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Articles

Cytoplasmic Juxtamembrane Region of the Insulin Receptor: A Critical Role in ATP Binding, Endogenous Substrate Phosphorylation, and Insulin-Stimulated Bioeffects in CHO Cells†

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ABSTRACT: We have expressed in CHO cells a mutant receptor ($IR_{\Delta 960}$) from which 12 amino acids in the juxtamembrane region (A954-D965), including Tyr_{960}, have been deleted. The mutant receptor bound insulin normally but exhibited an increased K_m for ATP during autophosphorylation. Upon prolonged incubation in vitro, or at high ATP concentrations such as those observed in vivo, autophosphorylation of $IR_{\Delta 960}$ was similar to wild type, and the in vitro phosphotransferase activity of the autophosphorylated $IR_{\Delta 960}$ was normal. These results suggest that the deletion did not cause a nonspecific structural disruption of the catalytic domain of $IR_{\Delta 960}$. In vivo autophosphorylation of the $IR_{\Delta 960}$ receptor was reduced by 30% after 2 min of insulin stimulation and was similar to the wild-type receptor after 30 min of insulin stimulation. However, the mutant receptor was defective in insulin-stimulated tyrosyl phosphorylation of the endogenous substrate pp185. In additon, $IR_{\Delta 960}$ was deficient in mediating insulin stimulation of glycogen and DNA synthesis. Thus, autophosphorylation of the insulin receptor is necessary but not sufficient for signal transmission. These data extend the hypothesis that the cytoplasmic juxtamembrane region of the insulin receptor is important for its interactions with ATP, intracellular substrates, and other proteins and is broadly necessary for biological signal transmission.

Insulin binding to the insulin receptor causes the autophosphorylation and activation of its β-subunit tyrosyl kinase (Kahn & White, 1988). In intact cells, the tyrosine kinase is responsible for the phosphorylation of endogenous substrates such as pp185 (White et al., 1985). Inactivation of the receptor by mutagenesis of its ATP binding site abolishes both receptor autophosphorylation and biological activity, suggesting that the kinase is essential for signal transmission (Chou et al., McClain et al., 1987). However, in mutant receptors from which tyrosyl phosphorylation sites required for full activation have been removed, some biological responses are retained despite reduced kinase activity (Ellis et al., 1986; Wilden et al., 1990). In light of these studies, as well as the insulin-mimetic properties of anti-receptor antibodies which weakly stimulate receptor autophosphorylation, the relation between kinase activity and biological activity of the insulin receptor remains unclear (Sung et al., 1989).

Previous studies in our laboratory suggest that the juxtamembrane region of the insulin receptor β-subunit, located just beyond the membrane-spanning region, is important for biological signaling. Conservative point mutations in this region had no effect on receptor autophosphorylation yet blocked endogenous substrate phosphorylation and biological activity (White et al., 1988). In addition, we have shown that the deletion of 12 amino acids (A954-D965) from this region results in a mutant receptor ($IR_{\Delta 960}$) which is markedly de-

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ficient in insulin-stimulated internalization (Backer et al, 1990).

In this study, we find that the velocity of in vitro autophosphorylation of the $IR_{\Delta 960}$ is slow due to an increase in the K_m for ATP. However, once the receptor is activated by autophosphorylation, the in vitro kinase activity of the receptor is normal. In vivo autophosphorylation of $IR_{\Delta 960}$ is reduced only 30% relative to the wild-type receptor after 2 min of insulin stimulation, presumably due to the high intracellular concentration of ATP. However, the $IR_{\Delta 960}$ does not phosphorylate the endogenous substrate pp185 and is deficient in mediating insulin stimulation of glycogen and DNA synthesis. These data suggest that the juxtamembrane region of the insulin receptor plays a general role in the interactions of the receptor with ATP, intracellular substrates, and related kinases, as well as its mediation of insulin internalization and other biological effects in the cascade of insulin action.

Experimental Procedures

Expression Plasmids. The normal human insulin receptor expression plasmid pCVSVHIRc and the expression plasmid encoding a mutant human insulin receptor in which Tyr_{960} was replaced with phenylalanine (IR_{F960}) have been previously described (White et al., 1988). However, recent sequencing of the juxtamembrane region revealed an unexpected mutation of Ser_{962} to Thr_{962}. Although the IR_{F960} receptor contains two mutations in the juxtamembrane region, the mutant molecule is still valid for assessing the effect of changes in the juxtamembrane region on insulin action, and the discordance between autophosphorylation, in vitro kinase activity, and in vivo signal transduction. The plasmid for the expression of the mutant insulin receptor containing a deletion of amino acids A954–D965 (pCVSVHIRc/Δ960) was generated by subcloning a *Bgl*II–*Hin*dIII fragment from pCVSHIRc into M13mp19, followed by oligonucleotide-directed mutagenesis as previously described (Backer et al., 1990). *Escherichia coli* strain JM101 was transformed with double-stranded circular DNA, and plaques were screened under stringent conditions using the mutagenesis primer as a probe. The mutation was confirmed by M13 dideoxy sequencing and introduced into the pCVSHIRc expression vector (Sanger et al., 1977); recent sequencing of the juxtamembrane region revealed no unexpected changes.

