Receptor-mediated Internalization of Insulin Requires a 12-Amino Acid Sequence in the Juxtamembrane Region of the Insulin Receptor \( \beta \)-Subunit*

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The juxtamembrane region of the insulin receptor (IR) \( \beta \)-subunit contains an unphosphorylated tyrosyl residue (\( \mathrm{Tyr}^{658} \)) that is essential for insulin-stimulated tyrosyl phosphorylation of some endogenous substrates and certain biological responses (White, M. F., Livingston, J. N., Backer, J. M., Lauris, V., Dull, T. J., Ullrich, A., and Kahn, C. R. (1988) Cell 54, 641-649). Tyrosyl residues in the juxtamembrane region of some plasma membrane receptors have been shown to be required for their internalization. In addition, a juxtamembrane tyrosine in the context of the sequence NPXT is required for the coated pit-mediated internalization of the low density lipoprotein receptor. To examine the role of the juxtamembrane region of the insulin receptor during receptor-mediated endocytosis, we have studied the internalization of insulin by Chinese hamster ovary (CHO) cells expressing two mutant receptors: IR\(_{\mathrm{A1018}}\), in which \( \mathrm{Tyr}^{658} \) has been substituted with phenylalanine, and IR\(_{\mathrm{A2890}}\), in which 12 amino acids (\( \mathrm{Ala}^{654}-\mathrm{Asp}^{665} \)), including the putative consensus sequence NPXT, were deleted. Although the \( \mathrm{in~vivo} \) autophosphorylation of IR\(_{\mathrm{A2890}}\) and IR\(_{\mathrm{A2890}}\) was similar to wild type, neither mutant could phosphorylate the endogenous substrate pp185. CHO/IR\(_{\mathrm{A2890}}\) cells internalized insulin normally whereas the intracellular accumulation of insulin by CHO/IR\(_{\mathrm{A2890}}\) cells was 20-30% of wild-type. However, insulin internalization in the CHO/IR\(_{\mathrm{A2890}}\) cells was consistently more rapid than that occurring in CHO cells expressing kinase-deficient receptors (CHO/IR\(_{\mathrm{A1018}}\)). The degradation of insulin was equally impaired in CHO/IR\(_{\mathrm{A2890}}\) and CHO/IR\(_{\mathrm{A1018}}\) cells. These data show that the juxtamembrane region of the insulin receptor contains residues essential for insulin-stimulated internalization and suggest that the sequence NPXT may play a general role in directing the internalization of cell surface receptors.

Insulin regulates cellular metabolism and growth by binding

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stimulated internalization. We have shown previously that substitution of Tyr105 with phenylalanine does not affect receptor autophosphorylation but abolishes insulin-stimulated bioeffects (19). We now show that deletion of 12 amino acids (Ala84–Asp95), including NPXTTM, from this region of the insulin receptor has minimal effects on receptor autophosphorylation in vivo but diminishes insulin internalization and degradation by 70–80%. These data point to a role for the juxtamembrane region of the receptor in mediating endocytosis.

MATERIALS AND METHODS

Expression Plasmids—The normal human insulin receptor expression plasmid pCVSHVIRc as well as expression plasmids encoding mutant human insulin receptors in which alanine replaced Lys105 (IR105A) or phenylalanine replaced Tyr105 (IR105F) have been described previously (8, 19). The plasmid for the expression of the mutant insulin receptor containing a deletion of amino acids Ala84–Asp95 (pCVSHVIRc/D960) was generated by subcloning a BglII-HindIII fragment from pCVSHVIRc into M13mp19. A uracil-rich template was prepared in E. coli strain JM101 was transformed with double-stranded circular plasmid pCVSVHIRc as well as expression plasmids encoding wild-type or mutant insulin receptors—CHO cells were washed twice in PBS to determine total cell-associated radioactivity or acidic PBS, pH 3.0, containing 0.1% bovine serum albumin and once in neutral PBS, pH 7.4. The cells were then solubilized, and the radioactivity was counted as described above.

