The Insulin Receptor Juxtamembrane Region Contains Two Independent Tyrosine/β-Turn Internalization Signals

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Abstract. We have investigated the role of tyrosine residues in the insulin receptor cytoplasmic juxtamembrane region (Tyr953 and Tyr960) during endocytosis. Analysis of the secondary structure of the juxtamembrane region by the Chou-Fasman algorithms predicts that both the sequences GPLY953 and NPEY960 form tyrosine-containing β-turns. Similarly, analysis of model peptides by 1-D and 2-D NMR show that these sequences form β-turns in solution, whereas replacement of the tyrosine residues with alanine destabilizes the β-turn. CHO cell lines were prepared expressing mutant receptors in which each tyrosine was mutated to phenylalanine or alanine, and an additional mutant contained alanine at both positions. These mutations had no effect on insulin binding or receptor autophosphorylation. Replacements with phenylalanine had no effect on the rate of [125I]insulin endocytosis, whereas single substitutions with alanine reduced [125I]insulin endocytosis by 40–50%. Replacement of both tyrosines with alanine reduced internalization by 70%. These data suggest that the insulin receptor contains two tyrosine/β-turns which contribute independently and additively to insulin-stimulated endocytosis.

The coated pit-mediated endocytosis of many cell surface receptors is dependent on structural features in their cytoplasmic domains. Although deletion of the entire domain inhibits the localization of receptors to coated pits (Goldstein et al., 1985; Lehrman et al., 1985; Iacopetta et al., 1988; Mostov et al., 1986). efficient endocytosis has been shown in several cases to require only 20–30 amino acids of the cytoplasmic region (Chen et al., 1990; Lobel et al., 1989; Girones et al., 1991). In a number of receptors, aromatic residues in the cytoplasmic juxtamembrane region are necessary for coated pit-mediated endocytosis (Davis et al., 1987; Lazzaritis and Roth, 1988; Lobel et al., 1989; Jing et al., 1990; Breitfeld et al., 1990; Johnson et al., 1990; Valiquette et al., 1990; McGraw et al., 1991; Fuhrer et al., 1991). Moreover, in several receptors short tyrosine-containing sequences have been identified which are required for rapid internalization including FXNPXY97 (LDL receptor; Chen et al., 1989), Y2oTRF (transferrin receptor; Collawn et al., 1990), and Y5XY2XKV (mannose 6-phosphate/IGF-II receptor; Canfield et al., 1991). These sequences contain little homology, yet appear to be interchangeable in some cases (Collawn et al., 1991). Structural analysis and peptide modeling/2-D NMR studies of putative internalization motifs in the LDL and transferrin receptors and a lysosomal membrane protein suggest that the internalization signal for cell surface receptors is an aromatic residue in a β-turn (Collawn et al., 1990; Eberle et al., 1991; Bansal and Giersasch, 1991) or short surface loop stabilized by hydrogen bonds (Kitistakis et al., 1990). Consistent with this hypothesis, the introduction of a tyrosine immediately distal to residues favoring turn formation in the cytoplasmic tail of glycoporphin caused this molecule, normally excluded from coated pits, to be efficiently internalized (Kitistakis et al., 1990).

Insulin receptor endocytosis is stimulated by insulin (reviewed in Bergeron et al., 1985) and utilizes coated pits in a number of cell types (Fan et al., 1982; Pilch et al., 1983; Carpentier et al., 1986; Backer et al., 1991b). Mutations in the ATP binding site of the receptor block receptor autophosphorylation and internalization (McClain et al., 1987; Hari and Roth, 1987; Russell et al., 1987; Yamamoto-Hionda et al., 1990). In contrast, deletions and mutations in the cytoplasmic juxtamembrane region of the receptor have minimal effects on in vivo receptor autophosphorylation, but block receptor internalization (Backer et al., 1990, 1991a,b; Thies et al., 1990; Rajagopalan et al., 1991). These mutations include the residues NPEY960, which are similar to the sequence NPVY97 required for low-density lipoprotein (LDL) receptor endocytosis (Chen et al., 1990). These data suggest that the juxtamembrane region of the insulin receptor contains information required for its efficient endocytosis.

