THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 268, No. 15, Issue of May 25, pp. 11256-11264, 1993
Printed in U.S.A.

Insulin Stimulates Serine and Tyrosine Phosphorylation in the Juxtamembrane Region of the Insulin Receptor*

(Received for publication, November 30, 1992, and in revised form, January 13, 1993)

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Insulin-stimulated autophosphorylation of the cytoplasmic juxtamembrane region of the human insulin receptor was examined by Tricine/SDS-PAGE. Various mutant receptor molecules were used to identify two tryptic phosphopeptides associated with the juxtamembrane region which accounts for 15% of the autophosphorylation of partially purified insulin receptor. These phosphopeptides were immunoprecipitated with an antipeptide antibody against the juxtamembrane sequence and were phosphorylated exclusively on tyrosine. Substitution of both Tyr^{960} and Tyr^{953} with alanine eliminated insulin-stimulated phosphorylation of the juxtamembrane region without affecting tyrosine autophosphorylation in the C terminus or regulatory regions. Monosubstitution of Tyr^{960} with phenylalanine or alanine reduced phosphorylation in the juxtamembrane region by more than 50%, and manual Edman degradation indicated that Tyr^{960} was phosphorylated in wild-type receptor. *In vivo*, phosphorylation of the juxtamembrane region accounts for one-third of the insulin receptor phosphorylation and contains both phosphoserine and phosphotyrosine. Deletion of Tyr^{960} and 11 adjacent amino acids eliminated insulin-stimulated phosphorylation of the juxtamembrane region. Substitution of Tyr^{960} reduced this phosphorylation by more than 50%. The insulin receptor also undergoes serine phosphorylation outside of the juxtamembrane region which depends on the presence of Tyr^{1151}. Together with our previous studies, this report suggests that phosphorylation of Tyr^{960} may play an important role in signal transduction by the insulin receptor.

The insulin receptor tyrosine kinase mediates mitogenic and metabolic signals during insulin stimulation. Insulin binding to the α-subunit of its receptor activates the tyrosine kinase in the β-subunit which stimulates autophosphorylation that further activates the tyrosine kinase (1–4). Although the insulin receptor may mediate signal transduction via multiple mechanisms, one pathway appears to involve tyrosine phosphorylation of cellular substrates such as IRS-1[1] (5–7). The functional elements within the cytosolic domain of the insulin receptor that are required for substrate phosphorylation include an intact ATP binding domain (8, 9), autophosphorylation sites (Tyr^{1146}, Tyr^{1150}, Tyr^{1151})[2] in the regulatory region (4, 10, 11), and Tyr^{960} in the intracellular juxtamembrane region (12–14) (Fig. 1). Substitution of Tyr^{960} with phenylalanine (IR^{F960}) or the deletion of Tyr^{960} along with 11 adjacent amino acids ($IR^{\Delta 960}$) inhibits insulin-stimulated phosphorylation of IRS-1 (6, 12, 13), decreases insulin stimulation of the phosphatidylinositol 3′-kinase (15, 16), and blocks insulin stimulation of glycogen and DNA synthesis (12–14). Previous studies failed to reveal any abnormalities in insulin-stimulated tyrosine autophosphorylation of IR^{F960}, so the role of Tyr^{960} in signal transmission has been difficult to explain.

The intracellular juxtamembrane region has a significant potential for phosphorylation. Within the 22 amino acids of the intracellular juxtamembrane region encoded by exon 16, there are 4 serine residues at positions 955, 956, 962, and 964, and 3 tyrosine residues at 953, 960, and 972. Tyr^{960} was originally predicted to be an insulin-stimulated autophosphorylation site because it is preceded by a glutamic acid residue in an NPEY motif; however, we have previously been unable to identify the phosphorylation of this residue (12). In addition to the well-defined sites of tyrosine phosphorylation in the regulatory and C-terminal regions, Tornqvist *et al.* (17–19) detected a 4–5-kDa tryptic fragment from the β-subunit which contains 15% of the phosphate in the activated insulin receptor. Moreover, Tavare and Denton identified two tryptic peptides that have electrophoretic mobilities expected for phosphopeptides containing Tyr^{953} and Tyr^{960} or Tyr^{972} (20); however, direct evidence for tyrosine phosphorylation in the juxtamembrane region is unavailable.

We utilized Tricine/SDS-PAGE to study the phosphorylation of wild-type and mutant insulin receptors expressed in Chinese hamster ovary (CHO) cells and partially purified preparations. Significant insulin-stimulated tyrosine autophosphorylation in the intracellular juxtamembrane region was readily observed by Tricine/SDS-PAGE after *in vitro*[3] and *in vivo* autophosphorylation. Mutation of Tyr^{960} eliminated a significant portion of the insulin-stimulated tyrosine phosphorylation observed in this region, and radiosequencing confirmed that Tyr^{960} was a major site of autophosphorylation in the juxtamembrane region during *in vitro* labeling. Autophosphorylation of the regulatory region (Tyr^{1146}, Tyr^{1150}, and Tyr^{1151}) and the C terminus (Tyr^{1316} and Tyr^{1322}) was unaffected by mutations in the juxtamembrane region (Fig. 1). *In*

* Funding for this work was from National Institutes of Health Grants DK 38712 (to M. F. W.), DK 36433 (to G. L. K.), DK 33201 (to C. R. K.), and DK 44541 (to J. M. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a postdoctoral fellowship from the Juvenile Diabetes Foundation.

[1] The abbreviations used are: IRS-1, insulin receptor substrate 1; IR, human insulin receptor; TPCK, tosylphenylalanyl chloromethyl ketone; HPLC, high performance liquid chromatography.

[2] The insulin receptor isoform used in this study is exon 11 minus and the amino acid numbering system is according to Ullrich *et al.* (21).

[3] *In vitro* and *in vivo* in this report refer to studies on partially purified receptors and in intact cells, respectively.

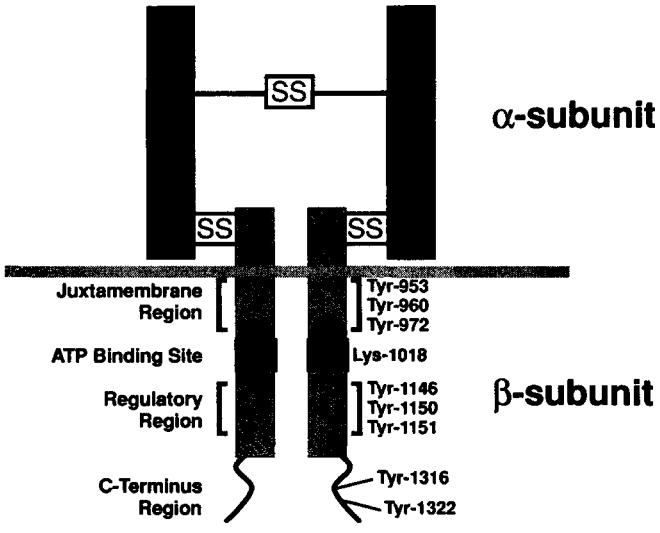

FIG. 1. **Schematic diagram of the human insulin receptor.** The functional regions within the cytoplasmic domain of the β-subunit includes tyrosine residues in the juxtamembrane, regulatory, and C-terminal regions. The insulin receptor mutants used in this study have mono- or bis-substitutions of Tyr^{953} and Tyr^{960} or deletion of Tyr^{960} along with 11 adjacent amino acids in the juxtamembrane region, mono-, bis-, or tris-substitutions of regulatory region tyrosines, or a C-terminal truncation which removes Tyr^{1316} and Tyr^{1322}.

vivo, the juxtamembrane region represented one-third of the insulin-stimulated phosphorylation and contained both phosphoserine and phosphotyrosine. Since the substitution of Tyr^{960} with phenylalanine also impairs the biological activity of the insulin receptor and the phosphorylation of IRS-1 (12, 14), these results suggest that phosphorylation in the juxtamembrane region may have an important role in insulin receptor signal transduction.

