An Irregularity in the Transmembrane Domain Helix Correlates with the Rate of Insulin Receptor Internalization†

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ABSTRACT: Internalization of insulin and its receptor via receptor-mediated endocytosis is an important step in insulin-induced signal transduction. To investigate the structural determinants underlying the enhanced internalization rate observed for the insulin receptor transmembrane mutant Gly^{933}-Pro^{934} → Ala-Ala (GP → AA), we have designed and chemically synthesized two peptides, IR(TM)-GP and IR-(TM)-AA, corresponding respectively to the N−terminal portion of the wild−type and the mutant insulin receptor TM segment containing these sites. Conformational studies by circular dichroism (CD) spectroscopy on these two peptides in their monomeric states revealed that peptide IR(TM)-GP forms an irregular helix in the membrane-mimetic environments of sodium dodecyl sulfate (SDS) micelles with a possible "kink" in the helix imposed by its Gly-Pro sequence, while peptide IR(TM)-AA assumes largely classical α-helical structure under corresponding conditions. The helical pattern of peptide IR(TM)-AA was maintained at elevated temperatures, while the shape of the CD curve for peptide IR(TM)-GP was found to alter as a function of temperature. At higher concentrations, both peptides formed high molecular weight aggregates in SDS micelles, as demonstrated by SDS−PAGE gels, but peptide IR(TM)-AA was shown to aggregate more readily and more extensively than peptide IR(TM)-GP. Fluorescent dye-leakage experiments indicated that peptide IR(TM)-GP produces an enhanced disruption of the membrane bilayer in phosphatidylglycerol vesicles *vs* that induced by IR(TM)-AA. The overall results are consistent with the notion that a Gly-Pro kink in the TM helix of the wild-type insulin receptor may submaximize the rate of its internalization by retarding its lateral movement through the plasma membrane and, accordingly, indicate an active role for the TM segment in receptor-mediated endocytosis.

The insulin receptor is an integral membrane glycoprotein composed of two extracellular α-subunits and two transmembrane β-subunits in a tetrameric form (Ullrich et al., 1985; Ebina et al., 1985). Binding of insulin to the α-subunit of its receptor has two major effects—activation of the tyrosine kinase domain in the cytoplasmic portion of the β-subunit and initiation of ligand-induced receptor internalization (Paccaud et al., 1992). Internalization of insulin and its receptor is believed to occur via receptor-mediated endocytosis (Bergeron et al., 1985; Brown et al., 1983; Hedo & Simpson, 1984; Pastan & Willingham, 1981). Specifically, binding of insulin to its receptor elicits a process that starts with the lateral migration of the receptor through the plasma membrane toward the clathrin-coated pits. As receptors become clustered in the coated pits, they pinch off from the plasma membrane to generate vesicles that transport the receptors to endosomes where insulin dissociates from its receptor (Brown et al., 1983; Pastan & Willingham, 1981). Insulin is subsequently delivered through endosomes to a compartment where it is largely degraded, while the receptors themselves are usually recycled back to the cell surface (Thies et al., 1989; McClain, 1990).

Although receptor internalization plays an important role in the biological action of insulin by controlling the receptor number on the cell surface and the degradation of the hormone (Paccaud et al., 1992; Bergeron et al., 1985), factors affecting this process are only beginning to be understood (Rajagopalan et al., 1991; Backer et al., 1992). In a previous study to address the functionality of the insulin receptor TM segment, we constructed a TM mutant receptor with the helix-breaking Gly^{933}-Pro^{934} pair of the wild-type (WT) TM segment replaced by a helix-promoting Ala-Ala pair (Gonclaves et al., 1992). The resulting mutant receptor (GP → AA) was found to be indistinguishable from WT receptor with respect to insulin binding ability and tyrosine kinase activity. However, it displayed an enhanced internalization rate about 2-fold that of the WT receptor (Conclaves et al., 1992). As the Gly-Pro pair occurs within the first two turns of the presumed TM α-helix of the insulin receptor (Ullrich et al., 1985; Ebina et al., 1985), it may reside close to the boundary region of the lipid head group and the acyl chains in the membrane. In view of the notable helix-breaking propensities of Gly and Pro residues—at least in soluble proteins (Chou & Fasman, 1978; Blaber et al., 1993; Williams & Deber, 1991)—we suggested that the rate of ligand-induced receptor internalization is normally retarded by a kinked helical structure within the TM domain of the

