Identification of the Insulin Receptor Tyrosine Residues Undergoing Insulin-stimulated Phosphorylation in Intact Rat Hepatoma Cells*

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Tyrosine-phosphorylated proteins were purified from extracts of insulin-treated rat hepatoma cells (H4-II-E-C3) by antiphosphotyrosine immunoaffinity chromatography. Two major insulin-stimulated, Tyr(P) proteins were recovered: an M, 95,000 protein (identified as the insulin receptor β subunit by its immunoprecipitation by a patient-derived anti-insulin receptor serum and several anti-insulin receptor (peptide) antisera) and an M, 180,000 protein (which was unreactive with all anti-insulin receptor antibodies).

After purification and tryptic digestion of the M, 95,000 protein, tryptic peptides containing Tyr(P) were purified by sequential antiphosphotyrosine immunoaffinity, reversed-phase, anion-exchange chromatography. The partial amino acid sequence obtained by gas- and solid-phase Edman degradation was compared to the amino acid sequence of the intracellular extension of the rat insulin receptor deduced from the genomic sequence. Approximately 80% of all β subunit [32P]Tyr(P) resides on two tryptic peptides: 50–60% of [32P]Tyr(P) is found on the tryptic peptide Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg from the tyrosine kinase domain, which is recovered mainly as the double phosphorylated species (predominantly in the form with Tyr(P) at residues 3 and 7 from the amino terminus; the remainder with Tyr(P) at residues 3 and 8), with 10–15% as the triple phosphorylated species. A second tryptic peptide is located near the carboxyl terminus, contains 2 tyrosines, and has the sequence, Thr-Tyr-Asp-Glu-His-Ile-Pro-Tyr-Thr.; this contains 20–30% of β subunit [32P]Tyr(P) and is identified primarily in a double phosphorylated form. Approximately 10% of β subunit [32P]Tyr(P) resides on an unidentified tryptic peptide of M, 4,000–5,000.

The insulin-stimulated tyrosine phosphorylation of the insulin receptor in intact rat hepatoma cells thus involves at least 6 of the 13 tyrosine residues located on the β subunit intracellular extension. These tyrosines are clustered in several domains in a distribution virtually identical to that previously found for partially purified human insulin receptor autophosphorylated in vitro in the presence of insulin. This multisite regulatory tyrosine phosphorylation is the initial intracellular event in insulin action.

The insulin receptor (IR) is a ligand-activated, tyrosine-specific protein kinase (1, 2) which phosphorylates tyrosine residues on its own β subunit (3–7) and subsequently on other proteins (8–10). The crucial importance of IR β subunit autophosphorylation/tyrosine kinase to the signaling function of the receptor has been established by several recent reports which demonstrate that interference with the receptor kinase function, either by introduction of inhibitory monoclonal antibodies into intact cells (11, 12) or via expression of genetically engineered receptors whose ATP-binding site has been inactivated (13, 14), prevents all of the cellular responses to insulin examined thus far (augmented glucose uptake, ribosomal S6 serine-protein kinase, glycogen deposition, and thymidine incorporation). The autophosphorylation and substrate phosphorylation reactions catalyzed by the IR have been extensively characterized with partially purified receptor and model substrates. A stimulatory, probably obligatory role for β subunit autophosphorylation in kinase activation has been established (15–18), and the majority of the multiple tyrosine residues which participate in β subunit autophosphorylation in vitro have been identified (20, 21). By contrast, less complete information is available concerning the tyrosine-specific phosphorylation of insulin receptors in situ in intact cells (22–27). Herein, we show that in response to insulin, the IR of intact rat hepatoma (H4) cells undergoes phosphorylation of at least 6 tyrosine residues, located on three domains of the IR β subunit. The pattern of autophosphorylation sites found in insulin-stimulated H4 hepatoma cells corresponds very closely, qualitatively and quantitatively, to that observed previously for partially purified human IR autophosphorylated in vitro in the presence of comparable insulin concentrations and saturating levels of ATP (21).

MATERIALS AND METHODS

32P Labeling of H4 Hepatoma Cells and Affinity Purification of 32P—H4-II-E-C3 rat hepatoma cells (American Tissue Culture Collection, CRL 1548) grown on 10-cm plates to late log phase in Swim's S-77 medium with 20% horse serum and 5% fetal bovine serum were deprived of serum for 72 h and labeled with 32P (0.2 mCi/ml, 1 Ci/mole).

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1 The abbreviations used are: IR, insulin receptor; EGTA, (ethylenebis(oxyethylenenitrilo)tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; PTH, phenylthiodydantoin; BSA, bovine serum albumin.

