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 (Tyr_{953} and Tyr_{960}) during endocytosis. Analysis of the secondary structure of the juxtamembrane region by the Chou-Fasman algorithms predicts that both the sequences $GPLY_{953}$ and $NPEY_{960}$ 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 [^{125}I]insulin endocytosis, whereas single substitutions with alanine reduced [^{125}I]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; 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). Moreover, in several receptors short tyrosine-containing sequences have been identified which are required for rapid internalization including $FXNPXY_{807}$ (LDL receptor; Chen et al., 1989), $Y_{20}TRF$ (transferrin receptor; Collawn et al., 1990), and $Y_{24}XY_{26}XKV$ (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 (Ktistakis et al., 1990). Consistent with this hypothesis, the introduction of a tyrosine immediately distal to residues favoring turn formation in the cytoplasmic tail of glycophorin caused this molecule, normally excluded from coated pits, to be efficiently internalized (Ktistakis 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 both 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 $NPEY_{960}$, which are similar to the sequence $NPVY_{807}$ 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-

stitutive 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 NPEY$_{960}$ motif (Backer et al., 1991b). Thus, the NPEY$_{960}$ 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 Tyr$_{953}$ and Tyr$_{960}$, which are present in GPLY$_{953}$ and NPEY$_{960}$ motifs, respectively, form tyrosine-containing β-turns. These predictions are supported by 2-D NMR analysis of peptides containing the GPLY$_{953}$ and NPEY$_{960}$ sequences. Substitution of alanine in place of either Tyr$_{953}$ or Tyr$_{960}$, 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, Tyr$_{953}$ and Tyr$_{960}$ appear to contribute independently and additively with respect to receptor internalization. The functional equivalence of Tyr$_{953}$ and Tyr$_{960}$ for internalization may be due to their similar conformational environment in the insulin receptor juxtamembrane region.

Materials and Methods

Peptide Synthesis

Octapeptides corresponding to the native and mutant insulin receptor sequences PLGPLY$_{953}$AS (GPLY), PLGPLA$_{953}$AS (GPLA), SSNPEY$_{960}$SA (NPEY), and SSNPEA$_{960}$SA (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

^{1}H-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 deuteroacetate (pH 4.5; 10% D$_2$O) 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 4°C. For each sample, double-quantum filtered correlated spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY) (mixing time 55 ms), and nuclear overhauser enhancement spectroscopy (NOESY) (mixing time 400 ms) were recorded and processed on Varian software. The water solvent resonance was attenuated by presaturation. For quantitative comparisons, NOESY spectra of octapeptide analogs were normalized relative to shared intra-residue cross-peaks, e.g., the intra-residue NH$_2$ crosspeak of glutamine, the ortho-meta crosspeak of tyrosine, etc. The absence of NOEs was determined relative to the observed signal-to-noise ratio.

Site-directed Mutagenesis of the Human Insulin Receptor cDNA and Expression of Mutant Receptors in CHO Cells

Numbering of the insulin receptor amino acid sequence is taken after Ullrich

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 Tyr$_{960}$ is replaced by phenylalanine (IR$_{F960}$) or from which residues Ala$_{954}$-Asp$_{965}$ have been deleted (IR$_{A960}$) were provided by A. Ullrich (Max Planck Institute für Biochemie); cDNA for a kinase-deficient mutant in which Lys$_{1018}$ is replaced by alanine (IR$_{A1018}$) was provided by O. M. Rosen (deceased). The IR$_{F960}$ receptor has been found to contain a second mutation, with Ser$_{962}$ substituted by Thr$_{962}$ (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/IR$_{F960}$ CHO/IR$_{A960}$; and CHO/IR$_{A1018}$) were maintained as previously described (Backer et al., 1991b). New point mutations consisting of the substitution of Phe or Ala in place of Tyr$_{953}$ (IR$_{F953}$ and IR$_{A953}$) or Ala in place of Tyr$_{960}$ (IR$_{A960}$) were introduced into the human insulin receptor cDNA by oligonucleotide-directed mutagenesis as previously described (White et al., 1988), using the primers GGACCGCTTGCCGCTTCTTCA (IR$_{A953}$), GGACCGCTTTTCGCTTCTTCA (IR$_{F953}$), and AACCCTGAGGCT-CTCAGTGCC (IR$_{A960}$). A double mutation, replacing both Tyr$_{953}$ and Tyr$_{960}$ with alanine (IR$_{A953/A960}$) was created by site-directed mutagenesis of the IR$_{A960}$ mutant, using the IR$_{A953}$ primer. CHO cells were cotransfected with pSVEneo, 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). Clonal 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 for 2 h with 0.5 mCi/ml [^{32}P]phosphate (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 aprotinin, 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 [^{125}I]Insulin by CHO Cells Expressing Wild-type or Mutant Insulin Receptors