EXPERIMENTAL PROCEDURES

Construction of IR Mutants—The human insulin receptor cDNA in pSVHIRc was characterized previously (21). pSG5HIRc was prepared by subcloning a *Hin*dIII/*Asu*II fragment from pSVHIRc into pSG5 (Stratagene). The template for mutagenesis was the 1.9-kilobase *Pst*I-*Pst*I fragment from pSVHIRc (A. Ullrich, Max Planck Institute, Munich) subcloned into M13mp18. The mutant expression vector was prepared by subcloning from M13mp18 into pSG5HIRc, and this was expressed in Chinese hamster ovary (CHO) cells as previously described (12). CHO cells containing equal amounts of wild-type and mutant insulin receptors were selected by fluorescence-activated cell sorting and repeated cloning. The cells were maintained in F-12 medium (GIBCO) containing 10% fetal bovine serum (Sigma) as described previously (12). Mutant insulin receptor molecules were prepared by oligonucleotide-directed mutagenesis (22). The IR^{F960} was described by White *et al.* (12); this mutant was subsequently found to contain an additional substitution of Ser^{962} to Thr^{962}. $IR^{\Delta 960}$, produced by deletion of amino acids Ala^{954} to Asp^{965} including Tyr^{960}, was characterized by Backer *et al.* (13, 23). Receptor mutants containing point mutations at Tyr^{953} and Tyr^{960} (IR^{A953}, IR^{F953}, IR^{A960}, and $IR^{A953,960}$) were recently described (24). The $IR^{\Delta CT}$, which lacks 43 amino acids at the C terminus of the β-subunit (Ala^{1301} to Ser^{1343}) was previously described (25, 26). The *single-Phe* (IR^{F1146}, IR^{F1150}, IR^{F1151}), *double-Phe* ($IR^{F1146,50}$, $IR^{F1146,51}$, $IR^{F1150,51}$), and *triple-Phe* (IR^{3F}) or *triple-Ser* (IR^{3S}) mutants of Tyr^{1146}, Tyr^{1150}, and Tyr^{1151} were prepared by Wilden *et al.* (1). The transmembrane mutant IR^{3A}, which has the substitution of Gly, Asn, and Ala for Arg^{941} to Arg^{943}, was prepared by Yamada *et al.*[4]

In Vitro Phosphorylation—CHO cells expressing wild-type or mutant insulin receptor were lysed in 50 mM HEPES (pH 7.6), 1% (v/v) Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 1 mg/ml aprotinin and centrifuged for 30 min at 100,000 × *g*. The soluble fraction was applied to a wheat germ agglutinin-Sepharose column (Sigma) and eluted with 0.3 M *N*-acetyl-D-glucosamine (Sigma). Partially purified insulin receptor was incubated with 17 nM insulin (Eli Lilly) and 5 mM $MnCl_2$ for 30 min at 4 °C followed by a 30-min incubation at 22 °C with 100 μM [^{32}P]ATP. Labeling was terminated by dilution into 50 mM HEPES containing 20 mM Na_3VO_4 and 100 mM EDTA and incubated with 5 μg of monoclonal anti-human insulin receptor antibody (83-14, a gift from Dr. K. Siddle) at 4 °C for 18 h and immunoprecipitated with pansorbin. Samples were eluted with Laemmli sample buffer and separated on 7.5% SDS-PAGE (27). Protein was transferred to nitrocellulose (Schleicher and Schuell) and visualized by autoradiography.

In Vivo Phosphorylation—Confluent monolayers of CHO cells in 15-cm plates were serum-starved for 18 h and then incubated for 2 h in phosphate-free Eagle's minimum essential medium (GIBCO) containing 0.3 mCi/ml [^{32}P]orthophosphate (Du Pont-New England Nuclear). Cells were then stimulated with 17 nM insulin for 10 min. Labeling was terminated with liquid N_2 and cells were thawed and scraped from the dish with 50 mM HEPES (pH 7.6), 1% (v/v) Triton X-100, 100 mM NaF, 2 mM sodium vanadate, 10 mM sodium pyrophosphate, 1 mg/ml aprotinin (Sigma), 4 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride (Sigma). Cell lysate was centrifuged at 100,000 × *g* for 30 min, and the labeled insulin receptor was isolated by wheat germ agglutinin-Sepharose and immunoprecipitated as described above.

Tryptic Phosphopeptide Analysis by Tricine/SDS-PAGE—The nitrocellulose containing ^{32}P-labeled β-subunit was excised and treated with 0.5% (w/v) PVP-40 (Sigma) in 100 mM acetic acid for 1 h at 37 °C. The paper was then washed extensively with water and digested with 10 μg of TPCK-trypsin (Worthington) in $NaHCO_3$ (pH 8.2) containing 5% acetonitrile at 37 °C for 24 h. An additional 10 μg of TPCK-trypsin was added, and the digestion was continued for an additional 24 h. This technique consistently eluted 90–95% of phosphopeptides. The trypsinization was terminated by the addition of sample buffer and boiling for 3 min. The phosphopeptides were separated by Tricine/SDS-PAGE, as described by Schagger and von Jagow (28), using a 32-cm acrylamide gel consisting of a 3% stacking, 10% spacing, and 16.5% resolving gel. After electrophoresis, the gels were sealed with plastic wrap and exposed to film at −80 °C. Autoradiograms were scanned and quantitated using ImageQuant™ software (version 3.15) on a Molecular Dynamics Densitometer (Sunnyvale, CA). The molecular weight of the phosphopeptides was estimated using Rainbow™ molecular weight markers (Amersham).

Phosphoamino Acid Analysis—Insulin receptor was labeled with [^{32}P]ATP, isolated, digested with TPCK-trypsin, and resolved by Tricine/SDS-PAGE as described above. Tryptic phosphopeptides were eluted from gel slices and dialyzed against 3 liters of water using Spectrapor 1000 dialysis tubing. The peptides were then evaporated in a Speed Vac, partially hydrolyzed in 100 μl of 6 N HCl at 110 °C for 2 h, diluted with 1 ml of water, and dried. Phosphoamino acid analysis was performed by electrophoresis on cellulose thin layer plates as described previously (29).

Manual Edman Degradation—Tryptic phosphopeptides I1, I2, I5, I6, and I8, from *in vitro* labeled receptor, were eluted from the Tricine/SDS-PAGE gel and dialyzed against water for 48 h. The phosphopeptides were lyophilized and I5, I6, and I8 were resuspended in 50% acetonitrile. I1 and I2 were dissolved in water, pooled, and digested with 7 μg of protease V8 (Boehringer Mannheim) in 25 mM phosphate buffer (pH 7.8) for 18 h at 22 °C. Peptides were then covalently coupled to Sequelon™-AA discs (Millipore) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and washed extensively with water, methanol, and trifluoroacetic acid. Manual Edman degradation of phosphopeptides was performed as described by Sullivan and Wong (30). Briefly, immobilized peptides were reacted with 0.5 ml of coupling reagent (methanol:water:triethylamine:phenylisothiocyanate, 7:1:1:1, v/v) at 55 °C for 10 min, and washed with methanol, and phenylthiohydantoin derivatives were eluted with 0.5 ml of trifluoroacetic acid (55 °C, 6 min) followed by a wash with trifluoroacetic acid and 42.5% phosphoric acid (Sigma; 9:1, v/v). The radioactivity released and associated with the disc was monitored by Cerenkov radiation. Edman degradation was repeated for 9 cycles (I5, I6, and I8) and 13 cycles (I1 and I2).

