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Receptor-mediated Internalization of Insulin Requires a 12-Amino Acid Sequence in the Juxtamembrane Region of the Insulin Receptor β-Subunit*

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The juxtamembrane region of the insulin receptor (IR) β-subunit contains an unphosphorylated tyrosyl residue (Tyr^{960}) 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 NP*X*T 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_{F960}, in which Tyr^{960} has been substituted with phenylalanine, and $IR_{\Delta960}$, in which 12 amino acids (Ala^{954}–Asp^{965}), including the putative consensus sequence NP*X*T, were deleted. Although the *in vivo* autophosphorylation of IR_{F960} and $IR_{\Delta960}$ was similar to wild type, neither mutant could phosphorylate the endogenous substrate pp185. CHO/IR_{F960} cells internalized insulin normally whereas the intracellular accumulation of insulin by CHO/$IR_{\Delta960}$ cells was 20–30% of wild-type. However, insulin internalization in the CHO/$IR_{\Delta960}$ cells was consistently more rapid than that occurring in CHO cells expressing kinase-deficient receptors (CHO/IR_{A1018}). The degradation of insulin was equally impaired in CHO/$IR_{\Delta960}$ and CHO/IR_{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 NP*X*T may play a general role in directing the internalization of cell surface receptors.

Insulin regulates cellular metabolism and growth by binding to the insulin receptor (1). Immediately after insulin binding, the β-subunit of the insulin receptor undergoes tyrosyl autophosphorylation and phosphorylates endogenous substrates on tyrosyl residues (1–3). Insulin binding also stimulates the internalization of the insulin receptor in a variety of cells, leading to a net increase in the intracellular receptor pool (reviewed in 4). Nearly all intracellular insulin receptor molecules are tyrosyl phosphorylated immediately after endocytosis (5). Kinase-deficient receptors are biologically inactive and do not undergo insulin-stimulated internalization, suggesting that tyrosine phosphorylation of the receptor is necessary for both signal transmission and endocytosis (6–8). However, inhibition of *in vivo* insulin receptor autophosphorylation by 80% does not inhibit insulin-stimulated endocytosis or insulin degradation in Fao hepatoma cells (9). Moreover, studies utilizing insulin-mimetic antibodies have also demonstrated autophosphorylation-independent internalization of the insulin receptor (10). Thus, tyrosyl phosphorylation of the insulin receptor may not be obligatory for insulin-stimulated endocytosis.

Studies of the coated pit-dependent internalization of many cell surface receptors suggest that specific amino acids in the juxtamembrane region of receptors may mediate endocytosis. A tyrosine 18 residues beyond the membrane-spanning region (Tyr^{807}) is required for internalization of the LDL[1] receptor; similar results have been obtained in other systems (11–16). Moreover, internalization of the LDL receptor requires that Tyr^{807} appears in the context of the sequence $NPXT^{807}$, suggesting that juxtamembrane tyrosines must be presented in a specific orientation to allow efficient endocytosis (17). The insulin receptor contains 2 tyrosine residues in the juxtamembrane region of the β-subunit, Tyr^{953} and Tyr^{960}; Tyr^{960} is present in the context of $NPXT^{960}$. Thus, the insulin receptor as well as the receptors for LDL and other ligands may share a common mechanism for receptor internalization. Consistent with this hypothesis, a chimeric receptor comprising the extracellular and transmembrane regions of the insulin receptor linked to the retroviral tyrosyl kinase v-*ros* undergoes insulin-stimulated autophosphorylation but not insulin-stimulated internalization (18); the kinase domain of v-*ros* is homologous to the insulin receptor but lacks tyrosine in its juxtamembrane region.

In this paper, we examine the role of the juxtamembrane region of the insulin receptor in the regulation of insulin-

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[1] The abbreviations used are: LDL, low density lipoprotein; IR, insulin receptor; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; αPY, antiphosphotyrosine antibody; αIR, antireceptor antibody.

stimulated internalization. We have shown previously that substitution of Tyr^{960} with phenylalanine does not affect receptor autophosphorylation but abolishes insulin-stimulated bioeffects (19). We now show that deletion of 12 amino acids (Ala^{954}–Asp^{965}), including $NPXT^{960}$, 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 Lys^{1018} (IR_{A1018}) or phenylalanine replaced Tyr^{960} (IR_{F960}) have been described previously (8, 19). The plasmid for the expression of the mutant insulin receptor containing a deletion of amino acids Ala^{954}–Asp^{965} (pCVSVHIRc/Δ960) was generated by subcloning a *Bgl*II-*Hind*III fragment from pCVSHIRc into M13mp19. A uracil-rich template was prepared in *Echerichia coli* BW313, and mutagenesis was carried out using a complementary mutagenesis primer containing a 36-base deletion coding for residues Ala^{954}–Asp^{965} as described previously (19). *E. coli* strain JM101 was transformed with double-stranded circular DNA, and plaques were screened under stringent conditions using the mutagenesis primer as a probe. The mutation was confirmed by M13 dideoxy sequencing and reintroduced into the pCVSHIRc vector (20).