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Table 1: Primary Structure of TM Regions of WT and Mutant ($G^{933}P^{934} \rightarrow AA$) Insulin Receptor (IR) and Corresponding Synthetic Peptides[a]

wild-type IR	...SNIAKIII**GP**LIFVFLFSVVIGSIYLFLRKR...
peptide IR(TM)-GP	SNIAKIII**GP**LIFVF*SKSKSK*
mutant IR	...SNIAKIII**AA**LIFVFLFSVVIGSIYLFLRKR...
peptide IR(TM)-AA	SNIAKIII**AA**LIFVF*SKSKSK*

[a] Putative transmembrane regions in the insulin receptor and corresponding hydrophobic cores of the synthetic peptides are underlined. Sites involved in mutation are bolded. The C-terminal hydrophilic ends of the peptides are italicized.

WT insulin receptor (Gonclaves et al., 1992).

To explore the above notion and to further elucidate the structural basis underlying the apparent "less than maximal" internalization rate observed for wild-type insulin receptor, we have synthesized two peptides designed to include, respectively, the N-terminal half of the WT and the mutant (GP-AA) insulin receptor TM domains (Table 1). Investigation of their conformational, aggregational, and membrane-interactive properties yielded results that suggest a structural role for the TM domain in the internalization of the insulin receptor.

MATERIALS AND METHODS

Peptide Synthesis. Peptides were synthesized on a Milligen/Biosearch 9600 synthesizer using an N^{α}-fluoren-9-ylmethoxycarbonyl (Fmoc)/*tert*-butyl side chain protecting group strategy. Peptide bond-forming reactions with 0.2 M N^{α}-Fmoc amino acid, 1-hydroxybenzotriazole, and benzotriazolyl-1-oxytris(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 reverse-phase HPLC using a Dynamax-300 A 12-μm C_8 column (41.4 × 250 mm). Results from analytical HPLC, amino acid composition, protein microsequencing, and mass spectrometric analyses were consistent for the desired peptides. To avoid aggregation, purified peptide samples were stored in lyophilized powder form. Alternatively, stock solutions of the peptides could be made in ddH_2O and aliquoted into small portions immediately prior to lyophilization.

Circular Dichroism Spectroscopy. CD measurements were performed on a Jasco J-720 spectropolarimeter equipped with computer-assisted data processing. Peptide spectra were obtained by averaging over six consecutive scans with the background subtracted. Cells with 0.1-cm path length were used to record spectra except for relatively concentrated (>100 μM) peptide samples, where cells with 0.01-cm path length were used instead. Peptide samples were prepared fresh immediately before CD measurements. Peptide concentrations in each experiment were determined by amino acid analysis. The aqueous buffer used to dissolve peptides consisted of 10 mM sodium chloride and 10 mM phosphate at pH 7.0. Sodium dodecyl sulfate (SDS) solutions were prepared from the same buffer.

Fluorescence Spectroscopy. Fluorescent dye-leakage experiments were conducted according to Mclean et al. (1990) with minor modifications. Appropriate amounts (~1 mg/mL) of phosphatidylglycerol (egg yolk PG, Sigma Chemical Co.) were added to 2 mL of a saturated solution of 6-carboxyfluorescein (Sigma) in 10 mM Tris-HCl buffer (pH 7.0), and the resulting lipid suspension was probe sonicated (~15 min) until a clear solution was obtained. Dye-entrapped PG vesicles were then separated from free fluorophores on a size-exclusion HPLC column (SEC 125-5, 300 × 7.8 mm, Bio-Rad) after eluting with 10 mM Tris-HCl buffer at 1.0 mL/min. The fluorescence (excitation 490 nm, emission 515 nm) of a 2-mL portion of the diluted vesicle sample was measured at 37 °C for 1 min before adding 6 μg (in 30 μL of Tris-HCl buffer) of peptide IR(TM)-GP or IR(TM)-AA. The fluorescence of the sample was measured for an additional 10 min, after which time 100 μL of Triton X-100 (10%) was added to burst the vesicles in order to establish the fluorescence for 100% dye leakage.