RESULTS

Phosphopeptide Mapping of the Insulin Receptor by Tricine/SDS-PAGE—Human insulin receptors expressed in CHO cells were purified by wheat germ agglutinin-Sepharose and

[4] K. Yamada, R. B. Cheatham, E. Gonclaves, C. R. Kahn, and S. E. Shoelson, manuscript in preparation.

autophosphorylated at 24 °C for various time intervals in the presence of 17 nM insulin and Mn^{2+}/[^{32}P]ATP. The phosphorylated β-subunit of the wild-type insulin receptor (IR) was isolated by SDS-PAGE, transferred to nitrocellulose, and digested exhaustively with trypsin. Eight tryptic phosphopeptides, with apparent molecular weights below 7,000 (I1–I8), were resolved by Tricine/SDS-PAGE and detected by autoradiography (Fig. 2*A*). The relative phosphate labeling of these peptides, after 30 min of autophosphorylation, was quantified by laser densitometry (Fig. 2*B*). The highest molecular weight peptides, I1 and I2, were poorly resolved in this profile but were separated in subsequent experiments. I7 was a minor tryptic peptide after 30 min of labeling, but was easily observed at earlier times (Fig 2*A*, *lanes a* and *b*).

Phosphoamino acid analysis revealed that phosphopeptides I1, I2, and I4–I8 contained phosphotyrosine exclusively, whereas I3 contained mainly phosphoserine and a trace of phosphotyrosine (Fig. 2*C*). Threonine phosphorylation was not observed in this analysis. I7 was the first phosphopeptide detected after 10 s, followed immediately by the accumulation of I6 and I8 at 30 s and the gradual loss of I7 (Fig. 2*A*). I1 and I2 also appeared at the earliest times and continued to accumulate up to 30 min. Phosphopeptides I4 and I5 were observed after 1 min. In contrast, serine phosphorylation of I3 occurred slowly, as it was first detected at 3 min.

Characterization of the Tricine/SDS-PAGE Profile—Previous reverse-phase HPLC analysis and radiosequencing indicated that the β-subunit contains three major autophosphorylation sites in the regulatory region (Tyr^{1146}, Tyr^{1150}, Tyr^{1151}) and two sites in the C terminus (Tyr^{1316} and Tyr^{1322}) (2, 4) (Fig. 1). These sites were also identified by direct sequencing and two-dimensional tryptic peptide mapping (17–20). In contrast, autophosphorylation of tyrosine residues in the juxtamembrane region is poorly defined. We used three methods to characterize the insulin receptor phosphorylation observed in the Tricine/SDS-PAGE profile, including comigration with peptides separated by reverse-phase HPLC, the use of mutant insulin receptor molecules which lack specific autophosphorylation sites, and manual Edman degradation.

A reverse-HPLC separation of phosphopeptides from a tryptic digest of the insulin receptor β-subunit phosphorylated *in vitro* is shown in Fig. 3*A*. Based on previous work (2), pY1 and pY1a are tris-phosphorylated peptides derived from the regulatory region which contains $Tyr(P)^{1146}$, $Tyr(P)^{1150}$, and $Tyr(P)^{1151}$ (Fig. 1); pY1 and pY1a migrated with I6 during Tricine/SDS-PAGE suggesting that I6 contains tris-phosphorylated peptides from the regulatory region (Fig. 3, C_2 and C_3). Manual Edman degradation of ^{32}P-labeled I6 released phosphate at cycles 3, 7, and 8 confirming this conclusion (Fig. 4). Oddly, I8 was not related to any peptides in the reverse-phase HPLC profile even though it was immunoprecipitated with an antibody (αPep1150) against residues 1143–1154 in the regulatory region (data not shown). Edman degradation revealed that I8 is also a tris-phosphorylated peptide from the regulatory region as radioactivity was released at cycles 3, 7, and 8 (Fig. 4).

There are two sites of autophosphorylation in the C terminus of the β-subunit at Tyr^{1316} and Tyr^{1322}. By HPLC analysis, pY2 and pY3 are bis-phosphorylated peptides derived from the C terminus of the β-subunit which contains $Tyr(P)^{1316}$ and $Tyr(P)^{1322}$ (2, 25); pY2 and pY3 migrated with I5 and I4, respectively, during Tricine/SDS-PAGE (Fig. 3, C_4 and C_5). Moreover, I5 and I4 were absent from the Tricine/SDS-PAGE profile of the $IR^{\Delta CT}$, a mutant receptor molecule that lacks the C-terminal phosphorylation sites (Fig. 2*B*).