Transfection of CHO Cells—CHO cells were grown in 10-cm dishes in F-12 medium containing 10% fetal bovine serum (GIBCO). Subconfluent CHO cells (10^6) were transfected by calcium phosphate precipitation with 1 μg of pSVEneo alone or together with 10 μg of pCVSVHIRc, pCVSVHIRc/F960, pCVSVHIRc/A1018, or pCVSVHIRc/Δ960 as described previously (19). After 72 h, 800 μg/ml of geneticin (GIBCO) was added to the medium to select for neomycin-resistant cells. Surviving cells were cultured in the presence of geneticin to amplify the cell line. CHO cells that expressed high levels of surface insulin receptors were selected by fluorescence-activated cell sorting (21), and clonal cell lines were obtained by plating at limiting dilution.

Scatchard Analysis of Insulin Binding to CHO Cells Expressing Wild-type or Mutant Insulin Receptors—Confluent monolayers of CHO cells were grown in 24-well dishes. The cells were incubated with 30,000 cpm of ^{125}I-insulin in the presence of varying concentrations of unlabeled insulin for 16 h at 4 °C. The cells were washed twice in PBS at 4 °C to remove unbound ligand, solubilized in 0.1% SDS, 0.1 N NaOH, and counted in a GammaTrac 1290 γ-counter (TM Analytic). Protein content was determined by the method of Bradford (22). Scatchard analysis was performed using LIGAND (23).

[^{35}S]Methionine, [^{125}I]Iodine, and [^{32}P]Phosphate Labeling of CHO Cells Expressing Wild-type or Mutant Insulin Receptors—Confluent monolayers of transfected CHO cells in 10- or 15-cm dishes (Nunc) at 37 °C were labeled for 24 h with 0.5 mCi/ml [^{35}S]methionine or for 2 h with 0.5 mCi/ml [^{32}P]phosphate (Du Pont-New England Nuclear) (19). Alternatively, cells were surface iodinated with lactoperoxidase/glucose oxidase using 1 mCi of [^{125}I]iodine (Amersham Corp.) per dish as described previously (5). The cells were incubated for additional periods of time in the absence or presence of 100 nM insulin and rapidly frozen with liquid nitrogen. The frozen cells were solubilized in 100 mM Tris, pH 8.2, containing 2 mM sodium vanadate, 3.4 mg/ml phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, 1 μg/ml leupeptin, and 1% Triton X-100. Tyr(P)-containing proteins 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 insulin receptor subunits was quantified by Cerenkov counting.

Uptake and Degradation of ^{125}I-Insulin by CHO Cells Expressing Wild-type or Mutant Insulin Receptors—Confluent monolayers of CHO cells were grown in six-well dishes. The cells were washed twice in phosphate-buffered saline, pH 7.4 (PBS), and incubated at 37 °C in binding buffer (F-12 medium containing 50 mM HEPES, pH 7.4, 0.1% bovine serum albumin, and 5 × 10^6 cpm/ml ^{125}I-insulin. At various times, the medium was removed, and intact ligand was precipitated by the addition of 10% trichloroacetic acid. The cells were rapidly chilled by immersion in ice-cold PBS, and washed twice with either PBS to determine total cell-associated radioactivity or acidic PBS (pH 3.5) to remove surface-bound radioactivity and to determine internalized radioactivity. The cells were then solubilized and counted as described above.