RESULTS

Peptide Design. Table 1 compares the amino acid sequences of the designed peptides with those of the corresponding TM domains of the wild-type and mutant insulin receptors. Ideally, the designed peptides should be the full length of the receptor TM segments (23 AAs). However, in practice, the facile synthesis and purification of hydrophobic peptides are limited by the length of hydrophobic segments (Li & Deber, 1992, 1993). The present design is thus a compromise among factors that define the physicochemical properties and biological relevance of the peptides. It may be noted that even for a peptide containing approximately half the length of the receptor TM domain, a hydrophilic $(Lys\text{-}Ser)_2$ moiety added to its C-terminus was required to ensure reasonable peptide solubility in water (Li & Deber, 1992). While peptides of similar length may be blocked at N- and C-termini to facilitate secondary structure formation (Lyu et al., 1990; Padmanabhan et al., 1990), we chose to maintain free amino carboxyl termini to increase peptide solubility in aqueous buffers at neutral pH. Even with these precautions, the present peptides tended to aggregate at relatively low concentrations (vide infra); exclusively monomeric states of the peptides, as confirmed by size exclusion HPLC, could only be obtained at peptide concentrations as dilute as 30 μM or below (data not shown). This phenomenon may reflect the intrinsic property of the insulin receptor TM segment itself, as peptides with similar overall design but different amino acid compositions were much less prone to aggregate (Li & Deber, 1993, 1994).

Peptide Conformation. As shown in Figure 1, circular dichroism (CD) spectroscopy was used to observe the discrete conformations of monomeric IR(TM)-GP and IR(TM)-AA in aqueous buffer and SDS solutions containing 20 μM peptides. CD curves obtained in aqueous buffer suggest that peptide IR(TM)-AA assumes a partial helical/partial random structure with a measurable content of β-structure, whereas the unique CD pattern of IR(TM)-GP under the same conditions is more compatible with a combination of β-strand/β-turn structures (Seetharama et al., 1992). Secondary structure estimation using the Chou−Fasman algorithm (Chou & Fasman, 1978) reveals that both peptides have a greater tendency to form β-strands than α-helices (data calculation not shown); this estimation appears to account for the significant amounts of β-structural elements observed in corresponding CD spectra. Furthermore, the established propensity of Gly and Pro residues as

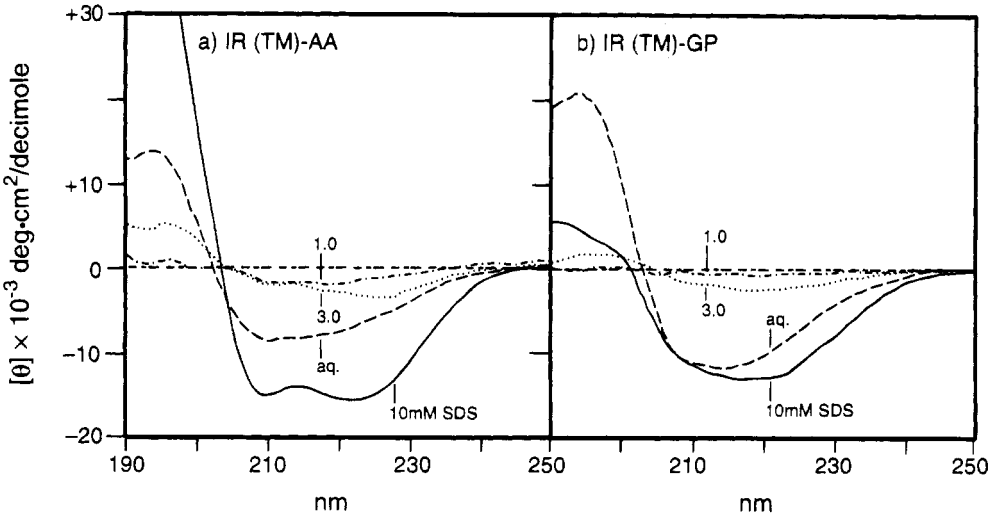

FIGURE 1: Circular dichroism spectra of transmembrane segment peptides corresponding to (a) mutant [IR(TM)-AA] and (b) wild-type [IR(TM)-GP] insulin receptors. Spectra are recorded at 20 μM peptide concentration in aqueous buffer (10 mM phosphate, pH 7.0) and in 1.0, 2.5, and 10.0 mM sodium dodecyl sulfate (SDS) solutions as indicated on the curves. See text for further experimental details.