We have previously shown that in CHO cells, insulin-stimulated receptor internalization occurs via a rapid, saturable pathway which utilizes coated pits (Backer et al., 1991b). In contrast, receptors also internalize by a slow, con-
stittive pathway which appears to be independent of coated pits. Saturable internalization has also been demonstrated for the EGF receptor (Lund et al., 1990), suggesting that the ligand-stimulated movement of the insulin and EGF receptors requires specific saturable interactions with components of the endocytic system. Entry of the insulin receptor into the rapid saturable endocytic pathway is inhibited by deletion of 12 amino acids from the juxtamembrane region, including the NPEY900 motif (Backer et al., 1991b). Thus, the NPEY900 motif may be required for interactions which mediate entry of the insulin receptor into the rapid coated pit–mediated pathway.

In this study, we have examined the role of putative internalization motifs during insulin receptor endocytosis. Analysis of the insulin receptor juxtamembrane region using the Chou-Fasman algorithms for prediction of secondary structure (Chou and Fasman, 1978) predicts that the two tyrosine residues Tyr953 and Tyr960, which are present in GPLY953 and NPEY900 motifs, respectively, form tyrosine-containing β-turns. These predictions are supported by 2-D NMR analysis of peptides containing the GPLY953 and NPEY900 sequences. Substitution of alanine in place of either Tyr953 or Tyr960, which destabilizes β-turn formation in the corresponding peptides, inhibits insulin receptor internalization by 50%. Substitution of both residues with alanine inhibits internalization by 70%, and blocks the entry of insulin receptors into the rapid saturable internalization pathway. Therefore, Tyr953 and Tyr960 appear to contribute independently and additively to receptor internalization. The functional equivalence of Tyr953 and Tyr960 for internalization may be due to their similar conformational environment in the insulin receptor juxtamembrane region.

Materials and Methods

Peptide Synthesis

Oligopeptides corresponding to the native and mutant insulin receptor sequences GPLGLY953AS (GPL), PLGPLA953AS (GPLA), SSNPEY900SA (NPEY), and SSNPEAY960SA (NPEA) were synthesized on an ABI 430A synthesizer. All peptides used for the NMR studies were >98 % pure, judged by analytical HPLC, and compositions were confirmed by amino acid analysis.

Solution Structure of Peptide

1H-nuclear magnetic resonance (NMR) spectra were observed at 500 MHz using a Varian VXR spectrometer, located at the Harvard Medical School NMR Facility. The peptides were dissolved in 700 μl of a buffer consisting of 50 mM sodium deuterocetate (pH 4.5; 10% D2O) and 50 mM KCl. The pH was chosen so as to retard base-catalyzed amide proton exchange while remaining above the pK of carboxylate groups. Spectra were recorded at a peptide concentration of 5 mM at pH 8.0, containing 2 mM sodium vanadate, 350 μg/ml PMSF, 100 μg/ml aprotonin, 1 μg/ml leupeptin, and 1% Triton X-100. Insulin receptors were immunoprecipitated with monoclonal anti-receptor antibody 83-14 (provided by Dr. K. Siddle, University of Cambridge), reduced with DTT, and analyzed by SDS-PAGE (Backer et al., 1991b). Phosphoproteins were identified by autoradiography. Data is representative of two separate experiments, and was confirmed in two additional experiments by immunoprecipitation with anti-receptor antibody followed by blotting with anti-phosphotyrosine antibody.

Uptake of [125I]Insulin by CHO Cells Expressing Wild-type or Mutant Insulin Receptors

Internalization of [125I]Insulin ([125I]iodotyrosyl A14-insulin, 2,000 Ci/mm) was determined as previously described during a 0–10-min incubation at 37°C (Backer et al., 1991b). Total cell-associated and intracellular radioactivity was determined by washes in PBS/0.1% BSA, pH 7.6 (neutral PBS), or PBS/0.1% BSA, pH 3.5 (acidic PBS), respectively. Nonspecific binding was determined in the presence of 10−6 M insulin, and was found to be ~0.25% of total at 37°C.

The internalization rate constant for each receptor mutant was determined during a 10-min incubation with [125I]Insulin as previously described (Lund et al., 1990; Backer et al., 1991b) using the equation:

\[ L_{max} = k_i \cdot (LR) \cdot \Delta t \]  \tag{1}  

where \( L_{max} \) represents internalized [125I]Insulin, \( LR \) represents surface-bound [125I]Insulin, and the slope of the resultant line is equal to the internalization rate constant \( k_i \). Integrals were approximated by the trapezoidal rule, using an interval of \( \Delta \tau = 2 \) min, and slopes were determined by linear regression. Data is representative of three separate experiments.