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mmol) for 2 h, except where indicated. Thereafter, insulin (1 μM) was added to one set of plates; after 5 min, the medium was aspirated, and the monolayer was washed once with ice-cold 0.15 M NaCl, 0.01 M NaP, (pH 7.4). All procedures thereafter were at 0-4 °C. A solubilization buffer (0.75 ml/plate) was added 1% (w/v) Triton X-100, 2 mM NaN3, 0.1 mM NaF, 2 mM EGTA, 10 mM EDTA, 50 mM Tris-HCl (pH 7.4), 100 kallikrein-inactivating units/ml aprotinin, 0.1 mM leupeptin, and 0.1 mM diisopropyl fluorophosphatase. The plates were scraped with a rubber policeman, and the extracts were combined and centrifuged at 10,000 × g for 60 min. The supernatant was filtered through glass wool and cycled twice over a column containing antiphosphotyrosine monoclonal antibody 1G2 coupled to Sepharose 4B (15 mg of IgG/ml of settled beads; 10-cm plate). The column was washed successively with 100 volumes of 0.2% Triton X-100, 50 mM Tris-HCl (pH 7.4), 0.25 M NaCl, followed by 10 volumes of the same buffer minus NaCl and finally 5 column volumes of 0.02% Triton X-100, 5 mM Tris-HCl (pH 7.4). Elution was performed with Triton 0.02% X-100, 5 mM Tris-HCl (pH 7.4), 40 mM phenyl phosphate either in 1 ml/fraction or batchwise with 3 column volumes. The column was regenerated by washing with 10 volumes of 0.2% Triton X-100, 50 mM Tris-HCl (pH 7.4), 1.0 M NaCl and re-equilibrated with the same buffer without NaCl.

The eluates were concentrated to 10% of original volume in an evacuated centrifuge (Speed-Vac); brought to 2% SDS, 0.1 M DTT, 5% (w/v) sucrose; boiled for 3 min, and subjected to SDS-PAGE. *P*-Proteins were detected by autoradiography of the unstained frozen gel. The segments containing the *P*-labeled M, 95,000 and 180,000 proteins were excised. *P*-Protein was eluted, concentrated by acetone precipitation, and subjected to extensive digestion with trypsin (Worthington) (21).

Fractionation of Tryptic Peptides Containing [*32P]Tyr(P)—Immunooaffinity purification of [*32P]Tyr(P)-containing tryptic peptides from columns containing monoclonal antiphosphotyrosine antibody 1G2 was carried out as described (21). The [*32P]Tyr(P) peptides eluted from the immunooaffinity column were diluted with 0.02 M NaH2PO4 (pH 3; adjusted with H3PO4) and applied to a C18 column (Pharmacia LKB Biotechnology Inc. Pro RPC, HR 5/10) in 0.02 M NaH2PO4 (pH 3) at 0.5 ml/min (20 °C). Elution was generally performed with a gradient of acetonitrile to 60% in 40 min. Recovery of [*32P] always exceeded 80% of applied counts/minute.

Prior to anion-exchange chromatography, pooled peaks from the C18 column were briefly evaporated to remove acetonitrile, diluted 2-fold or more with the starting buffer (0.02 M triethanolamine HCl (pH 7.0)), and neutralized with NaOH. Anion-exchange chromatography was carried out on a Pharmacia LKB Biotechnology Inc. Mono Q column (HR 5/5) with NaCl gradient elution.

Digestion of tryptic [*32P] peptides with Staphylococcus aureus V8 protease (20 μg/ml) was carried out in 50 mM ammonium bicarbonate (pH 7.8) at 37 °C for 1–2 h. Tryptic peptides eluted from the Mono Q column were digested further with the reaction solution containing 3.4 units/ml of diisopropyl fluorophosphatase to 1 mM. The digests were analyzed by anion-exchange chromatography on a Pharmacia LKB Biotechnology Inc. Mono Q column (HR 5/5) as described above.

Protein sequencing of [*32P]Tyr(P)-containing peptides was carried out on an Applied Biosystems Protein Sequencer 470A equipped with PTH analyzer 120A. Coupling of [*32P]Tyr(P) peptides to aminobenzylpolystyrene beads, manual Edman degradation of the coupled peptides, and identification of PTH-[*32P]Tyr(P) by thin-layer electrophoresis at pH 1.9 were carried out as described before (21).

Peptides were synthesized by the stepwise solid-phase method (28, 29) in a Applied Biosystems 430A peptide synthesizer and purified by reverse-phase HPLC on a C18 Vydac columns.

Antipeptide antibodies were elicited as follows. Synthetic peptide (1 mg), mixed with an equal weight of methylated BSA (Sigma detected by autoradiography, excised, and eluted three times into 0.5% SDS by boiling for 30 min each time. The protein, in the presence of carrier BSA (0.5 mg/ml), was precipitated by addition of 4 volumes of acetone at −20 °C and, after 30 min, was collected by centrifugation at −20°C (1.2 × 10^8 × g for 20 min). The pellet was resuspended with Nn and subjected to partial acid hydrolysis (6 N HCl, 110 °C, 2 h). The residue was redisolved in two-dimensional thin-layer electrophoresis as described (6) in the presence of carrier phosphoamino acids. The autoradiograph of the plate is shown.
RESULTS

Insulin receptors were solubilized from \(^{32}P\)-labeled, control and insulin-treated H4 cells into buffers containing Triton X-100 and phosphatase inhibitors. Over 90% of cellular insulin binding activity is recovered in the supernatant after centrifugation at 10,000 \(\times g\) for 60 min. Insulin receptors containing \(^{32}P\)Tyr(P) were partially purified by immunoaffinity chromatography on columns of monoclonal antiphosphotyrosine antibodies coupled to Sepharose 4B (Fig. 1). The haptens eluate of these columns contained two major insulin-stimulated \(^{32}P\)-labeled polypeptides which migrate at \(M_r 95,000\) and \(180,000\) (Fig. 1A); both proteins contain \(^{32}P\)Tyr(P) on enzymatic and acid hydrolysis, respectively (Figs. 3B and 1C).

