Optimizing transmembrane domain helicity accelerates insulin receptor internalization and lateral mobility

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ABSTRACT Transmembrane (TM) domains of integral membrane proteins are generally thought to be helical. However, a Gly-Pro sequence within the TM domain of the insulin receptor is predicted to act as a helix breaker. CD analyses of model TM peptides in a lipid-like environment show that substitution of Gly and Pro by Ala enhances helicity. On this basis, Gly^{933} and Pro^{934} within the TM domain of the intact human insulin receptor were mutated to Ala (G → A, P → A, GP → AA) to assess effects of altered helicity on receptor functions. Mutated and wild-type receptors, expressed stably in cultured CHO cells at equivalent levels, were properly assembled, biosynthetically processed, and exhibited similar affinities for insulin. Receptor autophosphorylation and substrate kinase activity in intact cells and soluble receptor preparations were indistinguishable. In contrast, insulin-stimulated receptor internalization was accelerated 2-fold for the GP → AA mutant, compared to a wild-type control or the G → A and P → A mutants. Insulin degradation, which occurs during receptor endocytosis and recycling, was similarly elevated in cells transfected with GP → AA mutant receptors. Fluorescence photobleaching recovery measurements showed that the lateral mobility of GP → AA mutant receptors was also increased 2- to 3-fold. These results suggest that lateral mobility directly influences rates of insulin-mediated receptor endocytosis and that rates of endocytosis and lateral mobility are retarded by a kinked TM domain in the wild-type receptor. Invariance of Gly-Pro within insulin receptor TM domain sequences suggests a physiologic advantage for submaximal rates of receptor internalization.

Surface receptors involved in ligand-mediated signal transduction contain discrete protein domains that traverse the lipid milieu of the plasma membrane. Whether these hydrophobic domains play a passive role as membrane anchors or participate directly in signal propagation, protein–protein interactions, or other cellular functions has been the subject of considerable speculation (1–5). From high-resolution structural analyses (6–8) and computer-assisted modeling and energy minimization studies (9–11), it is generally held that transmembrane (TM) domains of many membrane-spanning proteins adopt α-helical structures. For proteins that span the membrane once, a hydrophobic α-helix is an energetically favored structure. Therefore, amino acid compositions of some TM domains are somewhat surprising (1–5). For example, Gly and Pro, residues considered to be classical helix breakers (12, 13), are found within putative TM domains of integral membrane proteins with relatively high frequency. Pro and Gly are rarely found within α-helices of globular proteins, except at the N and C termini, respectively (14). When present within the body of an α-helix Pro creates

a kink (15). Although effects are positional, Gly substitutions destabilize α-helices of globular proteins by 0.4–2.0 kcal/mol (1 cal = 4.184 J), relative to Ala (16, 17).

The single TM of the insulin receptor (within each αβ-half-receptor) contains mainly hydrophobic amino acids that are flanked at appropriate positions by basic residues (18, 19) (K^{929}IIIGPLIFVFLFSVVIGSIYLFLRKR^{955}).¶ Whereas other regions of the insulin receptor vary between species, the putative 23-residue insulin receptor TM domain is invariant in sequences of human, mouse, and rat receptors that have been reported (16, 17, 20, 21). The presence of a Gly-Pro sequence (underline) suggests a possible function for these residues and provides an attractive model for testing the functional significance of helix breakers within the TM domain of a complex membrane-spanning protein. We have substituted Gly^{933} and Pro^{934} of the wild-type human insulin receptor with Ala (G → A, P → A, GP → AA) by using oligonucleotide-directed mutagenesis (22) and studied the effects on a variety of receptor functions.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. Peptides SNIAKIIIGPLIFVFSKSKSK and SNIAKIIIAALIFVFSKSKSK were synthesized on a Milligen/Biosearch 9600 synthesizer using an N^α-fluoren-9-ylmethoxycarbonyl (Fmoc)/*t*-butyl side chain protecting group strategy. Peptide-bond-forming reactions with 0.2 M N^α-Fmoc amino acid, 1-hydroxybenzotriazole, and benzotriazoyloxy tris-(dimethylamino)phosphonium hexafluorophosphate were conducted for 1–4 h. Peptides were cleaved from the resin and deprotected by treatment with trifluoroacetic acid/thioanisole/ethanedithiol/anisole, 90:5:3:2 (vol/vol), for 2 h at 22°C, precipitated with diethyl ether, desalted on a column of Bio-Gel P-2 (2.6 × 100 cm) in 3.0 M acetic acid, and purified by reversed-phase HPLC using a Dynamax-300Å 12-μm C$_8$ column (41.4 × 250 mm). Results from analytical HPLC, amino acid composition, protein microsequencing, and mass spectrometric analyses were as predicted.