Internalization of [^{125}I]insulin ([^{125}I]iodotyrosyl A14-insulin, 2,000 Ci/mmol) 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 [^{125}I]insulin as previously described (Lund et al., 1990; Backer et al., 1991b) using the equation:

$$L_{int} = k_e \int_0^t (LR)dt. \tag{1}$$

where L_{int} represents internalized [^{125}I]insulin, LR represents surface-bound [^{125}I]insulin, and the slope of the resultant line is equal to the internalization rate constant k_e. Integrals were approximated by the trapezoidal rule, using an interval of $\Delta t = 2$ 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 [^{125}I]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_e) was determined at each insulin concentration by linear regression as described above, and k_e 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-

mal ligand recycling during the period of measurement and a large number of receptors per cell, these data can be described by the equation:

$$k_e = -K_{IC}v + K_{IC}V_{max}. \qquad (2)$$

where k_e is the internalization rate constant, v is the velocity of ligand uptake, V_{max} is the maximum uptake velocity of the system, and 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 (Lund et al., 1990). The plot of k_e against v, as both parameters change with increasing ligand concentrations, is analogous to an Eadie-Scatchard plot, where the slope reflects the ability of a receptor to enter the saturable internalization pathway in CHO cells (Backer et al., 1991b). The slope of the saturable component in each cell line, K_{IC}, was determined by linear regression; the x-intercept of the calculated line yields the V_{max} for the saturable pathway. The half-saturating surface occupancy, $1/K_{IC}$ (Lund et al., 1990), was calculated from the negative reciprocal of the derived slope of the rapid component of the saturation plot.

Results

Structural Characterization of the Insulin Receptor Juxtamembrane Region

Several recent studies suggest that tyrosine residues in β-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 β-subunit contains three tyrosine residues in its cytoplasmic juxtamembrane region at Tyr953, Tyr960, and Tyr972. We have previously shown that the deletion of 12 amino acids, including Tyr960, reduces the rate of insulin receptor endocytosis (Backer et al., 1990, 1991b). To examine the possible role of tyrosine/β-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 GPLY953 and NPEY960 are both predicted to form β-turns with the tyrosine residue in the fourth position, whereas Tyr972 was not predicted to reside in a β-turn (data not shown).

To evaluate the conformational preference of these potential insulin receptor internalization signals, synthetic octapeptides corresponding to the native sequences PLGPLY953AS (GPLY),SSNPEY960SA (NPEY) and the mutant sequences PLGPLA953AS (GPLA) SSNPEA960SA (NPEY) were prepared and examined by NMR. The amide resonances of the two native sequences are shown in one-dimensional 1H-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 β-turn formation, as recently emphasized by Bansal and Giersasch (1991) and Eberle et al. (1991). No evidence for α-helix or β-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_{i+2}-HN_{i+3} contacts characteristic of a β-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 GPLY953 and NPEY960 have a strong preference to form β-turns in

solution. Furthermore, substitution of Tyr953 with alanine in the GPLA953 peptide, or Tyr960 with alanine in the NPEA960 peptide, destabilizes formation of the β-turn.

