A Domain of the Insulin Receptor Required for Endocytosis in Rat Fibroblasts

(Received for publication, January 26, 1990)

R. Scott Thies‡‡, Nicholas J. Webster‡, and Donald A. McClain¶¶

From the ‡Department of Medicine, University of California, San Diego, La Jolla, California 92039 and the ¶¶Veterans Administration Medical Center, Medical Research Service, San Diego, California 92161

Insulin action is mediated by the insulin receptor, a transmembrane glycoprotein consisting of two extracellular α- and two transmembrane β-subunits. Analysis of the structure and function of the insulin receptor has been greatly facilitated by the cloning of the receptor cDNA (1, 2) and gene (3). For example, analysis of the cDNA sequence has revealed the existence of several structural domains within the receptor, including a cysteine-rich domain in the α-subunit, a single transmembrane domain, and a region in the β-subunit with homology to tyrosine-specific protein kinases. The insulin receptor gene consists of 22 exons, and there is good correspondence between the domains described above and individual exons (3).

With the exception of the tyrosine kinase region itself, functional domains of the insulin receptor are less well characterized, and in particular the regions of the receptor responsible for signaling insulin action are incompletely understood. Site-directed mutagenesis has been used to begin this characterization. For example, a COOH-terminal region of the β-subunit has been shown to be required for signaling the metabolic but not mitogenic actions of insulin (4–6). The structural and functional requirements for another activity of the insulin receptor, namely endocytosis, have not been defined, however. There is evidence that the tyrosine kinase activity of the receptor is required for normal receptor internalization and down-regulation in fibroblastic cells (7–9), but this is apparently not the case for receptor internalization in other cell types (10). Analysis is made difficult not only by cell- or tissue-specific differences in the endocytotic itinerary of insulin receptors, but also by the multiplicity of pathways that a receptor can take even within a single cell (11, 12).

To begin to analyze the necessary and sufficient conditions for insulin receptor endocytosis, we have created by site-directed mutagenesis receptors that have altered endocytotic itineraries. In this paper we report the identification of a cytoplasmic domain of the insulin receptor that is required for internalization of the receptor in fibroblasts.

EXPERIMENTAL PROCEDURES

Materials—Porcine insulin and 125I-insulin, moniodinated at the alanine 14 position (300–400 μCi/μg), were kindly provided by Eli Lilly, Inc. Cell culture reagents and dialyzed fetal calf serum were purchased from Gibco, and cells were cultured in dishes from Costar (Cambridge, MA). Wheat germ agglutinin coupled to agarose was from Vector Labs, Inc. (Burlington, CA). Protein concentrations were determined using Bio-Rad protein assay dye. [*32P]ATP labeled in the γ position (∼5000 Ci/mmol, 10 mCi/ml) was from Amersham Corp. Other routine reagents were purchased from Sigma. A monoclonal anti-insulin receptor antibody was kindly provided by Dr. Kenneth Siddle (University of Cambridge, Cambridge, United Kingdom).

Construction of the Mutant—Deletion of the nucleotides encoding exon 16 of the HIR was achieved by oligonucleotide-directed muta-

‡‡ Current address: Genetics Inst., Cambridge, MA 02140.
¶¶ To whom correspondence should be addressed: Division of Endocrinology, University of Alabama at Birmingham, UAB Station, Birmingham, AL 35294.

1 The abbreviations used are: HIR, human insulin receptor; HIRc, human insulin receptor (wild type); HIRΔex16, human insulin receptor with the 16th exon deleted; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 125I-NAPA-DP-insulin, iodinated B2 (2-nitro-4-sydnonymethyl)-des-Phe31-insulin.
Insulin and Insulin Receptor Endocytosis and Metabolism—Cells were covalently labeled with \(^{125}\text{I}-\text{NAPA-DP-insulin}\) as described above and then warmed to 37 °C to allow internalization to proceed. At various times, cultures were removed and either exposed to trypsin (1-tosylamido-2-phenylethyl chloromethyl ketone, 1 mg/ml, 1 h on ice) or not. Trypsinization was stopped by the addition of 2.5 mg/ml soybean trypsin inhibitor, and the cells were solubilized in 1% Triton containing the inhibitors listed above. The human insulin receptors were immunoprecipitated using an antibody specific for the human receptor and fractionated by 7.5% reducing SDS-PAGE. The proportion of intact 135-kDa HIR α-subunit was determined by excising and counting gel bands visualized by autoradiography.