Application of the flow-through from the first antiphosphotyrosine monoclonal antibody column into a second, identical column gave <10\% additional recovery of these \(^{32}P\)Tyr(P)-containing proteins (Fig. 1A). The \(M_r 95,000\) \(^{32}P\)-labeled polypeptide is the \(\beta\) subunit of the rat IR; it is immunoprecipitated by a human (patient) serum which contains polyclonal antibodies reactive with the human IR, as well as by several rabbit anti-peptide antibodies raised against synthetic peptides corresponding to human IR precursor residues 40–48 (\(\alpha\) subunit), 1143–1153 (\(\beta\) subunit), and 1313–1322 (\(\beta\) subunit) (the latter two peptides encompass 5 tyrosine residues which correspond to two-thirds of all Tyr(P) in the human IR subjected to maximal autophosphorylation \(in vitro\)). By contrast, the \(M_r 180,000\) insulin-stimulated \(^{32}P\)Tyr(P)-containing protein is unreactive with all of these anti-receptor antibodies (Fig. 1B).

The recovery of \(^{32}P\)Tyr(P)-containing proteins in this one-step affinity purification was estimated by immunoblot analysis of nitrocellulose-immobilized hepatoma H4 proteins using a rabbit polyclonal antiphosphotyrosine antibody distinct from that employed in the immunoaffinity chromatography (Fig. 2). These studies indicate that: 1) insulin substantially

was incubated with a polyclonal IgG reactive with Tyr(P), washed, and reacted with \(^{125}I\)-protein A (31). The autoradiograph is shown. B, competition of polyclonal antiphosphotyrosine IgG by phosphoamino acids. Four replicate nitrocellulose sheets, each containing two lanes of transferred proteins from control and insulin-treated H4 hepatoma cells, corresponding to lanes 1 and 2 in A, were blocked with BSA and then incubated separately for 2 h with a polyclonal IgG reactive with phosphotyrosine in the presence of no added phosphoamino acid (lanes 1 and 2), 2 mM Ser(P) (lanes 3 and 4), 2 mM Thr(P) (lanes 5 and 6), or 2 mM Tyr(P) (lanes 7 and 8). The blots were washed and reacted with \(^{125}I\)-protein A. Tyr(P) blocks all immuno blotting by this IgG.

\(3\) A. R. Frackelton, Jr., M. R. Posner, S. Edelstein, and F. Meruelo, manuscript in preparation.
TABLE I

Purification of \(^{32}\)P/Tyr(P) tryptic peptides from insulin receptor autophosphorylated in intact \(^{32}\)P-labeled rat hepatoma cells

Rat H4 hepatoma cells (75-100 cm\(^2\) cell plates, 1.6 \(\times\) 10\(^6\) cells/plate), serum-starved for 72 h and labeled with carrier-free \(^{32}\)P (1 mCi/plate) for 2 h, were incubated with 1 \(\mu\)M insulin for 5 min. The cells were rinsed with cold (0-4 \(^\circ\)C) phosphate-buffered saline and solubilized as described under "Materials and Methods," and the \(^{32}\)P/Tyr(P)-containing proteins were immunoaffinity-purified using immobilized antiphosphotyrosine monoclonal antibodies as described under "Materials and Methods." The \(^{32}\)P-labeled \(M_1\), 95,000 \(\alpha\) subunit (Fig. 1, A and B) was isolated by SDS gel electrophoresis. The data from 2 of 12 preparations are shown. Steps 1-4 were at 0-4 \(^\circ\)C; steps 5 and 6 were at 23 \(^\circ\)C. Analysis of step 4 (preparation B) by SDS/urea-polyacrylamide gradient gel electrophoresis is shown in Fig. 3A. The chromatogram corresponding to steps 5 and 6 (preparation A) are shown in Fig. 4, A and C, respectively. Analysis of step 5, pool 2 (preparation A), by SDS/urea-polyacrylamide gradient electrophoresis is shown in Fig. 5.

| Step | Preparation A | Preparation B |
|------|---------------|---------------|
| 1. IR \(M_1\), 95,000 \(\alpha\) subunit in polyacrylamide gel | \(^{32}\)P cpm \(	imes 10^{-3}\) | \(^{32}\)P cpm \(	imes 10^{-3}\) |
| 2. IR \(M_1\), 95,000 \(\alpha\) subunit eluted from gel | 53.4 | 74.4 |
| 3. \(^{32}\)P-Peptides recovered from tryptic digest of \(M_1\), 95,000 \(\alpha\) subunit | 43.1 | 66.1 |
| 4. Immunoaffinity purification of \(^{32}\)P-peptides | 35.9 | 57.6 |
| Applied | Phenyl phosphate (40 mM) eluate | 15.1\(^a\) | 24.9\(^a\) |
| | 2 | 1.8 |
| | 3 | 1.1 |
| Flow-through fraction | 18.2 | 22.8 |
| Washes | NC\(^c\) | NC |
| Retained on column | NC | NC |
| 5. CI/CS reverse-phase chromatography | 15.1\(^b\) | 27.8 |
| Pool 1 (goes to step 6) | 10.6 | 20.9 |
| Pool 2 | 2.5 | 4.3 |
| 6. Anion-exchange chromatography | Applied | 10.6\(^b\) | 20.9\(^b\) |
| 0.17 M NaCl peak | 1.2 | 1.6 |
| 0.22 M NaCl peak | 2.5 | 4.9 |
| 0.29 M NaCl peak | 4.6 | 12.1 |
| 0.34 M NaCl peak | 1.0 | 1.6 |

\(^a\) Counts/minute were corrected for decay.

