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INSULIN RECEPTORS

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INTRODUCTION

Insulin interacts with its target tissues initially by means of highly specific, high-affinity receptors located at the cell surface. Over the past several years, progress in the investigation of these receptors has been rapid. Insulin receptors have been purified, and much has been learned about their structure and organization in the membrane. It has been demonstrated that after insulin binds to its receptor on the cell surface, both insulin and receptor are internalized by an endocytic process. The fate of each in regard to the mechanism of insulin action and receptor down-regulation has been intensively scrutinized. Closely related studies on the kinetics of insulin receptor biosynthesis and turnover have also helped explain the mechanism by which cell surface insulin receptors are regulated. Perhaps the most intransigent question about insulin action has been, what is the immediate consequence of the insulin-receptor interaction that signals its diverse metabolic effects? It appears that researchers are making some progress toward solving this most difficult problem.

RECEPTOR STRUCTURE

Insulin receptor is an intrinsic membrane protein, but it can be solubilized with a variety of non-ionic detergents in a state that retains its insulin-binding properties (1, 2). The solubilized receptor has been purified by affinity chromatography using insulin-Sepharose (3–5) or anti-insulin receptor antibodies linked to Sepharose (6). These very different methods give similar results. The purified product, when analyzed by SDS-polyacrylamide gel electrophoresis, is composed of three polypeptides with approximate molecular weights of 135,000, 90,000, and 45,000 (4–7). [These components have been named α, β, and β_1, respectively (8)]. Similar com-

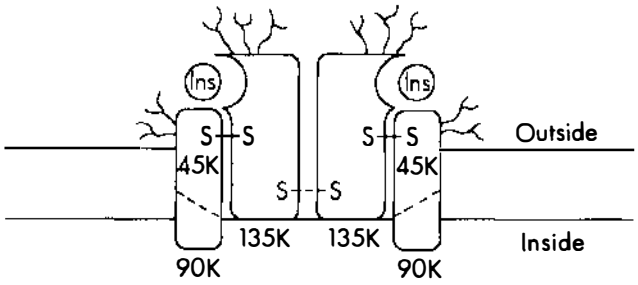

Figure 1 Schematic model of the proposed subunit structure of the insulin receptor. Branched structures symbolize complex carbohydrate chains. Broken lines connecting the disulfide bond between the 135K subunits indicate that this disulfide bond is sometimes reduced.

ponents have also been identified in affinity-labeling studies by using aryl azide derivatives of insulin to photoaffinity-label the receptor (7, 9–14), or by using the bifunctional reagent, disuccinimidyl suberate, to cross-link ^{125}I-insulin to its receptor (15–17). However, in affinity-labeling studies, α is the predominantly labeled component, and in some studies only that subunit is labeled.

Beta-1 appears to be a proteolytic fragment of β. The relative amounts of β_1 and β vary considerably in different preparations of purified receptor. If proteolysis is minimized by decreasing the length of the purification procedure and by including protease inhibitors in the buffers, there is a relative increase in the amount of β at the expense of β_1 (18). Furthermore, peptide maps show a considerable degree of homology between these two peptides (18, 19). In addition, treatment with elastase or lysosomal proteases clips β to form a peptide similar to β_1 (19).

Some data obtained from photoaffinity-labeling studies are difficult to reconcile with the hypothesis that β_1 is a proteolytic fragment of β (12). In isolated adipocytes, $N^{\epsilon\beta\text{-}29}$-(azidobenzoyl) insulin photoaffinity-labels both α and β, and in addition a polypeptide of 40,000 molecular weight, which is in a molecular weight range similar to that of β_1. In contrast, $N^{\alpha\beta 1}$-(azidobenzoyl) insulin photoaffinity-labels α and a 40,000 molecular weight polypeptide, but not β (12). Clearly, if the 40,000 molecular weight photoaffinity-labeled polypeptide is β_1, and β_1 is a proteolytic fragment of β, it is difficult to understand why $N^{\alpha\beta 1}$-(azidobenzoyl) insulin would label it but not β. One possible explanation is that the 40,000 molecular weight photoafffinity-labeled peptide is not β_1. Its molecular weight as determined by SDS-polyacrylamide gel electrophoresis is somewhat smaller than most laboratories have reported for β_1, and a 40,000 molecular weight proteolytic fragment of α is sometimes seen in affinity-labeling studies (19). Therefore, at the present time, the bulk of evidence indicates that β_1 is a proteolytic fragment of β.

