Review Articles

Internalization of Polypeptide Hormones

Mechanism, Intracellular Localization and Significance

Ph. Gorden¹, J.-L. Carpentier², P. Freychet³, and L. Orci²

¹Diabetes Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland, USA; ²Institute of Histology and Embryology, University of Geneva School of Medicine, Geneva, Switzerland; and ³Group INSERM U-145, Faculty of Medicine (Pasteur), Nice, France

Polypeptide hormones, growth factors and a variety of other naturally occurring ligands can be labelled with ¹²⁵I; the biologically active labelled material binds specifically to a component of the cell or a subcellular constituent. On the basis of specificity for the ligand the binding site has been called a receptor. For the polypeptide hormones it was inferred, from early studies, that the ligand bound to the receptor was exposed to the external environment of the cell, since it could be perturbed by enzymes and antibodies [1, 2].

The chemically well characterized hormone interacts with the poorly characterized receptor, an integral protein of the plasma membrane, in a saturable fashion to form a hormone-receptor complex. This complex represents the first step in the action of the hormone. Using equilibrium analytical methods the state of the receptor in terms of affinity and number of binding sites has been estimated in many conditions in both experimental animals and human subjects. It is important to emphasize that these studies, for the most part, have been carried out at low temperatures where equilibrium conditions are most closely simulated. At higher more physiological temperatures it has become clear that the hormone receptor interaction is more complex and cannot be described as a simple bimolecular reversible reaction. Furthermore, there are several diverse functions of the hormone-receptor complex: (a) both membrane and intracellular biological activities are linked in some way to the hormone-receptor complex [1, 3]; (b) degradation of the ligand is linked to the binding of the ligand [4]; and (c) ligand-induced regulation of

the receptor is linked to the initial binding process [5–7].

In this review we will explore how direct visual probes of the hormone-receptor interaction have extended our concepts of: (a) the nature of the cell surface binding; (b) the subsequent fate of the ligand; and (c) the possible relevance of this process to hormone action, degradation and receptor regulation. Finally, we will emphasize that polypeptide hormones and growth factors are involved in a similar qualitative process to other unrelated ligands that bind to specific cell surface receptors (Table 1).

The Hormone at the Cell Surface

When ¹²⁵I-insulin at physiological concentration is incubated with freshly isolated rat hepatocytes at 20 °C for 5 minutes and the cell pellet prepared for autoradiography, the labelled material can be both qualitatively and quantitatively localized to the plasma membrane of the hepatocyte (Figs. 1 and 2) [9, 33].

Earlier experiments demonstrated the localization of ferritin-insulin complexes to the membrane of the fat cell in thin section [34, 35] and to the true surface of hepatocyte membranes in freeze-etched preparations [36]. Ferritin cores appeared singly or in small groups and it was reported that in fat cells the ferritin groups were present in cells prefixed prior to incubation suggesting that these groups were naturally occurring [35]. More recently, using an elegant new method of video intensification microscopy, it was shown that insulin and epidermal growth factor conjugated to fluorescent lactalbumin bind diffusely to mouse fibroblasts at 4 °C but at higher temperatures the ligand is mobile and forms clusters [16].

¹ Much of this work was carried out while P. G. was a Visiting Professor, Institute of Histology and Embryology, University of Geneva

Table 1. Studies using direct morphologic probes of ligand-cell interaction^a

Ligand	Cell or organ	Probe	Reference
Insulin	Rat hepatocyte	EM autoradiography	[8–12]
Insulin	Intact rat liver	EM autoradiography	[13, 14]
Insulin	Cultured human lymphocyte	EM autoradiography	[2, 15, 7]
Insulin	Cultured mouse fibroblast	Silicon intensification fluorescence (SIT) optical microscopy	[16]
Anti-insulin receptor antibody	Cultured human lymphocyte	EM autoradiography	[17] ^b
EGF	Cultured human fibroblasts	EM autoradiography	[18, 19]
EGF	Cultured mouse fibroblasts	Silicon intensification fluorescence optical microscopy	[16]
EGF	Cultured A431 epidermal carcinoma	Optical fluorescence, Ferritin-EGF (EM), and EM autoradiography	[20, 21] ^c
EGF	Rat hepatocyte	EM autoradiography	^b
Human growth hormone	Cultured human lymphocytes	EM autoradiography	[22] ^b
Glucagon	Rat hepatocyte	EM autoradiography	[23] ^b
Human chorionic gonadotrop.	Cultured granulosa cells	EM autoradiography	[24, 25]
Low density lipoprotein (LDL)	Cultured human fibroblasts and circulating human mononuclear cells	Ferritin-LDL (EM) and EM autoradiography	[26–29]
α -2 macroglobulin	Cultured mouse fibroblast	Ferritin (EM), immunoperoxidase (EM), and silicon intensification fluorescence	[16, 30]
Asialoglycoprotein	Intact liver	EM autoradiography	[31, 32]

^a This list is partial and does not include conventional antibodies, lectins, toxins, transcabalin, lysosomal enzymes, transferrin and other ligands which are also internalized by cells

^b Manuscript submitted for publication

^c Manuscript in preparation

Since insulin behaved in a similar fashion to α -2 macroglobulin, it was suggested that insulin, like α -2 macroglobulin, is preferentially clustered in “coated pits” on the cell surface [30].

Anderson et al. have demonstrated that ferritin-conjugated low density lipoprotein (ferritin-LDL) is preferentially localized in “coated pits” in human fibroblasts and that this is true in cells fixed to prevent mobility prior to incubation [26]. By contrast in a mutant fibroblast cell line that binds but does not internalize the LDL, the ligand is not localized in “coated pits” [27, 28]. The coated segment of the membrane is made up primarily of the protein clathrin as well as several other minor components [37, 38].