CD Analysis. Spectra were obtained at 4°C using an Aviv spectropolarimeter with a 1.0-mm·pathlength cuvette; protein concentrations were 50 μM. Ellipticity (θ) values measured for 10 s at each wavelength (nm) were used to calculate molar ellipticities [θ].

Biochemistry: Goncalves *et al.*

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Receptor Mutagenesis and Isolation. Oligonucleotide-directed mutagenesis of the wild-type insulin receptor TM domain and transfection of CHO cells were as described (22). Clonal cell lines were obtained by limiting dilution and clones expressing equivalent receptor numbers were identified by insulin binding and Scatchard analyses (22). CHO cells expressing wild-type or mutated receptors were grown to confluence in culture and solubilized with 1.0% Triton X-100 in 50 mM Hepes containing various protease inhibitors; receptors were partially purified by lectin affinity chromatography (22, 23).

Receptor Quantitation and Insulin Binding Affinity. Intact CHO cells expressing wild-type or mutated receptors were grown to confluency in 24-well tissue culture plates and incubated with ^{125}I-labeled insulin ([^{125}I]insulin; 0.1 ng/ml) and various concentrations of unlabeled insulin at 15°C for 5 h. Medium was removed, cells were lysed with 1 M NaOH, and associated radioactivity was determined in a γ counter (22). Insulin binding assays for lectin-purified receptor preparations and Western blot experiments were as described (22, 23).

Receptor Kinase Activity, Receptor Internalization, and Cell-Mediated Insulin Degradation. Receptor autophosphorylation and substrate kinase assays in intact cells and with partially purified receptors were as described (22–24). Analyses of rates of insulin-induced receptor internalization in intact cells were as described (25). To quantify rates of [^{125}I]insulin degradation by transfected CHO cells, confluent 24-well dishes were washed with phosphate-buffered saline (PBS) and incubated with [^{125}I]insulin (10^5 cpm) for 18 h at 4°C. Cells were washed twice with chilled PBS and quickly warmed to 37°C by addition of warm F-12 medium containing 50 mM Hepes and 0.1% bovine serum albumin at pH 7.4. Cells were maintained at 37°C. At the indicated times, aliquots of medium were removed, and [^{125}I]insulin was precipitated with 7.5% (wt/vol) aqueous trichloroacetic acid (final concentration).

Fluorescence Photobleaching Recovery. Transfected CHO cells were grown to subconfluent densities on microscope coverslips in F-12 media supplemented with 10% (vol/vol) fetal bovine serum and transferred to phenol red-free medium 18 h prior to fluorescence photobleaching recovery experiments. Cells were washed twice with PBS, incubated with 0.1 μM B29$^\varepsilon$-carboxyfluorescein-insulin for 5 min at 21°C, and washed three additional times with PBS. Within 5 min of fluorescent labeling, each coverslip was inverted, sealed on a microscope slide, and transferred to the microscope stage, maintained at 21.0 $\pm$ 0.1°C. The apparatus and analytical methods were as described (26, 27).

B29$^\varepsilon$-carboxyfluorescein-insulin was prepared by trypsin-catalyzed semisynthesis (23). A protected octapeptide corresponding to residues B23–B30 of insulin (trifluoroacetyl-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr-OH; 0.2 M in dimethylformamide) was incubated with two equivalents of 5-(and 6)-carboxyfluorescein succinimide ester (Molecular Probes) for 24 h at 22°C. The product (>99% reacted) was precipitated with diethyl ether and incubated with 10% (vol/vol) aqueous piperidine to remove the trifluoroacetyl group; the resulting H-Gly-Phe-Phe-Tyr-Thr-Pro-Lys(ε-carboxyfluorescein)-Thr-OH was desalted on a column of LH-20 Sephadex in methanol and coupled with the assistance of trypsin to A1$^\alpha$,B1$^\alpha$-di-*t*-butoxycarbonyl-Des-(B23–B30)-insulin to yield, after deprotection and gel filtration (23), B29$^\varepsilon$-carboxyfluorescein-insulin (40% yield; purity >98%).