\(^b\) All counts/minute are \(^{32}\)P/Tyr(P) (cf. Fig. 3B).

\(^c\) NC, not counted.

Augments the immunoreactive Tyr(P) content of \(M_1\), 95,000 and 180,000 proteins in serum-starved rat hepatoma (H4) cells; 2) the increment in Tyr(P) is relatively stable after cell disruption in the extraction buffer at 0-4 \(^\circ\)C for 3 h; 3) all of the rat IR (which contains Tyr(P)), but none of the \(M_1\), 180,000 protein (which contains Tyr(P)), is adsorbed to a wheat germ agglutinin-Sepharose column; 4) all rat insulin receptors (which contain Tyr(P)) and approximately 50% of the \(M_1\), 180,000 Tyr(P)-containing protein are adsorbed by the monoclonal antiphosphotyrosine antibody column and are recovered in the phenyl phosphate eluate. Thus, antiphosphotyrosine immunoaffinity chromatography, followed by SDS-PAGE/gel elution, provides essentially complete recovery of the H4 cell insulin receptors whose \(\beta\) subunit contain \(^{32}\)P Tyr(P).

\(^{32}\)P-Labeled \(\beta\) subunit (SDS-denatured and acetone-precipitated) was subjected to complete digestion with trypsin. The digest was partially purified by immunoaffinity chromatography on monoclonal antiphosphotyrosine antibodies coupled to Sepharose 4B. Approximately equal fractions (40-50%) of the applied \(^{32}\)P were recovered in the phenyl phosphate eluate and nonadsorbed flow-through (Table I); replications of the flow-through fraction to a second antibody column did not give further adsorption of \(^{32}\)P (not shown). Analysis of the unfraccionated tryptic digest by SDS/urea gradient-PAGE (Fig. 3A) revealed that 70-75% of \(^{32}\)P migrates near the front, corresponding to peptides with \(M_1\) \(\leq\) 2000; whereas 25-30% of the \(^{32}\)P-peptide migrates at \(M_1\) 4000-5000. The bulk (~80%) of these \(M_1\), 4000-5000 \(^{32}\)P-labeled tryptic peptides are recovered in the flow-through of the antiphosphotyrosine antibody column, where they constitute 50% of all \(^{32}\)P-labeled peptide in this fraction. By contrast, 90% of the \(^{32}\)P-labeled peptides in the hapten eluate migrate at \(M_1\) \(\leq\) 2000, with 10% at \(M_1\), 4000-5000. Analysis of the eluate fraction by two-dimensional thin-layer electrophoresis after total enzymatic hydrolysis (Fig. 3B) indicates that \(^{32}\)P Tyr(P) is the only detectable \(^{32}\)P-amino-acid in the peptides eluted from the antiphosphotyrosine affinity column; \(^{32}\)P Ser(P)/\(^{32}\)P Thr(P) thus contribute negligibly to the overall \(^{32}\)P in this phenyl phosphate eluate. A parallel analysis of the flow-through fraction from the affinity column indicates that no \(^{32}\)P/Tyr(P) is detectable in this fraction (not shown).

![Fig. 3](image-url)

**FIG. 3.** A, antiphosphotyrosine immunoaffinity purification of IR \(^{32}\)P-labeled tryptic peptides: analysis by SDS/urea-polyacrylamide gradient electrophoresis. The tryptic peptides of \(^{32}\)P-labeled insulin receptor \(\beta\) subunit from insulin-stimulated rat H4 hepatoma cells were adsorbed to columns of antiphosphotyrosine monoclonal antibodies, coupled to Sepharose 4B, and washed, and \(^{32}\)P/Tyr(P)-containing peptides were specifically eluted with 40 mM phenyl phosphate as described under "Materials and Methods" (Table I, preparation B, step 4). Aliquots of the tryptic digest (0.5 \(\times\) volume, lane 1), the eluate (1 \(\times\) volume, lane 2), and the flow-through (1 \(\times\) volume, lane 3) were boiled in 2% SDS and 100 mM DTT and subjected to SDS-urea gradient electrophoresis (21) together with prestained CNBr fragments of myoglobin as markers. The gels were immediately frozen and subjected to autoradiography, which is shown. The \(^{32}\)P-labeled bands at \(M_1\), 2000 and 4000-5000 were cut out, and \(^{32}\)P counts/minute were determined; the values shown below for the tryptic digest have been multiplied by \(10^8\).
Thus, the \(^{32}\text{P}\)Tyr(P)-containing tryptic peptides are purified selectively and with high recovery by immunoaffinity chromatography; these \(^{32}\text{P}\)Tyr(P)-containing peptides lack \(^{32}\text{P}\)Ser(P)/\(^{32}\text{P}\)Thr(P), and 90% have \(^{32}\text{P}\)have. M.  