It is not clear if proteolytic clipping of β to generate β_1 is merely an in vitro artifact, or if it represents physiologically important processing of the receptor. Consistent with the latter possibility, β_1 is found in a variety of target tissues for insulin in several different species by using several different methods. Insulin receptor is internalized following hormone binding (see below) and may be exposed to lysosomal enzymes, providing a possible mechanism for proteolytic processing.

The molecular weight of the native insulin receptor, as determined by gel exclusion chromatography and sucrose density centrifugation under non-denaturing conditions, is about 350,000 (5, 21–23). This high molecular weight species is a disulfide-linked heterotetramer containing two copies of α and two copies of β (16–18, 20). Only when the receptor is denatured and treated with disulfide reducing agents are isolated subunits generated (16–20). The receptor exists in two states (8, 20). In the predominant state, all four subunits are covalently coupled by disulfide bonds. A smaller fraction of the receptor exists in a partially reduced state in which the disulfide bond linking two α-β heterodymers is reduced (8, 20).

The functional significance of these two states of the receptor is not clear. Several investigators have found that the native receptor will dissociate into a smaller, lower-affinity form (24–29). This form may represent partially reduced receptor (22), but this has not yet been demonstrated.

Treatment of receptor with disulfide reducing agents produces variable effects on the affinity of insulin binding, depending upon the tissue studied. In human placenta, there is a marked decrease (31); in rat fat cells and human erythrocytes, there is a marked increase (32, 33); in rat liver, there is only a small change (31, 32). In addition to interchain disulfide bonds, insulin receptor also appears to have intrachain disulfide bonds (8). In these studies, it is not clear which is the critical disulfide bond whose reduction results in the observed change in insulin binding affinity. In fact, it has been suggested that the effects of disulfide reduction on insulin binding may not directly involve the structure of the receptor, but rather the manner in which receptors are arranged in clusters in the membrane (32). This does not appear to be the case with placenta receptor, since similar changes in affinity are seen when insulin binds to the reduced receptor after it has been solubilized (31).

As discussed above, in affinity-labeling studies, α is predominantly labeled. Therefore, it is likely that this subunit is important in insulin binding. However, both β and its β_1 fragment are sometimes also labeled. Therefore, β and in particular its β_1 portion may contribute to the insulin binding site, or at least must be in close proximity to it. The contributory role of β_1 is also suggested by radiation inactivation studies (34). In a preparation of solubilized insulin receptors that was found by affinity labeling to be composed of $(\alpha\beta_1)_2$ tetramers, the functional target size of the insulin binding

unit was 170,000, which would correspond to the $\alpha\beta_1$ dimer (34). It is not clear from this type of study whether β participates directly in insulin binding by making contact with the insulin molecule, or whether it stabilizes the α subunit in the proper conformation for insulin binding.

The twofold symmetry of the subunit structure of the insulin receptor, and the fact that the target size of the insulin-binding unit is approximately one half the molecular weight of the receptor, suggest that each receptor can bind two molecules of insulin. Although there is no direct evidence for this, there is circumstantial evidence that the receptor is multivalent. Antibodies that bind to the insulin receptor and compete with insulin have been described (35). At high insulin concentrations antibody binding is inhibited; at high antibody concentrations insulin binding is inhibited. However, tracer amounts of ^{125}I-insulin bound to solubilized receptor can be quantitatively immunoprecipitated by appropriately low concentrations of the antibody (36). For this to occur, the ^{125}I-insulin must be binding to one available site on the receptor, and the antibody binding to a second unoccupied site on the same receptor.

Because of its susceptibility to glycosidases (37–39), its interaction with lectins (40, 41), and the fact that tunicamycin inhibits its biosynthesis (42–44), the insulin receptor is clearly a glycoprotein. Digestion of the receptor with neuraminidase alters the mobility of both the α and β subunits, as well as the β_1 fragment, which demonstrates that all three components contain sialic acid (20, 45, 46). Both the α and β subunits biosynthetically incorporate galactose, glucosamine, fucose, and mannose, although the proportion of the various sugars incorporated into each subunit is different (47). Thus, both α and β appear to be glycoproteins of the complex type. Glycosylation of the receptor may be necessary for delivery of newly synthesized receptor to the cell membrane and for some post-translational modification of the receptor through which it acquires insulin-binding activity, since in the presence of tunicamycin, receptor accumulates at some intracellular site in a nonglycosylated form, which is not able to bind insulin (44).