RESULTS

TM Domain Structural Analyses. Synthetic peptides corresponding to wild-type and mutated insulin receptor TM domains were designed to test regional helicity in aqueous
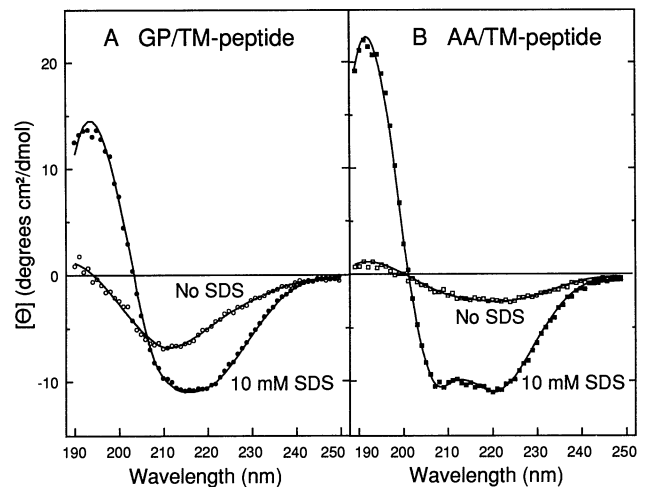

FIG. 1.   Far UV CD spectra. CD spectra of wild-type GP/TM- (*A*) and substituted AA/TM-peptides (*B*) were obtained in 10 mM sodium phosphate/10 mM NaCl, pH 7.0 (no SDS; open symbols), and in the same buffer containing 10 mM SDS (solid symbols).

and lipid-like environments, by strategies developed by Li and Deber (28). The GP/TM-peptide <u>SNIAKIIIGPLIFVF</u>-SKSKSK contains the N-terminal half of the native sequence (underline) plus an SKSKSK sequence at the peptide C terminus to enhance solubility;$^\parallel$ the AA/TM-peptide <u>SNIAKIIIAALIFVF</u>SKSKSK is identical except for a GP$\rightarrow$ AA substitution.

CD data obtained in the absence of detergent suggest that neither peptide is helical. Upon addition of 10 mM SDS, the AA/TM-peptide adopts CD spectral features that are classic for α-helices (Fig. 1), with characteristic minima at 208 nm and 222 nm and maximum at 198 nm (29) [the critical micelle concentration of SDS is 8 mM (30)]. Under identical conditions the GP/TM-peptide does not adopt defined minima at either 208 nm or 222 nm, having instead a broad minimum at $\approx$214 nm. Spectra exhibited little dependence on peptide concentration between 20 and 100 μM. These findings demonstrate enhanced helicity of the insulin receptor TM domain in a lipid-like environment due to the GP $\rightarrow$ AA substitution.

Receptor Expression and Insulin Binding Affinity. Transfected CHO cells exhibited high expression levels of the wild-type and all three (G $\rightarrow$ A, P $\rightarrow$ A, and GP $\rightarrow$ AA) mutated receptors (0.5–0.9 $\times$ 10^6 receptors per cell), as assessed by insulin binding (Fig. 2), Western blot analysis (Fig. 2 *Inset*), and metabolic labeling experiments (data not shown), vs. $\approx$10^4 endogenous hamster receptors per cell for cells that were transfected with the neomycin-resistance gene alone. The mutated receptors bound insulin with normal affinity, with calculated ED$_{50}$ values for competition with [^{125}I]insulin of 0.7–0.9 nM for mutant and wild-type receptors in intact cells (Fig. 2).

Receptor Autophosphorylation and Substrate Phosphorylation. The ability of the mutated receptors to transmit the insulin binding signal to the intracellular tyrosine kinase domain was also tested. Normal stimulation of β-subunit autophosphorylation was observed in each case, whether studied in intact cells by Western blot analysis with anti-phosphotyrosine antibodies (Fig. 3*A*) or by solubilization of cells with Triton X-100 and partial receptor purification on a column of wheat germ agglutinin-Sepharose (Fig. 3*B*) (22–24). Furthermore, insulin stimulated the *in vitro* phosphorylation of an exogenous peptide substrate by solubilized wild-type and mutated receptors 6- to 10-fold (Fig. 3*C*).