Transfection of CHO Cells. CHO cells were grown in 10-cm dishes in F12 medium containing 10% fetal bovine serum (Gibco). Subconfluent CHO cells (10^6) were transfected by calcium phosphate precipitation with 1 μg of pSVEneo alone or together with 10 μg of pCVSVHIRc, pCVSVHIRc/F960, or pCVSVHIRc/Δ960 as previously described (White et al., 1988; Backer et al., 1990). After 72 h, 800 μg/mL geneticin (GIBCO) was added to the medium to select for neomycin-resistant cells. Surviving cells were cultured in the presence of geneticin to amplify the cell line. CHO cells that expressed high levels of surface insulin receptors were selected by fluorescence-activated cell sorting (Maron et al., 1984), and clonal cell lines were obtained by plating at limiting dilution.

Expression and Autophosphorylation of the Normal and Mutant Insulin Receptors Expressed in Chinese Hamster Ovary (CHO) Cells. Insulin receptor mutants were constructed, using oligonucleotide-directed mutagenesis, in which Tyr_{960} was substituted with phenylalanine (IR_{F960}) or 12 amino acids (A954-D965) were deleted from the juxtamembrane region ($IR_{\Delta 960}$) (Figure 1). CHO/neo cells, expressing only pSVEneo, contained about 3000 hamster insulin receptors. Following transfection and selection by fluorescence-activated cell sorting, clonal lines of CHO/IR cells and mutant CHO/IR_{F960}, and $CHO/IR_{\Delta 960}$ cells were obtained which expressed approximately 1.2×10^6 receptors/cell (data not shown). Scatchard analysis of CHO/IR_{F960} and $CHO/IR_{\Delta 960}$ cells has been previously described, and indicated that binding was normal (White et al., 1988; Backer et al., 1990). The insulin dose response of in vitro autophosphorylation by partially purified receptors from CHO/IR, CHO/IR_{F960} and $CHO/IR_{\Delta 960}$ cells was identical; a half-maximal response was obtained at approximately 3 nM insulin for all three lines, identical with the insulin binding affinity of the receptors (data not shown). Thus, despite mutations near the transmembrane domain, the coupling of insulin binding to receptor autophosphorylation was normal in IR_{F960} and $IR_{\Delta 960}$.

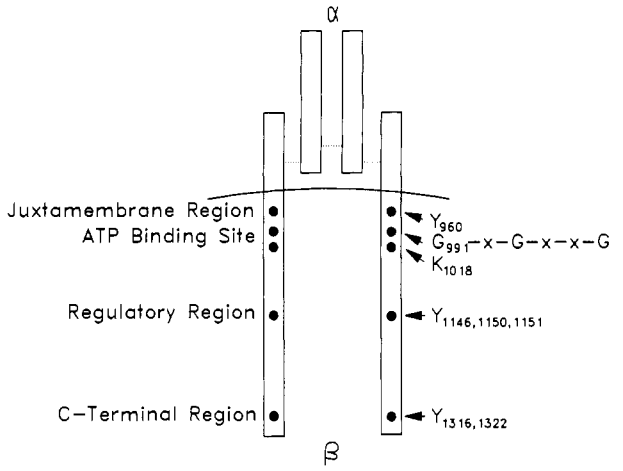

FIGURE 1: Model of insulin receptor functional regions. Major functional regions of the insulin receptor and the location of the juxtamembrane mutations in IR_{F960} and $IR_{\Delta 960}$ are depicted. Numbering is according to Ullrich et al. (1985).

In Vitro Insulin Receptor Autophosphorylation. Insulin receptors were purified on wheat germ agglutinin–agarose (Vector). The ^{125}I-insulin binding capacity of the purified receptors was determined by precipitation in poly(ethylene glycol) as previously described (Taylor et al., 1982). The $IR_{\Delta 960}$ and IR_{F960} receptors bind insulin with normal affinity (Backer et al., 1990; White et al., 1988), and the relative number of receptors in different wheat germ preparations could therefore be determined from the binding of ^{125}I-insulin to various amounts of wheat germ agglutinin purified protein. Aliquots of receptor, normalized to binding, were incubated in a final volume of 50 μL in the presence of 50 mM HEPES, pH 7.5, containing 0.1% Triton X-100, insulin, and $MnCl_2$ as indicated. The reaction was initiated by the addition of 20–30 μCi of [^{32}P]ATP (New England Nuclear) in the presence of unlabeled ATP as indicated. In experiments in which the concentration of ATP varied, a constant specific radioactivity was maintained. The autophosphorylation reactions were terminated at various times by boiling in Laemmli sample buffer. The samples were separated by reducing SDS–PAGE (6% resolving gels) and visualized by autoradiography, and the radioactivity in β-subunit bands was determined by Cerenkov counting. The kinetic parameters of the autophosphorylation reaction were fitted to the Michaelis–Menten equation using the Marquardt–Levenberg algorithm provided in SigmaPlot Version 4.0 (SigmaPlot Version 4.0, 1989), and were confirmed by using the Enzyme kinetic analysis program (Lutz et al., 1986).

In Vitro Kinase Activity Assays. The synthetic peptide substrate Thr-12-Lys, composed of amino acids 1143–1152 of the human insulin receptor [numbered as per Ullrich et al.

