceivable.

IDE, a neutral thiol-metalloendopeptidase also called insulysin [142], was described as an enzyme that intracellularly degrades glucagon with an apparent K_m of 6 μM (Table 1) [143]. Using a fluorescence assay, Baskin et al. [144] have proposed that the peptide bonds cleaved during the digestion of glucagon with highly purified IDE were Ser^{11}-Lys^{12}, Tyr^{13}-Leu^{14}, Arg^{18}-Ala^{19} and Trp^{25}-Leu^{26}. Contrary to this report, Rose et al. [145] have proposed that cleavage of glucagon by IDE occurs at only one region, namely the double-basic doublet sequence Arg^{17}-Arg^{18} with the release of glucagon-(1–17) and -(18–29). It has been shown that, although less active than glucagon-(19–29) at inhibiting the Ca^{2+} pump of the liver plasma membranes, glucagon-(18–29) was still 100-fold more active than glucagon itself [126], suggesting that IDE may release a hormone fragment having enhanced activity [145]. Moreover, an undefined aminopepti-

dase-type enzyme might exist to convert glucagon-(18–29) into glucagon-(19–29) by removal of Arg^{18} within the endosomal lumen. However, significant progress in understanding the physiological localization of IDE has toned down its putative role in endosomal proteolysis of internalized glucagon. Since both biochemical [146, 147] and morphological evaluations [148, 149] have revealed a dual peroxisomal and cytosolic location of the protease, it is likely that IDE is not readily available for internalized glucagon, which is located within the endo-lysosomal apparatus. Consequently, plasma membrane and endosome preparations likely contain IDE activity through nonspecific adsorption [150]. Thus, the potential biological significance of the proteolytic activity of IDE towards glucagon *in vivo* has not yet been established.

Although a significant rate of hydrolysis of glucagon was observed at pH 7, maximal degradation was obtained after endosomal acidification [139]. The endosomal acidic glucagonase was partially inhibited by cysteine protease inhibitors (E64 and iodoacetic acid) and aspartic acid protease inhibitor (pepstatin-A), whereas bacitracin blocked glucagon proteolysis at acidic pH almost completely [139]. Metal chelating agents (1,10-phenanthroline and EDTA) and the serine protease inhibitor PMSF were ineffective. Membrane-bound forms of cysteine protease cathepsin B and aspartic acid protease cathepsin D mediate endosomal proteolysis of glucagon at acidic pH (Table 1). The glucagon-processing cathepsins induce proteolytic modifications in the C terminus, internal and N terminus of the hormone, producing more than 30 endosomal metabolites [139].

No study has been directed towards the cell-mediated metabolism of glucagon analogs except for the des-His^1-[Glu^9]glucagon antagonist [151]. *In vitro* proteolysis of the antagonist and wild-type glucagon using endosomal lysates were comparable at acidic pH (pH 4), but a reduced rate of degradation was observed for des-His^1-[Glu^9]glucagon (less than 65 % of that of glucagon) at neutral pH.

GR endocytosis and phosphorylation

GR endocytosis was first demonstrated in intact rat liver using biochemical methods [23]. As measured using [^{125}I]iodoglucagon binding assay performed on subcellular fractions isolated from liver homogenates after acute administration of glucagon to rats, endosomal translocation of GR reached a maximum by 12–24 min, caused a 10–20 % decrease in glucagon binding at the plasma membrane and underwent reversal within 2 h [23]. The change in glucagon-binding activity in plasma membrane and endosomal fractions results from a change in receptor number, with receptor affinity remaining unaffected [23]. A time-dependent increase in GR content has also been demonstrated in hepatic endosomes at 5–45 min after glucagon injection using an antibody directed against the distal end of the intracellular C-terminal tail of GR [95, 151]. The loss of both [^{125}I]iodoglucagon binding [23] and immunoreactive GR [151] from plasma membranes is comparably partial, indicating that the changes in the subcellular distribution of GR induced by its ligand are quantitatively modest. In acutely glucagon-treated rats, no loss of hepatic GR is observed [23], whereas chronically hyperglucagonemic rats and isolated hepatic cells exposed to glucagon *in vitro* display a net decrease in total glucagon-binding activity [4]. The GR domains important for glucagon-mediated receptor internalization have been studied in CHO cells transfected with wild-type or truncated GRs [72]. In these studies, wild-type GRs internalize [^{125}I]iodoglucagon at 37°C such that 45–50 % of surface-associated radioactive ligand is within intracellular compartments after 10 min, with glucagon endocytosis decreasing slightly after 45 min. The internalization capacity of truncated mutants in which the C-terminal 56 or 62 amino acids have been deleted is greatly reduced with no measurable agonist-induced internalization after 45 min. Truncation of the C-terminal 24 amino acids results in a 10 % reduction in internalization capacity after 10 min relative to wild-type receptor [72]. Moreover, a mutant receptor containing a total of seven Ser-to-Ala mutations in the C-terminal 47 amino acids displays a dramatically decreased glucagon-dependent internalization and phosphorylation, suggesting a predominant role for phosphorylation of the C-terminal tail in GR endocytosis [72].

The post-endosomal fate of internalized GR and its lysosomal association has been evaluated in a cell-free rat liver endosome-lysosome fusion system following glucagon injection into rats [151]. A partial endo-lysosomal transfer of internalized GR occurred with no immunoreactive intermediate degradative products generated from the GR at the endosomal and lysosomal locus [151].