The major function of insulin receptors involves transmission of information from the exterior of the cell to the interior. The possible mechanisms for accomplishing this would be limited, depending upon whether or not the receptor is a transmembrane protein. Recent data suggest that the receptor is indeed a transmembrane protein. Since the carbohydrate portion of cell membrane glycoproteins is located exclusively on the external surface, α, β, and β_1 must all be exposed on the external surface. Consistent with this idea, α, β, and β_1 can be affinity-labeled with insulin under conditions in which insulin would not have access to the cytoplasmic surface of the membrane (11, 12, 14, 17, 19). In addition, α, β, and β_1 have been labeled

in intact cells by surface labeling techniques such as lactoperoxidase (46,48, 49), periodate, and galactose oxidase (47).

The β subunit also appears to be exposed on the internal surface of the membrane. Under certain conditions, a portion of the cell's insulin receptors is located in membranes of intracellular vesicles (see below). The orientation of the membrane of these vesicles is reversed so that the cytoplasmic surface is external. Lactoperoxidase surface labeling of these vesicles labels the β subunit of the insulin receptors (50). Under these conditions, the α subunit is not labeled. However, if the vesicles are disrupted, both subunits are labeled (50). This indicates that the β subunit spans the membrane and is exposed on the cytoplasmic surface. This arrangement is also suggested by the fact that the β subunit is phosphorylated in intact cells upon exposure to insulin (51).

RECEPTORS FOR INSULIN-LIKE GROWTH FACTORS

Insulin belongs to a family of biologically active polypeptides that share a considerable degree of similarity in primary and secondary structure, and probably have a common evolutionary origin (52). This family includes insulin-like growth factor I (IGF I), also known as somatomedin C, insulin-like growth factor II (IGF II), the human homologue of rat multiplication-stimulating activity, relaxin, and a very distant relative, nerve growth factor. Each peptide has its own receptor, but some peptides also cross-react with lower affinity with receptors for other members of the family. For example, IGF I binds to the IGF II receptor with a relatively high affinity, and vice versa (53–56). Both peptides bind to the insulin receptor, but with considerably lower affinity (53–56). Insulin binds to the IGF I receptor with low affinity (53–55), but has little or no affinity for IGF II receptors (54, 55, 57). Proinsulin has weak affinity for relaxin receptor (58). In view of this cross-reactivity, some structural similarities amongst the receptors for these peptides are perhaps to be expected, and have indeed been found.

Receptors for both IGF I and IGF II have been identified and characterized by affinity-labeling techniques. The IGF I receptor appears to have a subunit structure remarkably similar to that of the insulin receptor. It is a glycoprotein with a molecular weight of approximately 350,000 (59). When affinity-labeled IGF I receptors are analyzed by SDS-polyacrylamide gel electrophoresis without prior reduction, high molecular weight species with mobilities similar to unreduced forms of the insulin receptors are present (54, 55, 57, 59–61). After complete reduction, a prominent species with an apparent molecular weight of 135,000, which corresponds to the isolated α subunit of the insulin receptor, is present (54, 55, 57, 59–61). In some

studies, a faint band with an apparent molecular weight of approximately 90,000 is also seen (55). This band, which is more prominent when the receptor is labeled with lactoperoxidase and immunoprecipitated with antibodies directed against the IGF I receptor (F. C. Kull, S. Jacobs, P. Cuatrecasas, and J. J. Van Wyk, unpublished findings),
subunit of the insulin receptor, which is also only weakly labeled in
labeling studies. Treatment with low concentrations of reducing agents yields partially reduced forms of the receptor that have mobilities similar to those of partially reduced forms of the insulin receptor (60). Thus, the IGF I receptor, like the insulin receptor, is a disulfide-linked heterotetramer with a subunit structure $(\alpha\beta)_2$, where α is approximately 135,000 and β is approximately 90,000.

IGF I receptors also share immunological similarities with insulin receptors. Antisera from a patient with acanthosis nigricans and insulin resistance inhibit the binding of both insulin and IGF I to their respective receptors (62). Although these antisera are polyclonal, the relative titers for the two activities fluctuated
that both activities are due to a common population of antibodies (62). Recently, several monoclonal antibodies have been produced that preferentially recognize either insulin receptors or IGF I receptors [(49), unpublished observations]; however, each antibody does cross-react with both receptors, clearly demonstrating that the two receptors are immunologically related.

The IGF II receptor, at least superficially, appears to have a structure very different from the insulin and IGF I receptor. Analysis of the affinity cross-linked receptor indicates that it is a single polypeptide that is not disulfide-linked
molecular weight of 260,000 when reduced and 225,000 under nonreducing conditions (54, 55, 57). This difference in molecular weight is probably due to the presence of intrachain disulfide bonds, which prevent the complete unfolding of the receptor in SDS.