To study insulin internalization and degradation, the culture medium was aspirated from 35-mm dishes of cells (2 × 10⁶ cells/dish) and replaced with 1 ml of modified Eagle's medium (bicarbonate-free), 1% bovine serum albumin, 10 mM HEPES, pH 7.4. Cultures were held at 37 °C in a shaking water bath. \(^{125}\text{I}-\text{Insulin}\) (0.07 nM) was added, and at various times, cultures were assayed for insulin degradation and internalization as described (8). Degraded insulin was estimated by measuring trichloroacetic acid-soluble material, and internalized insulin was defined as that not removed by pH 4.0 buffer (21).

To study down-regulation, 2–4 × 10⁶ cells in 35-mm dishes were exposed to 170 nM insulin for 24 h at 37 °C. Cells were then washed twice (2 min each) at 4 °C with pH 4.0 buffer to remove bound insulin and returned to 37 °C for 1 h. During this period internalized receptors return to their pretreatment distribution, i.e. 80–90% at the cell surface so that the total cellular component of receptors can be estimated by surface binding of \(^{125}\text{I}-\text{Insulin}\).

RESULTS

Transfection of Cells and Characterization of Insulin Binding—A mutant insulin receptor with the 16th exon deleted was engineered as described under “Experimental Procedures.” The deletion encompasses 22 amino acids from residues 944–965 inclusive according to the numbering of Ullrich et al. (1). It should be pointed out that the wild type template was the HIR-A isoform of the receptor, that is the receptor that lacks the 12-amino acid insert encoded by the alternatively spliced exon 11 (22, 23). After verifying the construct by sequencing, the wild type human insulin receptor cDNA (HIRc) or the cDNA encoding the receptor with the deletion (HIRΔex16) were transfected into Rat 1 fibroblasts. After selection by growth in methotrexate, cells were cloned by limiting dilution and clones expressing high levels of insulin binding selected for further analysis. The host Rat 1 cells do not express endogenous insulin receptors (4, 24), but at levels sufficiently low (<2000 rat insulin receptors/cell) to make the selection of transfectants relatively straightforward.

Competition of tracer labeled insulin by cold insulin was studied in two clones of cells expressing similar levels of insulin receptors (4 × 10⁵ HIRc, 6 × 10⁵ HIRΔex16). Fig. 1 reveals that cells expressing the HIRΔex16 receptors bind insulin with an affinity somewhat lower than cells expressing wild type receptors. Half-maximal competition of tracer binding was seen at 0.2 nM insulin for the wild type HIR but at 1.1 nM for the mutant receptors. This result was verified in several other independent clones; five clones of HIRc (of the HIRc-A or minus 12-amino acid isoform) had average EC₅₀ values for competition of 0.24 nM, whereas four clones of HIRΔex16 cells all had EC₅₀ values of greater than 1 nM. Scatchard analysis of these competition curves reveals that the HIRΔex16 receptors, like normal receptors, still exhibit curvilinear binding but that the high affinity component of insulin binding is decreased in the cells carrying the mutant (not shown). Interestingly, when insulin binding is assayed in solution, the mutant receptors are very similar to normal HIR, with EC₅₀ values for competition of tracer insulin being 0.1 nM for both receptors. Both mutant and wild type receptors were processed to α₁β₂ glycoproteins, as determined by
and HIRex16 (Cl) cells were exposed to 8.3 nM cold insulin for 3 h at 12 °C. Competition of the label (as the percent maximum bound/free (B/F) ratio of 125I-insulin) was performed at specific binding.

Results are the means of two experiments each performed in triplicate. Experiments were performed at B/F = 10%, and the amount of labeled insulin bound in the presence of 330 nM insulin was subtracted to yield specific binding.

their ability to bind to lectins, and the sizes of their subunits (see below).