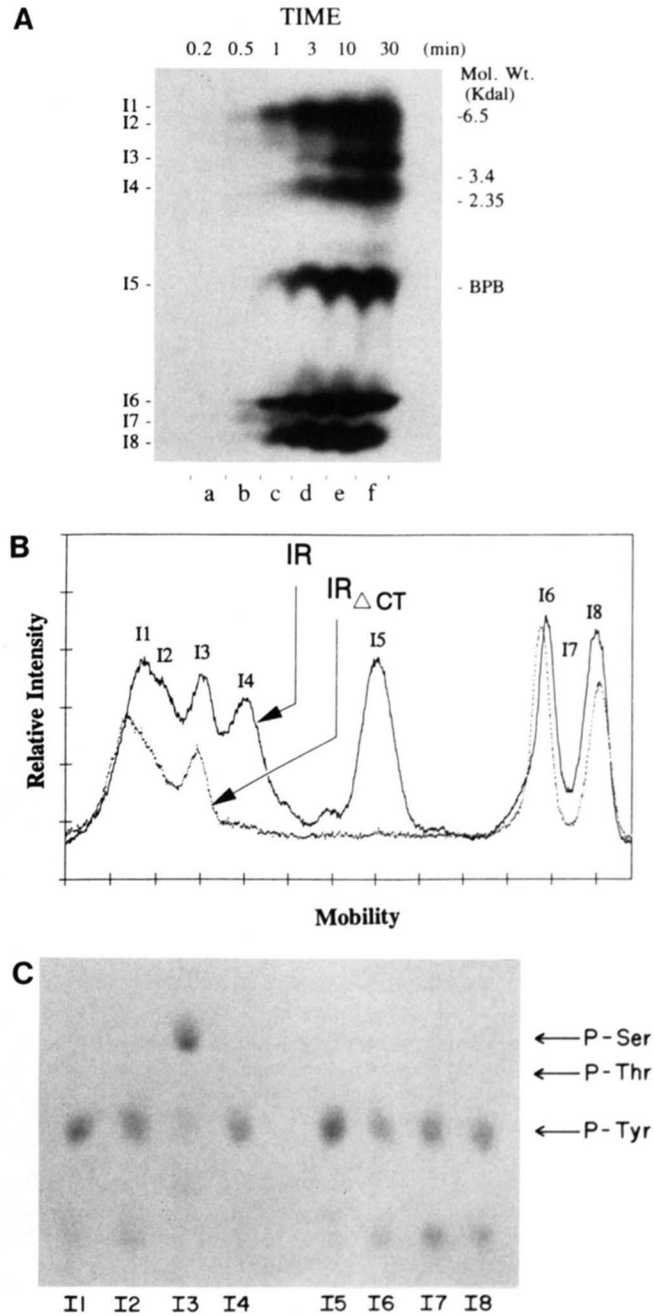

FIG. 2. **Tricine/SDS-PAGE separation of tryptic phosphopeptides from *in vitro* labeled insulin receptor.** *Panel A*, partially purified IR was pretreated with 17 nM insulin for 30 min at 4 °C and autophosphorylated in the presence of 100 μM [^{32}P]ATP for 10 s to 30 min at 24 °C. Labeling was terminated and receptors were immunoprecipitated with monoclonal anti-insulin receptor antibody 83–14. Labeled IR were run on SDS-PAGE and transferred to nitrocellulose, and the β-subunit was digested with TPCK-trypsin. Tryptic phosphopeptides, from equivalent amounts of receptors, were separated by Tricine/SDS-PAGE and visualized by autoradiography. Molecular weights were estimated with prestained markers and the location of bromphenol blue (*BPB*) is indicated. *Panel B*, tryptic phosphopeptides of IR (*solid line*) and $IR^{\Delta CT}$ (*broken line*) were quantitated by laser densitometric scanning of receptors labeled for 30 min. *Panel C*, tryptic phosphopeptides from *in vitro* labeled IR were eluted from the gel, hydrolyzed in 6 N HCl, and separated by thin layer electrophoresis. Labeled phosphoamino acids were visualized by autoradiography and identified by comparison with authentic phosphoamino acids.

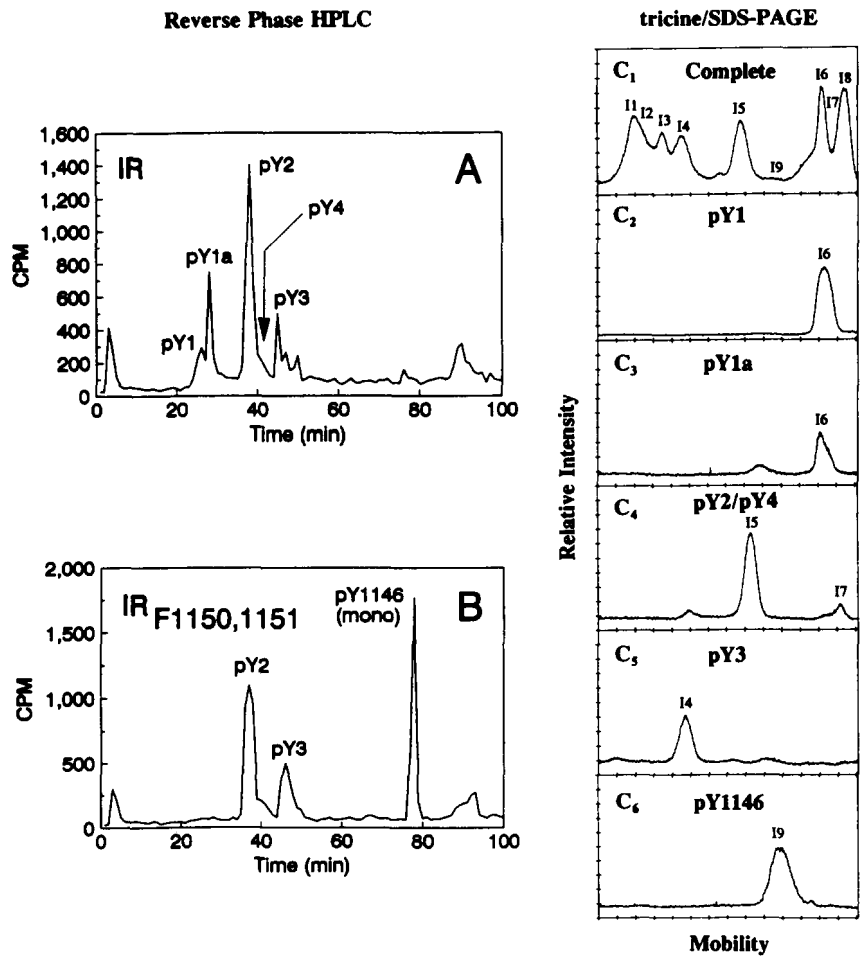

FIG. 3. **Comparison of HPLC and Tricine/SDS-PAGE tryptic peptide mapping of the insulin receptor.** Partially purified IR (*Panel A*) and $IR^{F1150,1151}$ (*Panel B*) were autophosphorylated for 30 min, isolated, and digested with TPCK-trypsin as described under "Experimental Procedures." The tryptic digests were resolved by HPLC using a RP-318 (Bio-Rad) reverse-phase column. Labeled fractions were dried, dissolved in sample buffer, and examined by Tricine/SDS-PAGE (*Panel C*) as described in Fig. 1.

Manual Edman degradation released radioactive phosphate from I5 at cycles 2 and 8, confirming that it is a bis-phosphorylated peptide derived from the C terminus (Fig. 4).

The Tricine/SDS-PAGE profile was further characterized by using a series of mutant insulin receptor molecules in which Tyr^{1146}, Tyr^{1150}, and Tyr^{1151} were replaced in various combinations with phenylalanine. I6 and I8 were lost from all of these mutants confirming that they contained tris-phosphorylated peptides from the regulatory region (Table I). I7 is ordinarily a minor phosphopeptide in the wild-type insulin receptor which migrates between I6 and I8 (Fig. 2*A*). I7 was significantly increased in all three *single-Phe* mutants, whereas it was absent from all three *double-Phe* mutants (Table I). These results suggest that I7 is a bis-phosphorylated peptide from the regulatory region. The *double-Phe* mutants contained a new peptide I9 (Table I), which co-migrates with a reverse-phase HPLC peak that was previously shown to contain a monophosphorylated peptide from the regulatory region (4) (Fig. 3, *B* and C_6). The mutants IR^{3F} and IR^{3S} eliminated I6, I7, I8, and I9 from the Tricine/SDS-PAGE profile. None of the regulatory region mutations altered the phosphorylation of I1 and I2 or the C-terminal derived peptides (I4 and I5).

Tyr^{1151} Is Essential for Serine Phosphorylation of the Insulin Receptor—Tricine/SDS-PAGE analysis of partially purified insulin receptor phosphorylated *in vitro* revealed a serine phosphopeptide which migrated at I3 (Fig. 2). I3 was observed in the $IR^{\Delta CT}$ (Fig. 2*B*), but it was absent from the triple mutants (IR^{3F} and IR^{3S}) (Table I). Moreover, the *single-Phe* and *double-Phe* mutants in which Tyr^{1151} was replaced with phenylalanine also lacked I3 (Table I). These data reveal a correlation between the presence of Tyr^{1151} and *in vitro* serine phosphorylation at I3. Kinetic analysis showed that bis- and tris-autophosphorylation of the regulatory region (I6–I8) preceded the serine phosphorylation at I3 (Fig. 2*A*), suggesting that tyrosine phosphorylation of Tyr^{1151} is necessary for serine phosphorylation at this position.

Autophosphorylation of the Juxtamembrane Region of the Insulin Receptor—After *in vitro* autophosphorylation, Tricine/SDS-PAGE revealed two phosphotyrosine-containing peptides, I1 and I2, with apparent molecular weights of approximately 6,000 (Fig. 5, *lane a*). Similarly sized tryptic peptides have been observed previously (17, 18). The presence of these phosphopeptides in IR^{3F} and $IR^{\Delta CT}$ (Table I and Fig. 2*B*) demonstrated that I1 and I2 were not derived from the regulatory or C-terminal tyrosines.