Considering the remarkable similarity between insulin and IGF I receptors, it is surprising that IGF II receptors would be so different. In fact, this difference may be more apparent than real. A 200,000 molecular weight peptide that is labeled early after cells are incubated with [^{35}S]methionine and is immunoprecipitated by antibodies to insulin receptor has been identified and proposed as a biosynthetic precursor of the insulin receptor (63, 64). It is possible that proteolytic processing of this putative precursor generates both α and β subunits. It might be expected, then, that this 200,000 molecular weight precursor would be homologous to the single polypeptide subunit of the IGF II receptor, which would contain regions corresponding to both the α and β subunits in a single linear sequence.

During posttranslational processing of insulin and IGF I receptors, but not IGF II receptors, two precursor molecules would be linked by disulfide bonds and then cleaved to generate a tetramer. This scheme is, of course, only hypothetical.

RECEPTOR-MEDIATED INTERNALIZATION OF INSULIN

After binding to receptors on the surface of the cell, insulin along with its receptor is internalized. Several lines of evidence indicate that this occurs. In intact cells, insulin is degraded by a receptor-mediated process (65–71). Receptor-mediated degradation occurs only after an absolute lag and is inhibited by agents that block the production of metabolic energy or disrupt microtubules, which suggests that translocation of insulin is required (65, 67, 72, 73). When incubated with intact cells, insulin gradually becomes resistant to dissociation by acid, is inaccessible to trypsin (74), and can be recovered along with intracellular vesicles when the cells are disrupted and fractionated by density centrifugation (75–79). Lysosomotropic agents, such as chloroquine and methylamine, block receptor-mediated degradation, which implicates lysosomes as the site of degradation (65, 67, 69, 72, 73, 80, 81). Furthermore, insulin labeled with fluorescent probes (82), ferritin (73), or ^{125}I (83–91) has been shown by fluorescent microscopy, electron microscopy, or electron microscopic autoradiography to be specifically internalized.

Several areas related to receptor-mediated internalization of insulin have been intensively investigated: What is the pathway and fate of internalized insulin? What is the pathway and fate of internalized receptor? How does this relate to insulin receptor down-regulation? What role does insulin internalization have in its mechanism of action?

Pathway and Fate of Internalized Insulin

Insulin initially binds to receptors on the cell surface (73, 82–85). Occupied receptors rapidly redistribute to form clusters or patches (73, 82, 89, 92, 93). In some cells [e.g. fibroblasts (82), IM-9 lymphocytes (92), and 3T3-L1 cells (93)], these clusters form in coated pits, which are invaginated regions of the cell membrane surrounded by an electron-dense cage composed primarily of clathrin, a high molecular weight protein. Coated pits rapidly pinch off or bud to form small, thin-wall vesicles that contain internalized insulin. In some cells (e.g. liver and adipocytes) insulin is internalized by a mechanism that has not been observed to involve coated pits (73, 90). Initially, the vesicles that contain internalized insulin are located adjacent to the plasma membrane. They then rapidly migrate to the Golgi region of the cell;

en route, they enlarge, presumably by fusing with each other or with other intracellular vesicles. In liver, at 37°, 10 minutes following a bolus of insulin most cell-associated insulin is in vesicles in the Golgi region of the cell (90).

The origin and nature of these vesicles is not entirely clear. Although many morphologically resemble lysosomes (73, 86–90, 92, 93), they do not appear to be classical lysosomes. Only a small fraction of these vesicles stain for lysosomal enzyme markers, such as acid phosphatase (90). On subcellular fractionation, they do not sediment with the major lysosomal fraction (76–79), nor is their density shifted by accumulation of Triton WR-1339 as is characteristic for lysosomes (77, 79, 92). It has been suggested that these are Golgi-derived vesicles (90). Many contain very low-density lipoprotein-like particles (76, 77, 90), which are thought to be markers of Golgi vesicles, and upon subcellular fractionation, they sediment with the Golgi fraction (76–79). However, this Golgi-enriched fraction is not homogeneous (77). It is possible that it might contain vesicles having a different origin (coalescence of endocytic vesicles) or a mixed origin (e.g. fusion of endocytic vesicles with elements of Golgi, GERL, or the lysosomal compartment). Consistent with this, when this Golgi-enriched fraction is further fractionated on a Percol gradient, a small population of dense vesicles, relatively enriched in lysosomal enzyme markers, is present (77). At later times, a significant fraction of internalized insulin is found in these vesicles, which may represent a transition to lysosomes (77, 94). Whatever the origin of these vesicles, it is important to emphasize that even those investigators who propose that Golgi vesicles are structures in which internalized insulin accumulates find little or no insulin in *cis* or *trans* Golgi stacks (90).