Insulin Receptor Tyrosine Kinase Activity—The ability of the HIRex16 receptor to autophosphorylate and to mediate tyrosine phosphorylation of other substrates was verified in a number of ways. First, receptors from transfected cells were solubilized in detergent and partially purified by lectin affinity chromatography. Receptor concentration was determined by measuring insulin binding activity, and equal numbers of HIRc and HIRex16 receptors were exposed to 330 nM insulin and then allowed to autophosphorylate as described. The reaction was stopped with 2 × SDS sample buffer and the receptors fractionated on a 7.5% gel. The positions of the wild type (○) and HIRex16 (●) β-subunits are indicated.

The mutant receptors also had kinase activity toward exogenous substrates in vitro. Insulin stimulation of the wild type receptor caused a 2.9 ± 0.8-fold increase in phosphorylation of a random copolymer of Glu:Tyr (4:1), whereas the HIRex16 led to a 2.0 ± 0.2-fold increase. Insulin sensitivities were similar, with half-maximal stimulation occurring at 0.7–1 nM insulin (not shown).

Autophosphorylation and kinase activity of the receptors were also intact in living cells. To measure autophosphorylation, receptor α-subunits were first covalently labeled in the cold (10 °C) with a photoreactive 125I-insulin derivative, NAPA-DP insulin (18). The cells were then briefly warmed to 37 °C to allow autophosphorylation of the occupied receptors to proceed and then solubilized in the presence of phosphatase inhibitors. The receptors were immunoprecipitated using either human insulin receptor-specific antibody or an antibody reactive to phosphotyrosine residues (19). As shown in Fig. 3, both HIRc and HIRex16 receptors have identical α-subunits that label with the 125I-insulin. Similar proportions of the labeled receptors are precipitated by the anti-phosphotyrosine antibody; in two experiments an average of 60% of HIRc receptors and 58% of HIRex16 receptors were recognized by the antiphosphotyrosine antibody. Analysis of the supernatants from the precipitation confirmed that the insulin receptor antibody recognized both normal and mutant receptors quantitatively.

Kinase activity was also assayed by Western blotting extracts of insulin-stimulated cells using the anti-phosphotyrosine antibody. In Fig. 4, it is clear that both HIRc and

- [Fig. 1. Insulin binding to transfected cell lines. HIRc (●) and HIRex16 (○) cells were exposed to 8.3 nM 125I-insulin and varying concentrations of cold insulin for 3 h at 12 °C. Competition of the label (as the percent maximum bound/free (B/F) ratio of 125I-insulin) is plotted as a function of total insulin concentration. Results are the means of two experiments each performed in triplicate. Experiments were performed at B/F = 10%, and the amount of labeled insulin bound in the presence of 330 nM insulin was subtracted to yield specific binding.]

- [Fig. 2. Autophosphorylation of wild type and mutant insulin receptors. Equal numbers (~400 fmol) of solubilized wheat germ agglutinin-agarose purified wild type or HIRex16 receptors were exposed to 330 nM insulin and not then allowed to autophosphorylate as described. The reaction was stopped with 2 × SDS sample buffer and the receptors fractionated on a 7.5% gel. The positions of the wild type (○) and HIRex16 (●) β-subunits are indicated.]

- [Fig. 3. Autophosphorylation of wild type and mutant insulin receptors in situ in living cells. Cells were covalently labeled with 125I-NAPA-DP insulin and warmed briefly (37 °C, 4 min) as described. After solubilization, labeled receptors were precipitated either with an anti-insulin receptor (nlR) antisera that recognized >95% of the total receptors or an anti-phosphotyrosine antibody (apY). Shown is an autoradiogram of a reducing SDS gel that reveals the precipitated labeled α-subunits.]