$^\parallel$Full-length TM-domain-derived peptides are insoluble (S.E.S., unpublished data).
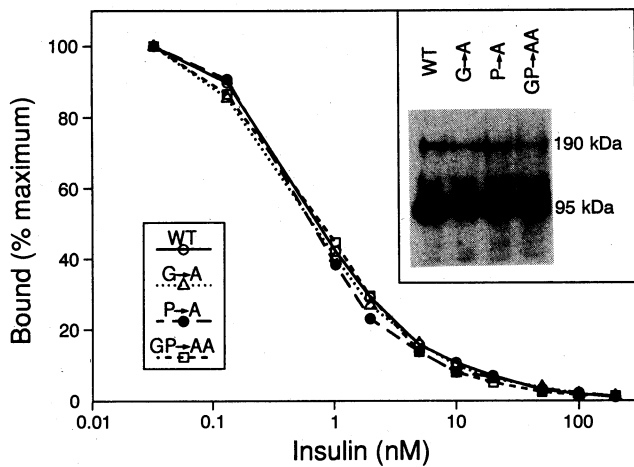
FIG. 2. Competition assays for [^{125}I]insulin binding to CHO cells transfected with wild-type or mutated receptors. Confluent cell monolayers in 24-well tissue culture plates were incubated with A14-[^{125}I]insulin (0.1 ng/ml) and various concentrations of unlabeled insulin in 0.5 ml of F-12 medium supplemented with 10 mM Hepes and 0.1% bovine serum albumin (pH 7.5) at 15°C for 5 h. Medium was removed, cells were washed and lysed with 1 M NaOH, and associated radioactivity was determined in a γ counter. (*Inset*) Western blot analysis of insulin receptors from transfected cells. Equivalent numbers of transfected cells were solubilized with Laemmli sample buffer and separated by SDS/PAGE. Proteins were transferred to nitrocellulose sheets, which were incubated sequentially with an anti-insulin receptor antibody directed against the β subunit (R1064, provided by Paul Pilch, Boston University) and ^{125}I-labeled protein A. Receptors were identified in autoradiograms; 95-kDa β subunits and 190-kDa proreceptors are identified. WT, wild type.

Insulin dose–response effects for activation of substrate kinase activity were similar for wild-type and mutant receptors, with calculated ED$_{50}$ values of 6–9 nM. Phosphotransferase activity of the mutated receptors was also normal in intact cells, as observed by normal phosphorylation of the endogenous substrate pp185 (data not shown). Thus, insulin binding and the abilities of the mutated receptors to transmit ligand binding signals to intracellular kinase domains appear to be entirely normal.

Insulin Receptor Internalization. Insulin-occupied receptors are internalized via clathrin-coated pits and enter the coated vesicle–endosome system where insulin dissociates and is degraded (31). We have shown (25) that, at physiological levels of insulin, normal human insulin receptors expressed in transfected CHO cells are internalized via this pathway as well. To determine effects of TM mutations on rates of insulin receptor endocytosis, CHO cells expressing equivalent numbers of wild-type or mutated receptors were incubated with subsaturating concentrations (0.2 nM) of [^{125}I]insulin at 37°C. To stop internalization, cells were chilled to 4°C at the appropriate times and washed with PBS at either neutral (7.6) or acidic (3.5) pH to assess total-cell-associated or internalized [^{125}I]insulin, respectively (25). Rate constants for internalization, k_e, were calculated using Eq. 1,

$$L_{int} = k_e \int_0^t (LR)dt, \qquad [1]$$

where L_{int} and LR represent internalized and receptor-bound [^{125}I]insulin, respectively, k_e is defined by the slope of the resulting line (25, 32), and t is time. CHO cells transfected with mutated G → A (k_e = 0.056 ± 0.004 min^{-1}; n = 4) and P → A (k_e = 0.060 ± 0.009 min^{-1}; n = 4) receptors internalized [^{125}I]insulin at rates indistinguishable from cells transfected with wild-type receptors (k_e = 0.065 ± 0.004
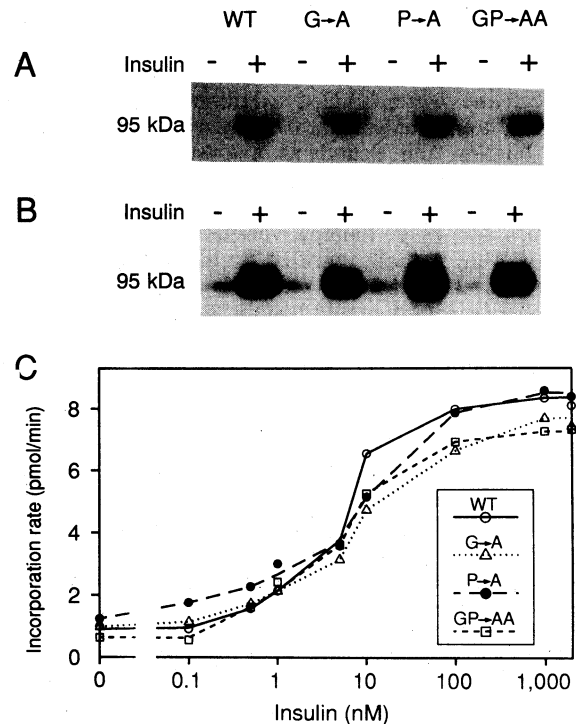