Short-term signal desensitization of GPCRs is generally characterized by a phosphorylation event catalyzed by a specific GPCR kinase, PKA or PKC (reviewed in [152]). Using the *in vivo* rat liver model and an immunological approach, it has recently been shown by Merlen et al. [151] that the hepatic GR was phosphorylated on Ser residues in response to glucagon, both at the cell surface (strong phosphoserine immunoreactivity) and after its internalization into the endosomal apparatus (low phosphoserine immu-

noreactivity). Specific signaling of the GR may be dispensable for GR endocytosis since the peptide antagonist des-His[1]-[Glu[9]]glucagon induces a detectable internalization of the GR, albeit less pronounced compared to that induced by wild-type glucagon [151]. These *in vivo* studies are in accordance with the *in vitro* experiments of Heurich et al. [153] in which a rapid, time- and concentration-dependent phosphorylation of GR on Ser residues induced by glucagon was demonstrated using ^{32}P labeling of a chinese hamster ovary (CHO) cell line expressing the human GR. Interestingly, neither forskolin nor phorbol ester increased glucagon-induced GR phosphorylation, suggesting that neither PKA nor PKC catalyze this phosphorylation event *in vivo* [153]. On the other hand, *in vitro* phosphorylation studies have recently shown that PKCα, PKCζ, and to a lesser extent, PKCδ, phosphorylate the GR *in vitro*, and that certain bile acids, such as chenodeoxycholic acid, might stimulate phosphorylation and heterologous desensitization of GR *via* a potential PKCα activation [154]. Other studies have implied that protein kinase D might regulate G-stimulated adenylyl cyclase activity in COS cells transfected to overexpress GRs [155]. Clearly, further studies are required to identify the serine kinase responsible for GR phosphorylation induced by glucagon. A strong correlation between the number of potential phosphorylation sites, GR phosphorylation and GR internalization has previously been demonstrated [72, 153], implying a role for phosphorylation in the process of agonist-induced GR internalization. Thus, C-terminal truncated GRs [72, 153] and a mutant GR containing seven Ser-to-Ala mutations in the cytoplasmic C-terminal tail [72] display reduced internalization capacities and phosphorylation rates. Comparably, the *in vivo* studies showing the glucagon-mediated endocytosis of Ser-phosphorylated GR support the idea that phosphorylation may regulate the endocytic process for the GR [151]. Similar regulation has been shown for other GPCRs and especially the family B GLP-1 receptor for which Ser phosphorylation of the C-terminal tail after agonist binding is required not only for homologous desensitization but also for internalization of the ligand-receptor complex [156].

Although the role of the endosomal association of internalized glucagon in signal down-regulation has clearly been demonstrated, the role of GR endocytosis in regulating GR signal transduction has been poorly investigated [72, 151]. Termination of GPCR-mediated signaling is regulated by kinase activity, leading to phosphorylation of the receptor and recruitment of intracellular scaffolding proteins such as β-arrestins from the cytosol, thereby excluding the receptor from further G protein interaction [152]. However, these cellular processes may not be conserved for the GR since glucagon treatment has no discernible effect on the subcellular location of β-arrestins in rat liver *in vivo* [151]. Interestingly, a similar regulation which is β-arrestin-independent appears to apply for the GLP receptors, which belong to GPCR family B [9]. Thus, mutant GLP-2 receptor, which failed to interact with β-arrestin-2, underwent ligand-induced endocytosis similar to that observed for the wild-type GLP-2 receptor [157]. With respect to the GLP-1 receptor, its interaction with β-arrestin-2 seems not to represent a determining or limiting factor for receptor internalization [158]. Despite the fact that other experiments using *in vitro* cell system and/or RNA interference are required to confirm a β-arrestin-independent endocytosis of GR [151], alternative endocytic mechanisms may account for the GR internalization. For instance, studies are required to evaluate the possibility that GR may also utilize alternative clathrin-independent internalization pathways such as lipid-raft-dependent receptor endocytosis.

Recent studies have suggested that glucagon signals may be generated and disseminated continually within the cell during and/or after GR endocytosis raising many additional issues concerning the relationship between GR endocytosis and signaling. Thus, Merlen et al. [151] observed that acute glucagon injection into rats was associated with the massive shift of both the 45- and 47-kDa Gsα proteins without any detectable change in the subcellular distribution of adenylyl cyclase (Fig. 5). Following administration of a saturating dose of glucagon to rats, a sustained endolysosomal transfer of the Gsα isoforms has been also demonstrated [151]. Despite the fact that adenylyl cyclase does not translocate to hepatic endosomes upon glucagon-GR endocytosis, adenylyl cyclase activity sensitive to *in vitro* added glucagon has been reported in rat liver endosomal fractions [23, 151], and adenylyl cyclase IV has recently been detected in hepatic endosomes using Western blot analysis [151, 159]. Importantly, it has been shown that *in vivo* glucagon treatment led to a time-dependent increase in basal and, to a lesser extent, glucagon-stimulated adenylyl cyclase activity in hepatic endosomes [23], suggesting a similar compartmentalization of a glucagon-sensitive adenylyl cyclase system and effective coupling between endosomal GR and the Gsα/adenylyl cyclase proteins. The similar kinetics of endolysosomal appearance of the internalized GR and the two Gsα isoforms, as well as the presence of a glucagon-sensitive adenylyl cyclase associated with the endosomal apparatus, suggest an extended signal transduction consequent to glucagon-mediated GR endocytosis. Importantly, in response to tetraiodoglu-

cagon, a GR agonist of enhanced biological potency [160], GR endocytosis, Gsα shift and activation of endosomal adenylyl cyclase are of higher magnitude and of longer duration confirming that glucagon signal transduction may occur *in vivo* at the locus of endosomal apparatus [151].

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