- [Fig. 4. Tyrosine phosphorylation in intact cells. Cells in culture dishes were exposed to insulin (5 min, 37 °C) and rapidly lysed in SDS sample buffer. Equal amounts of cellular protein (as determined in parallel dishes) were fractionated by SDS-PAGE and Western blotted using an antiphosphotyrosine antibody followed by 125I-protein A. The insulin receptor β-subunits (HIRc) as well as a phosphoprotein of 185 kDa (pp 185) are indicated.]
HIR\textsubscript{exx16} receptors tyrosine phosphorylate themselves and a previously described 185-kDa phosphoprotein (25) in intact cells. Equal amounts of protein from untransfected Rat 1 cells, cells expressing 4 \texttimes\ 10^{-4} normal HIR, or cells expressing 6 \texttimes\ 10^{-4} mutant HIR\textsubscript{exx16} were loaded on the gel. The fact that little insulin receptor or p185 tyrosine phosphorylation is seen in untransfected Rat 1 cells with their relatively few receptors confirms that the phosphorylation seen in the other cells is mediated by the human receptors encoded by the transfected cDNAs.

**Ligand and Receptor Internalization—**Having demonstrated that the HIR\textsubscript{exx16} receptors are expressed at the cell surface, bind insulin, and exhibit ligand-dependent tyrosine kinase activity, we next examined the endocytosis of normal and mutant receptors. Cell surface receptors were covalently labeled with \textsuperscript{125}I-NAPA-DP-insulin at 10 °C to prevent internalization during the labeling procedure. Cells were then warmed to 37 °C to allow endocytosis to proceed, and at different times the cells were trypsinized to degrade any receptors remaining on the cell surface. The number of intact (intracellular) labeled 135-kDa \(\alpha\)-subunits was then quantitated after SDS-PAGE. As can be seen in Fig. 5, surface receptors can be labeled and prior to warming these receptors are \(>95\%\) accessible to trypsin degradation. With time at 37 °C, however, normal receptors internalize and become trypsin-resistant such that 38\% of the receptors are intracellular at 60 min. The HIR\textsubscript{exx16} receptors do not undergo endocytosis to any appreciable degree, however. After 60 min at 37 °C, only 5\% have become trypsin-resistant.

A similar conclusion is suggested by the lack of \textsuperscript{125}I-insulin internalization and degradation mediated by HIR\textsubscript{exx16} cells (Fig. 6). Internalization of tracer insulin (0.07 nM) (A) proceeds rapidly in cells expressing 4 \times 10^{-4} normal HIR\textsubscript{c}, as measured by the inability of the internalized insulin to be released from the cells by acid pH. Very few counts are internalized in the HIR\textsubscript{exx16} cells, however. Because of the lower affinity of insulin for the mutant receptor, it was necessary to normalize these results for the lower total cell-associated counts in the HIR\textsubscript{exx16} cells. After subtracting the counts internalized in untransfected Rat 1 cells, the proportion of total cell-associated counts/min that are acid-resistant (intracellular) has been plotted as a function of time in panel B. By this analysis the HIR\textsubscript{exx16} receptors are defective in their ability to internalize insulin (B).

Wild type insulin receptors also deliver insulin to a degradative compartment, and the amount of degradation can be estimated by the appearance in the culture medium of trichloroacetic acid-soluble \textsuperscript{125}I. C shows that HIR\textsubscript{c} but not HIR\textsubscript{exx16} cells mediate significant amounts of insulin degradation. Normalizing the data for cell-associated insulin after subtracting the degradation caused by control untransfected cells confirms this conclusion (D). Nearly identical results (after normalization) were seen in another independently isolated clone each of HIR\textsubscript{c} and HIR\textsubscript{exx16} cells (not shown).

**FIG. 5. Internalization of labeled insulin receptors in transfected cells.** A, an autoradiogram, with the intact \(\alpha\)-subunit indicted, showing the total initial level of receptors (+ trypsin, time 0), the initial trypsin sensitivity of all of these receptors (+ trypsin, time 0), and the internalization or escape from trypsin sensitivity of these receptors with time at 37 °C (+ trypsin, 7.5 and 60 min) B, plot of internalization or escape from trypsin sensitivity of these receptors with time at 37 °C. The \(\alpha\)-subunit bands from the experiment in A were excised and counted. The total amount of receptor initially present on the cells (+ trypsin, time 0) was taken to represent 100%.