In addition to these vesicular structures, some investigators have found intracellular insulin to be concentrated in other intracellular structures, in particular the nuclear envelope and the endoplasmic reticulum (91, 95). Other investigators have not observed this (96). The reason for this discrepancy is not clear.

One fate of internalized insulin is degradation. The quantitative importance of degradation varies depending upon the cell type. In hepatocytes, the majority of internalized insulin is degraded. In adipocytes, only 30% of internalized insulin is degraded, the remainder being extruded into the medium intact (69). In IM-9 cells, only a small fraction of internalized insulin is degraded (97, 98). It has been generally assumed that lysosomes are the site of insulin degradation, as receptor-mediated degradation of insulin is blocked by lysosomotropic agents, such as chloroquine and methylamine. However, these lysosomotropic agents are not entirely specific for lysosomes. These agents are amines that are permeable to biological membranes when deprotonated but impermeable when protonated. They therefore diffuse into lysosomes where, because of the low pH, they are

protonated and trapped. This is thought to be a mechanism for their specificity for lysosomes. However, both endocytic vesicles and Golgi have an acidic pH (99). Therefore, it is not surprising that lysosomotropic agents have been shown to accumulate in these organelles and interfere with their function (100–102). When cells are treated with chloroquine, insulin degradation is inhibited, and internalized insulin accumulates in intracellular vesicles that do not appear to be classical lysosomes. They have low levels of acid phosphatase, contain very low-density lipoprotein particles, and have different sedimentation properties than lysosomes (100). This has been interpreted as indicating that insulin is degraded in these vesicles rather than in classical lysosomes, or that chloroquine blocks the normal transfer of insulin from these vesicles to lysosomes.

The Fate of Internalized Receptor

The receptor does not share the same fate as internalized insulin. This is quite clear from studies comparing the kinetics of receptor-mediated insulin degradation with the kinetics of insulin receptor turnover. For example, when hepatocytes are incubated with 10^{-8} M insulin at 30°C, 2×10^5 insulin molecules per hour per cell are degraded (103). If receptors were internalized and degraded at the same rate, 164% of receptors originally present on the cell surface would be destroyed in the first hour. Yet incubation of cells with this concentration of insulin for up to 2.5 hours does not cause decreased insulin binding, even if new receptor synthesis is inhibited by cycloheximide (103). Similar results have been obtained in a number of other tissues (104–106). In chick liver cells, the rate of turnover of insulin receptor has been measured directly by the heavy isotope density shift technique (104). It is more than 200-fold less rapid than the rate of receptor-mediated insulin degradation; and in this cell type, it is not altered by the presence of insulin (104).

This kinetic evidence suggests that the receptor either remains on the cell surface when insulin is internalized or is internalized along with insulin, but at some point dissociates from insulin, escapes degradation, and is recycled to the cell membrane. Evidence suggests the latter possibility. Incubation of adipocytes with insulin in simple buffers results in a tissue- and temperature-dependent decrease in the number of cell surface insulin receptors, but an increase in the number of intracellular receptors (107). If chloroquine is also present, loss of surface receptors is compensated by the increase in intracellular receptors. However, in the absence of chloroquine, the increase in intracellular receptors is not sufficient to account for the loss of surface receptors, and there is a decrease in the number of total cellular receptors, suggesting that when chloroquine is absent, a fraction of the internalized receptors are degraded, perhaps by a lysosomal process (107). Interestingly,

when fat cells are incubated with insulin in complete medium rather than in simple buffer, the number of cell surface receptors remains unchanged after exposure to insulin, although the cells bind, internalize, and degrade insulin in a similar fashion under both incubation conditions (105, 108). In the presence of chloroquine or ammonium chloride, however, there is a rapid net translocation of receptors from the cell surface of cells, even if they are incubated in complete medium (105). These results have been interpreted as follows (105): Both insulin and its receptor are rapidly internalized; however, in complete medium, internalized receptor is rapidly recycled to the cell membrane and there is no net change in the number of surface insulin receptors. Chloroquine and ammonium chloride interfere with the process of recycling of receptors, causing a rapid loss of receptors from the cell surface and their intracellular accumulation. Thus, lysosomotropic agents have two effects on insulin-receptor dynamics. They inhibit receptor degradation and block receptor recycling.