The \(^{32}\text{P}\)Tyr(P)-containing peptides were further purified on reverse-phase (C1/C8) chromatography (Fig. 4, A and B). Elution with a steep gradient of acetonitrile yielded 65–85% of applied counts/minute in a slightly asymmetrical peak, with the remainder of the \(^{32}\text{P}\) (ranging in various cell preparations from 10 to 30% of applied counts/minute) emerging later in a broad, heterogeneous zone (Fig. 4A). The initial major C1/C8 peak could be partially subfractionated on a shallow gradient of acetonitrile to 12% (Fig. 4B) into two major peaks (peaks 1 and 2) and several minor peaks (unlabeled) in a pattern very similar to that observed previously for the tryptic peptides containing \(^{32}\text{P}\)Tyr(P) derived from \(^{32}\text{P}\)-labeled human IR autophosphorylated in vitro (cf. Fig. 2B in Ref. 21). However, a more incisive subfractionation of the array of peptides in the major C1/C8 peak is achieved by anion-exchange chromatography (Fig. 4, C and D). When the initial C1/C8 peak is applied to a Mono Q anion-exchange column (Fig. 4C), over 90% of applied \(^{32}\text{P}\) is recovered in four peaks: two minor peaks eluting at 0.17 and 0.34 M NaCl, respectively (the 0.34 M NaCl peak is the predominant component of C1/C8 peak 1 shown in Fig. 4B), and two major peaks eluting at 0.22 and 0.29 M NaCl, respectively (both derived from C1/C8 peak 2 indicated above; cf. Fig. 4D). The chromatographic profile observed on anion-exchange chromatography is virtually indistinguishable from that previously described for \(^{32}\text{P}\)Tyr(P)-containing tryptic peptides derived from in vitro phosphorylated human IR (21). Gas-phase sequencing of the rat IR \(^{32}\text{P}\)Tyr(P)-containing tryptic peptides eluting from Mono Q at 0.29 M NaCl yielded the clear-cut partial sequence XIXEDTPYR (Table II); gas-phase sequence analysis of the peak of the \(^{32}\text{P}\)Tyr(P)-containing peptide eluting at 0.22 M NaCl yielded unequivocal signals only at cycle 4 (PTH-Glu) and cycle 6 (PTH-Ile) (Table II). Comparison of these partial sequences to the amino acid sequence of the intracellular extension of the rat IR \(\beta\) subunit

**Fig. 4. Separation of insulin receptor \(^{32}\text{P}\)Tyr(P)-containing tryptic peptides on reverse-phase and anion-exchange chromatography.** Immunoaffinity purified \(\beta\) subunit \(^{32}\text{P}\)Tyr(P)-containing tryptic peptides were applied to a C1/C8 column (Pharmacia LKB Biotecagogy Inc. Pro RPC, HR 5/10) in 20 mM sodium phosphate (pH 3.0) at 0.5 ml/min (20 °C) and eluted with a steep (0–60% in 40 min) (A) or biphasic (0–12% in 40 min; 12–60% in 20 min) (B) gradient of acetonitrile. Total recovery of applied \(^{32}\text{P}\) was 88 and 86%, respectively. In a second step (C and D), the major peaks from the C1/C8 column were subjected to anion-exchange chromatography. C, fractions 20-30 in A were pooled, centrifuged briefly in isooctano, diluted with an equal volume of 20 mM triethanolamine HCl (pH 7), adjusted to pH 7 with NaOH, and applied to an anion-exchange chromatographic column (Pharmacia LKB Biotecagogy Mono Q HR 5/5) equilibrated in 20 mM triethanolamine HCl (pH 7.0). Elution was performed with a 0–0.4 M NaCl gradient at 0.5 ml/min (22 °C). Recovery of \(^{32}\text{P}\) was 90%, D, peak 1 (---) and peak 2 (——) from B were each run separately on the same Mono Q column under identical conditions as for C. Recovery of \(^{32}\text{P}\) was 64% for peak 1 and 87% for peak 2. Note that A and C reflect steps 5 and 6 from preparation A in Table I, whereas B and D are different IR preparations.

| Cycle no. | Amino acid sequence | Amino acid sequence deduced from nucleotide sequence |
|-----------|---------------------|-----------------------------------------------------|
| 1         | X                   | Asp                                                 |
| 2         | Ile (14.9)          | Tyr                                                 |
| 3         | X                   | Tyr                                                 |
| 4         | Glu (5.4)           | Glu                                                 |
| 5         | Thr (9.0)           | Thr                                                 |
| 6         | Asp (5.7)           | Asp                                                 |
| 7         | X                   | Tyr                                                 |
| 8         | Tyr (6.0)           | Tyr                                                 |
| 9         | Arg (4.0)           | Arg                                                 |

*Peptides correspond to those shown in Fig. 4C. Numbers in parentheses are in picomoles.

Based on Footnote 4.
TABLE III

Tyrosine-containing tryptic peptides of the intracellular domain of the rat insulin receptor β subunit

| Peptide no. | No. of residues | Amino acid sequence* | Tyrosine at residue no. from amino terminus |
|------------|----------------|----------------------|------------------------------------------|
| 1          | 38             | QPDGPGMPLYASSNPEYLSASDVFPSSVYPDEWEVPR  | 10, 17, 29                              |
| 2          | 16             | ELGQGSGMVYGEGNAK       | 11                                       |
| 3          | 25             | PPPTLQEIMQMATAIDGMYLNK  | 21                                       |
| 4          | 9              | DIIYDDYYR              | 3, 7, 8                                 |
| 5          | 38             | DGVFTASSDMWSFGGVLWEITSLAEQPYQGLSNEVYKL | 28                                       |
| 6          | 17             | FVMQGGYLPDPNCRRK        | 7                                        |
| 7          | 19             | DDLHPFEPVSFFYSEENK      | 13                                       |
| 8          | 15             | TYDEHIYTHMNGGK          | 2, 8                                     |

* The tryptic peptides are numbered from the transmembrane region toward the carboxyl terminus.