Saturation Plots of Insulin Uptake by CHO Cells

CHO cells expressing wild-type or mutant insulin receptors were incubated at 37°C in "binding buffer" containing 100,000 CPM/ml [125I]Insulin plus varying concentrations of unlabeled insulin. At various times between 0–10 min, cell-associated and internalized radioactivity was determined as described above. The internalization rate constant \( k_i \) was determined at each insulin concentration by linear regression as described above, and \( k_i \) was plotted against the average velocity of insulin uptake between 0 and 10 min at each insulin concentration as previously described (Lund et al., 1990; Backer et al., 1991b). At steady-state internalization, assuming mini-

et al. (1985) and reflects the exon 11– version of the human insulin receptor cDNA. The wild-type human insulin receptor cDNA (exon 11–variant) cDNAs for mutant receptors in which Tyr953 is replaced by phenylalanine (IR953) or from which residues Ala953–Asp960 have been deleted (IR960) were provided by A. Ulrich (Max Planck Institute für Biochemie); cDNA for a kinase-deficient mutant in which Lys913 is replaced by alanine (IRA913) was provided by O. M. Rosen (deceased). The IR960 receptor has been found to contain a second mutation, with Ser962 substituted by Thr962 (Backer et al., 1990). However, this mutant internalizes normally, and the second mutation does not therefore compromise the conclusions of this study. CHO cells expressing these receptors (CHO/IR, CHO/IR953 CHO/IR960, and CHO/IR910) were maintained as previously described (Backer et al., 1991b). New point mutations consisting of the substitution of Phe or Ala in place of Tyr953 (IR953 and IR955) or Ala in place of Tyr960 (IR960) were introduced into the human insulin receptor cDNA by oligonucleotide-directed mutagenesis as previously described (White et al., 1988), using the primers GGAGGCGCTTCCCTTCCTCT (IR953), GGAGGCGCTTCCCTTCCTCT (IR955), and AACCTGAGCCTCTCA960 (IR960). A double mutation, replacing both Tyr953 and Tyr960 with alanine (IR953+960) was created by site-directed mutagenesis of the IR960 mutant, using the IR953 primer. CHO cells were cotransfected with pSVneo, selected for resistance to geneticin (Gibco Laboratories, Grand Island, NY), and sorted for high expression by FACS as previously described (Backer et al., 1990; Maron et al., 1984). Cisplastin cell lines were obtained by plating FACS-positive cells at limiting dilution.

In Vivo Insulin Receptor Autophosphorylation in CHO Cells

Confluent monolayers of transfected CHO cells in 15-cm dishes at 37°C were labeled with 2 μCi/ml [3H]bicarbonate (New England Nuclear, Boston, MA) (White et al., 1988). The cells were incubated for 2 min at 37°C 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 8.0, containing 2 mM sodium vanadate, 350 μg/ml PMSF, 100 μg/ml aprotonin, 1 μg/ml leupeptin, and 1% Triton X-100. Insulin receptors were immunoprecipitated with monoclonal anti-receptor antibody 83-14 (provided by Dr. K. Siddle, University of Cambridge), reduced with DTT, and analyzed by SDS-PAGE (Backer et al., 1991b). Phosphoproteins were identified by autoradiography. Data is representative of two separate experiments, and was confirmed in two additional experiments by immunoprecipitation with anti-receptor antibody followed by blotting with anti-phosphotyrosine antibody.
Results

Structural Characterization of the Insulin Receptor Juxtamembrane Region

Several recent studies suggest that tyrosine residues in $\beta$-turns may serve as signals for the localization of receptors to coated pits (Collawn et al., 1990; Bansal and Giersasch, 1991; Eberle et al., 1991). The insulin receptor $\beta$-subunit contains three tyrosine residues in its cytoplasmic juxtamembrane region at Tyr$^{953}$, Tyr$^{960}$, and Tyr$^{962}$. We have previously shown that the deletion of 12 amino acids, including Tyr$^{960}$, reduces the rate of insulin receptor endocytosis (Backer et al., 1990, 1991b). To examine the possible role of tyrosine/$\beta$-turn motifs in signaling insulin receptor endocytosis, we analyzed the insulin receptor juxtamembrane region using the Chou-Fasman algorithms for prediction of secondary structure (Chou and Fasman, 1978). Interestingly, the sequences GPLY$^{953}$ and NPEY$^{960}$ are both predicted to form $\beta$-turns with the tyrosine residue in the fourth position, whereas Tyr$^{962}$ was not predicted to reside in a $\beta$-turn (data not shown).