Mutagenesis of the Insulin Receptor Juxtamembrane Region at Tyr953 and Tyr960

Chou-Fasman analysis and peptide modeling studies suggest that both Tyr953 and Tyr960 should be present in tyrosyl-containing β-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 Tyr953 or Tyr960 was replaced singly by phenylalanine (IRF953, IRF960) or alanine (IRA953, IRA960); an additional mutant replaced both residues with alanine (IRA953/A960). The mutant receptors were expressed in CHO cells at $\sim$1.2 × 10^6 receptors/cell; the CHO/IRF960 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 IRA1018 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 [32P]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 β-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 β-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 IRF960 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 [125I]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 [125I]insulin rapidly, with a rate constant of k_e = 0.154 ± 0.01 min^{-1} (Fig. 4). In contrast, [125I]insulin internalization by the kinase-deficient CHO/IRA1018 cells was over 10-fold slower (k_e = 0.0135 ±
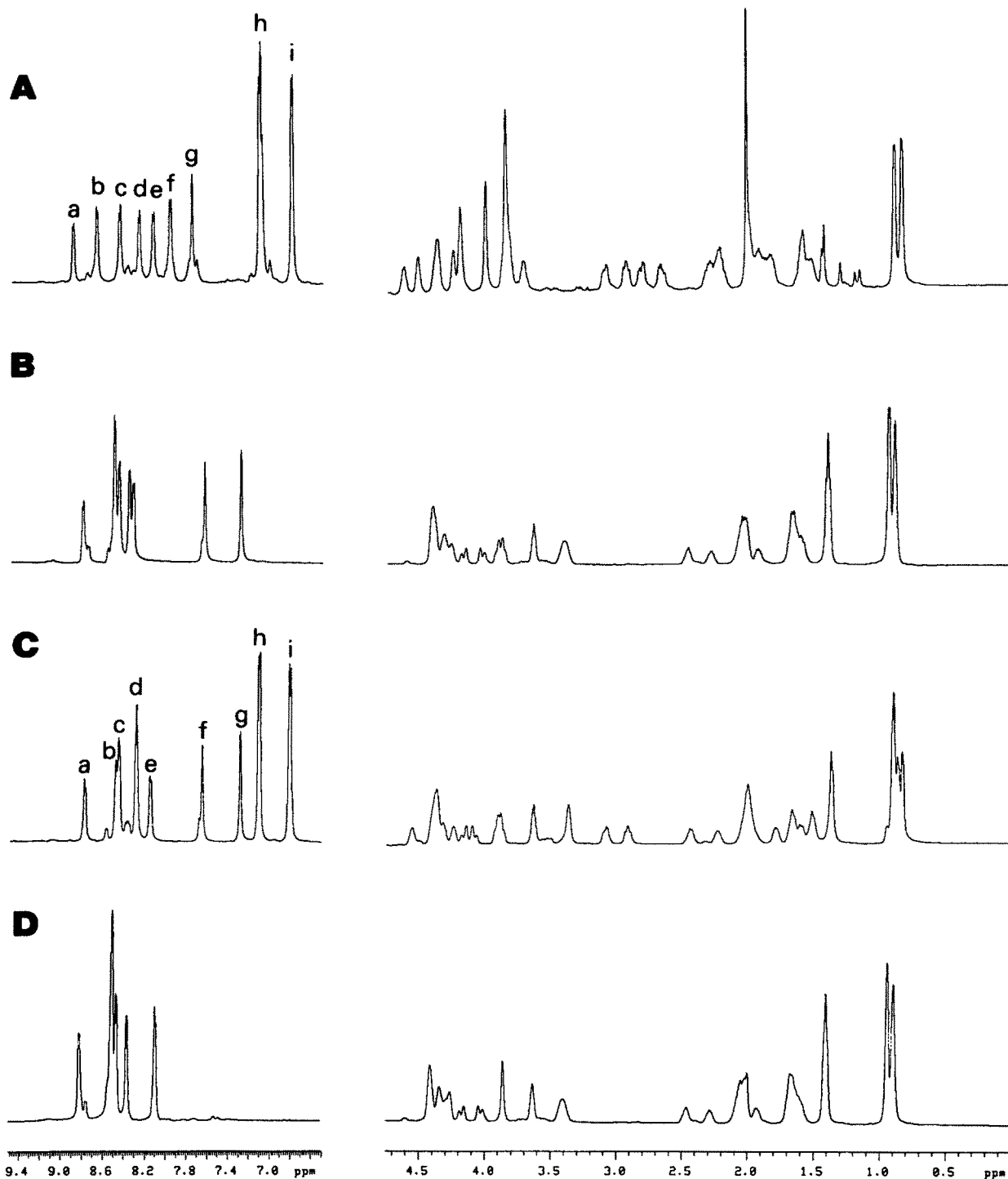
Figure 1. 1-D NMR spectra of synthetic peptides derived from the juxtamembrane region. One-dimensional ^{1}H-NMR spectra of NPEY (*A*), NPEA (*B*), GPLY (*C*), and GPLA (*D*) octapeptides at 4°C in 90% H_2O/10% D_2O and 50 mM NaCl (pH 4.5). The enhanced resonance dispersion seen in spectra *A* and *C* reflect the presence of a nonrandom conformational preference (β-turn; see Fig. 2). In each case, complete resonance assignment has been obtained by the 2-D sequential method. Resonances in the spectra of β-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$_{\delta1}$; (*h*) N3-NH$_{\delta2}$ and Y6-H$_\delta$ (unresolved); and (*i*) Y6-H$_\epsilon$. 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$_\delta$; and (*i*) Y6-H$_\epsilon$.

