Identification of Insulin Receptor Tyrosine Residues
Autophosphorylated in Vitro

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To identify the autophosphorylation sites on the human insulin receptor (IR), partially purified human IR was incubated in vitro in the presence of insulin and manganese [γ-32P]ATP so as to achieve near-maximal activation of the histone 2b kinase activity. Approximately 70% of all β subunit [32P]phosphotyrosine residues on two tryptic peptide segments identified by microsequencing as IR precursor (Ullrich, A., Bell, J. R., Chen, E.-Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Gronfeld, C., Rosen, O. M., and Ramachandran, J. (1985) Nature 313, 756–761) 1144–1152 (tyrosine at 1146, 1150, 1151, designated peptide 5) and 1315–1329 (tyrosine at 1316, 1322, designated peptide 8), which were recovered in approximately equal amounts. Half of the remaining unidentified [32P]phosphotyrosine residues reside on another tryptic peptide of M, 4000–5000. Assignment of [32P]phosphotyrosine to specific residues required subdigestion and Edman degradation of [32P]peptides covalently coupled to solid supports. Peptide 5 was recovered in triple and double phosphorylated forms in a molar ratio of about 2:1. Tyr-1146 contained [32P] in both forms of peptide 5; in the double phosphorylated form, phenylthiohydantoin-[32P]phosphotyrosine was recovered at both Tyr-1150 and Tyr-1151, in a ratio of about 1:2. Thus, the double phosphorylated peptide 5 is presumably a mixture of Tyr-P-1146/1150 and Tyr-P-1146/1151, predominantly the latter. Peptide 8 was recovered only as the double phosphorylated form. We conclude that autophosphorylation of human IR in vitro leads to the phosphorylation of at least 6 of the 13 tyrosine residues on the β subunit intracellular extension. Five of these tyrosines are clustered in two domains; one domain is in the structurally unique C-terminal tail and contains Tyr-1316 and -1322 which are both phosphorylated. The second domain is located in the segment of the tyrosine kinase region homologous to the major in vivo autophosphorylation site of pp60 v-src and contains Tyr-1146, which is fully phosphorylated, and Tyr-1150 and -1151; although the majority of IR β subunits exhibit phosphorylation of both tyrosine 1150 and 1151, up to 20–25% of Tyr-1150 remains unphosphorylated at complete kinase activation.

The insulin receptor is an insulin-activated tyrosine-specific protein kinase (1, 2). Although the role of this enzymatic activity in the signaling functions of the receptor remains uncertain, the properties of the kinase activity in vitro have been extensively characterized, both for the autophosphorylation of the β subunit (3, 4) and the phosphorylation of exogenous protein/peptide substrates (5–7). Autophosphorylation is an intramolecular reaction (4, 7), whose Me2+ATP requirements are identical to those for the phosphorylation of exogenous substrates (8). Insulin activates both reactions in direct proportion to insulin binding (3, 7). As to the mechanism of insulin activation, several reports have established that insulin-stimulated autophosphorylation of the β subunit is a necessary intermediate step for insulin activation of the phosphotransferase activity toward exogenous peptide/protein substrates (8–11). We have shown previously that overall β subunit 32P incorporation correlates closely with kinase activation, both over time and at a variety of Me2+ATP concentrations (8). Moreover, once the receptor is autophosphorylated in vitro, insulin can be removed from the binding site with no loss of kinase activity (8, 9). Thus, insulin binding transforms the receptor to a new functional state capable of a rapid intramolecular autophosphorylation; autophosphorylation transforms the receptor to yet another state capable of phosphorylating exogenous substrates. This presumably corresponds to the active conformation in vivo.

Several recent reports indicate that a similar stable activation of the receptor tyrosine kinase occurs in intact cells (12, 13). Consequent to insulin binding, the insulin receptor tyrosine kinase, measured in cell-free extracts, is activated; this activation is reversed in part by treatment of the partially purified insulin receptor with alkaline phosphatase. It is presumed that insulin-stimulated autophosphorylation of β subunit tyrosine residues underlies the activation in intact cells, as with purified receptor.

Based on these considerations, we sought to identify the tyrrosine residues which undergo autophosphorylation in vitro. We have developed a peptide-mapping approach which yields high recovery of [32P]phosphotyrosine-containing β subunit peptides and have directly sequenced those peptides which bear two-thirds of all [32P]phosphotyrosine generated in the presence of insulin in vitro. In addition, we have identified directly those tyrosine residues undergoing phosphorylation in vitro. Our results indicate that the human insulin receptor

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is autophosphorylated in vitro on two major β subunit domains and at least 6 tyrosine residues, but with differing efficiency, and in an asymmetric fashion.