FIG. 3. (*A*) Receptor autophosphorylation in intact cells. Confluent monolayers of transfected CHO cells in 10-cm culture dishes were incubated with or without 100 nM insulin for 10 min at 37°C. Cells were frozen with liquid N$_2$ and solubilized in 1 ml of a 1.0% Triton X-100 solution. Insulin receptors, isolated with monoclonal antibody 83-14 and separated by SDS/PAGE, were identified by Western blot analysis with anti-phosphotyrosine antibodies (22). (*B*) Autophosphorylation of solubilized receptors. Identical amounts of partially purified receptors (equalized for insulin binding) were incubated with or without 1.0 μM insulin for 45 min at 22°C. Receptors were phosphorylated with 5 mM MnCl$_2$/10 mM MgCl$_2$/0.10 mM [γ-^{32}P]ATP (final concentrations) for 10 min at 22°C, immunoprecipitated with antibody 83-14, and separated by SDS/PAGE. (*C*) Exogenous substrate phosphorylation. Partially purified receptor preparations catalyzed phosphorylation of exogenous peptide RDIYETFYYRK (insulin receptor sequence, residues 1155–1165). Equivalent amounts of mutated and wild-type receptors were activated by sequential incubation with various amounts of insulin (45 min at 22°C) and 100 μM unlabeled ATP (5 mM MnCl at 22°C for 10 min). Substrate phosphorylation reactions were with 0.5 mM peptide (final concentration) in 100 μM [γ-^{32}P]ATP as described (24). WT, wild type.

min^{-1}; n = 7) (Fig. 4*A*). In contrast, CHO cells transfected with the double mutant GP → AA receptor internalized [^{125}I]insulin at a significantly accelerated rate, k_e = 0.108 ± 0.012 min^{-1} (n = 4). The relative rate for GP → AA receptor internalization, determined using three cell clones, was consistently elevated over that observed for the wild-type receptor. Comparisons with the kinase-deficient receptor K1030A (k_e, of 0.010 ± 0.002 min^{-1}; n = 5) and untransfected cells (data not shown) confirm that the observed insulin internalization is receptor- and phosphorylation-dependent.