0.004 min^{-1}). We have previously shown that internalization of the IR$_{A1018}$ receptor does not occur through the kinase-dependent saturable route (Backer et al., 1991*b*). Therefore, these cells set a lower limit for [^{125}I]insulin in-

ternalization in cells which have a high level of insulin binding but are defective for insulin-stimulated internalization.

Substitution of either Tyr$_{953}$ or Tyr$_{960}$ with phenylalanine had little or no effect on the rate of 0.02 nM [^{125}I]insulin in-
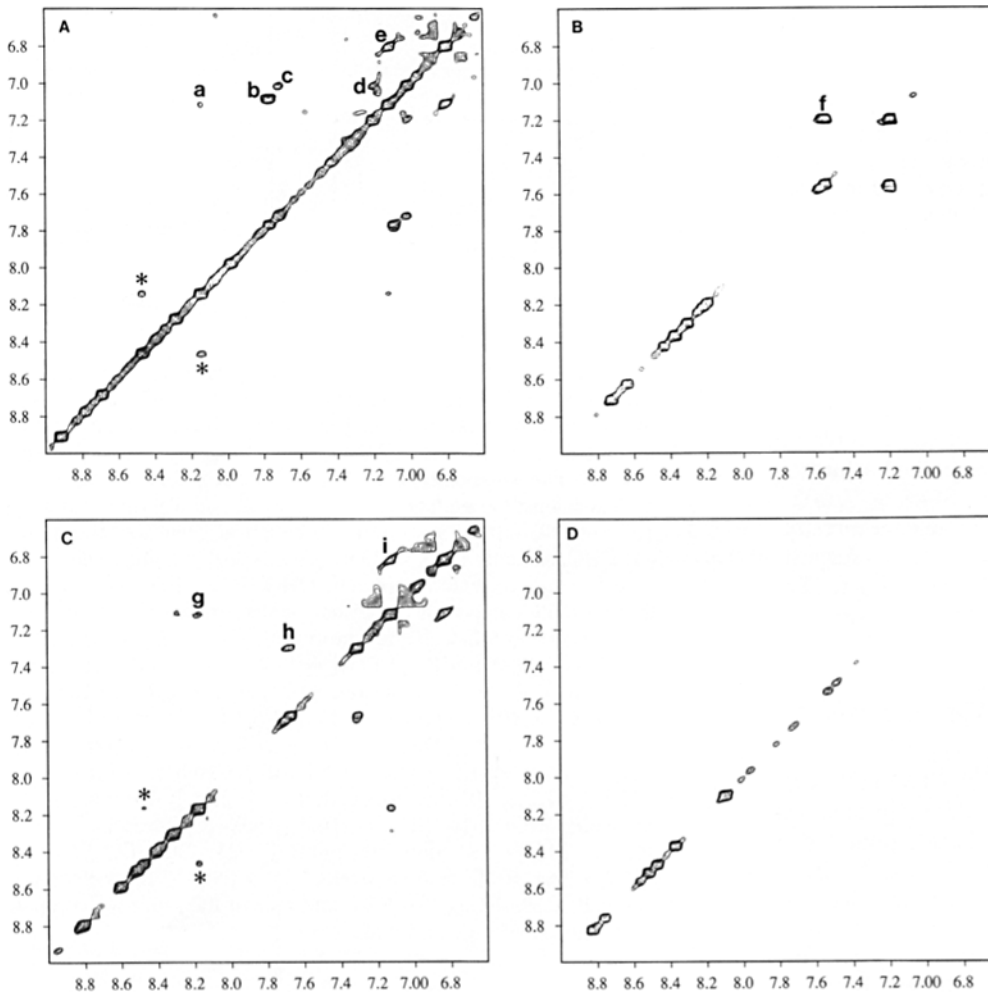
Figure 2. 2-D NMR spectra of synthetic peptides derived from the juxtamembrane region. Amide region of corresponding two-dimensional NOESY spectra of NPEY (*A*), NPEA (*B*), GPLY (*C*), and GPLA (*D*) octapeptides at 4°C in 90% H_2O/10% D_2O and 50 mM NaCl (pH 4.5). Characteristic $(i+2,i+3)$ NOE (*asterisks*) is observed in *A* and *C*, providing evidence of β-turn formation (Bansal and Gierasch, 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 upfield 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 $N_\delta H_2$ 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 NH_2 NOE from COOH-terminal amide; and (*i*) Y6 ortho-meta intra-residue NOE.