**MATERIALS AND METHODS**

The preparation of human placental microsomal membranes, solubilization of the insulin receptor, the preparation and storage of partially purified concentrated receptor, NaDodSO₄-PAGE, two-dimensional thin-layer electrophoresis, and insulin binding were performed according to previously described techniques (3, 7, 8).

**Phosphorylation Reactions—**Insulin receptor histone 2b tyrosine kinase was measured at 30 °C in the presence of manganese (10 mM), [γ-32P]ATP (2.0 mM), histone 2b (5.5 mg/ml), NaCl (0.24 M), Triton X-100 (0.02-0.07%, v/v), Tris-HCl (50 mM, pH 7.4) ± prior incubation with 0.1 μM insulin (15 min at 22°C) (7). To measure the extent of activation of IR histone 2b kinase caused by in vitro autophosphorylation, an aliquot of IR was assayed under conditions previously shown to minimize further autophosphorylation and activation (8): 10 mM magnesium, 0.20 mM [γ-32P]ATP, 5.5 mg/ml histone 2b at 30°C. This rate was multiplied by 3/2 to correct to the corresponding rate at 2.0 mM ATP (as extensively validated in Ref. 8, cf. Figs. 3-5), and the percent activation was calculated as follows.

\[
\frac{({H_2b\text{ kinase}}\text{ ATP}) \times \frac{3}{2}}{({H_2b\text{ kinase}}\text{ ATP, with insulin})} \times 100
\]

Autophosphorylation of insulin receptor preparatory to site analysis was carried out at 22°C in the presence of manganese (10 mM), [γ-32P]ATP (0.2 mM, 2-15 cpm/μmol), 0.1 μM insulin for 30 or 40 min. The reaction was terminated by addition of EDTA to 15 mM. These conditions led to near-maximal H2b kinase activation: 84% at 30 min (range 60-114% in six preparations) and 119% at 40 min (97, 125, 134%).

**Purification of 3P-IR β Subunit—**After autophosphorylation, preparations were taken to avoid unwanted receptor degradation and contamination; all procedures were at 4°C except as noted, glassware was autoclaved, and solutions were freshly prepared from ultrapure water and filter sterilized. Disopropyl fluorophosphate (0.1 mM) was added, all solutions were adjusted to trypsin digestion.

**3P-Insulin receptor was immunoadsorbed to antiphosphotyrosine monoclonal antibody 1G2 coupled to Sepharose 4B (15 mg of antibody/ml of settled beads). Antiphosphotyrosine antibody 1G2 is a high affinity IgG, κ monoclonal antibody that demonstrates high specificity for the phenyl phosphate moiety of phosphotyrosine proteins and which was affinity-purified and coupled to CNBr-activated Sepharose 4B as described previously (15). The capacity of these columns for 32P-insulin receptor exceeds 2 nmol of 32P/ml of beads, the highest amount of receptor tested. 32P-Insulin receptor was quantitatively adsorbed by rotating with beads for 45 min at 4°C. The column was washed extensively with 0.25 M NaCl, 0.2% Triton X-100, Tris-HCl (50 mM, pH 7.4), followed by 0.2% Triton X-100, Tris-HCl (50 mM, pH 7.4), and finally with this buffer diluted 10-fold, and 3P-receptor was eluted with 3 column volumes of phenyl phosphate (0.04 M) in Tris-X100 (0.02%), Tris-HCl (5 mM, pH 7.4) and evaporated to 19% of initial volume. The concentrate was brought to 2% NaDodSO₄, 100 mM dithiothreitol, and 5% sucrose, boiled for 3 min, and subjected to NaDodSO₄-PAGE. The gel segment containing the M₉₀₀₀₀-3P-β subunit, identified by autoradiography of the frozen gel, was excised, soaked in water for 20 min at 23°C, and transferred to 1.5 ml of ammonium bicarbonate (0.1 M), NaDodSO₄ (1%), dithiothreitol (50 mM) for 6-12 h at 37°C; this elution was repeated twice. The eluates were precipitated successively in 80% acetone at −20°C in a 15-ml siliconized glass tube, and the pellets were washed once with 80% acetone at −20°C.

**Tryptic Digestion and Isolation of 3P-Tyrosine Peptides—**Precipitated 3P-β subunit was dispersed in 2 μl of N-ethylmorpholine acetate (0.1 M, pH 8) by brief sonication followed by the addition of 100 μg/ml TPCK-trypsin (Worthington). Digestion was carried out at 37°C for 36-48 h with further additions of trypsin at 12 and 24 h. Trypsin was inactivated with disopropyl fluorophosphate, and the digest was assayed to the Sepharose 4B immobilized antiphosphotyrosine monoclonal antibody as above. After extensive washing with N-ethylmorpholine acetate (0.1 M, pH 8), 3P-tyrosine peptides were eluted with three column volumes of phenyl phosphate (0.04 M) in N-ethylmorpholine acetate (0.91 M, pH 8) (see Table III).