**FIG. 6. Insulin internalization and degradation by transfected cells.** 2 \times 10^{4} cells, either HIR\textsubscript{c} (C) or HIR\textsubscript{exx16} (C) were equilibrated at 37 °C in 35-mm dishes in 1 ml of buffer with 0.5% albumin. At time 0, \textsuperscript{125}I-insulin (0.06 nM) was added as described under "Experimental Procedures." A, the amount of intracellular insulin (counts/min, as determined by the cell-associated counts not removable by an acid wash, is plotted as a function of time. B, the amount of intracellular insulin was normalized as the percentage of total cell-associated insulin (cell bound counts/min after saline rinse but without an acid wash). The small amount of insulin internalized by untransfected Rat 1 cells (<200 cpm) has been subtracted to control for insulin metabolism by the endogenous rat insulin receptors. C, insulin degradation as estimated by the appearance of trichloroacetic acid-soluble counts/min in the medium. D, insulin degradation normalized as in B, that is, degraded counts/min expressed as a fraction of total cell-associated counts, after subtracting the counts/min degraded by an equal number of untransfected Rat 1 cells (<200 cpm).

**TABLE I**

<table>
<thead>
<tr>
<th>Receptor type</th>
<th>Clone</th>
<th>Receptors/cell</th>
<th>Down-regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIR\textsubscript{c}</td>
<td>8-9</td>
<td>4 \times 10^{4}</td>
<td>26%</td>
</tr>
<tr>
<td>HIR\textsubscript{c}</td>
<td>B</td>
<td>1.3 \times 10^{4}</td>
<td>41%</td>
</tr>
<tr>
<td>HIR\textsubscript{exx16}</td>
<td>14</td>
<td>5.9 \times 10^{4}</td>
<td>0</td>
</tr>
<tr>
<td>HIR\textsubscript{exx16}</td>
<td>19</td>
<td>6.4 \times 10^{4}</td>
<td>0</td>
</tr>
</tbody>
</table>
As a final index of receptor internalization, HIRc and HIRAexl6 cells were exposed to saturating concentrations of insulin for prolonged periods to assess whether receptor down-regulation occurred (26). As shown in Table I, the mutant HIRAexl6 receptors do not down-regulate. Whereas total receptor number decreases by 26-41% in cells with normal receptors exposed to 170 nM insulin for 24 h, no decrease is seen in HIRAexl6 cells.

DISCUSSION

The data presented here indicate that deletion of the immediately submembraneous region of the insulin receptor results in a molecule that maintains ligand specific activation of tyrosine kinase activity but does not undergo endocytosis in Rat 1 cells. That this receptor maintains normal transmembrane signaling of its kinase despite the deletion of the region connecting its extracellular and kinase domains is of interest. These results argue that exons of the insulin receptor may indeed encode structurally and functionally discrete domains as has been hypothesized for insulin (3) and low density lipoprotein (27) receptors.

The properties of the HIRAexl6 receptor have not been completely unaltered by the deletion, however. The insulin binding properties of the mutant receptors in cells (but not in solution) were changed from those of normal receptors. The decreased affinity of the HIRAexl6 receptor for insulin suggests that the submembraneous conformation of the receptor can affect ligand binding. A similar conclusion was reached by studying chimeras of the insulin and epidermal growth factor receptors, wherein it was demonstrated that the cytoplasmic region did affect the binding properties of the extracellular ligand-binding domain (28). This could occur because of intramolecular (i.e. β-subunit-β-subunit) or intermolecular interactions. A similar pattern of binding properties, that is, lower affinity on cells but normal affinity in solution, has also been reported for a kinase-defective receptor with a point mutation in the ATP binding site (8). The basis for these binding properties is unknown. Although both the HIRAexl6 and the kinase defective receptors are processed correctly into αβ₂ heterotetramers (data not shown), further details of their structure and their association with other receptors is not known.