RESULTS

Expression of the Normal and Mutant Insulin Receptors in Chinese Hamster Ovary (CHO) Cells—CHO/jeo cells, expressing only pSVEneo, contained about 3,000 hamster insulin receptors. Following transfection and selection by fluorescence-activated cell sorting, clonal lines of CHO/IR cells and mutant CHO/IR105A, CHO/IR105F, and CHO/IR105D Cells were obtained which expressed approximately 80,000 receptors/cell (data not shown). To determine the structure of these receptors, CHO cells expressing the wild-type IR and mutant IR105F were labeled with [35S]methionine or surface-labeled with [125I]iodine, immunoprecipitated with antiphosphotyrosine antibody (αPY) or antireceptor antibody (αIR), and analyzed by SDS-PAGE (Fig. 1, left panel). In [35S]methionine-labeled cells, the α- and β-subunits in CHO/IR cells were detected at 135 and 95 kDa after reducing SDS-PAGE (7.5% resolving gel). In [125I]iodine-labeled CHO/IR105F cells, the α-subunit was detected at 135 kDa whereas the β-subunit was slightly smaller (93 kDa). The radioactivity in the α-subunit was 50% of that in the β-subunit in both cell lines, consistent with the 1:2 ratio of methionine residues in α- and β-subunits, respectively (25). The smaller size of the CHO/IR105F β-subunit was more clearly seen in surface-iodinated receptors analyzed by reducing SDS-PAGE (6% resolving gel) and reflected the deletion of 12 amino acids. Scatchard analysis of CHO/IR105F and CHO/IR105D cells has been described previously and indicated that binding is normal (19, 26); the displacement of tracer [125I]insulin bound to CHO/IR and CHO/IR105F cells by unlabeled insulin is shown in Fig. 1 (right panel). Analysis of insulin binding data

FIG. 1. Expression of wild-type and mutant insulin receptors in CHO cells. Left panel, CHO cells expressing wild-type IR or the mutant IR105F, labeled with [35S]methionine or [125I]iodine as described and stimulated with 100 nM insulin. Labeled insulin receptors were immunoprecipitated with antiphosphotyrosine antibody (αPY), reduced with dithiothreitol, and analyzed by SDS-PAGE (24). Phosphoproteins were identified by autoradiography, and the radioactivity in the phosphotyrosine-containing subunits was quantitated by Cerenkov counting.

Uptake and Degradation of [125I]Insulin by CHO Cells Expressing Wild-type or Mutant Insulin Receptors—Confluent monolayers of CHO cells were grown in 24-well dishes. The cells were labeled with [35S]methionine or surface-labeled with [125I]iodine, immunoprecipitated with antiphosphotyrosine antibody (αPY) or antireceptor antibody (αIR), and analyzed by SDS-PAGE (Fig. 1).两条[125I]iodine-labeled CHO/IR105F cells, the α-subunit was detected at 135 kDa whereas the β-subunit was slightly smaller (93 kDa). The radioactivity in the α-subunit was 50% of that in the β-subunit in both cell lines, consistent with the 1:2 ratio of methionine residues in α- and β-subunits, respectively (25). The smaller size of the CHO/IR105F β-subunit was more clearly seen in surface-iodinated receptors analyzed by reducing SDS-PAGE (6% resolving gel) and reflected the deletion of 12 amino acids. Scatchard analysis of CHO/IR105F and CHO/IR105D cells has been described previously and indicated that binding is normal (19, 26); the displacement of tracer [125I]insulin bound to CHO/IR and CHO/IR105F cells by unlabeled insulin is shown in Fig. 1 (right panel). Analysis of insulin binding data.
for four CHO/IR,360 clones indicated a mean binding constant of 4.46 ± 0.3 (±S.D.) nM, as compared with 3.1 ± 0.4 (±S.D.) nM for CHO/IR cells; expression levels in the selected CHO/IR,360 clones were 1.13–1.27 times higher than those in CHO/IR cells. Thus, deletion of 12 amino acids from the juxtamembrane region of the insulin receptor did not affect its affinity for insulin or its expression in CHO cells.

In Vivo Autophosphorylation of the Wild-type and Mutant Receptors in CHO Cells—Before insulin stimulation, autophosphorylation of wild-type and mutant insulin receptors in CHO cells was undetectable by αPY (Fig. 2). Tyrosine phosphorylation of the wild-type insulin receptor β-subunit in CHO/IR cells was evident after 1 min of insulin stimulation (100 nM) (Fig. 2). Insulin also stimulated tyrosyl phosphorylation of pp185, an endogenous substrate of the insulin receptor, in CHO/IR cells (19). Autophosphorylation of the β-subunit from insulin-stimulated CHO/IRneo cells was normal (Fig. 2). However, as described previously, insulin-stimulated tyrosyl phosphorylation of pp185 in CHO/IR,960 cells was barely detectable (19). In the CHO/IR,360 cells, insulin stimulated the tyrosyl phosphorylation of the β-subunit but not pp185 (Fig. 2). Phosphorylation of pp185 in both CHO/IR,960 and CHO/IR,360 cells was similar to that seen in control CHO/neo cells (Fig. 2). Thus, like IR,960, IR,360 undergoes insulin-stimulated autophosphorylation but does not mediate tyrosyl phosphorylation of the intracellular substrate pp185.