strong β-turn promoters (Chou & Fasman, 1978) is apparently fully manifested in peptide IR(TM)-GP in the aqueous environment. The extent of folding/β-sheet formation, however, may be physically limited in the intact receptor in which N- and C-terminal ends of the TM domain carry periplasmic and cytoplasmic domains each containing hundreds of residues.

To determine whether the above characteristics of Gly and Pro are preserved in the membrane-mimetic (van de Ven et al., 1993; Hoyt & Gierasch, 1991; Gordon et al., 1992) environments of SDS solutions, both peptides were titrated with SDS up to a concentration of 10 mM SDS (above the critical micelle concentration). Although the current peptides are too short to traverse bilayer membranes, they are nevertheless of sufficient length to span the micelles formed by SDS, which has an acyl chain effectively half the length of those found in biological lipids. At submicellar concentrations of SDS—which can induce β-sheet structure in peptides (Zhong & Johnson, 1992)—we found that both peptides were largely unordered (Figure 1). However, the CD spectrum of peptide IR(TM)-GP in 10 mM SDS is significantly different from that of IR(TM)-AA (Figure 1). Given the high helical propensity of the Ala residue, peptide IR(TM)-AA undergoes a smooth transition from partial helical, partial β-structure in aqueous buffer to almost full helix in SDS micelles (Figure 1a). A similar, albeit less defined, transition is also observed for peptide IR(TM)-GP. However, caution must be taken in assigning the nonclassical CD pattern obtained for IR(TM)-GP in 10 mM SDS, in which the double absorption minima (208 and 222 nm) for classical α-helical structure (Chen et al., 1974) are partially obscured Figure 1b). Two competing factors may be considered influential to peptide IR(TM)-GP conformation: on the one hand, SDS micelles are strong helix-promoting agents (Li & Deber, 1992; Wu & Yang, 1990); yet, the mid-peptide Gly-Pro pair is highly detrimental to helical structure (Chou & Fasman, 1978; Blaber et al., 1993). It is possible that interplay of these two conflicting factors has created a kinked or partial helix for peptide IR(TM)-GP, which is manifested by its irregular CD spectrum. Energy-minimized conformations for Pro-containing helices [modeled by $(Ala)_8$-Leu-Pro-Phe-$(Ala)_8$ in a nonpolar environment] similarly indicate that the lowest energy structures correspond to (*trans*-Gly-Pro peptide bond) kinked α-helices, with the kink centered at the Pro ψ angle (Polinsky et al., 1992).

Table 2: Estimated Secondary Structure Content[a] for Peptides in Various Media

	aqueous buffer					10 mM SDS				
peptide	α	β	t	r	RMS	α	β	t	r	RMS
IR(TM)-GP	28	16	22	35	22	45	14	19	22	8
IR(TM)-AA	21	62	0	17	15	59	19	10	11	6

[a] Secondary structure contents were estimated from CD spectra taken on 20 μM peptide samples (see Figure 1) using Yang's program (Yang et al., 1986) provided by Jasco, Japan Spectroscopic Co. Ltd. Fractional percentages of secondary structures are given. Notations are as follows: α, α-helix; β, β-strand; t, turn; r random; RMS, root mean square value for curve fitting.