Our results suggest that I1/I2 are derived from the cytoplasmic juxtamembrane region of the insulin receptor. The slow mobility of I1 and I2 on Tricine/SDS-PAGE is consistent with the large tryptic peptide predicted from this region of the β-subunit which contains a minimum of 38 amino acids, including Tyr^{953}, Tyr^{960}, and Tyr^{972} (21). Deletion of Tyr^{960} and 11 adjacent residues ($IR^{\Delta 960}$) eliminated I1/I2 without affecting the other sites (Fig. 5, *lanes a* and *c*). An antipeptide antibody against residues 952–967 (AbP4) (31) immunoprecipitated I1/I2 from a tryptic digest of the wild-type IR, whereas nothing was immunoprecipitated from a parallel digest of the $IR^{\Delta 960}$ (data not shown). Moreover, substitution of the presumed amino-terminal tryptic cleavage sites (Arg^{941}, Lys^{942}, and Arg^{943}) with Gly, Asn, and Ala in IR^{3A}, increased the apparent size of I1/I2 without affecting the other peptides, as this manipulation is predicted to add 26 residues to the

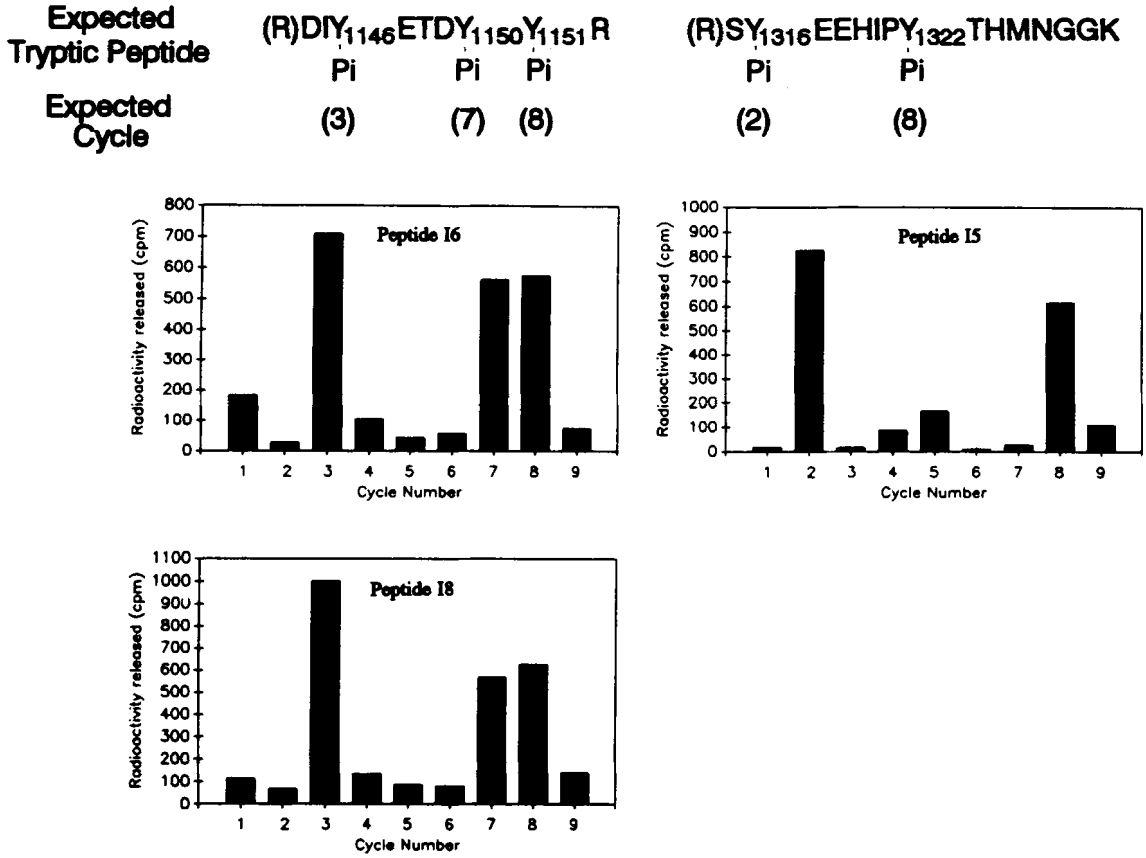

FIG. 4. **Manual Edman degradation of phosphopeptides.** Tryptic phosphopeptides from *in vitro* labeled IR were isolated by Tricine/SDS-PAGE, eluted from the gel, and coupled to arylamine-Sequelon discs. Peptides were subjected to Edman degradation as described under "Experimental Procedures," and radioactivity eluted from each reaction cycle was measured by Cerenkov counting. The radiosequence for peptide I6 (*Panel A*), I8 (*Panel B*), and I5 (*Panel C*) are compared to the predicted sequences for the tris-phosphorylated regulatory and bis-phosphorylated C-terminal tryptic peptides.

TABLE I

Phosphopeptide analysis of in vitro autophosphorylated regulatory domain mutants

The autophosphorylation of wild-type IR was compared with that of IR mutants containing *single-Phe*, *double-Phe*, *triple-Phe*, and *triple-Ser* substitutions of the regulatory domain tyrosines. Partially purified receptors were labeled with [^{32}P]ATP for 30 min at 24 °C and immunoprecipitated, and β-subunits were isolated by SDS-PAGE and trypsinized for 48 h. Tryptic phosphopeptides were separated by tricine/SDS-PAGE, and the symbols + or − indicate the presence or absence of ^{32}P-labeled phosphopeptides described in Figs. 2 and 3.

Tryptic phosphopeptides		IR	Insulin receptor regulatory domain mutants							
			IR_{3F}	IR_{3S}	$IR_{F1146,50}$	$IR_{F1146,51}$	$IR_{F1150,51}$	IR_{F1146}	IR_{F1150}	IR_{F1151}
Juxtamembrane	I 1/I2	+	+	+	+	+	+	+	+	+
Serine	I3	+	−	−	+	−	−	+	+	−
C-terminal	I4	+	+	+	+	+	+	+	+	+
	I5	+	+	+	+	+	+	+	+	+
Regulatory	I6 (tris)	+	−	−	−	−	−	−	−	−
	I7 (bis)	−	−	−	−	−	−	+	+	+
	I8 (tris)	+	−	−	−	−	−	−	−	−
	I9 (mono)	−	−	−	+	+	+	+	+	+

juxtamembrane tryptic peptide (Fig. 5, *lanes a* and *b*). The reason for two tryptic fragments from the juxtamembrane region (I1/I2) is unknown, but is similar to the two fragments observed for both the C-terminal (I4 and I5) and regulatory (I6 and I8) regions, which has been attributed to heterogenous tryptic cleavage at the C-terminal end (2, 20).

To identify the major autophosphorylation site in the juxtamembrane region, I1/I2 were analyzed by manual Edman degradation. I1/I2 were eluted from the polyacrylamide gel and digested with V8 protease under conditions that cleave after glutamate and aspartate residues. A maximum of three phosphotyrosine-containing peptides was predicted in which [^{32}P]phosphate would be released at cycle 1 or 7 during Edman degradation (Fig. 6). The mixture of V8 fragments were subjected to Edman degradation without further purification. Radioactivity was released only at cycle 1, which is consistent with the phosphorylation of Tyr^{960} (Fig. 6).

Point mutations at Tyr^{960} and Tyr^{953} have no effect on autophosphorylation in the regulatory or C-terminal regions (Fig. 5), as previously shown by HPLC analysis (12); however, these mutations altered the recovery of I1/I2. Oddly, the substitution of Tyr^{960} by phenylalanine eliminated I1, whereas substitution with alanine eliminated I2 (Fig. 5, *lanes e* and *f*). In contrast, substitution of Tyr^{953} with phenylalanine had no effect on recovery of I1 and I2, whereas substitution with alanine caused I1/I2 to migrate as a single band located

between I1 and I2 (Fig. 5, *lanes g* and *h*). Densitometric quantitation of the total juxtamembrane phosphorylation in I1 and I2 revealed that substitution of Tyr^{960} with phenylalanine or alanine reduces this more than 50%, whereas the substitution of Tyr^{953} alone had little or no effect on the phosphorylation in this region. However, substitution of both Tyr^{953} and Tyr^{960} almost completely eliminated the labeling at I1/I2 (Fig. 5, *lane d*). Thus, *in vitro* tyrosine autophosphorylation of the intracellular juxtamembrane region appears to occur primarily at Tyr^{960} in wild-type receptor, whereas Tyr^{953} may also be involved when Tyr^{960} is absent.