To evaluate the conformational preference of these potential insulin receptor internalization signals, synthetic octapeptides corresponding to the native sequences PLGPLA$^{953}$AS (GPLA), SSNPEAGE$^{960}$SA (NPEY) and the mutant sequences PLGLA$^{953}$AS (GLRA) SSNPEA$^{960}$SA (NPRA) were prepared and examined by NMR. The amide resonances of the two native sequences are shown in one-dimensional $^1$H-NMR spectra (Fig. 1, A and C) and exhibit a dispersion of chemical shifts suggestive of nonrandom structure. Further analysis by 2-D-NMR demonstrates a single strong contact between the amide protons of E and Y in the NPEY and between amide protons L and Y in GPLY (Fig. 2, A and C). This close contact is characteristic of $\beta$-turn formation, as recently emphasized by Bansal and Giersasch (1991) and Eberle et al. (1991). No evidence for $\alpha$-helix or $\beta$-sheet formation by these peptides could be detected. In contrast to the native peptides, the mutant peptides GPLA and NPEA exhibited more limited dispersion of amide resonances (Fig. 1, B and D) and no strong contacts between amide protons (Fig. 2, B and D). In the case of the key HN$_{12}$HN$_{13}$ contacts characteristic of a $\beta$-turn, the relative intensities in the mutant peptides (if present) were reduced by at least a factor of ten. These data demonstrate that peptides containing the sequences GPLY$_{93}$ and NPEY$_{960}$ have a strong preference to form $\beta$-turns in solution. Furthermore, substitution of Tyr$^{953}$ with alanine in the GPLA$_{953}$ peptide, or Tyr$^{960}$ with alanine in the NPEA$_{960}$ peptide, destabilizes formation of the $\beta$-turn.

Mutagenesis of the Insulin Receptor Juxtamembrane Region at Tyr$^{953}$ and Tyr$^{960}$

Chou-Fasman analysis and peptide modeling studies suggest that both Tyr$^{953}$ and Tyr$^{960}$ should be present in tyrosyl-containing $\beta$-turns, which may serve as a signal for localization to coated pits (Collawn et al., 1990). To test the importance of these residues for insulin receptor endocytosis, mutants were constructed in which either Tyr$^{953}$ or Tyr$^{960}$ was replaced singly by phenylalanine (IR$_{P953}$, IR$_{P960}$) or alanine (IR$_{A953}$, IR$_{A960}$); an additional mutant replaced both residues with alanine (IR$_{A953A960}$). The mutant receptors were expressed in CHO cells at $\sim$1.2 x 10$^6$ receptors/cell; the CHO/IR$_{960}$ cells (White et al., 1988) expressed $\sim$50% fewer receptors. Scatchard analysis revealed normal insulin binding affinity for all the cell lines, with a $K_d$ for each line of 2–5 nM.

Autophosphorylation is required for rapid endocytosis of the human insulin receptor expressed in CHO cells, as the kinase-deficient mutant receptor IR$_{A1018}$ is defective for internalization (Russell et al., 1987; Hari and Roth, 1987). It was therefore critical to measure the insulin-stimulated autophosphorylation of the mutant receptors. Cells expressing similar numbers of wild-type or mutant receptors were labeled with $[^{32}$P]orthophosphate for 2 h and incubated in the absence or presence of 100 nM insulin for 2 min. Insulin receptors were immunoprecipitated from cell lysates with a human-specific anti-receptor antibody (Ab 83-14) and separated by SDS-PAGE (Fig. 3). In the absence of insulin, the 95-kD $\beta$-subunit of the insulin receptor was weakly and variably detected in all the transfected cell lines, indicating a small amount of basal phosphorylation. After insulin stimulation, a marked increase in the phosphorylation of the 95-kD $\beta$-subunit occurred in the CHO/IR cells, whereas it was undetectable in the control CHO/neo cells. Insulin-stimulated receptor autophosphorylation in cells expressing mutant receptors was similar to that seen in the CHO/IR cells; the lower level of receptor autophosphorylation in the IR$_{960}$ cells reflected the 50% lower expression level of the mutant receptor in these cells. Receptor autophosphorylation was stimulated five to eightfold by insulin in all cases, suggesting that mutations in the juxtamembrane region of the receptor did not affect receptor autophosphorylation.