ternalization. The k_e for internalization in the CHO/IR_{F953} and CHO/IR_{F960} cells was 0.137 ± 0.008 and 0.156 ± 0.005 min^{-1}, respectively. These data are consistent with previous observations that the substitution of phenylalanine for Tyr807 has no effect on LDL receptor internalization (Davis et al., 1987) or formation of the NPVY807 β-turn (Bansal and Giersasch, 1991). In contrast, substitution of insulin receptor Tyr953 with alanine caused a 50% decrease in the
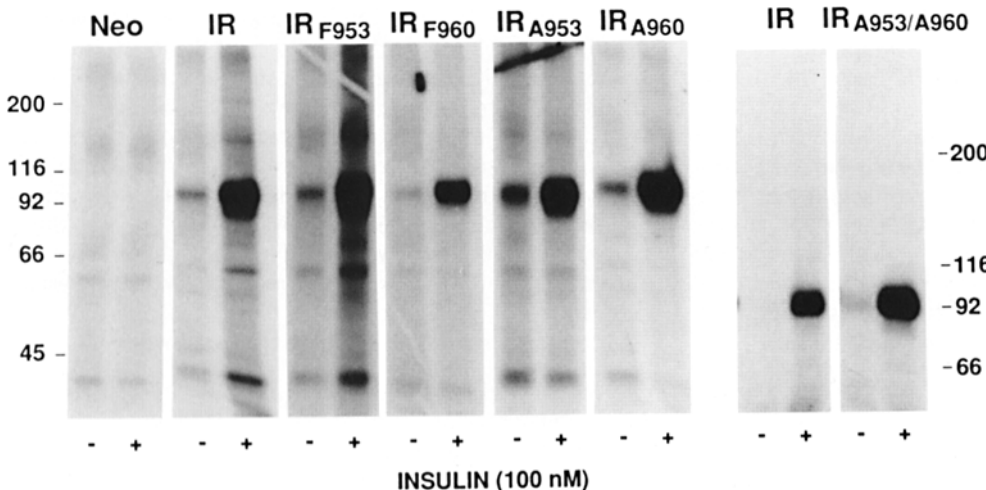