(1985)], was purchased from Dr. David Coy, Tulane University, New Orleans, LA. Lectin-purified insulin receptors, normalized to insulin binding activity, were incubated for various periods of time in a final volume of 50 μL containing 50 mM HEPES, pH 7.5, 0.1% Triton X-100, and 5 mM $MnCl_2$ in the absence or presence of 100 nM insulin. Substrate phosphorylation was initiated by the addition of varying concentrations of peptide for 5 min at 22 °C. Reactions were terminated by the sequential addition of 20 μL of 1% bovine serum albumin (BSA) and 50 μL of 10% trichloroacetic acid (TCA). Precipitated protein was removed by centrifugation, and the supernatant, containing the phosphorylated peptide, was applied to a 1 × 1 in. piece of phosphocellulose paper (Whatman). The papers were washed with four changes (1 L each) of 75 mM phosphoric acid, and the retained radioactivity was measured by Cerenkov counting.

[^{32}P]Phosphate Labeling of CHO Cells Expressing Wild-Type or Mutant Insulin Receptors. Confluent monolayers of transfected CHO cells in 10- or 15-cm dishes (Nunc) at 37 °C were labeled 2 h with 0.5 mCi/mL [^{32}P]phosphate (New England Nuclear) (White et al., 1988). The cells were incubated for additional periods of time in the absence or presence of 100 nM insulin and rapidly frozen with liquid nitrogen. The frozen cells were solubilized in 100 mM Tris, pH 8.2, containing 2 mM sodium vanadate, 3.4 mg/mL PMSF, 100 μg/mL aprotinin, 1 μg/mL leupeptin, and 1% Triton X-100. Tyr(P)-containing proteins were immunoprecipitated with anti-phosphotyrosine antibody (αPY), reduced with dithiothreitol, and analyzed by SDS–PAGE (White and Backer, 1991). Phosphoproteins were identified by autoradiography, and the radioactivity in the insulin receptor subunits was quantified by Cerenkov counting.

Thymidine Incorporation. Subconfluent CHO cells, grown in 12-well trays, were incubated for 24 h in 1 mL of F-12 medium containing 1% BSA without fetal bovine serum. The cells were washed and incubated for 15 h in F-12/1% BSA containing insulin as indicated, followed by an additional 1-h incubation in F-12/1% BSA containing 20 mM HEPES (pH 7.4) and 0.5 μCi/mL [^{3}H]thymidine. The cells were then washed 3 times in ice-cold phosphate-buffered saline and solubilized in 1 mL of 0.1% SDS. DNA was precipitated by the addition of 2 mL of 20% TCA at 4 °C, collected on glass filters (Whatman), and washed with 20 mL ice-cold 10% TCA. The retained radioactivity was counted in a scintillation counter using ACS cocktail (Amersham).

Glucose Incorporation into Glycogen. Confluent CHO cells, grown on 12-way trays, were washed in PBS and incubated at 37 °C for 3 h in F-12 medium containing 1% BSA and 400 mg/L glucose. The medium was removed, and fresh F-12 medium containing 1% BSA, 400 mg/L glucose, 25 mM HEPES (pH 7.4), and various concentrations of insulin was added. After 30 min, [^{14}C]glucose (10 μCi/mL final) was added for an additional 1 h. Finally, the cells were washed twice in ice cold PBS, solubilized in 0.4 mL of 20% KOH for 1 h at 37 °C, transferred to glass tubes, and boiled for 20 min. Glycogen was precipitated from this extract by adding 100 μL of 5% Na_2SO_4 containing 1 mg/mL glycogen followed by 2.5 mL of ethanol and an overnight incubation of 0 °C. The precipitate was collected on glass filters (Whatman) and washed with 10 mL of ice-cold 66% ethanol, and the retained radioactivity was measured by liquid scintillation counting in ACS cocktail.

RESULTS

Autophosphorylation of $IR_{\Delta 960}$ Exhibits an Increased K_m for ATP. The time course of in vitro insulin-stimulated autophosphorylation was identical for the IR and IR_{F960} receptors (data not shown). In contrast, autophosphorylation of the $IR_{\Delta 960}$ was slower than that of the wild-type receptor when autophosphorylation was measured at 25 μM ATP and 5 mM Mn^{2+} (Figure 2A). Incorporation of [^{32}P]phosphate into the wild-type receptor reached steady state within 10–15 min, whereas $IR_{\Delta 960}$ was not even maximal after 30 min. However, the rate of $IR_{\Delta 960}$ autophosphorylation at 200 μM ATP was similar to the wild-type receptor, with both receptors reaching half-maximal autophosphorylation in under 5 min (Figure 2B). These data suggest that the slow autophosphorylation of the $IR_{\Delta 960}$ at low concentrations of ATP reflected a decreased affinity of the receptor for ATP.

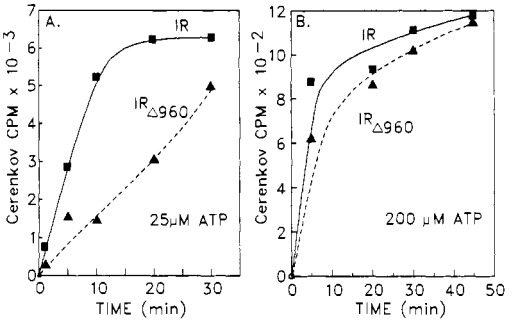

FIGURE 2: Time course of insulin receptor autophosphorylation. Lectin-purified receptors from CHO/IR and CHO/$IR_{\Delta 960}$ cells were incubated at 22 °C for various periods of time in the presence of 5 mM $MnCl_2$, 100 nM insulin, 30 μCi of [^{32}P]ATP, and (A) 25 or (B) 200 μM ATP. Autophosphorylation reactions were stopped by boiling in Laemmli sample buffer, and proteins were separated by SDS–PAGE and visualized by autoradiography. Radioactivity in insulin receptor β-subunit bands was measured by Cerenkov counting.