Photoaffinity-labeling studies also provide evidence that occupied insulin receptors are internalized and recycled to the cell membrane. When isolated adipocytes or hepatocytes are photoaffinity-labeled with aryl azide derivatives of insulin at 15° and the cells are then warmed to 37°, the label receptors are rapidly internalized, as indicated by their loss of sensitivity to trypsin (109, 110) and by electron microscopic radioautography (109). In hepatocytes, SDS polyacrylamide gel electrophoresis of the labeled receptors, at times after internalization has occurred, reveals no evidence for processing (14, 109). Instead, there appears to be recycling of internalized receptor back to the cell surface (109). This recycling is apparent both by electron miscroscopic radioautography and by the fact that labeled receptors that had become resistant to trypsin once again became sensitive at a later time. In fat cells, a portion of the internalized receptors is proteolytically processed (110). Interestingly, in IM-9 cells, photoaffinity-labeled insulin receptor does not appear to be internalized, but instead is shed into the medium (112).

It may be argued that photoaffinity-labeling the receptor may drastically alter the pathway it will follow. Since insulin and receptor are covalently coupled, they must follow each other. This objection is overcome by directly labeling cell surface receptors with ^{125}I by lactoperoxidase and then immunoprecipitating it with anti-receptor antibodies. When these studies are carried out in fat cells (50), surface-labeled receptor is found initially almost exclusively in the plasma membrane fraction. When cells are incubated with insulin after 30 minutes, a significant portion of labeled receptor is found in the Golgi fraction. If the cells are incubated in the absence of insulin, labeled receptors remain with the plasma membrane fraction, suggesting that receptor internalization is mediated by insulin binding. With this tech-

nique (50), in contrast to photoaffinity labeling (110), no proteolytic degradation of internalized receptor is found.

The evidence cited above suggests the following pathways for receptor: After binding insulin, surface receptors are internalized. In most cells, the major fraction of internalized receptor is recycled to the cell membrane; internalized receptor can also be sequestered in an intracellular pool or proteolytically degraded, perhaps in lysosomes. The flux of receptors through each of these possible pathways depends upon the cell type and the conditions of incubation.

Although insulin and receptor enter the cell together, their ultimate fate within the cell may diverge. A hypothetical scheme that would explain this is illustrated in Figure 2. Possibly of central importance is the finding that endocytic vesicles develop an acid pH (5.0) as they mature (99). Since insulin-receptor binding is very pH-sensitive (113), this would cause insulin to dissociate from its receptor. It is then postulated that receptors are sequestered in one region of the vesicle, which would eventually bud off. This could result from an interaction of the receptor with cytoplasmic peripheral membrane proteins or elements of the cytoskeleton. The derived vesicle, enriched in receptor, would ultimately be destined for recycling to the cell membrane. This recycling may occur directly, or perhaps with some delay that might involve mixing with an intracellular pool of receptors. The remaining vesicle, depleted of receptors, but containing free insulin, would fuse with lysosomes. If the process of separating receptors from free insulin is not complete, some free insulin might accompany the receptor-enriched vesicle to the cell surface and be released into the medium. Similarly, some receptors may be incorporated into the receptor-depleted vesicle and be delivered to lysosomes for proteolytic degradation.

Receptor Down-Regulation

Exposure to insulin either in vivo or in vitro results in a time- and insulin concentration-dependent decrease in the number of functioning cell surface insulin receptors (114–122). There are two possible mechanisms by which insulin-mediated receptor internalization could result in this process of down-regulation: intracellular sequestration of internalized receptor and degradation of internalized receptor. Evidence has been described for both of these mechanisms. Down-regulation in chick hepatocytes results from a translocation of cell surface receptors to an internal pool, with no change in the total number of cellular receptors, and no change in either the rate of synthesis or degradation of receptors (117). In several other tissues, down-regulation is associated with a decrease in the total number of cellular receptors (46, 107, 123–127). This results from an increased rate of degradation of receptors with no change in their rate of synthesis (125–127).