Studies in cultured human lymphocytes have also demonstrated that ¹²⁵I-insulin and ¹²⁵I-anti-insulin receptor antibody are redistributed in the plane of the membrane as a function of temperature. The hormone or specific immunoglobulin binds initially at low temperatures in a diffuse fashion around the plasma membrane but at higher temperatures the ligand redistributes toward the Golgi pole of the cell in a form analogous to capping [39, 40].

While the “coated pit” is an important structure for polypeptide hormones, growth factors and LDL in fibroblasts, preferential initial localization of the ligand to these structures is not found for ¹²⁵I-insulin in rodent hepatocytes [11] or cultured human lymphocytes [39] nor for ferritin-LDL in circulating lymphocytes [29].

Several important generalizations can be drawn from these studies: (a) Insulin receptors may exist in naturally occurring groups on the cell surface but this conclusion depends totally on the monomeric nature of ferritin-insulin [35]. Either the natural groups or isolated receptors can be redistributed in the plane of the membrane. (b) Receptors may be localized in specialized structures on the cell surface such as the “coated pits” or they may localize randomly and cluster in these specialized structures. (c) While the coated pit or invagination is an excellent model of a type of specificity that is undoubtedly important in fibroblasts, has not yet been demonstrated to be responsible for the internalization of the ligand in other cell types. It is clear, however, that these studies are at an early stage of development and additional data will be required to define more precisely the role of the “coated pit”.

It seems clear that the hormone or other ligand receptor can exist in a specific domain or microenvironment on the cell surface; this microenvironment, therefore, provides a mechanism whereby specific ligands may regulate the concentration of their unique receptor.

Fig. 1. Thin section of isolated hepatocyte with developed autoradiographic grains (circles)

The Hormone is Internalized by the Cell

Following the initial binding of ^{125}I -insulin to the cell surface, the ligand is progressively internalized by the cell in a time- and temperature-dependent fashion (Fig. 3). At apparent steady-state, approximately 60% of the ligand is on the plasma membrane of the cell and 40% is internalized. It is, therefore, clear that what is usually referred to as binding is actually cell-associated radioactivity (i. e., plasma membrane bound + internalized ligand).

The process by which a macromolecule enters an intact cell is endocytosis or more specifically pinocytosis [41]. In the case of a ligand that binds to the cell and is concentrated on the surface the process is known as adsorptive pinocytosis [41]. Macromolecules that do not bind to the cell surface may be taken up by fluid-phase pinocytosis in proportion to their concentration in the incubation media but this

Fig. 2. Grain distribution histogram of ^{125}I -insulin binding to isolated hepatocytes. Note symmetrical distribution of grains around plasma membrane (PM). The universal curve refers to the distribution of ^{125}I around a defined line source of irradiation. For details see [2, 9, 33]

Fig. 3. Grain distribution histograms of ^{125}I -insulin binding to isolated hepatocytes as a function of time and temperature. Note progressive shift in grain distribution at 37°C indicating internalization of the ligand. For details see [9, 33]

Fig. 4. Relationship of receptor binding to internalization of ^{125}I -insulin in isolated rat hepatocytes. "r" is the coefficient of correlation for each condition shown. The 3 separate lines represent experiments at 20°C , 37°C , or combined 20°C and 37°C data. For details see [33]

process is much less efficient than adsorptive pinocytosis.

Pinocytosis involves the invagination of a segment of the membrane (size of the invagination depends on the cell type). The invaginated membrane then fuses and pinches off the plasma membrane in an energy consuming step or steps to form a small pinocytotic vesicle. The ligand is, therefore, internalized in a membrane-bounded compartment. Qualitative images of these events have been demonstrated for ferritin-EGF in A-431 cells [21] and for ferritin-LDL in human fibroblasts [26].

The rate controlling steps for the internalization process are unclear. We have found that the amount of the ligand internalized is proportional to the concentration of the ligand bound to the plasma membrane and suggested that binding per se may be the rate controlling step of internalization (Fig. 4) [33].

While many different ligands that bind to the cell surface are internalized by cells, the rate and magnitude of the internalization process is different for different ligands in different cell types. For instance, in the cultured human lymphocyte at steady-state we find that only about 15 to 20% of ^{125}I -insulin is inter-

Fig. 5. Thin section of isolated hepatocyte with developed autoradiographic grains. "Ly" refers to lysosome-like structures where grains have been shown to preferentially localize. For details see [11, 12]

nalized [2] while in the hepatocyte under similar conditions 40–50% of the ligand is internalized [9, 33]. On the other hand, human growth hormone in the cultured human lymphocyte is internalized in a similar fashion to insulin in the hepatocyte [22]. Thus, neither the specific ligand nor the cell determines the rate or magnitude of the internalization process.