Receptor-Mediated Insulin Degradation. Additional experiments were performed to determine whether the increase in internalization rate was accompanied by a concomitant increase in insulin degradation. After incubating cells with [^{125}I]insulin at 4°C for 18 h and removal of unbound [^{125}I]insulin by washing, cells were warmed to 37°C for the indicated times (Fig. 4*B*). Trichloroacetic acid was added to the supernatant medium at a final concentration of 7.5%, and soluble ^{125}I radioactivity was used to estimate insulin degradation. Whereas untransfected CHO cells degraded little [^{125}I]insulin, significant linear rates of degradation were observed for
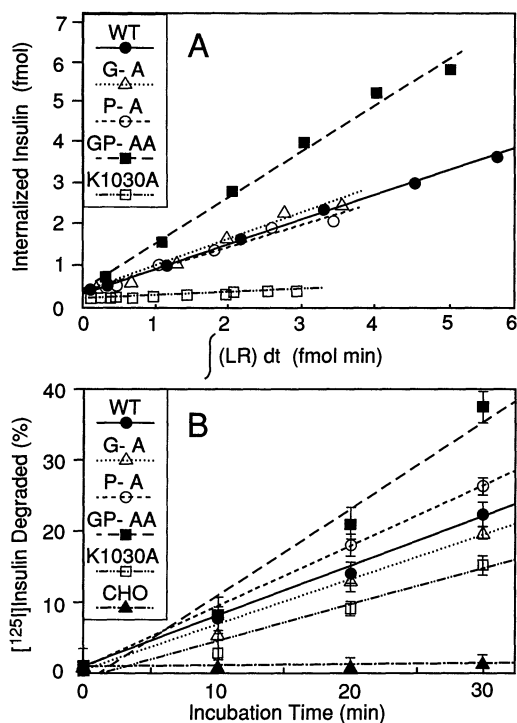
FIG. 4. (*A*) [^{125}I]Insulin internalization in CHO cells expressing wild-type and mutant receptors. Confluent cells in 24-well dishes were washed with PBS and incubated at 37°C with 5.0 × 10^5 cpm of A14-[^{125}I]insulin (2.0 Ci/μmol; 1 Ci = 37 GBq) in F-12 medium containing 50 mM Hepes and 0.1% bovine serum albumin (pH 7.4). At times from 0 to 10 min, medium was removed and cells were washed at 4°C with PBS at pH 7.6 or pH 3.5 to determine total cell-associated or intracellular radioactivity, respectively. Data shown represent values for single clones of mutated receptors and the average value for two clones of wild-type receptors; similar data were obtained in four experiments. (*B*) Insulin degradation by transfected CHO cells. Confluent cells in 24-well dishes were washed with PBS and incubated with [^{125}I]insulin (10^5 cpm) for 18 h at 4°C. Cells were washed twice with chilled PBS and quickly warmed to 37°C by addition of warm F-12 medium containing 50 mM Hepes and 0.1% bovine serum albumin (pH 7.4). Cells were maintained at 37°C and, at the indicated times, aliquots of medium were removed and combined with an equal volume of 15% aqueous trichloroacetic acid (final concentration, 7.5%); acidified mixtures were incubated at 4°C for 30 min and centrifuged. Degraded insulin was determined as (supernatant radioactivity/initial bound radioactivity) × 100%. Due to low initial binding for untransfected CHO cells (CHO), values expressed are a percentage of initial bound to cells transfected with wild-type (WT) receptors. Data shown are the means (±SEM) of two experiments, and each experiment was done in triplicate.

each of the transfected cell lines. Cells transfected with wild-type receptors or either of the single mutants degraded insulin at intermediate rates. Cells expressing the kinase-deficient K1030A receptors degraded insulin more slowly, whereas cells transfected with GP → AA receptors degraded [^{125}I]insulin ≈2-fold more rapidly than cells expressing wild-type receptors, consistent with an accelerated rate of internalization.

Lateral Mobilities of Receptors in Intact Cells. To differentiate possible mechanisms for the observed acceleration in rates of receptor internalization and insulin degradation, lateral mobilities of wild-type and GP → AA receptors were assessed by the fluorescence photobleaching recovery technique (26, 27, 33). Transfected cells were incubated with B29$^\varepsilon$-carboxyfluorescein-insulin for 5 min at 21°C and washed to remove excess fluorescent insulin. Single cells were observed in a fluorescence microscope using a focused laser beam as the excitation source and a 1.5-μm^2 area of

Table 1. Lateral mobilities of wild-type and mutated GP → AA receptors in transfected CHO cells

Receptor	*D*	*f*	*n*
Wild type	6.0 ± 0.5	58 ± 3	46
GP → AA mutant	17.1 ± 1.5	51 ± 2	59

Individual transfected CHO cells that had been incubated with B29$^\varepsilon$-carboxyfluorescein-insulin were pulsed with an intense 1.5-μm^2 laser beam. Recovery of the fluorophore within the bleached zone was followed by fluorescence microscopy as described (27); *D* is the diffusion coefficient (×10^{10} cm^2/s), *f* is the fractional mobility (%), and *n* is the number of independent measurements. Values represent mean ± SEM.

membrane was exposed to a brief intense (2 mW) laser pulse to irreversibly bleach the fluorophore in that area. Analyses of fluorescence recovery curves yielded the fraction of insulin receptors that were free to diffuse in the plane of the plasma membrane (mobile fraction, *f*) and the diffusion coefficients (*D*) of the mobile fractions. Whereas fractional mobilities for wild-type and GP → AA receptors were similar (Table 1), the diffusion coefficient for the mutated GP → AA receptor (17.1 ± 1.5 × 10^{-10} cm^2/s) was elevated 2.8-fold compared to that for the wild-type receptor (6.0 ± 0.5 × 10^{-10} cm^2/s). Values determined in this study for the diffusion coefficient of the wild-type insulin receptor were similar to those determined previously for native receptors in cultured fibroblasts (34, 35) (3–5 × 10^{-10} cm^2/s). Therefore, an increased rate of lateral diffusion for the mutated receptor correlated directly with enhanced rates of receptor internalization and insulin degradation.