Internalization of Wild-type and Mutant Receptors

To determine the effect of the juxtamembrane mutations on insulin receptor endocytosis, CHO cells expressing similar numbers of wild-type or mutant receptors were incubated with $[^{32}$P]insulin for 0–10 min at 37°C, chilled to 4°C, and washed in neutral or acidic PBS to measure total cell-associated or internalized radioactivity, respectively. The rate constant for insulin receptor endocytosis was calculated for each cell line as previously described (Lund et al., 1990; Backer et al., 1991b). CHO cells expressing wild-type receptors internalized 0.02 nM $[^{32}$P]insulin rapidly, with a rate constant of $k_e = 0.154 \pm 0.01$ min$^{-1}$ (Fig. 4). In contrast, $[^{32}$P]insulin internalization by the kinase-deficient CHO/IR$_{A1018}$ cells was over 10-fold slower ($k_e = 0.0135 \pm$
Figure 1. 1-D NMR spectra of synthetic peptides derived from the juxtamembrane region. One-dimensional $^1$H-NMR spectra of NPEY (A), NPEA (B), GPLY (C), and GPLA (D) octapeptides at 4°C in 90% H$_2$O/10% D$_2$O and 50 mM NaCl (pH 4.5). The enhanced resonance dispersion seen in spectra A and C reflect the presence of a nonrandom conformational preference ($\beta$-turn; see Fig. 2). In each case, complete resonance assignment has been obtained by the 2-D sequential method. Resonances in the spectra of $\beta$-turn containing peptides (A and C) are labeled a–i, with assignments as follows. In spectrum A: (a) S2-HN; (b) N3-HN; (c) E5-HN; (D) L7-HN; (e) Y6-HN; (f) S8-HN; (g) N3-NH$_2$, and Y6-H$_2$ (unresolved); and (i) Y6-H$_2$. In spectrum B: (a) L2-HN; (b) G3-HN; (c) L5-HN; (d) A7-HN and S8-HN (unresolved); (e) Y6-HN; (f and g) COOH-terminal amide resonances; (h) Y6-H$_2$, and (i) Y6-H$_2$.

0.004 min$^{-1}$). We have previously shown that internalization of the IR$_{41218}$ receptor does not occur through the kinase-dependent saturable route (Backer et al., 1991b). Therefore, these cells set a lower limit for $[^{35}S]$insulin internalization in cells which have a high level of insulin binding but are defective for insulin-stimulated internalization.

Substitution of either Tyr$_{93}$ or Tyr$_{96}$ with phenylalanine had little or no effect on the rate of 0.02 nM $[^{35}S]$insulin in-
Figure 2. 2-D NMR spectra of synthetic peptides derived from the juxtamembrane region. Amide region of corresponding two-dimensional NOESY spectra of NPEY (A), NPEA (B), GPLY (C), and GPLA (D) octapeptides at 4°C in 90% H2O/10% D2O and 50 mM NaCl (pH 4.5). Characteristic (i+2,i+3) NOE (asterisks) is observed in A and C, providing evidence of β-turn formation (Bansal and Giersasch, 1991). In each case, the upfield amide resonance is assigned to Y, and the downfield resonance to the preceding residue. Intra-residue NOE crosspeaks are also observed in A and C from the ortho tyrosine ring resonance to its amide proton (crosspeaks a and g, respectively). The unlabeled crosspeak seen in the spectra of the NPEA peptide (crosspeak f in B), which contains no tyrosine, arises from a COOH-terminal amide group not present in the other peptides. Other labeled crosspeaks are: A: (b and c) major and minor intra-residue NH2 crosspeaks from residue 3, presumed to represent trans and cis X-Pro conformers, respectively; (d) unassigned minor state; and (e) Y6 ortho-meta intra-residue NOE. In C: (h) Intra-residue NH2 NOE from COOH-terminal amide; and (i) Y6 ortho-meta intra-residue NOE.