Intracellular Localization of the Hormone

If insulin is internalized by the process of adsorptive pinocytosis, the ligand enters the intracellular compartment by way of a pinocytotic vesicle. As previously mentioned, in some instances these vesicles are coated, but in other cases as for insulin internalization in hepatocytes, we have not found localization to coated invaginations on the cell surface [11]. Typically endocytic vesicles fuse with lysosomes [41] and we have found that ^{125}I -insulin is preferentially localized in lysosomes in isolated hepatocytes [10, 11] and in perfused rat liver (Fig. 5) [14]. We have also found preferential localization of other inter-

Fig. 6. Analysis of hepatocyte associated radioactivity from 0 to 60 min of incubation at 37° C. The cell associated radioactivity was extracted and filtered on Sephadex G-50. Peak II is the elution profile of intact ^{125}I -insulin, peak I is from the void volume (i. e. high molecular weight) and peak III elutes past the ^{125}I - marker in the region of iodotyrosyle peptides (i. e. low molecular weight). Note the various fractions are relatively constant as a function of incubation time. For details see [11]

nalized ligands to lysosomes. These include anti-insulin receptor antibody [17] and human growth hormone in cultured human lymphocytes [22], epidermal growth factor in human fibroblasts [19] and rat hepatocytes [42], and glucagon in rat hepatocytes [23]. In addition we have compared the EM autoradiographic method with two other systems that have used ferritin-coupled ligands. Ferritin-LDL is localized in lysosomes of human fibroblasts [26] and ferritin-EGF is localized in lysosomes of A-431 cells (Table 1). We have made essentially identical observations using ^{125}I -labelled ligands in each of these cell types [28].

In the perfused rat liver, Bergeron et al. have found that labelled insulin is primarily localized in lysosome-like structures but also finds localization to defined Golgi elements [13]. While we find labelled insulin and other ligands localized in the Golgi region of the cell which is rich in lysosomes [12], we cannot preferentially localize insulin or other ligands to morphologically identifiable Golgi structures.

By contrast to these studies, Goldfine et al. have found ^{125}I -insulin preferentially localized to endoplasmic reticulum and nuclear membrane in the cultured human lymphocyte [15]. The explanation for these different experimental findings is unclear.

We interpret our data to indicate that the ligand binds to the cell surface, is internalized in membrane-bounded vesicles and these vesicles fuse with lysosomes. Lysosomes are typically concentrated in the Golgi region of the cell. Thus, under continuous incubation conditions the ligand moves from the cell surface, through a vesicular compartment to lysosomes. Lysosomes are identified both on the basis of their morphologic characteristics and by cytochemical techniques (Fig. 5).

Intracellular Receptors

Receptors for insulin have been reported on Golgi structures [43–45], nuclei [46, 47] and endoplasmic reticulum [3]. The binding sites on these structures are similar to the plasma membrane receptor with respect to specificity for insulin and insulin analogues. The nuclear binding site is different in several respects from plasma membrane receptors in that it has a different pH optimum, shows no accelerated dissociation of labelled insulin in the presence of an excess of unlabelled insulin and binding to this site is not inhibited by anti-insulin receptor antibody [48, 49].

Golgi receptors have been characterized in detail both biochemically or morphologically and have very similar properties to plasma membrane receptors

[44, 45]. These receptors are probably located on the inner membrane of the Golgi cisternae and binding to these structures is enhanced by manoeuvres that disrupt membranes such as freezing and thawing [44]. This strongly suggests that these sites may represent the biosynthetic precursor of the plasma membrane receptor. Evidence for this concept has been presented for the acetylcholine receptor [50]. This could also be true for the receptor in endoplasmic reticulum. The function of the nuclear receptors is less clear, but it has been suggested that it may mediate some of the long-term effects of insulin [46, 47].

In our studies, we have been unable to show that the hormone that initially binds to the plasma membrane subsequently becomes localized in defined Golgi elements, endoplasmic reticulum or nuclei.

Implications for Hormone Action

The binding of the hormone to its receptor is necessary but not sufficient for the full expression of hormone action. For some polypeptide hormones such as glucagon all the required components for the expression of the hormone's action appear to be contained in the plasma membrane including the receptor, adenylate cyclase and the nucleotide regulatory component. Glucagon is a classic hormone with its intracellular effects presumably mediated by a second messenger. Insulin, growth hormone and the various growth factors and somatomedins also regulate many intracellular events and it has been widely felt that there must be a second messenger system for these agents as well. While enthusiasm has waxed and waned there has been no clear second messenger identified for these hormones and it is reasonable to question whether or not internalization of these ligands is a component of their biologic action.

Before considering the polypeptide hormones, it is useful to review the components of the best studied biological model of receptor binding, internalization and intracellular regulation. There are three important components necessary for LDL to regulate cholesterol metabolism in fibroblasts [51, 52]. First, there must be a cell surface receptor to recognize and bind LDL; second, the receptor must be localized in coated pits to allow the LDL receptor complex to be internalized; and third, the vesicles internalizing the LDL must fuse with lysosomes to allow lysosomal enzymes to release free cholesterol from the protein complex.

We have shown that all of these events occur for insulin, growth hormones and epidermal growth factor, but to what extent are they required for hormone action? While it is possible to speculate that the

entire insulin molecule is necessary for receptor binding and that the molecule is subsequently internalized to release an active fragment [53, 54], or that the internalized receptor may mediate some intracellular response [55], there is little direct evidence to support these speculations. In fact, in limited studies it appears that when internalization is impaired [56], or lysosomal enzymes inhibited [57, 58] there is no inhibition of hormone action. In addition, hormones with classic second messengers are internalized in a similar fashion to insulin and related ligands [23–25]. Thus, at the present time, there is no definitive experiment to prove that internalization is not a necessary step in hormone action, but there is little positive evidence for this concept.