Insulin Uptake and Degradation by Wild-type and Mutant Insulin Receptors in CHO Cells—CHO cells expressing wild-type and mutant insulin receptors bound insulin rapidly at 37 °C, and the total cell-associated radioactivity increased over 30 min in all lines (Fig. 3A). CHO/IR and CHO/IR,960 cells accumulated [125I]-insulin faster than did the CHO/IR,360 and CHO/IR,360 cells. This difference was because of differences in the rates of [125I]-insulin internalization (Fig. 3B). Approximately 40–50% of the [125I]-insulin associated with CHO/IR and CHO/IR,960 cells was in an intracellular compartment and resistant to removal by an acid wash (Fig. 3B). In contrast, only 10% of the [125I]-insulin associated with CHO/IR,360 cells was intracellular, which was barely increased over that seen with the internalization-defective CHO/IR,360 cells.

The internalization and processing of a single cohort of insulin were measured to examine the kinetics of insulin uptake and degradation. [125I]-Insulin was bound to the surface of CHO cells during incubation at 4 °C and then allowed to internalize during incubation at 37 °C (Fig. 4). CHO/IR-cells rapidly internalized the single cohort of [125I]-insulin, with 50–60% of surface-bound insulin inside the CHO/IR cells after 10–15 min (Fig. 4A). The amount of internalized insulin declined gradually after 20 min because of the release of degraded insulin into the medium. CHO/IR,960 cells internalized surface-bound insulin with kinetics that were similar to that of the wild-type cells although the maximum level of internalized insulin was 20% lower (Fig. 4A). Insulin internalization by the CHO/IR,960 cells, however, was reduced by 60–70% relative to the CHO/IR cells. Interestingly, internalization of insulin by the CHO/IR,960 cells was consistently higher than that seen in the kinase-deficient CHO/IR,360 cells (Fig. 4A). Release of degraded insulin into the medium was also diminished in the CHO/IR,360 and CHO/IR,360 cells whereas insulin degradation by the CHO/IR,960 cells was identical to wild type (Fig. 4B). Thus, the CHO/IR,360 cells were defective in insulin uptake under both steady-state and single cohort conditions.
DISCUSSION

The internalization of insulin at physiological concentrations occurs primarily by endocytosis of insulin-receptor complexes [4]. Our data suggest that the juxtamembrane region of the insulin receptor is required for internalization of the insulin receptor. The IR
\text{960}
 shows a marked reduction in receptor-mediated insulin uptake and degradation even though insulin-stimulated in vivo autophosphorylation is normal. Internalization of the IR
\text{960}
 is inhibited nearly as much as that of the kinase- and internalization-deficient ATP binding site mutant IR
\text{1018}. Thus, an intact ATP binding site may be necessary but not sufficient for insulin-stimulated insulin receptor internalization; amino acids in the juxtamembrane region of the insulin receptor are also required for the rapid uptake of physiological levels of insulin.

Davis et al. [11] have shown previously that the constitutive internalization of the LDL receptor requires the presence of a cytoplasmic aromatic residue (Tyr
\text{957}) 18 amino acids away from the membrane-spanning region [11]. Tyrosine residues in similar positions are important for internalization of several different receptors [12-16]. Furthermore, Chen et al. [17] have shown recently that the sequence NPXT, located at residues 904-907 of the LDL receptor, is required for internalization. The insulin receptor contains the sequence NPXT
\text{960}
 at residues 957-960, and this sequence is deleted in the internalization defective mutant IR
\text{960}
 (Fig. 5). In contrast, IR
\text{960}
 internalizes normally, as does the LDL receptor mutant in which Tyr
\text{957} is replaced by phenylalanine [11]. Thus, our findings are consistent with those from the LDL receptor system and suggest that NPXT may be involved in a general mechanism by which cell surface receptors are endocytosed.

Insulin receptor internalization is ligand stimulated in most cell types whereas internalization of the LDL receptor is constitutive [4]. Insulin binding and autophosphorylation cause conformational changes in the insulin receptor β-subunit, which may promote interactions between the juxtamembrane region and other cellular proteins [27, 28]. However, antipeptide antibodies prepared against the peptide Leu-Tyr-Lys-His-Ile (derived from insulin receptor residues 952-962) immunoprecipitate the Triton X-100-solubilized insulin receptor regardless of autophosphorylation, suggesting that the juxtamembrane region is always exposed [27, 29]. Therefore, it is unclear how the juxtamembrane region of the insulin receptor cycles between internalization competent and incompetent conformations in vivo.