Figure 3. Insulin-stimulated autophosphorylation of wild-type and mutant insulin receptors in CHO cells. CHO cells were incubated in [32P]orthophosphate for 2 h and for an additional 2 min in the absence or presence of insulin (100 nM). Insulin receptors were immunoprecipitated from solubilized cells with a monoclonal anti-receptor antibody 83-14, separated by SDS-PAGE, and visualized by autoradiography.
Saturation Plots for Insulin Receptor Internalization in CHO Cells Expressing Wild-type and Mutant Receptors

We have previously shown that in CHO cells expressing ~10^6 receptors/cell, insulin-stimulated internalization of wild-type receptors occurs via a rapid, saturable pathway that utilizes coated pits (Backer et al., 1991b). In contrast, the kinase-deficient IR^A1018 receptor and the IR^953 receptor, which lacks the NPEY_960 sequence, are unable to enter the rapid saturable pathway. The ability of a receptor mutant to enter the rapid coated pit-mediated internalization pathway can be analyzed by plotting the internalization rate constant k_e, measured at increasing receptor occupancies, against the velocity of insulin uptake at each occupancy level (Lund et al., 1990). The resulting saturation plot is analogous to an Eadie-Scatchard plot in which the slope of the saturable component is negative K_{IC}, the X-intercept is V_{max} and 1/K_{IC} is the half-saturating occupancy for the rapid pathway. K_{IC} is a combination of individual rate constants for formation and dissociation of receptor/coated pit complexes, the dissociation of ligand from receptor/pit complexes, and the actual rate of coated pit internalization, and Lund et al. (1990) have interpreted K_{IC} as reflecting the affinity of a given receptor for the endocytic apparatus. In any case, K_{IC} estimates the ability of different receptor mutants to enter the rapid, saturable internalization pathway.

Internalization saturation plots were constructed for the wild-type and mutant receptors (Fig. 5). As previously described, the wild-type receptor yields a biphasic plot, with a rapid saturable component and a slow nonsaturable component, whereas the juxtamembrane deletion mutant IR^A960 shows only the nonsaturable component (Fig. 5 A) (Backer et al., 1991b). The calculated values for K_{IC} and V_{max} of the saturable component in CHO/IR cells were somewhat variable between experiments; the means from six experiments were slightly higher than previously reported values (ED_{50} = 150,000 ± 35,500 receptors/cell; V_{max} = 22,800 ± 3,000 molecules/min/cell). Substitution of either Tyr_953 or Tyr_960 with phenylalanine had no effect on either the slope of the rapid, saturable component of the plot or the calculated V_{max} (Fig. 5 B, Table I). Substitution of Tyr_953 with alanine caused a 25% decrease in the slope and a 20% decrease in the V_{max} for the saturable component. Substitution of Tyr_960 with alanine caused a 40% reduction in internalization (k_e = 0.003 ± 0.007 min^{-1}). Thus, both Tyr_953 and Tyr_960 were required for rapid insulin receptor internalization. Furthermore, the effect of substitutions at these residues was partially additive, as replacement of both tyrosine residues with alanine caused a further reduction in internalization rate (k_e = 0.048 ± 0.002 min^{-1}). However, the double mutant still retained a threefold faster rate of internalization relative to the IR^A1018, suggesting that another feature of the receptor in addition to Tyr_953 and Tyr_960 may contribute to insulin receptor endocytosis.

Discussion

We have previously shown that the ligand-stimulated internalization of the human insulin receptor in CHO cells occurs...
Table I. Relative Rate Constants for Internalization of Insulin Receptor Mutants

| Cell line | $K_c$ (% of WT) | $V_{max}$ (% of WT) | $n$ |
|-----------|-----------------|---------------------|-----|
| CHO/IR^953| 89.5 ± 10.5     | 111.0 ± 1.5         | 2   |
| CHO/IR^960| 95.8 ± 3.5      | 103.6 ± 6.5         | 2   |
| CHO/IR^A953| 40.1 ± 14.0    | 152.2 ± 46.8        | 6   |
| CHO/IR^A960| 76.8 ± 10.2    | 81.5 ± 2.8          | 5   |
| CHO/IR^A953A960| 0 | ND | 2 |
| CHO/IR^A960| 0 | ND | 3 |

The values for $K_c$, the negative slope of the saturable component of internalization, were calculated by linear regression from saturation plots similar to those shown in Fig. 5; the $V_{max}$ for the saturable component of internalization was calculated from Eq. 2. To pool data from multiple experiments, the values for the slope and $V_{max}$ for each mutant were expressed as a percentage of the values obtained with cells expressing wild-type receptors on the same day (% of WT). The $V_{max}$ values for the IR^960 and IR^A953A960 receptors could not be determined (ND), as the slope of the saturable component of internalization was approximately zero in these cell lines.