An immunoglobulin has been isolated from the plasma of a group of severely insulin resistant patients [59]. This immunoglobulin behaves like an antibody to the insulin receptor; it competes with insulin for receptor binding and it can produce all of the biologic effects of insulin [60–62]. Recently it has been shown that the univalent Fab component is an insulin antagonist but can be converted to an agonist by the addition of anti-Fab antibody [63]. This suggests that cross-linking of the antibody is in some way involved in its agonistic properties. Small concentrations of anti-insulin antibody will also increase to a small extent the effect of insulin bound to adipocytes while large concentrations of antibody are known to inhibit. These studies raise the possibility that cross-linking of either the ligand, their receptors or some other component of the membrane may be one of the post-binding steps involved in hormone action. It is interesting that labelled anti-receptor antibody displays all of the same morphologic events as does insulin; these include binding to the plasma membrane, redistribution in the plane of the membrane, internalization and lysosomal association. Thus, some form of cross-linking may be involved in hormone action and in the internalization process per se but whether these phenomenon are related is unknown at present.

Implications for Hormone Degradation

Using a perfused liver system Mortimer and Teitze showed 20 years ago that at low temperatures labelled insulin was “trapped” by the liver but not degraded, while at higher temperatures the hormone was “trapped” and following a brief delay the hormone was degraded [64]. Terris and Steiner confirmed these studies in the perfused rat liver [65] and extended the studies to the isolated hepatocyte where they demonstrated that insulin degradation by the

Fig. 7. Analysis of cell associated radioactivity as a function of dissociation time. Cells were incubated for 30 min at 37 °C, washed and diluted 100-fold. The nature of the hepatocyte associated (left) and medium radioactivity (right) is shown as a function of dissociation time (i. e. from 0 to 60 min of dissociation). For details see [11]

hepatocyte had all the characteristics of a receptor linked process [4]. On the other hand, it has also been shown in liver membranes and in isolated hepatocytes that a soluble degradative system exists which does not require binding nor even the continued presence of the cell [66, 67]. This form of degradation can be inhibited by bacitracin and other protease inhibitors and if present in the system must be neutralized before receptor linked degradation can be studied (Figs. 6 and 7) [11].

As previously discussed, following binding of insulin to isolated hepatocytes, the ligand is internalized as a function of time and temperature. The ligand is internalized in a membrane-bounded compartment and these pinocytotic vesicles subsequently fuse with lysosomes [11]. Since these morphologic observations, and the studies of Carpenter and Cohen showing that EGF degradation was inhibited by lysosomal stabilizing agents [68], many investigators have shown that agents such as chlorquine and ammonium chloride inhibit cell related insulin degradation but not soluble proteases previously discussed [69–74].

Thus, it seems reasonable that the internalization pathway for insulin in hepatocytes is primarily a degradative pathway involved in removing the ligand

Table 2. Relationship of internalization of ligand to concentration of ligand that induces receptor loss^a

Ligand	Cell	Proportion of ligand internalized	Concentration of ligand necessary to induce receptor loss	Reference
Insulin	Cultured human lymphocyte	Low	High	[2] [5]
Insulin	Cultured rat hepatocyte	Intermediate	Intermediate	[9] [89]
EGF	Cultured human fibroblast	High	Low	[19] [68]

^a These relationships are expressed in arbitrary terms in regard to the 3 ligands listed

from the cell surface and is very similar to the LDL system.

If receptor-linked degradation is a major physiological process then it appears to be mediated by receptor-linked internalization. This would mean that the degradation of insulin and other polypeptide hormones and growth factors is primarily a lysosomal process. Since most in vitro studies of insulin degradation have been carried out in homogenates at physiological pH, a lysosomal process would not have been detected; very few studies have considered this point [75, 76]. Thus it remains unclear to what extent and by what mechanisms membrane-associated proteases or transhydrogenase are involved in the physiological degradation of insulin.

It was first pointed out by Kahn and Baird that at 37 °C insulin becomes relatively less dissociable from adipocytes as a function of the length of incubation time and the labelled material that dissociates from the cell progressively appears as degradation products [77]. We have confirmed these findings in isolated hepatocytes and have further shown that dissociation and degradation can be delayed by the lysosomal stabilizing agent ammonium chloride [69]. This is consistent with the idea that the labelled ligand once internalized by the cell can not be released until it is degraded.

Implications for Receptor Regulation

It was first shown in severely insulin resistant hyperinsulinaemic obese mice that the concentration of insulin receptors on the cell accessible for binding was reduced compared to normal controls [78, 79].

Fig. 8. Diagrammatic view of polypeptide hormone or growth factor interaction with a cell. According to this view the ligand initially binds to a specific cell surface receptor, and the ligand receptor complex may then be redistributed in the plane of the membrane. The ligand is then internalized by the cell by adsorptive pinocytosis into a membrane bounded vesicle which fuses with lysosomes. The ligand is then degraded and the membrane vesicle containing the receptor is either degraded or reutilized

These studies were extended to obese, hyperinsulinaemic man and similarly reduced receptor concentrations were found [80–84]. It was shown in both mice and men that the receptor concentration was inversely proportional to the ambient insulin concentration [81, 85]. These studies were extended to in vitro systems where it was shown that the insulin receptor concentration of the cultured human lymphocyte was reduced as a function of time, temperature, and insulin concentration in the media and that this reduction was specific for the insulin receptor [5]. Again, receptor binding is necessary but not sufficient for the expression of this effect. Growth hormone can mediate the loss of its unique receptor but has no effect on the insulin receptor [6]. Hormone-induced receptor loss or “down regulation” represents a new steady-state where binding is reduced up to 20% of maximal. This process has now been demonstrated for many hormones and growth factors.

If the hormone is internalized by adsorptive pinocytosis then the membrane segment containing the receptor should be internalized with the ligand. This has led us to suggest that receptor-linked hormone degradation and receptor-linked hormone-induced receptor loss are coupled processes [7, 9].