Due to the lack of reference spectra for transmembrane peptides, it is difficult to assess the secondary structure components represented by the CD spectra. In an attempt to analyze the CD data in further detail, we applied Yang's method (Yang et al., 1986) for secondary structure estimation to the CD spectra of both peptides in aqueous buffer and SDS micelles. Results thus obtained are given in Table 2. While the Yang method produces relatively good fitting to the CD spectra of peptide IR(TM)-AA in SDS micelles where α-helical conformation predominates, it does not result in a reliable fit of the spectra for either peptide in aqueous solution or for peptide IR(TM)-GP in SDS as indicated by the usually great RMS values (Table 2). In particular, β-structure elements may be overestimated by Yang's method, viz., an estimation of 62% of β-structure for peptide IR(TM)-AA in aqueous buffer is inconsistent with the Chou—Fasman prediction which indicates only a slight preference for β-sheet formation ($\langle P_\beta \rangle$, 1.10) over that for α-helix ($\langle P_\alpha \rangle$, 1.07) (calculation not shown). Such ambiguity in secondary structure estimation further suggests that the nonclassic CD patterns obtained in the present study are not a simple combination of classical secondary structure elements.

The irregularity of the helical structure for peptide IR-(TM)-GP in SDS micelles was also readily observable when the conformations of both peptides were compared at different temperatures. As shown in Figure 2a, helicity of

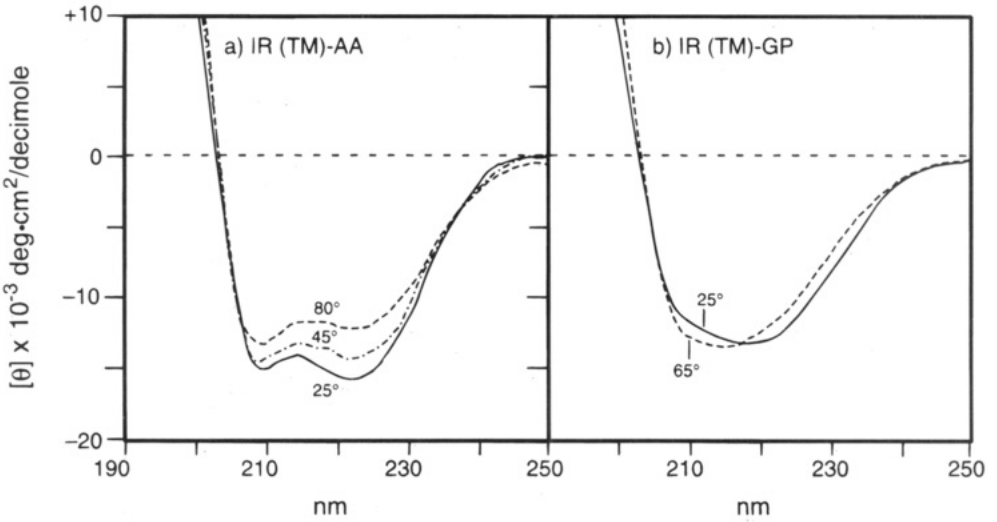

FIGURE 2: Temperature dependence of CD spectra for (a) peptide IR(TM)-AA and (b) peptide IR(TM)-GP in SDS micelles. Curves corresponding to different temperatures are as indicated on the diagram.

peptide IR(TM)-AA in SDS micelles decreases as the temperature rises, and the double minima at 208 and 222 nm, indicative of classical α-helical conformation, remain through the range of incremental temperatures. In contrast to this typical thermal denaturation of peptide IR(TM)-AA, peptide IR(TM)-GP responds to elevated temperatures by shifting its minimum around 220 nm to lower wavelengths, *e.g.*, to 216 nm at 65 °C (Figure 2b). Possibly the conformational rigidity/flexibility of the Pro residue (Williams & Deber, 1991) is sensitive to elevated temperatures in that it results in slightly increased populations of less favored structures at the local Gly-Pro-containing segment in peptide IR(TM)-GP. It is also conceivable that this peptide associates less well with SDS micelles at elevated temperatures such that the Gly-Pro pair becomes partially exposed to the bulk aqueous environment.