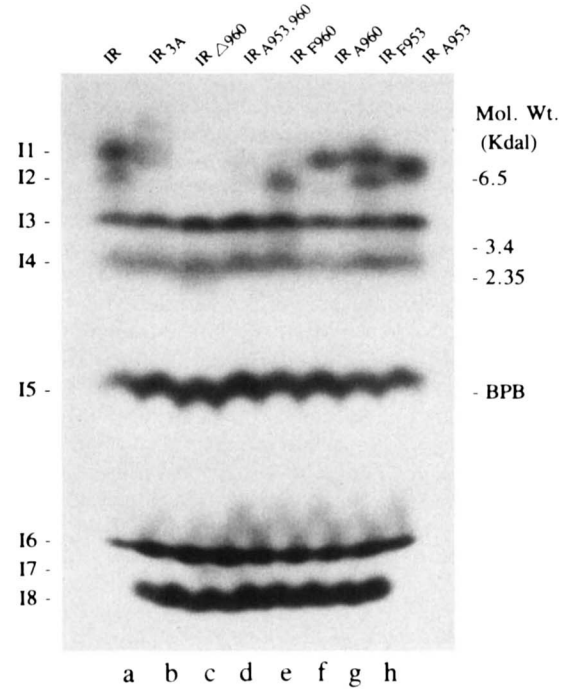

FIG. 5. **Phosphopeptide analysis of *in vitro* labeled juxtamembrane mutants.** Tryptic phosphopeptides from partially purified IR and IR juxtamembrane mutants with substitutions or deletion of Tyr^{953} and/or Tyr^{960} were prepared and separated by Tricine/SDS-PAGE as described in Fig. 1. Equal amounts of ^{32}P-labeled tryptic peptides were loaded in each lane. The results were visualized by autoradiography.

Phosphorylation of the Insulin Receptor in Intact CHO Cells—CHO/IR cells were labeled with [^{32}P]orthophosphate, stimulated with insulin, and β-subunit phosphorylation was characterized by Tricine/SDS-PAGE. The *in vivo* insulin dose-response revealed several novel aspects of IR phosphorylation (Fig. 7*A*). The juxtamembrane region located at 6.5 kDa (I1 and I2) contained most of the basal phosphorylation before insulin stimulation (Fig. 7*A*, *lane a*). Moreover, the juxtamembrane region was the most sensitive site of insulin-stimulated phosphorylation, as an increased phosphorylation of I1/I2 was detected at 0.17 nM insulin. In contrast, phosphorylation at the C terminus (I4 and I5) was observed only at the highest insulin concentrations (17 nM), suggesting that it is the least sensitive autophosphorylation site *in vivo*. The *in vivo* phosphorylation of the juxtamembrane region occurred mainly on Ser(P) and to a lesser extent on Tyr(P) (Fig. 7*B*); however, precise quantitation of the relative amounts of these phosphoamino acids is not possible because of their differential stabilities during acid hydrolysis. Finally, at high insulin concentrations (17 nM), the regulatory region (Tyr^{1146}, Tyr^{1150}, and Tyr^{1151}) was mainly bis-phosphorylated, as I7 was the major phosphopeptide band detected by Tricine/SDS-PAGE, which is consistent with previous HPLC analysis (2). However, stimulation at low insulin concentrations (1.7 nM) resulted in a more equal distribution of phosphate in I6, I7, and I8. Thus, tris-phosphorylation of the regulatory region predominated at physiological insulin levels, whereas bis-phosphorylation predominated at higher insulin concentrations.

In vivo phosphorylation of the juxtamembrane mutants was compared with wild-type IR (Fig. 8). Deletion of Tyr^{960} and the 11 adjacent amino acids ($IR^{\Delta 960}$) resulted in abnormalities in insulin-stimulated phosphorylation in both the regulatory

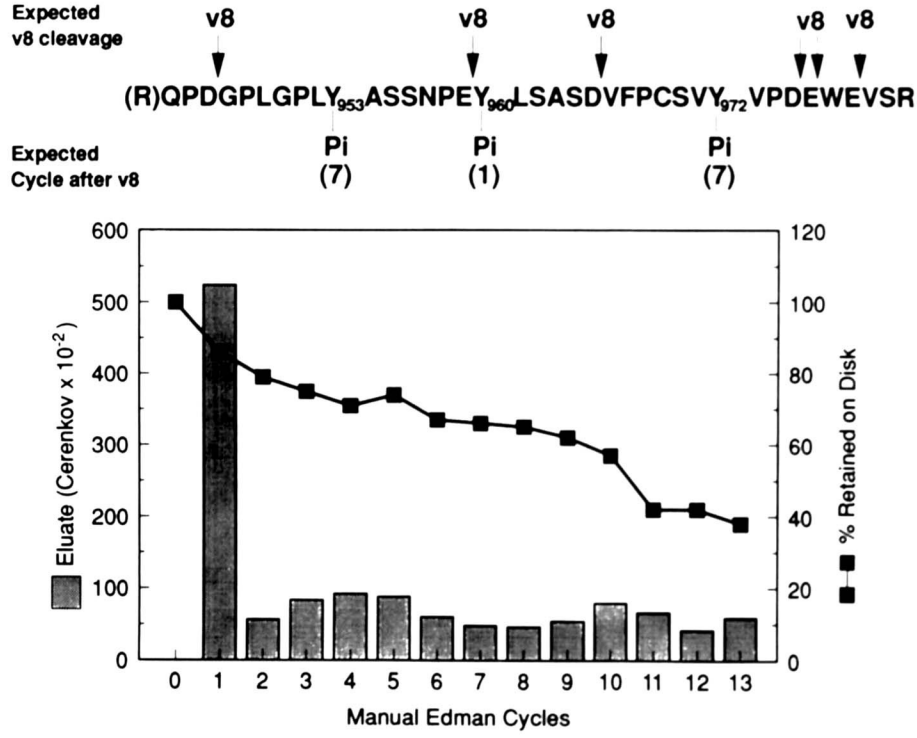

FIG. 6. **Manual Edman degradation of the juxtamembrane phosphopeptide.** The tryptic phosphopeptides I1 and I2, from *in vitro* labeled IR, were prepared and isolated by Tricine/SDS-PAGE as described in Fig. 1. Peptides were eluted from the gel, pooled, and digested with protease V8 for 18 h at 22 °C. The digest was coupled to Sequelon™-AA discs and subjected to Edman degradation. Radioactivity released from and retained on the disc was monitored after each cycle by Cerenkov counting. This radiosequence was compared with the expected cycle for phosphotyrosine released from the juxtamembrane region at residues 953, 960, and 972.