We examined the [ATP] dependence of insulin-stimulated autophosphorylation for the wild-type and mutant receptors (Figure 3A). The apparent K_m values for ATP of the IR and IR_{F960} receptors were similar at 27 ± 7 and 35 ± 10 μM, respectively. Notably, the K_m for ATP of $IR_{\Delta 960}$ was nearly 7-fold greater at 209 ± 74 μM. The apparent V_{max} values for each receptor were similar, consistent with the fact that the major sites of insulin receptor autophosphorylation are intact in the IR_{F960} and $IR_{\Delta 960}$ receptors (White et al., 1988; Backer and White, unpublished observations). The observed increase in the K_m for ATP in $IR_{\Delta 960}$ was not due to an altered requirement for Mn^{2+} (White et al., 1984). The [Mn^{2+}] dependence of receptor autophosphorylation was similar for the wild-type and mutant receptors (Figure 3B), with the K_m in the range of 2–3 mM $MnCl_2$. Furthermore, the ATP K_m determinations were performed at 10 mM $MnCl_2$, above the K_m for Mn^{2+}. Thus, the removal of 12 amino acids from the juxtamembrane region of the insulin receptor increased the K_m for ATP of autophosphorylation, thereby reducing the velocity of $IR_{\Delta 960}$ autophosphorylation at nonsaturating ATP concentrations.

In Vitro Kinase Activity of Wild-Type and Mutant Insulin Receptors. Autophosphorylation of the insulin receptor increases its kinase activity toward exogenous substrates (Rosen et al., 1983). We examined the autophosphorylation-induced activation of the wild-type and $IR_{\Delta 960}$ receptors toward the peptide substrate Thr-12-Lys. Lectin-purified receptors were allowed to autophosphorylate in the absence or presence of insulin (100 nM) for various times at 16 °C, followed by a 5-min incubation with peptide substrate (Figure 4A). In the absence of insulin, both wild-type and $IR_{\Delta 960}$ receptors displayed minimal kinase activity. Insulin stimulated the kinase activity of the wild-type receptor, which reached steady-state levels by 5–10 min. This time course was similar to that of

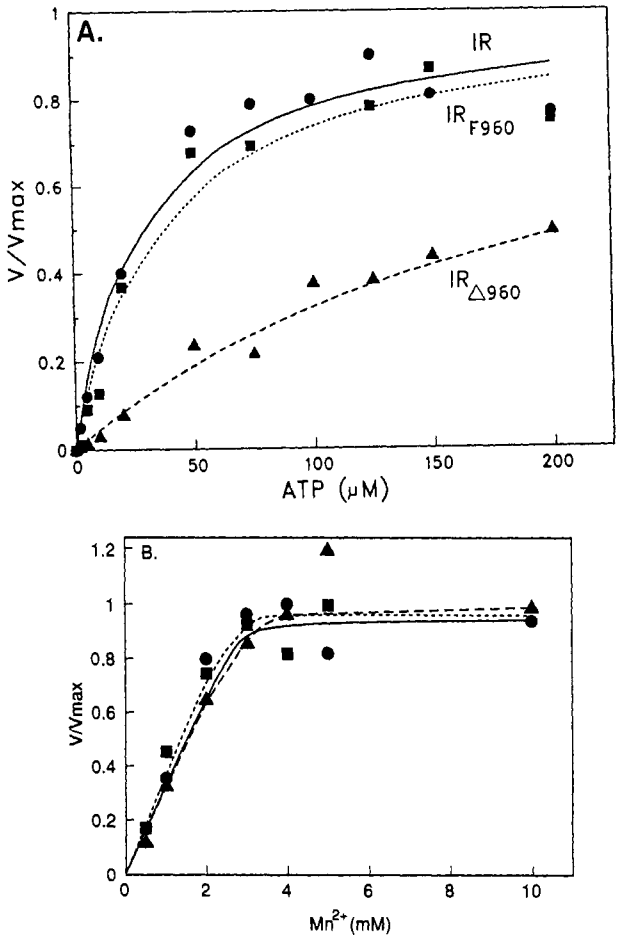

FIGURE 3: [ATP] and [Mn^{2+}] dependence of insulin receptor autophosphorylation. (A) Lectin-purified receptors from CHO/IR, IR_{F960}, and CHO/$IR_{\Delta 960}$ cells were incubated at 22 °C for 2 min in the presence of 100 nM insulin, 10 mM $MnCl_2$, and varying concentrations of ATP at constant specific radioactivity. Autophosphorylation reactions were stopped by boiling in Laemmli sample buffer, and incorporation of radioactivity into the insulin receptor β-subunit was measured as described in Figure 2. The data were analyzed and curves were fit by using Sigmaplot software (Jandel); the calculation of kinetic parameters was confirmed by using the Enzyme program (Lutz et al., 1986). The V_{max} values for autophosphorylation of the IR, IR_{F960}, and $IR_{\Delta 960}$ receptors were 62.6 ± 4.3, 46.3 ± 3.9, and 54.5 ± 8 fmol $min^{-1}/\mu g^{-1}$, respectively. The K_m values for ATP were 27 ± 7, 35 ± 10, and 209 ± 74 μM for autophosphorylation of the IR, IR_{F960}, and $IR_{\Delta 960}$ receptors, respectively. (B) Lectin-purified receptors from CHO/IR, IR_{F960}, and CHO/$IR_{\Delta 960}$ cells were incubated as described above in the presence of 100 nM insulin, 150 μM ATP containing 30 μCi, and varying concentrations of $MnCl_2$. Reactions were stopped, and ^{32}P incorporation into the β-subunit was measured as described above.