If the down regulation process is a new steady-state of reduced receptor concentration and if this process is mediated by internalization then net receptor concentration on the cell surface will represent a dynamic equilibrium between the rate of membrane internalization, receptor degradation, membrane recycling and the rate receptors are synthesized, transported, and inserted into the membrane. Quantitative data on these processes is essentially non-existent and it is, therefore, not surprising that if hormone degradation and receptor loss are coupled, they do not occur in concert. Hormone degradation is rapid, usually occurring in minutes, whereas, down regulation of receptors usually requires hours.

Several factors suggest that hormone-induced receptor loss is mediated by internalization: (a) membrane microheterogeneity, where classes of receptors occur in specific domains or move in the plane of the membrane by clustering and patching, could explain the exquisite specificity of the process; (b) the concentration of ligand necessary to induce receptor loss is inversely proportional to the magnitude and rate of internalization of the ligand (Table 2); and (c) the conditions that induce receptor loss are associated with an increase in the pinocytotic rate of the cell [7, 86].

It seems reasonable to conclude that internalization may be the major mechanism by which cell surface receptors are regulated, but it is clear that this is a complex process with many potential control points.

While it is known that receptor affinity can be experimentally modified, for instance, by starvation, decrease in pH or by a variety of physical agents the mechanism of these changes is unclear. Available data suggest that receptors may exist as a single affinity class, as multiple affinity classes [84] or change as a function of occupancy (negative cooperativity) [88]. At present there is no information to implicate the internalization process in the regulation of receptor affinity. Negative cooperativity is seen most prominently at low temperatures of dissociation but is not significantly influenced by the temperature of association of the ligand to the cell. By contrast the temperature of association strongly influences the rate and magnitude of internalization.

Conclusions and Speculations

In this review we have illustrated the interaction of ^{125}I -insulin with isolated hepatocytes, since the liver is the major target of all the actions of insulin. We have emphasized throughout that the events involved in the interaction of insulin with hepatocytes is qual-

itatively similar to the events involved in the interaction of many other polypeptide hormones, growth factors, lipoproteins and other ligands with cells. These general mechanisms include: (a) binding of the ligand to cell surface receptors, in some instances to specialized domains of the membrane such as coated pits; (b) redistribution of the ligand in the plane of the membrane providing a mechanism for the segregation of a given class of receptors; (c) internalization of the ligand by adsorptive pinocytosis into a membrane-bounded compartment; and (d) transfer of the ligand and probably its receptor to lysosomes where the ligand is degraded and the receptor either degraded or recycled back to the membrane (Fig. 8).

The binding of the Hormone (H) to Receptor (R) is a requirement for hormone action. Thus, the magnitude of $\overline{HR}$ at the cell surface is a major determinant of the magnitude of response. The concentration of H at any given time at the cell surface is a function of the concentration of H bound and H internalized. Similarly the concentration of R is critically determined by the amount of membrane internalized; internalization, therefore, provides a simple mechanism that couples hormone degradation with receptor regulation. If this is true, from a clinical view point, we can begin to think of mechanisms to maintain $\overline{HR}$ on the cell surface.

Finally, if one considers these general mechanisms common to many ligands and many cells, it is not surprising that peripheral blood cells and other non-target cells mirror the status of the insulin receptor on major target tissues.

References

1. Roth J (1973) Peptide hormone binding to receptors: A review of direct studies in vitro. *Metabolism* 22:1059-1073
2. Carpentier J-L, Gorden P, Amherdt M, Van Obberghen E, Kahn CR, Orci L (1978) ^{125}I -insulin binding to cultured human lymphocytes. Initial localization and fate of hormone determined by quantitative electron microscopic autoradiography. *J Clin Invest* 61:1057-1070
3. Kahn CR (1976) Membrane receptors for hormones and neurotransmitters. *J Cell Biol* 70:261-286
4. Terris S, Steiner DF (1975) Binding and degradation of ^{125}I -insulin by rat hepatocytes. *J Biol Chem* 250:8389-8398
5. Gavin JR III, Roth J, Neville DM Jr, De Meyts P, Buell DN (1974) Insulin-dependent regulation of insulin receptor concentrations: A direct demonstration in cell culture. *Proc Natl Acad Sci USA* 71:84-88
6. Lesniak MA, Roth J (1976) Regulation of receptor concentration by homologous hormone. Effect of human growth hormone on its receptor in IMq lymphocytes. *J Biol Chem* 251:3720-3729
7. Gorden P, Carpentier J-L, Van Obberghen E, Barazzzone P, Roth J, Orci L (1979) Insulin-induced receptor loss in the cultured human lymphocyte: Quantitative morphological perturbations in the cell and plasma membrane. *J Cell Sci* 39:77-88