Peptide Aggregation. We then examined how this discrepant conformational behavior of the two peptides would affect their aggregation tendencies in water and SDS micelles. To promote peptide aggregation, a relatively concentrated (~300 μM) sample of each peptide in either aqueous buffer or 10 mM SDS was incubated for 15 min at 25 °C. Aliquots of these samples were then analyzed on SDS−PAGE gels (Figure 3). As judged from the band distribution of high (aggregates) and low (monomers) molecular weight species on the gels, peptide IR(TM)-GP aggregates more easily in water than IR(TM)-AA, yet the latter peptide has a greater tendency to aggregate in SDS micelles. Presumably, the smooth helical structure formed by peptide IR(TM)-AA—containing two small Ala residues—should allow close packing (Deber et al., 1993) of this peptide chain with itself in SDS micellar solutions. In contrast, the kinked helix of IR(TM)-GP would make it difficult for the corresponding peptide chain to self-associate in such a favorable fashion, especially when surrounded by excess lipid in SDS solutions. In aqueous buffer, peptide IR(TM)-GP may circumvent its packing problem in SDS by folding upon itself to form a β-sheet structure facilitated by a β-turn structure at the Gly-Pro locus. The discrete aggregational properties of these two peptides in SDS micelles may have relevance to the aggregation/clustering of receptors in biological membranes.

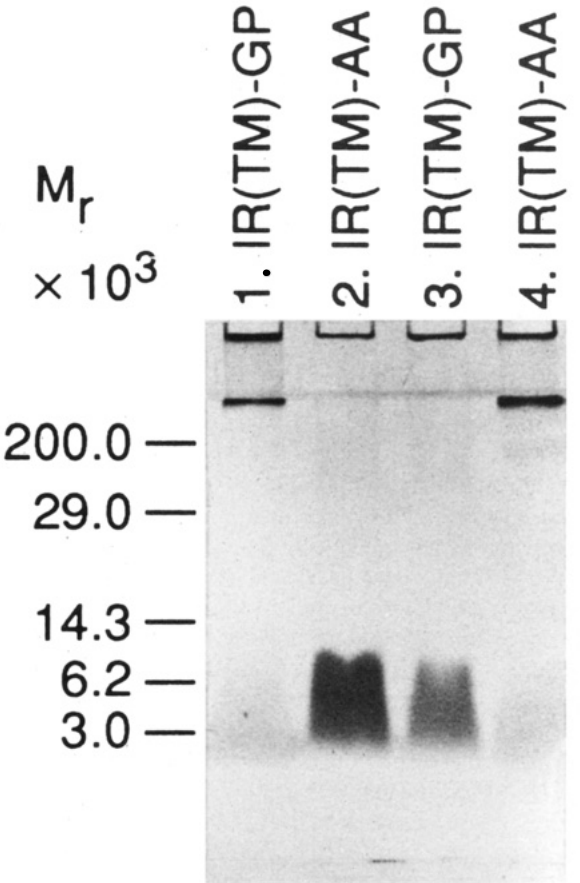

FIGURE 3: SDS polyacrylamide gel electrophoresis (SDS−PAGE) of wild-type and mutant insulin receptor TM segment peptides IR-(TM)-GP and IR(TM)-AA. Peptides were applied to the gel after incubation at 25 °C for 15 min in aqueous phosphate buffer (lanes 1 and 2) or 10 mM SDS solutions (lanes 3 and 4). Peptide concentration: 300 μM. Loading volume: 10 μL. Molecular weight markers are shown to the left side of the gel. Bands at the very top of the gel in lanes 1−4 indicate peptide precipitates; bands at the interface between stacking and running gel (appearing above 200 kDa in lanes 1 and 4) indicate peptide aggregates (see also text).

Peptide-Induced Dye Leakage. Fluorescence photobleaching recovery techniques have shown that the mobility of WT insulin receptors in membrane bilayers is 2−3 times slower than that of the GP → AA mutant (Gonclaves et al., 1992);