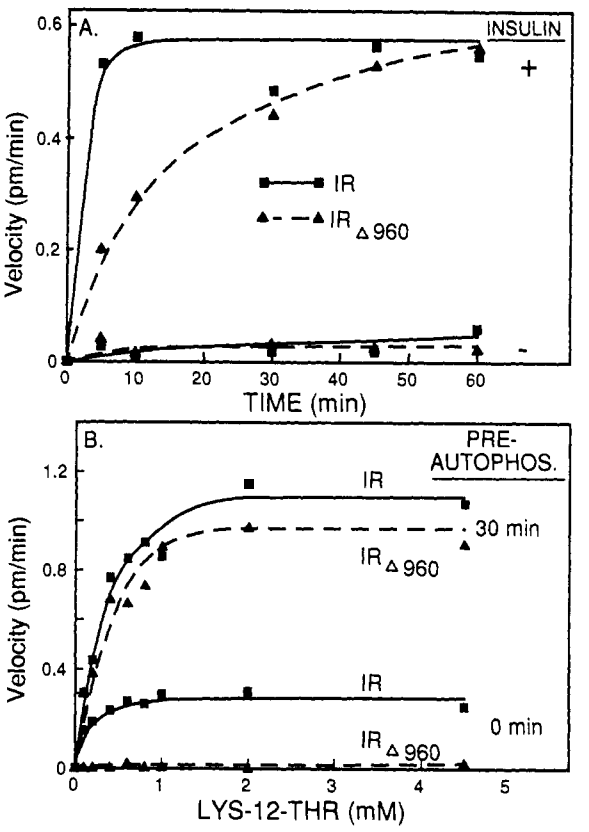

FIGURE 4: Activation of insulin receptor kinase activity by autophosphorylation. (A) Equal numbers of wheat germ agglutinin purified receptors from CHO/IR and CHO/$IR_{\Delta 960}$ cells were incubated at 16 °C for various times in 5 mM $MnCl_2$, 25 μM ATP, 20 μCi of [^{32}P]ATP, and the absence or presence of 100 nM insulin. Substrate phosphorylation was initiated by the addition of 1 mM peptide substrate (Thr-12-Lys), and after 5 min, the reaction was stopped by adding 20 μL of 1% BSA and 50 μL of 10% TCA. (B) Equal numbers of wheat germ agglutinin purified receptors from CHO/IR and CHO/$IR_{\Delta 960}$ cells were incubated at 22 °C for various times in 5 mM $MnCl_2$, 25 μM ATP, 20 μCi of [^{32}P]ATP, and 100 nM insulin. Substrate phosphorylation was initiated by the addition of various concentrations of Thr-12-Lys. After 5 min, the reaction was stopped, and the incorporation of radioactivity into the peptide was determined as described above.

receptor autophosphorylation, which in a separate experiment reached steady state by 10–15 min (Figure 2). Insulin-stimulated kinase activity of the $IR_{\Delta 960}$ increased more slowly, corresponding to its decreased rate of autophosphorylation. Interestingly, the kinase activity of the $IR_{\Delta 960}$ reached normal levels after 45 min of autophosphorylation, suggesting that the receptor functioned normally once the kinase was autophosphorylated.

To examine the kinase activity of the fully autophosphorylated $IR_{\Delta 960}$, substrate velocity curves for the wild-type and mutant receptors were obtained after 0 or 30 min of preautophosphorylation (Figure 4B). When equal numbers of mutant and wild-type receptors were compared without prior autophosphorylation, the $IR_{\Delta 960}$ receptor displayed only basal activity whereas the IR receptor was partially activated. This partial activation reflected autophosphorylation of the IR receptor during the 5-min assay period. However, after 30 min of autophosphorylation, both receptors were completely activated; substrate phosphorylation by the IR and $IR_{\Delta 960}$ receptors was similar with regard to K_m (0.311 ± 0.04 and 0.276 ± 0.04 mM, respectively) and V_{max} (1.23 ± 0.07 and 1.02 ± 0.06 pmol/min, respectively) even at 25 μM ATP. These experiments suggest that autophosphorylation normalizes the K_m for ATP of the $IR_{\Delta 960}$ such that no defect is seen during in vitro substrate phosphorylation by the autophosphorylated $IR_{\Delta 960}$ receptor.

In Vivo Autophosphorylation in CHO Cells Expressing Wild-Type and $IR_{\Delta 960}$ Receptors. The kinetics of autophosphorylation of the wild-type IR and mutant IR_{F960} and $IR_{\Delta 960}$ receptors were measured in [^{32}P]orthophosphate-labeled CHO cells. Before insulin stimulation, autophosphorylation of wild-type and mutant insulin receptors was undetectable by immunoprecipitation with anti-phosphotyrosine antibody (αPY) (Figure 5). Tyrosine phosphorylation of the wild-type insulin receptor β-subunit was evident after 2 min of insulin stimulation (100 nM) and remained elevated up to 30 min. The tyrosyl phosphorylation of the endogenous substrate pp185 is also evident in the CHO/IR cells (Figure 5, left panel). Insulin stimulation of $IR_{\Delta 960}$ autophosphorylation was slightly slow, reaching 70% of wild type by 2 min but equal to wild