DISCUSSION

Upon insulin binding the insulin receptor is autophosphorylated and the activated receptor sends its message into the cell. In addition to being phosphorylated, insulin-occupied receptors move laterally within the plasma membrane and into clathrin-coated pits (25, 31, 34–37). Thus in the presence of insulin, there is a net flux of occupied receptors into coated pits, from which they enter the coated vesicle–endosome continuum. The bulk of internalized insulin is degraded, whereas most of internalized receptors are recycled back to the cell surface. Degradation of insulin by target tissues is the major mechanism for insulin clearance from the bloodstream. The potential role of the TM domain of the insulin receptor in insulin binding and kinase activity and rates of lateral mobility, internalization, and insulin degradation have received little attention.

For reasons discussed above, TM domains of many membrane-spanning proteins are assumed to adopt α-helices. Of the four high-resolution three-dimensional structures of integral membrane proteins that have been reported—the *Rhodopseudomonas viridis* reaction center (1), bacteriorhodopsin (2), the plant light-harvesting chlorophyll–protein complex (3), and bacterial porin (38)—three contain >20 membrane-spanning α-helices (porin contains a membrane-spanning β-barrel). At least four of the membrane-spanning helices contain Pro and in every case Pro induces a kink in the helix. Thus Pro alone appears to be sufficient to bend a helix, even when buried in lipid. These types of analyses have led to considerable speculation about the function of Pro-induced kinked helices and Gly residues in multispanning integral membrane proteins (1–5). Mutagenesis of TM Pro residues in multispanning proteins can have a deleterious effect on growth and various transport and photocycling functions (for review, see ref. 8).

Studies presented here demonstrate that the α-helical content of peptides modeled after the insulin receptor TM

domain can be increased by replacing Gly and Pro with Ala. Our findings and additional data from other studies (1–14, 28) support the notion that the Gly-Pro sequence within the insulin receptor TM might force an imperfect kinked helical structure. We refined the structure of the TM domain in the intact insulin receptor by similar substitution of potent helix breakers with Ala, a potent helix former, to remove the putative kink. We propose that the observed functional correlates for optimized helicity—accelerated rates of internalization, insulin degradation, and lateral diffusivity—are the direct result of altered TM domain structure. That is, an optimized helix results in decreased resistance to lateral motion of the insulin receptor because it is more streamlined. This could be due to direct protein–lipid interactions between the TM domain helix and plasma membrane lipid components or to protein–protein interactions with other membrane-associated proteins.

Our findings may provide an insight into the mechanism of ligand-mediated receptor internalization. The correlations between rates of lateral diffusion and internalization suggest that diffusion rate influences entry into the internalization pathway. Biophysical models of capture rates for laterally diffusing molecules (39) support this conclusion, regardless of whether internalization occurs via (*i*) directed entry of occupied receptors into clathrin-coated pits by a postulated internalization machinery; (*ii*) random encounters with coated pits, followed by selective trapping of occupied receptors due to some signal [e.g., tyrosine within a β-turn (25, 36, 40)]; or (*iii*) selective release of occupied receptors from microvillar surfaces followed by migration into coated pits (37). Whichever of these mechanisms is correct, and they need not be mutually exclusive, results presented here demonstrate that rates of lateral diffusion within the plasma membrane directly influence rates of insulin-mediated receptor endocytosis.

These findings along with the invariance of known mammalian insulin receptor TM domain sequences (18–21) raise the question of what physiologic or evolutioⁿary advantage might result from a slower than maximal rate of endocytosis to the cell or the organism. An increased rate of internalization should lead directly to accelerated metabolic clearance of insulin. Furthermore, accelerated internalization is accompanied by an increased rate of receptor down-regulation (unpublished observation). Therefore, we speculate that the physiologic consequence of submaximal rates of internalization observed for the wild-type receptor is a prolonged insulin signal due to (*i*) a decreased rate of ligand clearance over the short term and (*ii*) an increase in surface receptor number after prolonged ligand exposure. Protein structural influences on rates of endocytosis and lateral motion observed in this study may provide a general mechanism for regulating trafficking of cell surface proteins.

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