via a rapid, saturable coated pit-dependent pathway that requires receptor autophosphorylation and an intact cytoplasmic juxtamembrane region (Backer et al., 1991b). In this study, we provide evidence that a critical determinant for rapid insulin receptor endocytosis is the presence of tyrosine-containing $\beta$-turns in the juxtamembrane region. We have used the Chou-Fasman algorithms as well as 2-D NMR studies of model peptides to identify two tyrosine residues, Tyr^953 and Tyr^960, as potential sites of $\beta$-turn formation in the juxtamembrane region. Tyr^960 is present in the sequence NPXY^960, which is similar to the LDL receptor sequence NPVY^907, and a peptide derived from the insulin receptor sequence NPEY^960 has been previously shown to form a $\beta$-turn in solution (Bansal and Giersasch, 1991). The sequence GPLY^953 is only 50% homologous with the LDL receptor NPVY^907 sequence. However, it is also predicted by Chou-Fasman analysis and 2-D NMR studies to form a $\beta$-turn. Mutagenesis of the insulin receptor at both Tyr^953 and Tyr^960 shows that both residues are independently and additively important for rapid insulin receptor endocytosis. Moreover, substitution of alanine at both positions completely blocks entry of the insulin receptor into the rapid, saturable internalization pathway. The predicted structural equivalence of Tyr^953 and Tyr^960 is thus paralleled by their functional equivalence for insulin receptor endocytosis, suggesting that insulin receptor endocytosis requires the presence of tyrosine-containing $\beta$-turns in the cytoplasmic juxtamembrane region.

Despite significant progress in the past few years, a unifying model for the association of cell surface receptors with coated pits has not yet emerged. The requirement for a tyrosine or other aromatic residue near the membrane has been demonstrated in many systems (Davis et al., 1987; Lazrovits and Roth, 1988; Lobel et al., 1989; Jing et al., 1990; Breitfeld et al., 1990; Johnson et al., 1990; Valiquette et al., 1990; McGraw et al., 1991; Fuhrer et al., 1991), but no consensus sequence for internalization is apparent from these studies. The LDL receptor sequence NPXY^907, which is required for rapid endocytosis (Chen et al., 1990), is present in the juxtamembrane region of the insulin receptor and IGF-I receptor and the COOH-terminal tail of the EGF receptor but not in other receptors known to associate with coated pits. Furthermore, deletion of the NPXY sequence inhibits endocytosis of the insulin receptor (Backer et al., 1990; Thies et al., 1990) but not the EGF receptor (Chen et al., 1989). In an alternative approach, Collawn et al. (1990) suggested that the sequence required for rapid transferrin receptor internalization is a tyrosine residue in a $\beta$-turn. Similarly, Kitstaks et al. (1990) have proposed that the internalization motif was a tyrosine-containing loop stabilized by hydrogen bonding at its base. Two recent reports show that peptides derived from the lysosomal acid phosphatase sequence PPGY^4 and the LDL receptor sequence NPVY^907 form $\beta$-turns in solution (Eberle et al., 1991; Bansal and Giersasch, 1991). Furthermore, analysis of peptides derived from LDL receptor mutants, containing alanine substitutions in the NPVY^907 sequence, showed a striking correlation between $\beta$-turn formation by the peptides in vitro and internalization of the corresponding receptor mutant in vivo (Bansal and Giersasch, 1991). Our peptide modeling and mutagenesis studies are consistent with these data, and suggest that the tyrosine/$\beta$-turn hypothesis is applicable to the insulin receptor as well.

Substitution of Tyr^960 with alanine in the LDL receptor inhibits endocytosis and is predicted to destabilize $\beta$-turn formation, whereas a phenylalanine substitution has no effect on internalization (Davis et al., 1989) and retains the $\beta$-turn (Bansal and Giersasch, 1991). Thus, in the LDL receptor the requirement for an aromatic residue (Tyr, Phe, or Trp) at this position cannot be separated from the requirement for the turn. Similarly, replacements of Tyr^953 or Tyr^960 with alanine in the insulin receptor sequences GPLY^953 and NPEY^960 destabilize the $\beta$-turn in receptor-derived peptides and inhibit endocytosis in intact cells. Thus, in both the insulin receptor and the LDL receptor the requirement for a juxtamembrane tyrosine may reflect the role of aromatic residues in stabilizing $\beta$-turn formation. Nonetheless, it seems unlikely that the simple presence of a juxtamembrane $\beta$-turn provides an adequate signal for the entry of receptors into coated pits. Additional mutagenesis studies, in which the juxtamembrane region is altered so as to retain the $\beta$-turn but remove the aromatic residue, will be required to resolve this question.