Internalization of a Single Cohort of Surface-bound Insulin by CHO Cells Expressing Wild-type or Mutant Insulin Receptors—CHO cells were washed twice in PBS containing 0.1% bovine serum albumin, and then incubated at 4 °C in binding buffer containing 10^5 cpm of ^{125}I-insulin for 2 h. The cells were then washed in cold PBS to remove unbound ligand and rapidly warmed by the addition of binding buffer at 37 °C. At the indicated time intervals, the medium was removed, and insulin degradation was measured by precipitation of intact insulin in 10% trichloroacetic acid. The cells were washed two times in 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/neo 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/IR_{F960}, CHO/IR_{A1018}, and CHO/$IR_{\Delta960}$ 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 $IR_{\Delta960}$ were labeled with [^{35}S]methionine or surface-labeled with [^{125}I]iodine, immunoprecipitated with antiphosphotyrosine antibody (αPY) or antireceptor antibody (αIR), and analyzed by SDS-PAGE (Fig. 1, *left panel*). In [^{35}S]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 [^{35}S]methionine-labeled CHO/$IR_{\Delta960}$ 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/$IR_{\Delta960}$ 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/IR_{F960} and CHO/IR_{A1018} cells has been described previously and indicated that binding is normal (19, 26); the displacement of tracer ^{125}I-insulin bound to CHO/IR and CHO/$IR_{\Delta960}$ cells by unlabeled insulin is shown in Fig. 1 (*right panel*). Analysis of insulin binding data

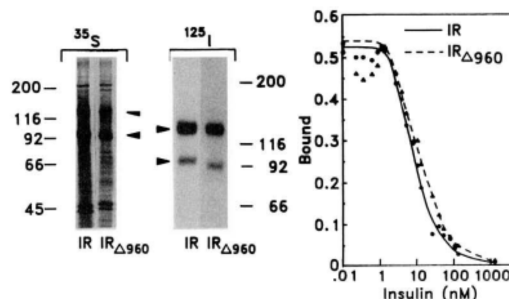

FIG. 1. **Expression of wild-type and mutant insulin receptors in CHO cells.** *Left panel*, CHO cells expressing wild-type IR or the mutant $IR_{\Delta960}$ labeled with [^{35}S]methionine or ^{125}I-iodine as described and stimulated with 100 nM insulin. Labeled insulin receptors were immunoprecipitated with αPY (*^{35}S lanes*) or αIR (*^{125}I lanes*), separated by SDS-PAGE (7.5% resolving gel for *^{35}S lanes*, 6% for *^{125}I lanes*), and visualized by autoradiography. *Right panel*, CHO cells were grown in 24-well dishes and the binding of A-14 monoiodinated ^{125}I-insulin in the presence of varying concentrations of unlabeled insulin was determined as described under "Materials and Methods."

for four CHO/IR$_{\Delta960}$ 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$_{\Delta960}$ 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/IR$_{F960}$ cells was normal (Fig. 2). However, as described previously, insulin-stimulated tyrosyl phosphorylation of pp185 in CHO/IR$_{F960}$ cells was barely detectable (19). In the CHO/IR$_{\Delta960}$ cells, insulin stimulated the tyrosyl phosphorylation of the β-subunit but not pp185 (Fig. 2). Phosphorylation of pp185 in both CHO/IR$_{F960}$ and CHO/IR$_{\Delta960}$ cells was similar to that seen in control CHO/neo cells (Fig. 2). Thus, like IR$_{F960}$, IR$_{\Delta960}$ 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. 3*A*). CHO/IR and CHO/IR$_{F960}$ cells accumulated ^{125}I-insulin faster than did the CHO/IR$_{\Delta960}$ and CHO/IR$_{A1018}$ cells. This difference was because of differences in the rates of ^{125}I-insulin internalization (Fig. 3*B*). Approximately 40–50% of the ^{125}I-insulin associated with CHO/IR and CHO/IR$_{F960}$ cells was in an intracellular compartment and resistant to removal by an acid wash (Fig. 3*B*). In contrast, only 10% of the ^{125}I-insulin associated with CHO/IR$_{\Delta960}$ cells was intracellular, which was barely increased over that seen with the internalization-defective CHO/IR$_{A1018}$ cells.

The internalization and processing of a single cohort of insulin were measured to examine the kinetics of insulin uptake and degradation. ^{125}I-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 ^{125}I-insulin, with 50–60% of surface-bound insulin inside the CHO/IR cells after 10–15 min (Fig. 4*A*). The amount of internalized insulin declined gradually after 20 min because of the release of degraded insulin into the medium. CHO/IR$_{F960}$ 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. 4*A*). Insulin internalization by the CHO/IR$_{\Delta960}$ cells, however, was reduced by 60–70% relative to the CHO/IR cells. Interestingly, internalization of insulin by the CHO/IR$_{\Delta960}$ cells was consistently higher than that seen in the kinase-deficient CHO/IR$_{A1018}$ cells (Fig. 4*A*). Release of degraded insulin into the medium was also diminished in the CHO/IR$_{A1018}$ and CHO/IR$_{\Delta960}$ cells whereas insulin degradation by the CHO/IR$_{F960}$ cells was identical to wild type (Fig. 4*B*). Thus, the CHO/IR$_{\Delta960}$ cells were defective in insulin uptake under both steady-state and single cohort conditions.