Also interesting is the failure of the HIRAexl6 receptors to internalize. Given the otherwise near-normal properties of the mutated receptor, this suggests that the deleted region includes a domain involved in ligand-dependent endocytosis. Chen, Goldstein, and Brown have recently described an amino acid sequence, NPXY, that is required for normal internalization of the low density lipoprotein receptor (35). One and only one copy of this sequence exists in the insulin receptor and that NPXY is in the deleted exon of the mutant reported here. This supports the hypothesis of the above-named workers that this sequence is important for signaling some (but not necessarily all) endocytotic pathways, although it should be emphasized that the current studies do not directly implicate the NPXY sequence itself in causing the defect of the HIRAexl6 receptor. The receptor for insulin-like growth factor I has an NPXY sequence in a similar submembraneous position (29). As pointed out by Chen et al. (35) however, other receptors of the tyrosine kinase family either have NPXY sequences in their cytoplasmic tails (e.g. the receptor for epidermal growth factor, Ref. 30) or have no NPXY sequence (e.g. the receptors for platelet-derived growth factor or for colony-stimulating factor I, that have interrupted tyrosine kinase domains (31)). Thus, the requirement for a submembraneous NPXY sequence is not universal for all receptors; alternate endocytotic mechanisms may operate in different cell types for different receptors. Interestingly, an epidermal growth factor receptor mutant lacking the cytoplasmic tail, which deletes that receptor's NPXY sequences, has been described (32). This mutant retains biologic and kinase activity but does not internalize.

The domain encoded by the 16th exon (and by implication possibly the NPEY sequence) is required for endocytosis in Rat 1 cells. However, understanding insulin receptor endocytosis will require the integration of a much larger and often contradictory body of evidence. For example, in these same cells, tyrosine kinase activity is also required for ligand-dependent endocytosis (7-9). In other cell types, notably liver-derived cells, kinase activity may not be required, however. For example, Backer et al. (10) have found that deletion of cellular ATP by treatment with 2,4-dinitrophenol sufficient to block receptor autophosphorylation does not block ligand-dependent endocytosis of insulin receptors. The mechanism underlying these differences among cell types in their requirement for kinase activity to initiate internalization is not known. Ligand-dependent internalization of receptors does proceed through different pathways that can be differentiated on kinetic (33) or pharmacologic (12) grounds. Ultrastructurally, insulin receptor endocytosis is largely via noncoated pit pathways in adipocytes and liver-derived cells (11), whereas embryonic cells (11) and, to different degrees, fibroblastic lines (34) may make more use of coated pit pathways. Such data, combined with the fact that the NPXY sequence is required for coated pit-mediated internalization of the low density lipoprotein receptor, which is devoid of kinase activity suggest: 1) there are coated pit-dependent and -independent modes of endocytosis for insulin receptors; 2) there are tyrosine kinase-dependent and -independent pathways. However, for insulin receptors the interrelationships among these pathways and the requirement for the deleted domain (or the NPXY sequences) are unclear. Further experiments, including both expressing the mutant receptor in other cell types and combining the deletion with other mutations such as those leading to tyrosine kinase defects, are needed. These studies, as well as assessing the ability of the noninternalizing receptors to activate and deactivate different aspects of insulin action, should shed further light on the mechanism and role of ligand-dependent endocytosis of insulin receptors.

Acknowledgments—We would like to acknowledge Dr. Axel Ullrich (Max Planck Institute, Munich) for making the wild type HIR cDNA available and Dr. Jerrold Olefsky (Veterans Administration Medical Center and University of California, San Diego) for helpful discussions. Dr. Dietrich Brandenburg provided the NAPA insulin. Clonon Tate prepared the manuscript.

REFERENCES
Endocytosis-defective Insulin Receptors

A domain of the insulin receptor required for endocytosis in rat fibroblasts.
R S Thies, N J Webster and D A McClain


Access the most updated version of this article at [http://www.jbc.org/content/265/17/10132](http://www.jbc.org/content/265/17/10132)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/265/17/10132.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/265/17/10132.full.html#ref-list-1](http://www.jbc.org/content/265/17/10132.full.html#ref-list-1)