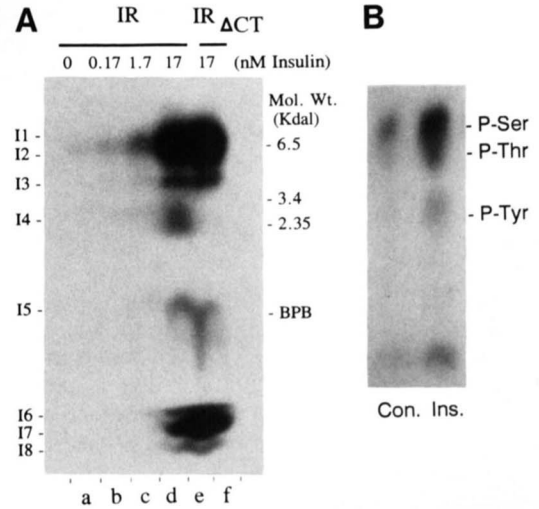

FIG. 7. ***In vivo* dose-response of insulin on the phosphorylation of insulin receptor's juxtamembrane, regulatory, and C-terminal domains.** CHO expressing wild-type IR of $IR^{\Delta CT}$ were labeled with [^{32}P]orthophosphate for 3 h. Cells were then stimulated for 10 min with 0 to 17 nM insulin (*IR*) or 17 nM insulin ($IR_{\Delta CT}$). Labeling was terminated with liquid nitrogen, and receptors were isolated by wheat germ agglutinin-Sepharose and immunoprecipitation. *Panel A*, phosphopeptide analysis of these receptors was performed as described in Fig. 1. Receptor β-subunit tryptic digests from equal numbers of cells were loaded in each lane. *Panel B*, phosphoamino acid analysis of the *in vivo* labeled juxtamembrane tryptic phosphopeptide. The 6-kDa tryptic phosphopeptide was eluted from the Tricine/SDS-PAGE gel slice, partially hydrolyzed in 6 N HCl, and separated by thin layer electrophoresis. Labeled phosphoamino acids were visualized by autoradiography and identified by comparison with ninhydrin-stained authentic phosphoamino acids.

and juxtamembrane regions. Most of the *in vivo* labeled $IR^{\Delta 960}$ contained a tris-phosphorylated (I6 and I8) regulatory domain compared with wild-type IR which is predominantly bis-phosphorylated (I7) (Fig. 8, *panels A* and *B*). This abnormality was observed in three separate experiments and contrasts the normal phosphorylation of the regulatory region observed *in vitro* for this mutant receptor (Fig. 5, *lanes a* and *c*). The juxtamembrane deletion in $IR^{\Delta 960}$ also eliminated insulin-stimulated phosphorylation in the juxtamembrane peptides (I1/I2). Since this region is phosphorylated on both serine and tyrosine (Fig. 7*B*), it would appear that both forms of phosphorylation are reduced in this mutant. Tyrosine phosphorylation of the juxtamembrane region was assessed by mono- and bis-substitution of Tyr^{953} and Tyr^{960}. Quantitation by densitometry revealed that phosphorylation of the juxtamembrane region (I1/I2) contained 36% of the total phosphate for wild-type IR, and this was reduced to 13, 18, and 25% for $IR^{A953,960}$, IR^{F960}, and IR^{A960}, respectively (Fig. 8, *panels C–E*). In contrast, monosubstitution of Tyr^{953} (IR^{F953} and IR^{A953}) did not reduce phosphorylation of I1/I2 (Fig. 8, *panels F* and *G*). These results suggest that Tyr^{960} is the major juxtamembrane region tyrosine phosphorylation site *in vivo*. Point mutations at Tyr^{960} and Tyr^{953} did not significantly alter autophosphorylation at the other sites in the β-subunit.

DISCUSSION

The tyrosine kinase activity of the insulin receptor is essential for insulin action, but the mechanism of signal transmission has been difficult to define. Tyrosine autophosphorylation of the insulin receptor plays an important role for signal transmission, as it stimulates the tyrosine kinase (2, 3, 32). Cellular proteins which undergo tyrosine phosphorylation during insulin stimulation, such as IRS-1, have been the focus of recent attention because they are expected to be important molecular elements in the insulin signal cascade. However, the structural features of the insulin receptor kinase that mediate interactions with cellular substrates remain poorly defined. Site-directed mutagenesis has been used to evaluate the importance of the cytoplasmic juxtamembrane region for insulin action. The substitution of Tyr^{960} with phenylalanine significantly inhibits the phosphorylation of IRS-1 (pp185) and blocks insulin action without affecting tyrosine kinase activity during *in vitro* assays (12, 14).

Autophosphorylation of Tyr^{960} and other amino acids in the cytoplasmic juxtamembrane region has been inferred by several studies, but never directly demonstrated. Although Tyr^{960} appears to be critical for insulin-stimulated phosphorylation of IRS-1 (pp185) (6, 12, 13), the role of this residue in signaling is unknown. Using Tricine/SDS-PAGE, we found that the cytoplasmic juxtamembrane region is a major site of insulin-stimulated phosphorylation both *in vitro* and *in vivo*.

In vitro, substitution of Tyr^{960} with phenylalanine or alanine reduces phosphorylation of the juxtamembrane region by more than 50%, without affecting autophosphorylation of the regulatory or C-terminal regions. Edman degradation of V8 protease digests of the juxtamembrane region (I1/I2) suggests that Tyr^{960} is a major phosphorylation site in the wild-type receptor. The remaining phosphorylation of I1/I2 in IR^{F960} suggests additional phosphorylation sites exist in the juxtamembrane region. Substitution of both Tyr^{960} and Tyr^{953} ($IR^{A953,960}$) eliminated nearly all of this phosphorylation, whereas the substitution of Tyr^{953} alone had little or no effect on reducing *in vitro* phosphorylation of the juxtamembrane region. These results suggest that Tyr^{953} may also be a phosphorylation site, but only when Tyr^{960} is unavailable. The absence of Tyr^{953} phosphorylation in $IR^{\Delta 960}$ may be due to structural changes associated with this 12-amino acid deletion.

In vivo, the juxtamembrane region (I1/I2) accounts for one-third of the total insulin-stimulated phosphorylation of the β-subunit. This increased labeling of I1/I2, compared to *in vitro*-labeled receptor, appears to be due to additional serine phosphorylation in this region *in vivo*. Single point mutations at Tyr^{960} significantly reduce insulin-stimulated phosphorylation of the juxtamembrane region, whereas point mutations at Tyr^{953} have little or no effect. These results are similar to the *in vitro* phosphorylation and suggest that Tyr^{960}, but not Tyr^{953}, is phosphorylated *in vivo*. Moreover, unlike Tyr^{960}, the substitution of Tyr^{953} with phenylalanine has no effect on insulin action, suggesting that phosphorylation of Tyr^{953} is not required for the receptor's biological activity (14). The amount of phosphorylation of the juxtamembrane region is comparable to that of the regulatory or C-terminal regions. In fact, *in vitro* kinetic analysis indicates that the juxtamembrane region (I1/I2) is phosphorylated more rapidly than the C-terminal region (I4/I5). *In vivo*, phosphorylation of the juxtamembrane region (I1/I2) is very sensitive to insulin, appearing before phosphorylation of the other regions. Additionally, the fraction of tris-phosphorylated insulin receptor was also increased in receptors stimulated *in vivo* with low insulin levels. This pattern of phosphorylation in intact CHO cells is similar to that which was observed in intact rat liver (33).