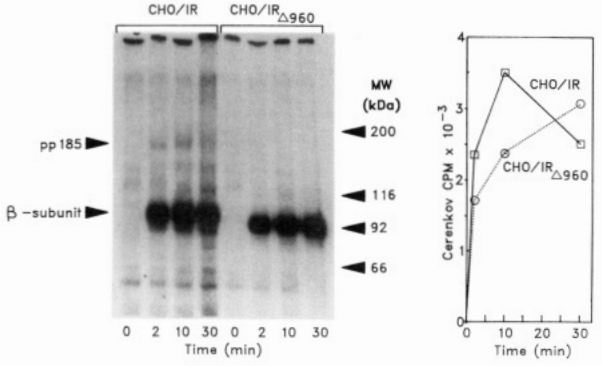

FIGURE 5: In vivo autophosphorylation of wild-type and mutant insulin receptors. (Left panel) CHO/IR and CHO/IR$_{\Delta 960}$ cells were labeled with [^{32}P]orthophosphate for 2 h and stimulated with 100 nM insulin for the indicated times. Tyrosyl-phosphorylated receptors were immunoprecipitated with αPY, separated by SDS–PAGE, and visualized by autoradiography. (Right panel) Tyrosyl-phoshorylated receptors were quantified by Cerenkov counting of β-subunit bands. (□) CHO/IR; (○) CHO/IR$_{\Delta 960}$.

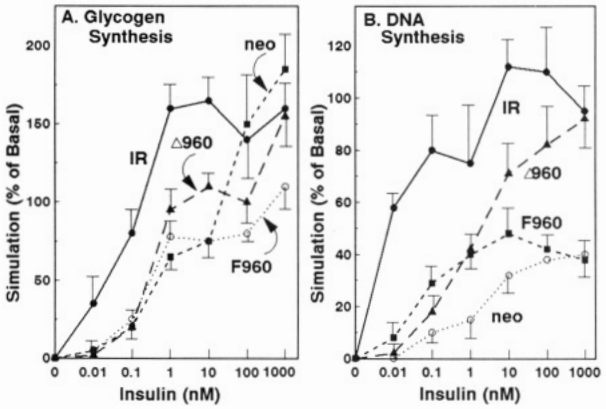

FIGURE 6: Biological activity of normal and mutant insulin receptors. The incorporation of [^{14}C]glucose into glycogen (A) and the incorporation of [^{3}H]thymidine into DNA (B) were measured after 30 min or 15 h of insulin stimulation, respectively. Results are expressed as the percentage stimulation above basal activity. Each point represents the mean of three replicates ± SEM; each experiment is representative of three to four separate experiments.

type by 30 min (Figure 5, right panel). Despite these altered kinetics, the overall level of autophosphorylation of IR$_{\Delta 960}$ was therefore similar to that of the wild-type IR in CHO cells. However, phosphorylation of pp185 in the CHO/IR$_{\Delta 960}$ cells was undetectable even after prolonged insulin stimulation. Thus, the deletion of 12 amino acids surrounding Tyr$_{960}$ caused significant defects in the ability of the insulin receptor to phosphorylate intracellular substrates.

Biological Activity of the IR$_{F960}$ and IR$_{\Delta 960}$ Mutant Insulin Receptors. The biological activity of the wild-type and mutant insulin receptors was evaluated by measuring the incorporation of glucose into glycogen after 30 min of insulin stimulation, and the incorporation of thymidine into DNA after 15 h of insulin stimulation (Figure 6). Incorporation of glucose into glycogen in control CHO/neo cells was stimulated by insulin, although an accurate determination of ED$_{50}$ was not possible. Expression of wild-type receptors in CHO/IR cells markedly increased the sensitivity of the response (ED$_{50}$ = 0.1 nM) (Figure 6A). Unlike the wild-type receptor, expression of the IR$_{\Delta 960}$ and IR$_{F960}$ receptors in CHO cells caused only a slight increase in the sensitivity of insulin-stimulated incorporation of glucose into glycogen over that seen in control cells.

The ability of the mutant receptors to mediate a mitogenic stimulus was examined by measuring the incorporation of [^{3}H]thymidine into DNA (Figure 6B). Although control CHO/neo cells exhibited insulin-stimulated thymidine incorporation, expression of the wild-type insulin receptor increased both the sensivity (ED$_{50}$ = 0.01 nM) and the magnitude of the response. In contrast, the sensitivity of insulin-stimulated thymidine incorporation in cells expressing IR$_{F960}$ and IR$_{\Delta 960}$ receptors was only slightly increased over the control CHO/neo cells. The magnitude of the response in CHO/IR$_{F960}$ cells was decreased relative to the CHO/IR cells, although the CHO/IR$_{\Delta 960}$ cells reached levels of incorporation similar to the CHO/IR cells at extremely high insulin levels (1000 nM). Thus, both juxtamembrane mutations interfered with the ability of the insulin receptor to transmit mitogenic and metabolic signals.