Figure 3. Insulin-stimulated autophosphorylation of wild-type and mutant insulin receptors in CHO cells. CHO cells were incubated in [^{32}P]orthophosphate for 2 h and for an additional 2 min in the absence or presence of insulin (100 nM). Insulin receptors were immunoprecipitated from solubilized cells with a monoclonal anti-receptor antibody 83-14, separated by SDS-PAGE, and visualized by autoradiography.
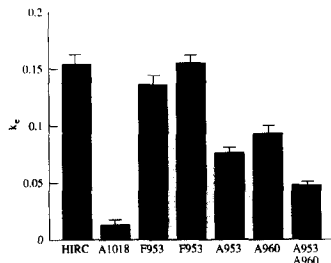
Figure 4. [125I]insulin internalization in CHO cells expressing wild-type or mutant insulin receptors. Cells were incubated with [125I]insulin (0.02 nM) for 0–10 min, and intracellular radioactivity was determined by acid washing. The rate constant for insulin receptor internalization (k_e) in each cell line was calculated as described in Materials and Methods.

[125I]insulin internalization rate (k_e = 0.076 ± 0.005 min⁻¹), and substitution of Tyr960 with alanine caused a 40% reduction in internalization (k_3 = 0.093 ± 0.007 min⁻¹). Thus, both Tyr953 and Tyr960 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 Tyr953 and Tyr960 may contribute to insulin receptor endocytosis.

Saturation Plots for Insulin Receptor Internalization in CHO Cells Expressing Wild-type and Mutant Receptors

We have previously shown that in CHO cells expressing ~10⁶ 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$_{A960}$ receptor, which lacks the NPEY960 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
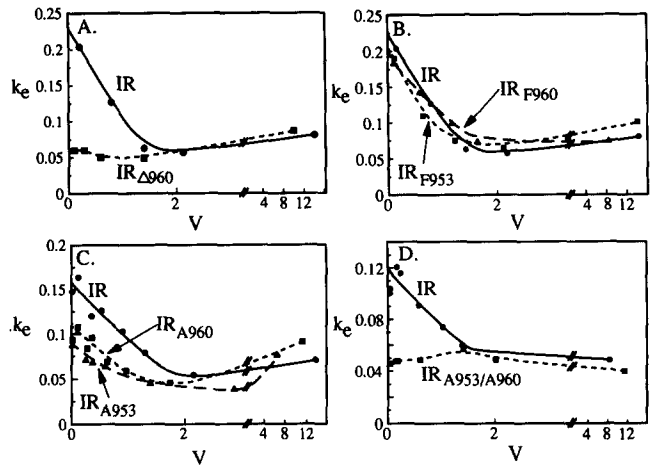

Figure 5. Saturation plots of [125I]insulin internalization in cells expressing wild-type or mutant insulin receptors. The rate constant for internalization (k_e) was determined in each cell line during 10-min incubations at 37°C in the presence of varying insulin concentrations. The value of k_e (min⁻¹) at each insulin concentration was plotted against the average velocity of insulin uptake (molecules × 10⁴/min/cell) during a 10-min incubation at each concentration. (A) CHO/IR cells; ED50 = 33.4 ± 9.2 × 10³ receptors/cell, V_{max} = 6.5 × 10³ molecules/min/cell; CHO/IR$_{A960}$: ED50 and V_{max} of the saturable component could not be determined (B). CHO/IR; ED50 = 33.4 ± 9.2 × 10³ receptors/cell, V_{max} = 6.5 × 10³ molecules/min/cell; CHO/IR$_{F953}$; ED50 = 42.4 ± 14.7 × 10³ receptors/cell, V_{max} = 7.2 × 10³ molecules/min/cell; CHO/IR$_{F960}$: ED50 = 33.7 ± 2.1 × 10³ receptors/cell, V_{max} = 6.3 × 10³ molecules/min/cell. (C) CHO/IR; ED50 = 158.6 ± 26.0 × 10³ receptors/cell, V_{max} = 24.3 × 10³ molecules/min/cell; CHO/IR$_{A953}$: ED50 = 276.5 ± 67.2 × 10³ receptors/cell, V_{max} = 23.8 × 10³ molecules/min/cell; CHO/IR$_{A960}$: ED50 = 191.6 ± 55.6 × 10³ receptors/cell, V_{max} = 20.1 × 10³ molecules/min/cell. (D) CHO/IR; ED50 = 261.7 ± 56.8 × 10³ receptors/cell, V_{max} = 29.3 × 10³ molecules/min/cell; CHO/IR$_{A953/A960}$: ED50 and V_{max} of the saturable component could not be determined.