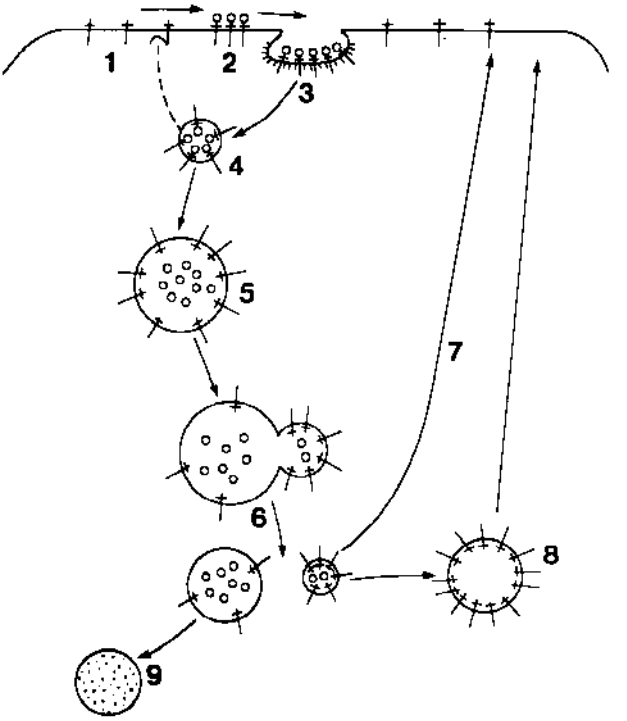

Figure 2 Possible pathways for internalized insulin and receptor. 1. Unoccupied receptors distributed diffusely over the cell surface. In some cells, receptors are preferentially localized to the microvillous surface. 2. Insulin binding induces clustering of receptors. 3. In some cells clusters form in coated pits. 4. Insulin bound to receptors in an endocytic vesicle adjacent to the cell surface. It is possible that a small fraction of these vesicles may be short circuited to the cell membrane. 5. Endocytic vesicles rapidly move to the Golgi region of the cell. As they do so, they enlarge, probably fusing with other intracellular vesicles, and the pH falls. It is postulated that the low pH would cause insulin and receptors to dissociate. 6. It is postulated that receptors would be sequestered in one region of the vesicle, which would pinch off. 7. The derived vesicle, enriched in receptor, but perhaps containing a small amount of insulin, would then migrate to the cell surface and fuse with the plasma membrane. 8. Alternatively, it might fuse with vesicles comprising an intracellular pool of receptors, and be delivered to the cell surface after a delay. 9. The derived vesicle, depleted of receptors, but perhaps containing some, would fuse with lysosomes, resulting in the degradation of its contents.

CONSEQUENCES OF INSULIN RECEPTOR BINDING: TRANSMEMBRANE SIGNALING

The effects of insulin on cellular metabolism are global. A wide variety of metabolic pathways and substrate fluxes are altered by insulin; a number of enzymes are altered shortly after cells are exposed to insulin. The action

of insulin on these enzymes is not direct. The intervening steps between their modification and insulin-receptor binding is unknown. However, recent findings may be important in clarifying this area.

Tyrosyl Phosphorylation

The β subunit of the insulin receptor is phosphorylated in intact cells, and insulin stimulates its phosphorylation (51). Under basal conditions, only phosphoserine residues are present; however, insulin stimulates the phosphorylation of tyrosine residues (128). This may be particularly significant because in other systems, tyrosine phosphorylation has been implicated in initiating a cellular proliferative response. Several viral oncogenes and their cellular homologs code for tyrosine-specific protein kinases (129–132). All of these are autophosphorylated and also phosphorylate other cellular proteins. In addition, epidermal growth factor stimulates tyrosine phosphorylation of its own receptor, as well as other membrane proteins (133–136). This effect is due to intrinsic tyrosine-specific protein kinase activity of the receptor (135, 136).

It is not yet clear if the insulin receptor is a protein kinase or if it is only a substrate for an extrinsic protein kinase. The functional significance of receptor phosphorylation is also unclear. It is possible that it alters the affinity of insulin binding, is a signal for insulin receptor internalization and down-regulation, or plays a role in initiating some of the biological responses to insulin. In support of the latter possibility, vanadate, a potent inhibitor of tyrosine-specific phosphoprotein phosphatase (137), mimics many of the biological effects of insulin (138–140).