Discussion

Activation of the insulin receptor kinase by insulin-stimulated autophosphorylation and the subsequent phosphorylation of cellular substrates are implicated in the mechanism of insulin action (Kahn & White, 1988). Studies utilizing kinase-deficient receptors suggest that insulin receptor kinase activity is necessary for some if not all insulin-stimulated bioeffects (Chou et al., 1987; McClain et al., 1987; Ellis et al., 1986; Wilden et al., 1990). Moreover, our studies with juxtamembrane mutants of the insulin receptor suggest that autophosphorylation and kinase activity are not sufficient for signal transmission, as the insulin receptor must interact with and phosphorylate appropriate cellular substrates to regulate growth and metabolism. Our previous studies showed that mutations in the juxtamembrane region diminished in vivo substrate phosphorylation (White et al., 1988). We now provide additional evidence for the importance of the juxtamembrane region in the activity of the receptor. Although the IR$_{\Delta 960}$ binds insulin normally and has a normal dose response of insulin-stimulated autophosphorylation, autophosphorylation of IR$_{\Delta 960}$ in vitro is slower than that of the wild-type receptor due to an elevated K_m for ATP. Thus, the juxtamembrane deletion in IR$_{\Delta 960}$ reduces the affinity of the unphosphorylated insulin receptor kinase for one of its substrates, ATP. Although the Gly$_{991}$-x-Gly-x-x-Gly consensus sequence for ATP binding begins 31 amino acids C-terminal to Tyr$_{960}$, these findings show that mutations in the juxtamembrane domain can nonetheless affect substrate interactions in the active site of the receptor kinase.

Despite altered in vitro autophosphorylation kinetics, the kinase activity of the IR$_{\Delta 960}$ toward an in vitro peptide substrate at low ATP concentrations is normal once the receptor is autophosphorylated. This strongly suggests that the deletion of 12 amino acids from the juxtamembrane region causes a relatively mild perturbation in the overall structure of the receptor. In this regard, mutant receptors containing much larger deletions behave normally with regard to autophosphorylation and kinase activity (McClain et al., 1988). The changes in ATP binding we observed during autophosphorylation of the IR$_{\Delta 960}$ in vitro are therefore likely to be specifically related to the deletion of sequences from the juxtamembrane region and not to gross conformational changes throughout the β-subunit.

The ability of autophosphorylation to largely normalize the ATP binding affinity of the receptor is surprising, as the autophosphorylation and phosphotransferase activities of the receptor presumably utilize the same ATP binding site. It is possible that conformational changes induced by autophosphorylation overcome the decreased avidity or accessibility of ATP to the active site in the IR$_{\Delta 960}$. In this regard, changes in insulin receptor conformation caused by autophosphorylation have been observed in the regulatory region surrounding

Tyr_{1146}–Tyr_{1151}, although not in the region surrounding Tyr_{960} (Perlman et al., 1989; Herrera & Rosen, 1986; Herrera et al., 1985). A second possibility is that autophosphorylation of the insulin receptor lowers the K_m for ATP in phosphotransferase activity toward exogenous substrates. Such a change might play a role in the in vitro activation of the receptor kinase by autophosphorylation. In any case, autophosphorylation of $IR_{\Delta 960}$ in intact cells is relatively normal because the intracellular ATP concentration of 1–3 mM saturates the mutant receptor. The $IR_{\Delta 960}$ receptor thus should be activated in vivo. Its inability to phosphorylate pp185 in intact cells is not simply a reflection of an inactive kinase, but may reflect a decreased interaction with endogenous substrates.

The juxtamembrane region of the insulin receptor may be important for the recognition and phosphorylation of endogenous substrates (White et al., 1988). Tyrosyl phosphorylation of pp185 is inhibited by mutations in the juxtamembrane region. Furthermore, the $IR_{\Delta 960}$ has a reduced ability to mediate insulin stimulation of glycogen and DNA synthesis. These findings support the importance of the juxtamembrane region of the insulin receptor for insulin-stimulated mitogenic and metabolic responses. The insulin and IGF-I receptors, both of which stimulate pp185 phosphorylation in vivo, share extensive amino acid sequence homology in the juxtamembrane region (Izumi et al., 1987). Furthermore, a chimeric receptor composed of the extracellular insulin receptor binding domain and the transmembrane and intracellular domains of v-ros, which is 50% homologous to the insulin receptor kinase domain but with a distinct juxtamembrane region, was unable to stimulate glucose uptake or thymidine incorporation (Ellis et al., 1987). However, the insulin receptor-v-ros chimera does stimulate S-6 kinase and MAP-2 kinase; it is not known whether the IR_{F960} or $IR_{\Delta 960}$ receptors stimulate these activities (Boulton et al., 1990). Interestingly, Thies et al. have reported that the insulin-stimulated phosphorylation of pp185 is normal in rat-1 fibroblasts expressing a mutant receptor from which exon 16 (Q_{944}–D_{965}) has been deleted (Thies et al., 1990). The reason for this disparity with our results is not clear.

We have recently reported that the receptor-mediated internalization of insulin by the IR_{F960} was normal whereas internalization of the $IR_{\Delta 960}$ was defective (Backer et al., 1990); similar results were obtained with a larger juxtamembrane deletion mutant (Thies et al., 1990). The internalization defect in the $IR_{\Delta 960}$ receptor may result from the deletion of the putative consensus sequence $NPXY_{960}$, which is required for the internalization of the LDL receptor (Chen et al., 1990). It is possible that the defective biological activity of the $IR_{\Delta 960}$ reflects its decreased internalization. However, the biological activity of the IR_{F960} receptor is also diminished despite the fact that it internalizes normally. It therefore seems likely that the defects in internalization and signaling in the $IR_{\Delta 960}$ represent distinct effects of the juxtamembrane deletion on receptor function.