8. Carpentier J-L, Gorden P, LeCam A, Freychet P, Orci L (1977) Limited intracellular translocation of ^{125}I -insulin in isolated rat hepatocytes. *Diabetologia* 13:386
9. Gorden P, Carpentier J-L, Freychet P, LeCam A, Orci L (1978) Intracellular translocation of iodine-125-labeled insulin: Direct demonstration in isolated hepatocytes. *Science* 200:782-785
10. Gorden P, Carpentier J-L, Freychet P, LeCam A, Orci L (1978) ^{125}I -insulin: Direct demonstration of binding, internalization, and lysosomal association in isolated rat hepatocytes. *Diabetes* 27:450
11. Carpentier J-L, Gorden P, Freychet P, LeCam A, Orci L (1979) Lysosomal association of internalized ^{125}I -insulin in isolated rat hepatocytes. Direct demonstration by quantitative electron microscopic autoradiography. *J Clin Invest* 63:1249-1261
12. Carpentier J-L, Gorden P, Freychet P, LeCam A, Orci L (1979) ^{125}I -insulin is preferentially internalized in regions of the hepatocytes rich in lysosomal and Golgi structures. *Experientia* 35:904-905
13. Bergeron JJM, Sikstrom R, Hand AR, Posner BI (1979) Binding and uptake of ^{125}I -insulin into rat liver hepatocytes and endothelium. An in vivo radioautographic study. *J Cell Biol* 80:427-443
14. Carpentier J-L, Gorden P, Barazzone P, Freychet P, LeCam A, Orci L (1979) Intracellular localization of ^{125}I -labeled insulin in hepatocytes from intact rat liver. *Proc Natl Acad Sci USA* 76:2803-2807
15. Goldfine ID, Jones AL, Hradek GT, Wong KY, Mooney JS (1978) Entry of insulin into human cultured lymphocytes; electron microscope autoradiographic analysis. *Science* 202:760-763
16. Schlessinger J, Schechter Y, Willingham MC, Pastan I (1978) Direct visualization of binding, aggregation, and internalization of insulin and epidermal growth factor on living fibroblastic cells. *Proc Natl Acad Sci USA* 75:2659-2663
17. Carpentier J-L, Van Obberghen E, Gorden P, Orci L (1979) ^{125}I -anti-insulin receptor antibody binding to cultured human lymphocytes: Morphological events are similar to the binding of ^{125}I -insulin. *Diabetes* 28:354
18. Gorden P, Carpentier J-L, Cohen S, Orci L (1978) Interaction du facteur de croissance épidermique avec des fibroblastes humains en culture: une étude autoradiographique à l'échelon ultrastructural. *C R Acad Sci [D] (Paris)* 286:1471-1474
19. Gorden P, Carpentier J-L, Cohen S, Orci L (1978) Epidermal growth factor: Morphological demonstration of binding, internalization, and lysosomal association in human fibroblasts. *Proc Natl Acad Sci USA* 75:5025-5029
20. Haigler HT, Ash JF, Singer SJ, Cohen S (1978) Visualization by fluorescence of the binding and internalization of epidermal growth factor in human carcinoma cells A-431. *Proc Natl Acad Sci USA* 75:3317-3321
21. Haigler HT, McKanna JA, Cohen S (1979) Direct visualization of the binding and internalization of epidermal growth factor in human carcinoma cells A-431. *J Cell Biol* 81:382-395
22. Carpentier J-L, Lesniak MA, Barazzone P, Van Obberghen E, Gorden P, Orci L (1979) Direct demonstration of binding and internalization of ^{125}I -hGH in cultured human lymphocytes: A mechanism linking degradation of the hormone with down regulation of the receptor (abstract). Presented at the 61st Annual Meeting of the Endocrine Society, Anaheim, CA, p 80
23. Barazzone P, Carpentier J-L, Gorden P, Caniver B (1979) ^{125}I -glucagon: Direct demonstration of binding, internalization and lysosomal association in isolated rat hepatocytes. *Int Congr Ser* 481, 16. Excerpta Medica, Amsterdam
24. Chen TT, Abel JH Jr, McCellan MI, Sawyer HR, Diekman MA, Niswender GD (1977) Localization of gonadotropic hormones in lysosomes of ovine luteal cells. *Cytobiologie* 14:412-420
25. Amsterdam A, Nimrod A, Lamprecht SA, Burstein Y, Lindner HR (1979) Internalization and degradation of receptor-bound hCG in granulosa cell cultures. *Am Physiol Soc* E129-E138
26. Anderson RGW, Goldstein JL, Brown MS (1976) Localization of low density lipoprotein receptors on plasma membrane of normal human fibroblasts and their absence in cells from a familial hypercholesterolemia homozygote. *Proc Natl Acad Sci USA* 73:2434-2438
27. Anderson RGW, Brown MS, Goldstein JL (1977) Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. *Cell* 10:351-364
28. Carpentier J-L, Gorden P, Goldstein JL, Anderson RGW, Brown MS, Orci L (1979) Binding and internalization of ^{125}I -LDL in normal and mutant human fibroblasts: A quantitative autoradiographic study. *Exp Cell Res* 121:135-142
29. Bilheimer DW, Ho YK, Brown MS, Anderson RGW, Goldstein JL (1978) Genetics of the low density lipoprotein receptor. Diminished receptor activity in lymphocytes from heterozygotes with familial hypercholesterolemia. *J Clin Invest* 61:678-696
30. Willingham MC, Maxfield FR, Pastan I (1979) α -2 macroglobulin binding to the plasma membrane of cultured fibroblasts: Diffuse binding followed by clustering in coated regions. *J Cell Biol* 82:614-625
31. Hubbard AL, Wilson G, Ashwell G, Stukenbrok H (1979) An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. I. Distribution of ^{125}I -ligands among the liver cell types. *J Cell Biol* 83:47-64
32. Hubbard AL, Stukenbrok H (1979) An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. II. Intracellular fates of the ^{125}I -ligands. *J Cell Biol* 83:65-81
33. Carpentier J-L, Gorden P, Freychet P, LeCam A, Orci L (1979) Relationship of binding to internalization of ^{125}I -insulin in isolated rat hepatocytes. *Diabetologia* 17:379-384
34. Jarett L, Smith RM (1975) Ultrastructural localization of insulin receptors on adipocytes. *Proc Natl Acad Sci USA* 72:3526-3530
35. Jarett L, Smith RM (1977) The natural occurrence of insulin receptors in groups on adipocyte plasma membranes as demonstrated with monomeric ferritin-insulin. *J Supramol Struct* 6:45-59
36. Orci L, Rufener C, Malaisse-Lagae F, Blondel B, Amherdt M, Bataille D, Freychet P, Perrelet A (1975) A morphological approach to surface receptors in islet and liver cells. *Isr J Med Sci* 11:639-655
37. Pearse BMF (1976) Clathrin: A unique protein associated with intracellular transfer of membrane by coated vesicles. *Proc Natl Acad Sci USA* 73:1255-1259
38. Woods JW, Woodward MP, Roth TF (1978) Common features of coated vesicles from dissimilar tissues: Composition and structure. *J Cell Sci* 30:87-97
39. Barazzone P, Carpentier J-L, Gorden P, Van Obberghen E, Orci L (Submitted for publication) Redistribution of ^{125}I -iodine-labeled insulin on the plasma membrane of cultured human lymphocytes.
40. Carpentier J-L, Van Obberghen E, Gorden P, Orci L (Submitted for publication) ^{125}I -anti-insulin receptor antibody binding to cultured human lymphocytes: Morphological events are similar to the binding of ^{125}I -insulin.
41. Silverstein SC, Steinman RM, Cohn ZA (1977) Endocytosis. *Annu Rev Biochem* 46:669-722