Inspection of the amino acid sequence of the intracellular extension of the rat IR β subunit, as deduced from genomic sequence, indicates that there are 13 tyrosines on this segment, distributed on eight potential tryptic peptides (Table III). Two of these peptides are 38 residues in length, and it is likely that the M<sub>f</sub> of tryptic fragment eluting later in the C1/C8 gradient (Figs. 4A and 5) is one or both of these peptides. Conversely, since all of the 32P-labeled peptides in the initial C1/C8 peak (Fig. 4A) and therefore the Mono Q peaks in Figs. 4 (C and D) derived therefrom are entirely M<sub>f</sub> ≤ 2000 on SDS/urea gradient gel electrophoresis, peptides 1 and 5 cannot contribute to this array. Each of the potential tyrosine-containing tryptic tryptic peptides shown in Table III contains 1 or more glutamic acid residues and should be susceptible to cleavage by S. aureus V8 protease. Thus, the [32P]Tyr(P)-containing peptide eluting at 0.29 M NaCl (identified above as DIIYDDYYR, peptide 4, Table III) was cleaved completely by S. aureus V8 protease into two [32P]-labeled peptide products, each containing essentially equal amounts of [32P], eluting at 0.24 and 0.13 M NaCl on Mono Q anion-exchange chromatography (Fig. 6B), respectively. This indicates that [32P]Tyr(P) is distributed in equal amounts on both sides of the S. aureus V8 cleavage site (presumably the glutamic acid at residue 4 from the amino-terminal residue) and strongly suggests that the 0.29 M NaCl [32P]-labeled peptide is a double phosphorylated species, with [32P]Tyr(P) at residues 3 and at residue 7 or 8 from the amino terminus (or some mole fraction of residue 7 and 8 equaling a single site). Additional information is provided by solid-phase manual Edman degradation of the 0.29 M NaCl [32P]-labeled peptide, a strong peak of PTH-[32P]Tyr(P) is observed at the third cycle from the amino terminus, and a second, lesser peak of PTH-[32P]Tyr(P) release occurs at cycle 7, carrying over into cycle 8. The origin of PTH-[32P]Tyr(P) in cycle 8 cannot be defined unequivocally; due to the increase in asynchrony during the manual Edman procedure, it is likely that a fraction of PTH-[32P]Tyr(P) in cycle 8 originates from Tyr(P) at residue 7. Taken together, the V8 cleavage and manual Edman degradation indicate that the 0.29 M NaCl [32P]Tyr(P)-containing peptide is the double phosphorylated form of DIIYDDYYR, predominantly in the form with Tyr(P) at residues 3 and 7 from the amino terminus, probably admixed with the species containing Tyr(P) at residues 3 and 8. The double phosphorylated variant with Tyr(P) at residues 7 and 8 is absent (since two, not three, [32P]-fragments are observed on S. aureus V8 digestion of the 0.29 M NaCl tryptic peptide).

The identity of the minor Mono Q peak eluting at 0.34 M NaCl is established as tryptic peptide 4 by the results of solid-phase manual Edman degradation; a peak of PTH-[32P]Tyr(P) is observed at cycle 3 from the amino terminus (Fig. 7, upper). Only tryptic peptide 4 contains a tyrosine at this location (Table III). A second, broad peak of PTH-[32P]Tyr(P) release is observed in the later cycles of this degradation, indicating phosphorylation at Tyr<sup>7</sup> and/or Tyr<sup>8</sup>; quantitative assignment of [32P]Tyr(P) at these residues is established by the results of S. aureus V8 protease cleavage of the 0.34 M NaCl [32P]-labeled peptide. Two new [32P]-labeled V8 fragments are observed, eluting at 0.21 and 0.24 M NaCl and containing [32P] in a ratio of 2:1, respectively (Fig. 6A). The 0.24 M NaCl S. aureus V8 fragment of the 0.34 M NaCl tryptic peptide coelutes with one of the V8 fragments derived from the 0.29 M tryptic NaCl peptide, identified above as the double phosphorylated form of tryptic peptide 4 (compare Fig. 6, A to B). This V8 fragment (eluting at 0.24 M NaCl) is likely the peptide Asp-Ile-Tyr(P)-Glu, the phosphorylated amino-terminal V8 fragment of tryptic peptide 4. Thus, the 0.21 M NaCl S. aureus V8 fragment of the 0.34 M NaCl tryptic peptide (Fig. 6A) represents the carboxyl-terminal V8 fragment of tryptic peptide 4 (TDYDDYR). Inasmuch as this carboxyl-terminal V8 fragment, which contains 2 tyrosines, also contains twice as much [32P] as the amino-terminal V8 fragment (Fig.

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* R. E. Lewis, M. P. Czech, and M. A. Tepper, personal communication.
peptide than that observed for the double phosphorylated form of tryptic peptide 4 (identified independently as the tryptic $^{32}$P-peptide eluting at 0.29 M NaCl) and with the comparative features of the S. aureus V8 cleavage products of the 0.29 and 0.34 M NaCl peptides. Moreover, the triple phosphorylated form of this peptide derived from the human insulin receptor (21), which also has the same sequence DI-YETDYYR, elutes at pH 7 from Mono Q at 0.34 M NaCl.