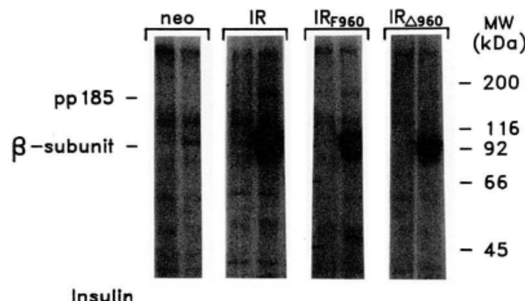

FIG. 2. ***In vivo* autophosphorylation of wild-type and mutant insulin receptors.** CHO/IR, CHO/IR$_{F960}$, CHO/IR$_{\Delta960}$, and CHO/neo cells were labeled with [^{32}P]orthophosphate for 2 h and incubated in the absence or presence of 100 nM insulin for 1 min. After solubilization, tyrosyl-phosphorylated receptors were immunoprecipitated with αPY, separated by SDS-PAGE, and visualized by autoradiography.

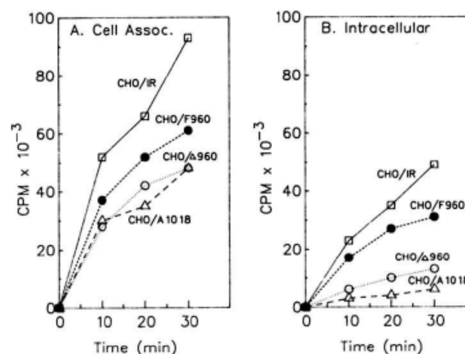

FIG. 3. **Internalization of ^{125}I-insulin by CHO cells expressing wild-type and mutant insulin receptors.** CHO/IR (□), CHO/IR$_{F960}$ (●), CHO/IR$_{\Delta960}$ (○), and CHO/IR$_{A1018}$ (△) cells were incubated at 37 °C in the presence of ^{125}I-insulin. *A*, at various times, the cells were washed with PBS and solubilized to determine cell-associated radioactivity. *B*, after incubations as above, the cells were washed with acidic buffer and solubilized to determine intracellular radioactivity.

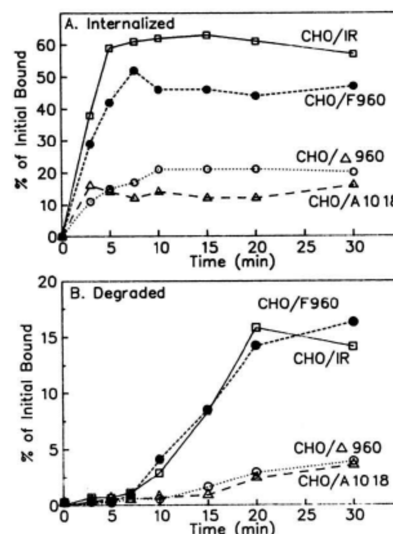

FIG. 4. **Internalization and degradation of a single cohort of surface-bound insulin by CHO cells expressing wild-type and mutant insulin receptors.** CHO/IR (□), CHO/IR$_{F960}$ (●), CHO/IR$_{\Delta960}$ (○), and CHO/IR$_{A1018}$ (△) cells were incubated at 4 °C with ^{125}I-insulin for 2 h. The cells were washed to remove unbound ligand and rapidly warmed to 37 °C. At various times, the medium was removed, and degraded insulin was determined by solubility in 10% trichloroacetic acid. The cells were washed with acidic buffer and solubilized to determine intracellular radioactivity. *A*, internalization of ^{125}I-insulin into CHO cells. *B*, release of degraded ^{125}I-insulin into the medium was determined by solubility in 10% trichloroacetic acid.