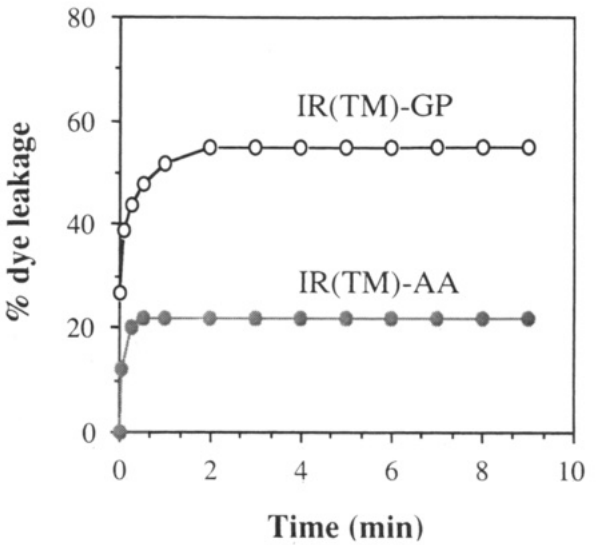

FIGURE 4: Leakage of vesicle-entrapped fluorescent dye (6-carboxyfluorescein) induced by wild-type and mutant insulin receptor transmembrane segment peptides IR(TM)-GP and IR(TM)-AA, respectively, as indicated on the diagram. Lipid: dimyristoylphosphatidylglycerol (DMPG). Bulk peptide concentration: 6 μM. See Materials and Methods section for experimental details.

this observation was attributed as well as to the kinked TM helix of the WT receptor. To explore further the mechanism underlying this phenomenon, we examined the membrane-disrupting ability of the present model peptides by assessing their efficiency to release vesicle-entrapped 6-carboxyfluorescein. As seen in Figure 4, peptide IR(TM)-GP induces dye leakage from phosphatidylglycerol vesicles about twice as efficiently as peptide IR(TM)-AA. It is likely that the kinked helix resulting from the Gly-Pro diad disturbs the packing of the membrane bilayer more profoundly than its relatively linear Ala-Ala counterpart and, thus, hinders the lateral movement through the membrane of the segment bearing the kink. This effect may then contribute directly to the slowdown of receptor internalization, which is found to be linearly correlated with receptor mobility in the plasma membrane lipid bilayer (Chen et al., 1974).

DISCUSSION

It is known that molecular environments may have profound impacts on peptide conformations (Zhong & Johnson, 1992; Li & Deber, 1993). In the present study, we are able to observe the diverse effects of adding SDS to aqueous media on the conformations of peptide IR(TM)-GP and IR(TM)-AA. While it is inappropriate to relate peptide structures in aqueous buffer to their function in the context of a transmembrane domain, results obtained here suggest that the structures observed in SDS micelles for the two peptides may be relevant to the mechanism of internalization of the wild-type and mutant insulin receptors.

Proline is a frequently found residue in the transmembrane segments of various membrane proteins (Brandl & Deber, 1986; von Heijne, 1991), and Pro-induced bending (kinking) of α-helices has been implicated in various Pro-containing TM segments (Polinsky et al., 1992; Seetharama et al., 1992; Woolfson et al., 1991). It is possible that the irregular CD spectrum observed for peptide IR(TM)-GP in SDS micelles in our study represents a kinked helical structure imposed by the Gly-Pro pair, although no CD spectrum characteristic of a kinked helix has been reported specifically. The impact of the unique structural properties of the Gly-Pro pair on peptide−peptide association is also apparent. The irregular helix formed by peptide IR(TM)-GP does not allow its close packing, whereas peptide IR(TM)-AA is able to form aggregates in 10 mM SDS possibly in the α-helical conformation. Since the α-helix is considered to be the native structure for most TM segments (Gordon et al., 1992) and insulin receptor internalization involves a step that requires the clustering of receptors in coated pits (Pastan & Willingham, 1981), the ready helical aggregation of peptide IR(TM)-AA, albeit observed in the *in vitro* membrane-mimetic environment of SDS micelles, may contribute to the accelerated internalization rate of the mutant GP → AA receptor.

Our overall results are consistent with the concept that irregularity in the insulin receptor TM helix submaximizes its rate of internalization by retarding its migration through the plasma membrane. Although the functional significance of a submaximal rate of receptor internalization is yet to be deciphered, it is interesting to note that most TM segments of functional membrane proteins contain irregularities of one kind or another in their primary sequences, *e.g.*, significant Gly and/or Pro content, high content of β-branched residues, etc. (Li & Deber, 1992; Brandl & Deber, 1986). Why nature recruits such a great proportion of apparent helix-destabilizing residues into transmembrane helices remains a subject of active investigation.

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