The peak of $^{32}$P-peptide eluting from Mono Q at 0.22 M NaCl exhibited, on gas-phase microsequencing (Table 11), PTH-[32P]Tyr(P) at cycle 2; tyrosine is found at residue 2 only in peptide 8. These data establish tryptic peptide 8 as the major component of the 0.22 M NaCl peak. S. aureus V8 protease cleavage of the 0.22 M peptide yields two $^{32}$P-labeled fragments, indicating phosphorylation of tyrosine at residue 8 (carboxyl-terminal to the putative V8 cleavage site at residue 4), as well as at residue 2. Residue 8 is not, however, recovered cleanly in the manual Edman degradation (Fig. 7, lower). It is noteworthy that the double phosphorylated form of the tryptic peptide from the human insulin receptor (SYEEHIPYTHMNGGK) which corresponds to rat insulin receptor tryptic peptide 8 (TYDEHIPYTHMNGGK) also elutes at pH 7 from Mono Q at 0.22 M NaCl (21).

The minor peak of $^{32}$P-peptide eluting at 0.17 M NaCl (Fig. 4C) is unidentified. Recovery of PTH-$^{32}$P[Tyr(P)] on manual Edman degradation was too low for unequivocal assignment. S. aureus V8 protease digestion of two different preparations of the 0.17 M NaCl $^{32}$P-peptide gave only a single, new $^{32}$P-labeled fragment, eluting at 0.09 M NaCl (Fig. 6D). We surmise that the 0.17 M NaCl peak is a monophosphorylated form of tryptic peptide 4 (Tyr(P)) at residue 7 or 8 or more likely tryptic peptide 8 (Tyr(P) at residue 8), or both.

**DISCUSSION**

The present studies demonstrate that when serum-starved rat hepatoma cells are exposed to supramaximal concentrations of insulin for 5 min, the insulin receptor, initially devoid of immunoreactive Tyr(P) (Figs. 1A and 2A), undergoes in-
sulin-stimulated autophosphorylation on at least 6 tyrosine residues, clustered on three separate domains of the β subunit intracellular extension. One domain (peptide 4, Table II), defined by the tryptic peptide DIYETDYYR, is identical to human IR precursor residues 1144–1152 (1) and is situated in the "tyrosine kinase" domain of the β subunit; residue 1150 is homologous to the major in vitro autophosphorylation site of the Rous sarcoma virus-transforming antigen. This peptide is recovered predominantly in a double phosphorylated form (Mono Q peak eluting at 0.29 M NaCl; the amount of [32P]Tyr(P) at residue 3 = the amount found at residues 7 and 8 combined) and to, a lesser extent, as a triple phosphorylated species (Mono Q peak eluting at 0.34 M NaCl). Taken together, these isoforms of peptide 4 (cf. Table III) account for perhaps 50–60% of all [32P]Tyr(P) present in the rat IR β subunit. A second phosphorylated domain is defined by a tryptic peptide derived from the carboxyl-terminal tail of the β subunit, peptide 8 (Table III), recovered predominantly in a double phosphorylated form; this is a region through which the insulin receptor shares virtually no similarity in amino acid sequence with the epidermal (34) and platelet-derived (35) growth factor receptor tyrosine kinases and is the IR β subunit region least similar to the insulin-like growth factor receptor β subunit (36). A tentative estimate of the relative abundance of IR kinase domains 4 and 8 is obtained from eluates of the partially purified human IR autophosphorylated in intact hepatoma cells exposed to a supramaximal dose of insulin; the conditions chosen for this analysis is in effect a "snap shot" of the autophosphorylation on at least 6 tyrosine residues undergoing dephosphorylation. These considerations, coupled with the high recoveries at every step (Fig. 2 and Table I), indicate that the distribution of [32P]Tyr(P) observed reflects with fair accuracy the products of the insulin-stimulated IR intramolecular autophosphorylation as it occurs within the cell.

The immunoblotting data presented in Fig. 2 provide some evidence that tyrosine phosphatase action was effectively inhibited after cell disruption and during the immunoadfinity purification of the IRs which bear Tyr(P). Fig. 2 also verifies that affinity chromatography of crude cell extracts on columns of antiphosphotyrosine monoclonal antibody 1G2 adsorbs all of the IR which bear Tyr(P), which are then recovered in the hapten (phenyl phosphate) eluate. We have not attempted to determine whether the cell extracts contain [32P]-labeled IR which did not bind to the antiphosphotyrosine monoclonal antibody column; previous observations (23) suggest that such a population of IR phosphorylated exclusively on Ser/Thr residues may be present after insulin stimulation. Moreover, we have no definitive information on the fractional recovery of total cellular IR achieved by antiphosphotyrosine immunoadfinity chromatography.