Intracellular Mediators

Addition of insulin to a broken cell preparation containing plasma membranes and mitochondria results in activation of mitochondrial pyruvate dehydrogenase (141–143). Activation of pyruvate dehydrogenase is not due to a direct effect of insulin on mitochondria, since it does not occur when insulin is added directly to purified mitochondria; cell membranes are required (141). This finding suggests that interaction of insulin with its receptor on the cell membrane generates a mediator that activates pyruvate dehydrogenase. Indeed, insulin stimulates the production of a substance, which can be extracted from fat cells or fat cell membranes, that, when added to purified mitochondria, stimulates pyruvate dehydrogenase (144, 145). Similar substances have been isolated from skeletal muscle and liver (146–148). In addition to their effects on pyruvate dehydrogenase, these substances activate glycogen synthase (146) and low K_m cyclic AMP phosphodiesterase (149, 150), and inhibit cyclic AMP-dependent protein kinase (146). A low molecular weight substance that stimulates RNA polymerase

II when added to isolated nuclei has also been extracted from liver perfused with insulin (151), and in a mixed membrane, ribosomal preparation from 3T3-L1 adipocytes, insulin stimulates phosphorylation of ribosomal S_6 protein (152). The effects on pyruvate dehydrogenase and glycogen synthase appear to result from activation of phosphoprotein phosphatases, which convert these enzymes to a dephosphorylated, active form (146, 153, 154).

The chemical nature of this putative mediator substance or substances is not clear. Most investigators agree that it has a molecular weight in the range of 1000–2000 (146, 148, 154). Early studies found that it is partially sensitive to proteases, which suggests that it is a polypeptide (146, 155). Treatment of membranes with low concentrations of trypsin stimulated its production, whereas protease inhibitors blocked its production by insulin, and also inhibited the action of insulin (155, 156). This suggested that insulin-receptor interaction might activate a protease that cleaves the mediator from a precursor present in membranes. More recently, phospholipids have been found in partially purified preparations of mediator and have been shown to have certain properties similar to mediator (157). However, it is not clear if insulin stimulates the production of these phospholipids.

The effects of crude preparations of mediator on glycogen synthase and pyruvate dehydrogenase are biphasic (141, 145–148). When high concentrations of insulin are used to produce mediator, or when concentrated preparations of mediator are used, less activation or even inhibition of these enzymes occurs. One possible explanation for these findings is that crude preparations of mediator contain an inhibitor of these enzymes, as well as an activator. Consistent with this explanation, inhibitory and stimulatory activities have been resolved from these preparations for glycogen synthase and pyruvate dehydrogenase (158, 159). Insulin stimulates the production of both these substances, and after their resolution, the effects of insulin are no longer biphasic. Fractions containing the resolved inhibitor of pyruvate dehydrogenase also contain an inhibitor of adenylate cyclase whose production is stimulated by insulin (159). It is likely that the same substance possesses both activities.

Insulin-Receptor Internalization

The clear demonstration that both insulin and its receptor are internalized has revived the question of whether a direct interaction of insulin with intracellular organelles is required for some of its effects. It should be appreciated that this possibility is not incompatible with the two possible mechanisms of insulin action discussed above. For example, it is possible that following internalization, insulin, perhaps bound to its receptor, would be transported to an intracellular organelle where local production of a

mediator or phosphorylation of tyrosine residues of a protein present in that organelle would alter its behavior.

Several antibodies that react with cell surface receptors for insulin have been shown to mimic virtually all the cellular biological activities of insulin itself (160, 161). This cannot be taken as evidence that the action of insulin is exclusively on the cell surface, as these antibodies are internalized in a manner similar to insulin (162) and could also interact with intracellular receptors to produce their effects. However, these results do indicate that if insulin must be internalized to produce some of its biological responses, it must do so by interacting with intracellular receptors that are the same as or at least immunologically similar to receptors on the cell surface.

Two intracellular organelles that have been considered as sites of insulin action are the Golgi apparatus (163) and the nucleus (164). Insulin receptors are found in subcellular fractions highly enriched in Golgi membranes (165, 166), and internalized insulin has been shown to accumulate in these fractions (76–79). Insulin stimulation of glucose transport results from a translocation of glucose transport protein from vesicles present in this Golgi-enriched fraction to the cell membrane (167, 168). The presence of both insulin and receptor in this same Golgi-enriched fraction raises the possibility that a direct action of insulin at this site may be responsible for translocation of glucose transport proteins (163). The time course of these two processes casts doubt on this possibility. Insulin is found in the Golgi region of the cell only minutes after exposure to insulin (90). Stimulation of glucose transport occurs more rapidly (169).

Some investigators have found insulin receptors present on the nuclear membrane (170–173) and have reported that a portion of internalized insulin accumulates over the nucleus (91, 95). Other workers have found no evidence for this. Recently, there have been preliminary reports that insulin added directly to purified nuclei stimulates the efflux of mRNA and activates a nuclear membrane nucleoside triphosphatase (174, 175). If these findings hold up, they will be of obvious importance.

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