Our data suggest that tyrosyl-$\beta$-turns serve as internalization motifs in the insulin receptor. While extrapolations from peptide modeling studies to the structure of intact proteins must be made with caution, the point mutations in the present study do not affect insulin binding or receptor autophosphorylation and are unlikely to cause significant nonspecific structural perturbations. The effect of the juxtamembrane mutations on internalization is not due to the loss of potential tyrosine phosphorylation sites, as the phenylalanine mutants internalize normally. Similarly, the data cannot be explained by alterations in the insulin signaling cascade, as the IR^960 mutant is defective for insulin signal transduction (White et al., 1988) but normal for internalization. We also note that although the preference for $\beta$-turn formation by the sequences GPLY and NPEY is clear from peptide modeling studies and from analysis of known crystal structures (Chou and Fasman, 1978), the physical interactions that stabilize these turns are not yet defined. Bansal and Giersasch (1991) noted the importance of an aromatic residue in the i+3 position of the NPEY sequence, and suggested that hydrogen bonding between the side chain amide group of Asn and the aromatic ring of Tyr stabilizes the $\beta$-turn. Although an aromatic residue in the i+3 position is also important in the GPLY $\beta$-turn, the lack of a side group in the i position sug-
suggests that the turn must be stabilized by different interactions, whose delineation will require further study.

It is interesting that the insulin receptor contains an apparently redundant internalization signal. Two independent internalization signals have also been detected in the cytoplasmic domain mannose 6-phosphate receptor (Johnson et al., 1990), the lipoprotein receptor-related protein/α2-macroglobulin receptor (Herz et al., 1988) and the polymeric immunoglobulin receptor (Okamoto et al., 1992). The internalization of cell surface receptors through coated pits may involve direct interactions between their cytoplasmic domains and coated pit-associated proteins (Pearshe, 1988; Beltzer and Spiess, 1991). If such interactions require multivalent interactions with one or more receptor tails, then the presence of two internalization motifs in the same receptor would be expected to increase the apparent affinity of that receptor for the endocytic apparatus, and might increase the efficiency of endocytosis. In this regard, a mutant insulin receptor containing an additional NPEY sequence appears to internalize faster than the wild-type receptor (Carpentier et al., 1992).

The substitution of alanine at Tyr93 has a greater effect on internalization than the substitution of alanine at Tyr91, although the importance of Tyr91 residue is clearly seen in the IR93/IR91 double mutant. The smaller effect caused by the single Ala90 substitution may explain a recent report which could not detect altered internalization in a mutant from which the NPEY91 sequence alone was deleted (Berhanu et al., 1991); our ability to detect this deficit may reflect the greater sensitivity of the initial rate methods developed by Wiley and co-workers (Lund et al., 1990). However, it should be noted that human insulin receptor exists in two isoforms, which arise from the alternative splicing of exon 11 and result in the absence or presence of 12 amino acids at the COOH terminus of the α-subunit (Moller el al., 1989). The study by Berhanu et al. (1992) uses the Exon 11+ form of the insulin receptor, whereas this study uses the Exon 11− form. Although the physiological role of the alternative splicing is not clear, we have recently shown that the internalization rate constant of Exon 11+ receptor is 25% lower than that of the Exon 11− receptor (Yamaguchi et al., 1991); these data may reflect conformational differences in the juxtamembrane regions of the two receptor isoforms which affect endocytosis.

In summary, secondary structure predictions and peptide modeling studies suggest that the insulin receptor juxtamembrane region contains two sequences (GPLY91 and NPEY96) which are predicted to form tyrosine-containing β-turns. Moreover, mutagenesis studies show that Tyr93 and Tyr90 are independently and additively important for rapid endocytosis. The functional and predicted structural equivalence of Tyr93 and Tyr90 suggest that a tyrosyl β-turn serves as a structural motif mediating specific interactions between the insulin receptor and the endocytic system.

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The Journal of Cell Biology, Volume 118, 1992 838
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