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 $IR_{\Delta960}$ shows a marked reduction in receptor-mediated insulin uptake and degradation even though insulin-stimulated *in vivo* autophosphorylation is normal. Internalization of the $IR_{\Delta960}$ is inhibited nearly as much as that of the kinase- and internalization-deficient ATP binding site mutant IR_{A1018}. 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^{807}) 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 NP*X*T, located at residues 804–807 of the LDL receptor, is required for internalization. The insulin receptor contains the sequence $NPXT^{960}$ at residues 957–960, and this sequence is deleted in the internalization-defective mutant $IR_{\Delta960}$ (Fig. 5). In contrast, IR_{F960} internalizes normally, as does the LDL receptor mutant in which Tyr^{807} is replaced by phenylalanine (11). Thus, our findings are consistent with those from the LDL receptor system and suggest that NP*X*T 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-LYASSNPEYLS (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_{F960} 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 $IR_{\Delta960}$ 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, $IR_{\Delta960}$ mediates insulin internalization at a level faster than that of the kinase-deficient IR_{A1018}. 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 NP*X*T motif, may involve such interactions. The importance for insulin receptor internalization of residues 954–965, which contain the NP*X*T sequence, suggests that insulin receptors utilize pathways of internalization which are similar to the coated pit-mediated pathway used by the LDL receptor. However,

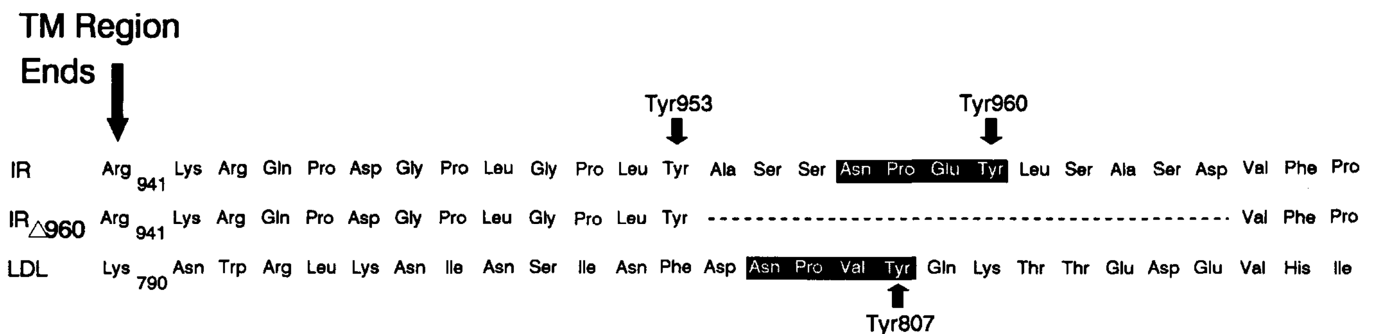

FIG. 5. **Juxtamembrane domains of the insulin and LDL receptors.** The amino acid sequences of the juxtamembrane regions of the insulin and LDL receptors, beginning at the end of the transmembrane region (*TM region*), are shown. The positions of Tyr^{953} and Tyr^{960} in the insulin receptor and Tyr^{807} in the LDL receptor are indicated with *arrows*, and the NP*X*T sequences are *boxed*. The 12-amino acid deletion in $IR_{\Delta960}$ is depicted by a *dashed line*.

insulin receptors can internalize in cells that contain few coated pits, and studies have suggested that insulin receptors may internalize by coated pit-independent pathways (37, 38). It is not clear whether the NP*X*T sequence is required for coated pit-independent internalization; such a requirement would necessitate a different explanation for the role of NP*X*T. The sequence might be involved in receptor aggregation in the plasma membrane. Alternatively, the *in vivo* phosphorylation of a juxtamembrane tyrosyl residue which may be important for internalization of the asialoglycoprotein receptor has been described (39). However, Tyr^{960} of the insulin receptor is not tyrosyl phosphorylated during insulin stimulation of CHO or Fao hepatoma cells, and substitution of this residue with phenylalanine does not diminish internalization (19). Thus, tyrosine phosphorylation in the juxtamembrane region 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. The deletion includes the residues NP*X*T, 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.

Acknowledgments—We would like to thank Drs. K. Siddle and S. E. Shoelson for helpful discussions.

Note Added in Proof—We have recently identified a second mutation in the cDNA used to transfect the IR_{F960} cells. The mutation is in codon 962, changing it from AGT to ACT. This mutation puts Thr in place of Ser^{962}. Therefore the IR_{F960} cells contain two mutations in the insulin receptor juxtamembrane region. However, since the behavior of the IR_{F960} mutant is normal with regard to internalization, this does not alter the conclusions of the study.

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