42. Carpentier J-L, Gorden P, Freychet P, Canivet B, Orci L (Submitted for publication) The fate of ^{125}I -EGF in isolated hepatocytes: A quantitative electron microscopic autoradiographic study.
43. Bergeron JJM, Evans WH, Geschwind II (1973) Insulin binding to rat liver Golgi fractions. *J Cell Biol* 59:771–776
44. Posner BI, Josefsberg Z, Bergeron JJM (1978) Intracellular polypeptide hormone receptors: Characterization of insulin binding sites in Golgi fractions from the liver of female rats. *J Biol Chem* 253:4067–4072
45. Bergeron JJM, Posner BI, Josefsberg Z, Sikstrom R (1978) Intracellular polypeptide hormone receptors: The demonstration of specific binding sites for insulin and hGH in Golgi fractions isolated from the liver of female rats. *J Biol Chem* 253:4058–4066
46. Goldfine ID, Smith GJ (1976) Binding of insulin to isolated nuclei. *Proc Natl Acad Sci USA* 73:1427–1431
47. Goldfine ID, Smith GJ, Wong KY, Jones AL (1977) Cellular uptake and nuclear binding of insulin in human cultured lymphocytes: Evidence for potential intracellular site of insulin action. *Proc Natl Acad Sci USA* 74:1368–1372
48. Vigneri R, Goldfine ID, Wong KY, Smith GJ, Pezzino V (1978) The nuclear envelope: The major site of insulin binding in rat liver nuclei. *J Biol Chem* 253:2098–2103
49. Goldfine ID, Vigneri R, Cohen D, Plam NB, Kahn CR (1977) Intracellular binding sites for insulin are immunologically distinct from those on the plasma membrane. *Nature* 269:698–700
50. Fambrough DM, Devreotes PN (1978) Newly synthesized acetylcholine receptors are located in the Golgi apparatus. *J Cell Biol* 76:237–244
51. Goldstein JL, Anderson RG, Brown MS (1979) Coated pits, coated vesicles, and receptor-mediated endocytosis. *Nature* 279:679–685
52. Brown MS, Goldstein JL (1979) Receptor-mediated endocytosis: Insights from the lipoprotein receptor system. *Proc Natl Acad Sci USA* 76:3330–3337
53. Steiner DF (1977) Insulin today. *Diabetes* 26:322–340
54. Goldfine ID (1977) Does insulin need a second messenger. *Diabetes* 26:148–155
55. Das M, Fox CF (1978) Molecular mechanism of mitogen action: Processing of receptor induced by epidermal growth factor. *Proc Natl Acad Sci USA* 75:2644–2648
56. LeCam A, Maxfield F, Willingham M, Pastan I (1979) Insulin stimulation of amino acid transport in isolated rat hepatocytes is independent of hormone internalization. *Biochem Biophys Res Commun* 88:873–881
57. Hammons GT, Jarrett L (1979) Dissociation of insulin stimulated glucose transport from insulin degradation by lysosomes in rat adipocytes. *Diabetes* 28:389
58. Marshall S, Olefsky JM (1979) Effects of lysosomotropic agents on insulin interactions with adipocytes. Evidence for a lysosomal pathway for insulin processing and degradation. *J Biol Chem* 254:10153–10160
59. Flier JS, Kahn CR, Roth J, Bar RS (1975) Antibodies that impair insulin receptor binding in an unusual diabetic syndrome with severe insulin resistance. *Science* 190:63–65
60. Jarrett DB, Roth J, Kahn CR, Flier JS (1976) Direct method for detection and characterization of cell surface receptors for insulin by means of ^{125}I -labeled autoantibodies against the insulin receptor. *Proc Natl Acad Sci USA* 73:4115–4119
61. Kahn CR, Baird K, Flier JS, Jarrett DB (1977) Effects of autoantibodies to the insulin receptor on isolated adipocytes: Studies of insulin binding and insulin action. *J Clin Invest* 60:1094–1106
62. Le Marchand-Brustel Y, Gorden P, Flier JS, Kahn CR, Freychet P (1978) Anti-insulin receptor antibodies inhibit insulin binding and stimulate glucose metabolism in skeletal muscle. *Diabetologia* 14:311–317
63. Kahn CR, Baird KL, Jarrett DB, Flier JS (1978) Direct demonstration that receptor crosslinking or aggregation is important in insulin action. *Proc Natl Acad Sci USA* 75:4209–4213
64. Mortimore GE, Tietze F (1959) Studies on the mechanism of capture and degradation of insulin- ^{131}I by the cyclically perfused rat liver. *Ann N Y Acad Sci* 82:239–337
65. Terris S, Steiner DF (1976) Retention and degradation of ^{125}I -insulin by perfused livers from diabetic rats. *J Clin Invest* 57:885–896
66. Freychet P, Kahn R, Roth J, Neville DM Jr (1972) Insulin interactions with liver membranes: Independence of binding of the hormone and its degradation. *J Biol Chem* 247:3953–3961
67. LeCam A, Freychet P, Lenoir P (1975) Degradation of insulin by isolated rat liver cells. *Diabetes* 24:566–573
68. Carpenter G, Cohen S (1976) ^{125}I -labeled human epidermal growth factor binding, internalization and degradation in human fibroblasts. *J Cell Biol* 71:159–171
69. Gorden P, Carpentier J-L, Freychet P, Orci L (1979) Receptor linked ^{125}I -insulin degradation is mediated by internalization. *Clin Res* 27:485
70. Ascoli M, Puett D (1978) Degradation of receptor-bound human choriogonadotropin by murine Leydig tumor cells. *J Biol Chem* 253:4892–4899
71. Marshall S, Olefsky JM (1979) Effects of lysosomotropic agents on insulin interactions with adipocytes. Evidence for a lysosomal pathway for insulin processing and degradation (abstract). Presented at the 61st Annual Meeting of the Endocrine Society, Anaheim, CA, p 118
72. Terris S, Hofmann C, Steiner DF (1979) Mode of uptake and degradation of ^{125}I -labelled insulin by isolated hepatocytes and H4 hepatoma cells. *Can J Biochem* 57:459–468
73. Suzuki K, Kono T (1979) Internalization and degradation of fat cell-bound insulin. *J Biol Chem* 254:9786–9794
74. Hammons GT, Jarrett L (1979) Uptake of ^{125}I -insulin into a lysosomal compartment in rat adipocytes. *Fed Proc* 38:1024
75. Grisolia S, Wallace R (1976) Insulin degradation by lysosomal extracts from rat liver: Model for a role of lysosomes in hormone degradation. *Biochem Biophys Res Commun* 70:22–27
76. Ansorge S, Bohley P, Kirschke J, Langner J, Hanson H (1971) Metabolism of insulin and glucagon: Breakdown of radioiodinated insulin and glucagon in rat liver cell fractions. *Eur J Biochem* 19:283–288
77. Kahn CR, Baird KL (1978) The fate of insulin bound to adipocytes: Evidence for compartmentalization and processing. *J Biol Chem* 253:4900–4906
78. Kahn CR, Neville DM Jr, Roth J (1973) Insulin-receptor interaction in the obese-hyperglycemic mouse. A model of insulin resistance. *J Biol Chem* 248:244–250
79. Freychet P, Laudat MH, Laudat P, Rosselin G, Kahn CR, Gorden P, Roth J (1972) Impairment of insulin binding to the fat cell plasma membrane in the obese hyperglycemic mouse. *FEBS Lett* 25:339–342
80. Archer JA, Gorden P, Roth J (1975) Defect in insulin binding to receptors in obese man. Amelioration with calorie restriction. *J Clin Invest* 55:166–174
81. Bar RS, Gorden P, Roth J, Kahn CR, De Meyts P (1976) Fluctuations in the affinity and concentration of insulin receptors on circulating monocytes of obese patients. Effects of starvation, refeeding, and dieting. *J Clin Invest* 58:1123–1135
82. Olefsky JM (1976) Decreased insulin binding to adipocytes and circulating monocytes from obese subjects. *J Clin Invest* 57:1165–1172
83. Harrison LC, Martin FIR, Melick RA (1976) Insulin receptor