were slightly higher than previously reported values (ED50 = 150,000 ± 35,500 receptors/cell, V_{max} = 22,800 ± 3,000 molecules/min/cell). Substitution of either Tyr953 or Tyr960 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 Tyr960 with alanine caused a 25% decrease in the slope and a 20% decrease in the V_{max} for the saturable pathway, whereas substitution of Tyr953 with alanine caused a 60% decrease in the slope without significantly affecting the V_{max} (Fig. 5 C; Table I). Strikingly, substitution of both tyrosine residues with alanine eliminated the rapid saturable component (Fig. 5 D, Table I), resulting in a saturation plot similar to that seen with the IR$_{A960}$ cells (Fig. 5 A). None of the mutations affected internalization velocity through the slow, nonsaturable internalization pathway observed at high receptor occupancies (Fig. 5, A–D). Thus, Tyr953 and Tyr960 play an independent and additive role in the entry of insulin receptors into the rapid saturable internalization pathway.

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_{IC} (% of WT)	V_{max} (% of WT)	n
CHO/IR$_{F953}$	89.5 ± 10.5	111.0 ± 1.5	2
CHO/IR$_{F960}$	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$_{A953/A960}$	0	ND	2
CHO/IR$_{\Delta960}$	0	ND	3

The values for K_{IC}, 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$_{\Delta960}$ and IR$_{A953/A960}$ 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., 1991*b*). In this study, we provide evidence that a critical determinant for rapid insulin receptor endocytosis is the presence of tyrosine-containing β-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 β-turn formation in the juxtamembrane region. Tyr$_{960}$ is present in the sequence NPEY$_{960}$, which is similar to the LDL receptor sequence NPVY$_{807}$, and a peptide derived from the insulin receptor NPEY$_{960}$ has been previously shown to form a β-turn in solution (Bansal and Giersasch, 1991). The sequence GPLY$_{953}$ is only 50% homologous with the LDL receptor NPVY$_{807}$ sequence. However, it is also predicted by Chou-Fasman analysis and 2-D NMR studies to form a β-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 β-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$_{807}$, 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 β-turn. Similarly, Ktistakis 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$_8$ and the LDL receptor sequence NPVY$_{807}$ form β-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$_{807}$ sequence, showed a striking correlation between β-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/β-turn hypothesis is applicable to the insulin receptor as well.

Substitution of Tyr$_{807}$ with alanine in the LDL receptor inhibits endocytosis and is predicted to destabilize β-turn formation, whereas a phenylalanine substitution has no effect on internalization (Davis et al., 1989) and retains the β-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 β-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 β-turn formation. Nonetheless, it seems unlikely that the simple presence of a juxtamembrane β-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 β-turn but remove the aromatic residue, will be required to resolve this question.

Our data suggest that tyrosyl/β-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$_{F960}$ mutant is defective for insulin signal transduction (White et al., 1988) but normal for internalization. We also note that although the preference for β-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 β-turn. Although an aromatic residue in the i+3 position is also important in the GPLY β-turn, the lack of a side group in the i position sug-

gests 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 cation-dependent 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 (Pearse, 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 Tyr_{953} has a greater effect on internalization than the substitution of alanine at Tyr_{960}, although the importance of Tyr_{960} residue is clearly seen in the $IR_{A953/A960}$ double mutant. The smaller effect caused by the single Ala_{960} substitution may explain a recent report which could not detect altered internalization in a mutant from which the $NPEY_{960}$ 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 et 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 ($GPLY_{953}$ and $NPEY_{960}$) which are predicted to form tyrosine-containing β-turns. Moreover, mutagenesis studies show that Tyr_{953} and Tyr_{960} are independently and additively important for rapid endocytosis. The functional and predicted structural equivalence of Tyr_{953} and Tyr_{960} 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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