In summary, we have examined the effect of a 12 amino acid deletion (A954–D965) in the juxtamembrane region of the insulin receptor ($IR_{\Delta 960}$). The $IR_{\Delta 960}$ displays an increased K_m for ATP during autophosphorylation in vitro, demonstrating that the juxtamembrane deletion alters the affinity of the insulin receptor kinase for this substrate. The $IR_{\Delta 960}$ undergoes near-normal autophosphorylation at higher concentrations of ATP in vitro and in intact cells. Moreover, the in vitro kinase activity of the autophosphorylated $IR_{\Delta 960}$ is normal. However, $IR_{\Delta 960}$ is unable to stimulate the tyrosyl phosphorylation pp185, and it is defective in mediating insulin stimulation of glycogen or DNA synthesis. These data suggest that the juxtamembrane region of the insulin receptor is a component of the active site of the receptor kinase and is necessary for phosphorylation of endogenous substrates and transmission of the insulin signal in vivo.

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Registry No. ATP, 56-65-5; insulin, 9004-10-8; phosphotransferase, 9031-09-8; insulin receptor kinase, 88201-45-0; glycogen, 9005-79-2; D-glucose, 50-99-7.

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Free Fatty Acid Accumulation in Secretagogue-Stimulated Pancreatic Islets and Effects of Arachidonate on Depolarization-Induced Insulin Secretion†

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ABSTRACT: Free fatty acids in isolated pancreatic islets have been quantified by gas chromatography–mass spectrometry after stimulation with insulin secretagogues. The fuel secretagogue D-glucose has been found to induce little change in islet palmitate levels but does induce the accumulation of sufficient unesterified arachidonate by mass to achieve an increment in cellular levels of 38–75 μM. Little of this free arachidonate is released into the perifusion medium, and most remains associated with the islets. Glucose-induced hydrolysis of arachidonate from islet cell phospholipids is reflected by release of the arachidonate metabolite prostaglandin E_2 (PGE_2) from perifused islets. Both the depolarizing insulin secretagogue tolbutamide (which is thought to act by inducing closure of β-cell ATP-sensitive K^+ channels and the influx of extracellular Ca^{2+} through voltage-dependent channels) and the calcium ionophore A23187 have also been found to induce free arachidonate accumulation within and PGE_2 release from islets. Surprisingly, a major fraction of glucose-induced eicosanoid release was found not to require Ca^{2+} influx and occurred even in Ca^{2+}-free medium, in the presence of the Ca^{2+}-chelating agent EGTA, and in the presence of the Ca^{2+} channel blockers verapamil and nifedipine. Exogenous arachidonic acid was found to amplify the insulin secretory response of perifused islets to submaximally depolarizing concentrations of KCl, and the maximally effective concentration of arachidonate was 30–40 μM. These observations suggest that glucose-induced phospholipid hydrolysis and free arachidonate accumulation in pancreatic islets are not simply epiphenomena associated with Ca^{2+} influx and that arachidonate accumulation may play a role in the signaling process which leads to insulin secretion.

The β cells of pancreatic islets can be induced to secrete insulin by a variety of secretagogues. D-Glucose is an example of a fuel secretagogue and must be metabolized in order to induce secretion (Malaisse et al., 1979; Hedeskov, 1980; Ashcroft, 1980; Wollheim & Scharp, 1981; Meglasson & Matschinsky, 1986). Tolbutamide and other hypoglycemic sulfonylureas are depolarizing secretagogues which appear to interact primarily with an ATP-sensitive K^+ channel in the β-cell plasma membrane and to induce closure of this channel, which results in membrane depolarization (Henquin & Meissner, 1982; Sturgess et al., 1985; Trube et al., 1986).

Stimulation of islets from rodents and from humans with glucose has been found to result in the rapid generation of mediators derived from the hydrolysis of membrane phospholipids, including arachidonic acid and its metabolites and inositol trisphosphates (Prentki & Matschinsky, 1987; Biden et al., 1987; Turk et al., 1987a; Robertson, 1988; Metz, 1988b). Such substances participate in the transduction of extracellular signals into cellular responses in other systems (Berridge, 1987; Needleman et al., 1986) and might be suspected to play a similar role in β cells (Turk et al., 1987a; Metz, 1988b). In particular, D-glucose has been found to induce the accumulation of substantial amounts of unesterified, unmetabolized arachidonic acid in islets with a time course that closely parallels that of glucose-induced insulin secretion (Wolf et al., 1987a,b; Turk et al., 1987b). At the concentrations which accumulate in glucose-stimulated islets (Turk et al., 1987b), arachidonate has also been found to induce the release of Ca^{2+} sequestered in islet endoplasmic reticulum (Wolf et al., 1987a,b). This effect is exerted by arachidonic acid itself and not by one of its metabolites (Wolf et al., 1987a). It is not yet known whether glucose induces accumulation of free fatty acids other than arachidonate in islets. Other unsaturated, but not saturated, fatty acids have been found to mimic the Ca^{2+}-mobilizing effect of arachidonate in other tissues (Beaumier et al., 1987; Knepel et al., 1988; Naccache et al., 1989; Tohmatsu et al., 1989; Chan & Turk, 1987). These fatty acids might, if present in sufficient concentrations, also participate in the regulation of the islet cytosolic Ca^{2+} levels and the secretion of insulin.

The islet endoplasmic reticulum is believed to play an important role in maintaining the cytosolic Ca^{2+} concentration in the range of 100 nM (Prentki et al., 1984), and stimulation of islets with insulin secretagogues, including glucose, has been

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