Studies using kinase-deficient ATP binding site mutants suggest that receptor autophosphorylation is required for insulin receptor internalization [6-8]. Furthermore, internalization is deficient in mutant receptors with substitutions at tyrosine autophosphorylation sites in the regulatory region of the β-subunit which are required for full activation of the receptor [30-32]. Autophosphorylation may lead to an unfolding of the β-subunit, exposing domains involved in internalization. Alternatively, phosphorylation at particular tyrosyl residues may be necessary for internalization. Insulin receptor mutants deficient in insulin internalization are also deficient in some or all insulin bioeffects [8, 26, 30, 33]. However, it is unlikely that receptor internalization requires an insulin-stimulated signal since the IR
\text{960} does not appear to mediate biological responses but internalizes normally [19]. Thus, the insulin receptor itself appears to be the predominant locus for regulating insulin receptor internalization.

Insulin binding alone, in the absence of autophosphorylation, may induce β-subunit conformational changes that are sufficient to allow internalization. In this regard, insulin receptor internalization was normal in hepatoma cells in which receptor autophosphorylation was inhibited with 2,4-dinitrophenol although it is possible that transient and undetectable autophosphorylation of these receptors still occurred [9]. Internalization was also normal in CHO cells expressing a truncated kinase-deficient insulin receptor [34]. However, the truncation itself may expose regions of the receptor required for internalization, as has been suggested by epidermal growth factor receptor truncation mutants [35]. Although the present study does not bear directly on the role of autophosphorylation in insulin receptor internalization, the IR
\text{960} is defective in internalization despite nearly normal in vivo autophosphorylation. Thus, although the role of insulin receptor autophosphorylation in ligand-stimulated internalization is not understood fully, our data suggest that phosphorylated tyrosyl residues are not a sufficient signal to drive receptor internalization. On the other hand, IR
\text{960} mediates insulin internalization at a level faster than that of the kinase-deficient IR
\text{1018}. Thus it is possible that autophosphorylation provides an alternative or additional stimulus to internalization.

The ability of various receptors to undergo coated pit-mediated internalization correlates with their ability to bind clathrin-associated proteins in vitro [36]. The role of cytoplasmic receptor sequences, in particular the NPXT motif, may involve such interactions. The importance for insulin receptor internalization of residues 954-965, which contain the NPXT sequence, suggests that insulin receptors utilize pathways of internalization which are similar to the coated pit-mediated pathway used by the LDL receptor. However,

![FIG. 5. Juxtamembrane domains of the insulin and LDL receptors. The amino acid sequences of the juxtamembrane regions of the insulin and LDL receptors, beginning at the end of the transmembrane region (TM region), are shown. The positions of Tyr
\text{807} and Tyr
\text{860} in the insulin receptor and Tyr
\text{907} in the LDL receptor are indicated with arrows, and the NPXT sequences are boxed. The 12-amino acid deletion in IR
\text{960} is depicted by a dashed line.](http://www.jbc.org/)

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our data suggest that the insulin receptor internalizes by a phosphorylation of the receptor is not a sufficient signal to mechanism similar to that of the LDL receptor. Tyrosine drive its internalization, which requires the presence of specific sequences in the juxtamembrane domain. This does not appear to be involved in insulin receptor internalization.

In summary, we have shown that deletion of 12 amino acids from the juxtamembrane domain of the insulin receptor inhibits receptor-mediated internalization and degradation of insulin, despite minimal effects on in vivo autophosphorylation of the receptor. This deletion includes the residues NPXT, which are required for internalization of the LDL receptor. Our data suggest that the insulin receptor internalizes by a mechanism similar to that of the LDL receptor. Tyrosine phosphorylation of the receptor is not a sufficient signal to drive its internalization, which requires the presence of specific sequences in the juxtamembrane domain.

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Note Added in Proof — We have recently identified a second mutation in the cDNA used to transfect the IRr960 cells. The mutation is in codon 962, changing it from AGT to ACT. This mutation puts Thr in place of Ser206. Therefore the IRr960 cells contain two mutations in the insulin receptor juxtamembrane region. However, since the behavior of the IRr960 mutant is normal with regard to internalization, this does not alter the conclusions of the study.

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Receptor-mediated internalization of insulin requires a 12-amino acid sequence in the juxtamembrane region of the insulin receptor beta-subunit.

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