The rapid and sensitive phosphorylation of the juxtamembrane region (I1/I2) *in vitro* and *in vivo* suggests that it may play an early role in receptor signaling. Phosphorylation of the juxtamembrane region may create a specific binding site that mediates interactions between the insulin receptor and IRS-1. The recognition of IRS-1 by the receptor may occur at

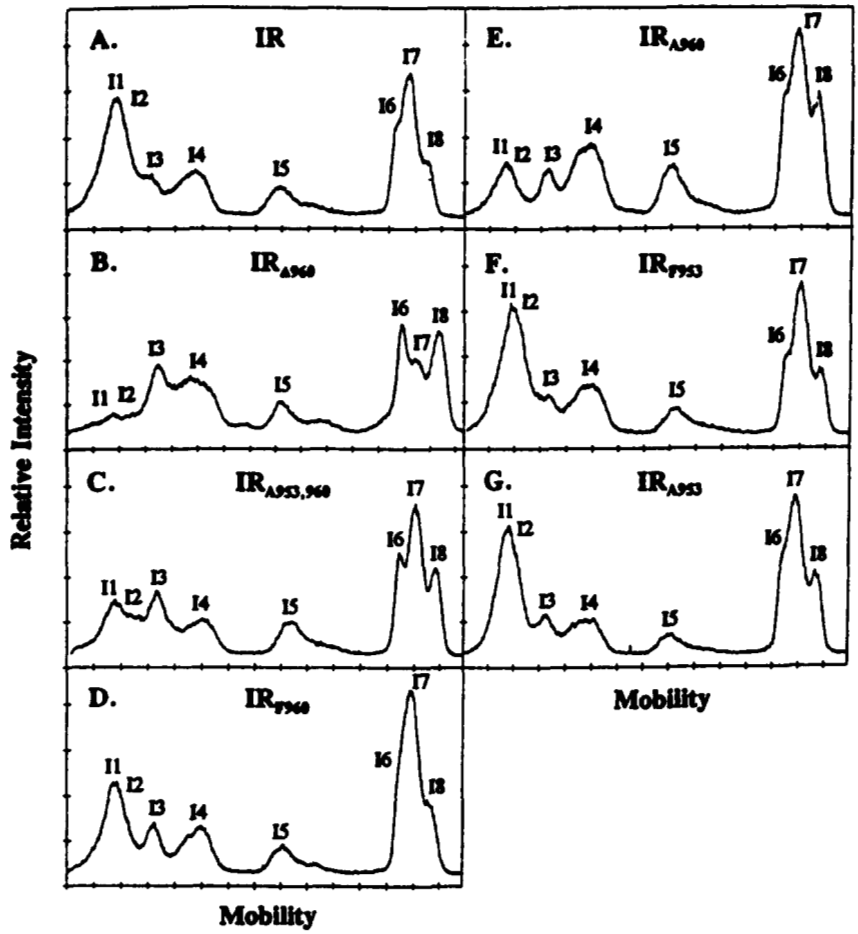

FIG. 8. **Phosphopeptide analysis of *in vivo* insulin-stimulated juxtamembrane mutants.** CHO cells expressing juxtamembrane IR mutants were labeled with [^{32}P]orthophosphate and stimulated with 17 nM insulin for 10 min. Labeling was terminated and receptors were isolated and digested for 48 h with trypsin as described in Fig. 7. Results are presented as densitometric scans of phosphopeptides separated by Tricine/SDS-PAGE.

the phosphorylated Tyr^{960}, or at a distal site due to a conformational change associated with its phosphorylation. Together with our previous results (12), we suggest that phosphorylation of the juxtamembrane region, in particular Tyr^{960}, may be critical for insulin signal transmission and the phosphorylation of IRS-1 by wild-type insulin receptor. However, we cannot rule out the possibility that there are additional structural changes in the juxtamembrane region of these mutant receptors which may inhibit normal signaling.

We have previously shown that the ligand-stimulated internalization of the human insulin receptor in CHO cells occurs via a rapid, saturable coated pit-dependent pathway that requires receptor autophosphorylation and an intact cytoplasmic juxtamembrane region (34). A critical determinant for rapid insulin receptor endocytosis is the presence of tyrosine-containing β-turns in the juxtamembrane region. Tyr^{953} and Tyr^{960} are potential sites of β-turn formation in the juxtamembrane region based on NMR analysis of model peptides (24). Tyr^{960} is present in the sequence NPEY^{960}, which is similar to the low density lipoprotein receptor sequence NPVY; both sequences have been previously predicted to form a β-turn (24, 35). The sequence GPLY^{953} is also predicted by Chou-Fasman analysis and two-dimensional NMR studies to form a β-turn (24). Mutagenesis of the insulin receptor at both Tyr^{960} and Tyr^{953} shows that both residues are independently and additively important for rapid insulin receptor endocytosis (24, 36). Substitution of alanine at both positions completely blocks entry of the insulin receptor into the rapid, saturable internalization pathway, whereas substitution with phenylalanine has no effect on endocytosis rate. Therefore, although aromatic residues at these positions appear to be required for rapid endocytosis, phosphorylation of these tyrosine residues, in particular Tyr^{960}, is not required for endocytosis. However, the predicted structural equivalence of Tyr^{960} and Tyr^{953} for endocytosis suggests that one of them (Tyr^{953}) may primarily mediate endocytosis, whereas the other (Tyr^{960}) primarily mediates insulin action.

In vivo, juxtamembrane region phosphopeptides (I1/I2), which are eliminated in IR$^{\Delta 960}$, are also phosphorylated on serine. The juxtamembrane region is not a site of serine phosphorylation *in vitro* and, therefore, is clearly different from the serine phosphorylation sites which have been identified in the C terminus (37). The *in vitro* serine phosphorylation, which may correspond to Ser^{1293}/Ser^{1294}, is located in band I3 in the Tricine/SDS-PAGE map and is present in all the juxtamembrane mutants, including IR$^{\Delta 960}$. Since the juxtamembrane peptides I1 and I2 are the major sites of phosphorylation *in vivo*, these results suggest that most of the *in vivo* serine phosphorylation on the IR occurs in the juxtamembrane region. The 12-amino acid deletion in IR$^{\Delta 960}$ eliminates 4 serine residues at positions 955, 956, 962, and 964. Thus, one or more of these serines may contribute to the phosphorylation observed in I1 and I2. These results agree with Tavare *et al.* (38) which have shown that the majors site(s) of insulin-stimulated serine phosphorylation *in vitro* are only a small component of the serine phosphorylation observed *in vivo*. The role of this insulin-stimulated serine phosphorylation in the juxtamembrane region is not known. However, IR$^{\Delta 960}$ exhibited an increased level of tris-phosphorylation of the regulatory region *in vivo*. This important finding suggests that serine phosphorylation of the juxtamembrane region may inhibit the autophosphorylation that is responsible for acti-

vating the tyrosine kinase. This is consistent with our early finding that phorbol ester-stimulated serine phosphorylation inhibits the insulin response (39, 40).

Interestingly, the *in vitro* serine phosphorylation observed in peptide I3 requires the presence of Tyr^{1151} in the regulatory domain. Tyrosine phosphorylation of the regulatory domain tyrosines precedes this serine phosphorylation, suggesting that the phosphorylated form of Tyr^{1151} is required for *in vitro* phosphorylation of partially purified IR. A similar phenomenon was observed by Tavare and Dickens (41), in which Tyr^{1150} was required for threonine phosphorylation of IR in intact cells. Thus, it appears that regulatory domain tyrosines may have an important role in insulin-stimulated Ser/Thr phosphorylation.

In summary, the juxtamembrane region of the insulin receptor undergoes insulin-stimulated phosphorylation. This phosphorylation occurs on Tyr^{960} *in vitro* and probably *in vivo*, and it does not require autophosphorylation of the regulatory or C-terminal domains. *In vivo*, the juxtamembrane region is also serine-phosphorylated. The phosphorylation of Tyr^{960} and other residues in the cytoplasmic juxtamembrane region may be essential for cellular signaling and endogenous substrate phosphorylation during insulin stimulation.

Acknowledgments—We thank Drs. K. Siddle and M. Murakami for providing us with anti-insulin receptor antibodies.

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