- binding in isolated fat cells and insulin sensitivity in obesity. *J Clin Invest* 58:1435–1441
84. De Fronzo RA, Soman V, Sherwin RS, Hendler R, Felig P (1978) Insulin binding to monocytes and insulin action in human obesity, starvation, and refeeding. *J Clin Invest* 62:204–213
85. Soll AH, Kahn CR, Neville DM Jr, Roth J (1975) Insulin receptor deficiency in genetic and acquired obesity. *J Clin Invest* 56:769–780
86. Haigler HT, McKanna JA, Cohen S (1979) Rapid stimulation of pinocytosis in human carcinoma cells A-431 by epidermal growth factor. *J Cell Biol* 83:82–90
87. Pollet RJ, Standaert ML, Haase BA (1977) Insulin binding to the human lymphocyte receptor. *J Biol Chem* 252:5828–5834
88. De Meyts P, Bianco AR, Roth J (1976) Site-site interactions among insulin receptors. *J Biol Chem* 251:1877–1888
89. Blackard WG, Guzelian PS, Small ME (1978) Down regulation of insulin receptors in primary cultures of adult rat hepatocytes in monolayer. *Endocrinology* 103:548–553

Dr. Ph. Gorden
Diabetes Branch
National Institute of Arthritis,
Metabolism and Digestive Diseases
National Institute of Health
Bethesda, MD 20205
USA