In any event, the high intensity of the insulin-stimulated IR autophosphorylation site greatly favors recovery of the IR Tyr(P)-containing product. The distribution of rat IR β subunit [32P]Tyr(P) provided by this analysis is in effect a "snap shot" of the autophosphorylation reaction in situ. We have examined in detail the effect of a supramaximal dose of insulin at one time point; analysis of overall insulin-stimulated β subunit Tyr(P) in intact H4 cells, by immunoblot with polyclonal antiphosphotyrosine IgG, indicates that the conditions chosen give maximal steady-state tyrosine phosphorylation (not shown). We have no information on the temporal progression of site-specific Tyr(P) phosphorylation. Thus, assignment of the relative abundances of various [32P]Tyr(P) residues must be tempered by the possibility that the continued activity of tyrosine phosphatase in the intact cell may have altered the relative occupancy of various tyrosines in a differential manner and conceivably resulted in the complete dephosphorylation of certain residues within the 5-min period of insulin stimulation. However, it should be emphasized that the distribution of [32P]Tyr(P) on the rat IR β subunit in intact hepatoma cells exposed to a supramaximal dose of insulin for 5 min is virtually identical to the pattern observed for partially purified human IR autophosphorylated in vitro in the presence of comparable concentrations of insulin and saturating concentrations of MnATP (21). In each case, 6 or more phosphotyrosines, clustered on three separate domains, are observed; and the specific tyrosines participating appear to be the same. During in vitro autophosphorylation, tyrosine phosphatase was fully suppressed, and the distribution of [32P]Tyr(P) observed reflected the autokinase reaction only. The similarity in the distribution of [32P]Tyr(P) on the IR β subunit purified from insulin-stimulated hepatoma cells, as compared to IR autophosphorylated in vitro, suggests that tyrosine phosphatase action probably did not contribute significantly to the pattern of rat IR [32P]Tyr(P) observed in the intact cell. It seems likely that the high intensity of the insulin stimulus employed in these studies greatly favored repro-
sequence obtained, coupled with the amino acid sequence deduced from the nucleotide sequence, permits unequivocal identification of tryptic peptides. This information is augmented by the direct identification of PTH-[³²P]Tyr(P) at specific sites on solid-phase sequencing. The possibility of coeluting peptides cannot be eliminated by the data from gas-phase sequencing, but is addressed effectively by the more sensitive analysis of PTH-[³²P]Tyr(P) provided by solid-phase sequencing.

Two circumstances greatly facilitated this analysis. First, there is very great similarity between the tyrosine-containing peptides in the human and rat insulin receptor β subunits. Lewis et al. recently completed the sequence of a segment of rat genome encompassing the intracellular extension of the rat IR β subunit. They found the amino acid sequence (carboxyl-terminal to the residue equivalent to human insulin proreceptor residue 943 (1)) to be 95% identical to the corresponding regions of the human IR. With regard to possible autophosphorylation sites, the intracellular extensions of the rat IR β subunit contain 13 tyrosines, distributed on eight potential tryptic peptides. Seven of these eight peptides (encompassing 12 of the 13 tyrosines) are identical in length, charge, and location of the tyrosines from the amino terminus and are 95% identical in amino acid sequence to tyrosine-containing tryptic peptides derived from the human IR β subunit intracellular extension. One tyrosine-containing tryptic peptide is completely dissimilar in that the tyrosine at 1075 in the human IR is replaced by a histidine in the rat IR, whereas the histidine at 1266 in the human IR is a tyrosine in the rat IR. Thus, exclusive of this one peptide (which contains a tyrosine not among those shown to be phosphorylated in vitro in the human IR; Ref. 21), the rat and human IR β subunits share virtually identical tyrosine-containing tryptic peptides. This provides the basis for the very similar chromatographic profiles of tryptic digests of [³²P]Tyr(P) human IR (21) and [³²P]Tyr(P) rat IR peptides. A second useful feature is the negligible amounts of [³²P]Ser/[³²P]Thr(P) on the [³²P]Tyr(P)-containing tryptic peptides (Fig. 3B) derived from the rat IR autophosphorylated in the intact cell. This circumstance increased the accuracy of estimates of relative distribution of [³²P]Tyr(P), as well as assignment to specific tyrosine residues.

In conclusion, in intact rat hepatoma cells, insulin stimulates the autophosphorylation of at least six of the 13 tyrosine residues on the intracellular extension of the IR β subunit. The Tyr(P) residues are clustered and distributed on separate domains, including a peptide which contains 3 Tyr(P) residues within 6 residues, located in the tyrosine kinase segment, and a second major peptide segment near the carboxyl terminus, which contains 2 Tyr(P) residues. This distribution corresponds closely to that observed previously for the partially purified human IR autophosphorylated in vitro in the presence of insulin and MnATP. Whereas much evidence indicates that overall β subunit autophosphorylation is critical to kinase activation and to intracellular signaling, the functional role of each phosphorylated domain cannot be rigorously defined at present. For example, the data of Ellis et al. (38) indicated that replacement of the tyrosine corresponding to residue 7 on peptide 4 (or both residues 7 and 8) with phenylalanine by site-directed mutagenesis gave mutant insulin receptor species with a diminished (Phe7 + Phe8 = abolished) insulin-stimulated component of β subunit autophosphorylation (despite substantial basal autophosphorylation) and substrate phosphorylation when examined in vitro with cell-free preparations of insulin receptor. Yet, when expressed in the intact cell, these two mutant IRs (Chinese hamster ovary YF1 and YF3) exhibited persistent insulin-stimulated β subunit tyrosine phosphorylation and continued (albeit impaired) ability to signal cellular activation in response to insulin. These rather confounding results are probably attributable, in part, to the complexity dictated by the multiplicity of tyrosines participating in the autophosphorylation reaction shown in this report and, in part, to the likelihood that mutant insulin receptor may interact with and potentially be transphosphorylated by endogenous wild-type insulin receptors, especially in the intact cell. Additional mutagenesis (preferably with expression in cells lacking endogenous insulin receptor) and further characterization of the kinetics of autophosphorylation in conjunction with kinase activation and biologic signaling will be necessary for the elucidation of the operation of this complex cascade of regulatory tyrosine phosphorylation.

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