**Fractionation of 3P-Tyrosine Tryptic Peptides—**The immunoadsorption-purified mixture of 3P-peptides was diluted with NaH₂PO₄ (0.02 M, pH 3, adjusted with H₂PO₄) and applied to a C18 reversed-phase column (Pharmacia ProRPC HR 5/10); the column was washed 30 mL of 30% acetonitrile. Recovery of applied 3P always exceeded 80%. Poorly peaks were briskly evaporated to remove acetonitrile; for subsequent anion exchange chromatography, samples were diluted 2-fold or more with the starting buffer, trifluoroacetic acid (0.02 M, pH 7) and neutralized with NaOH. Anion exchange chromatography was carried out on a Pharmacia MonoQ column (HR 5/5) with NaCl gradient elution. Prior to gas-phase sequence analysis, peaks were reapplied to the C18 column, eluted in a steep acetonitrile gradient containing 1.0% trifluoroacetic acid, and concentrated in an evacuated centrifuge.

**Protein Sequence Analysis—**3P-Peptides were sequenced in an Applied Biosystems Protein Sequencer 470A equipped with PTI Analyzer 129A. In attempting to recover PTH-phosphotyrosine, we observed that ATZ and PTH-phosphotyrosine were not extracted into the solvents usually employed (butyl chloride/ethyl acetate/heptane), but were quite soluble in water and aqueous buffers. Since such polar solvents lead to unacceptable losses of peptide, the peptide was first coupled covalently to a solid support. 3P-Peptides dissolved in a 1:1 mixture of pyridine HCl (1 M, pH 5)/dimethylformamide were mixed with 35-50 mg of dimethylformamide-washed amionex-zypolypropylene or aryl-NH₂-derived glass beads. Twenty mg of 1-ethyl-3-(3-dimethylaminopropyl) cardoamide HCI (0.2 mg/ml in 80% dimethylformamide) was added. The mixture was incubated for 1-2 h, the supernatant decanted, washed successively with dimethylformamide and dimethylalcoholamine (1 M, pH 8.5), and finally residual NH₄ groups were bichromic in water and aqueous buffers. Since such polar solvents lead to unacceptable losses of peptide, the peptide was first coupled covalently to a solid support. 3P-Peptides dissolved in a 1:1 mixture of pyridine HCl (1 M, pH 5)/dimethylformamide were mixed with 35-50 mg of dimethylformamide-treated amionex-zypolypropylene or aryl-NH₂-derived glass beads. Twenty mg of 1-ethyl-3-(3-dimethylaminopropyl) cardoamide HCI (0.2 mg/ml in 80% dimethylformamide) was added. The mixture was incubated for 1-2 h, the supernatant decanted, washed successively with dimethylformamide and dimethylalcoholamine (1 M, pH 8.5), and finally residual NH₄ groups were bichromic in water and aqueous buffers. Since such polar solvents lead to unacceptable losses of peptide, the peptide was first coupled covalently to a solid support. 3P-Peptides dissolved in a 1:1 mixture of pyridine HCl (1 M, pH 5)/dimethylformamide were mixed with 35-50 mg of dimethylformamide-washed amionex-zypolypropylene or aryl-NH₂-derived glass beads. Twenty mg of 1-ethyl-3-(3-dimethylaminopropyl) cardoamide HCI (0.2 mg/ml in 80% dimethylformamide) was added. The mixture was incubated for 1-2 h, the supernatant decanted, washed successively with dimethylformamide and dimethylalcoholamine (1 M, pH 8.5), and finally residual NH₄ groups were bichromic in water and aqueous buffers.
gradient gel electrophoresis, 15–20% of the $^{32}$P-peptide migrated at an apparent $M$, of ~4000–5000, while the remainder ran at the front, corresponding to $M$, < 2000 (Fig. 1A). After complete enzymatic hydrolysis (via papain and aminopeptidase M) of the tryptic digest, all $^{32}$P comigrated with phosphotyrosine. Complete enzymatic hydrolysis (via papain and aminopeptidase M) of the tryptic digest, all $^{32}$P comigrated with phosphotyrosine, and phosphoserine, $P$-Tyr, phosphothreonine, $P$-Ser, phosphoserine, $P$-Tyr, phosphothreonine, and phosphoserine.

Preliminary purification of the tryptic digest was accomplished by anti-phosphotyrosine affinity chromatography on two-dimensional thin-layer electrophoresis (Reg). Preliminary purification of the tryptic digest was accomplished by anti-phosphotyrosine affinity chromatography on two-dimensional thin-layer electrophoresis (Reg). Gas-phase sequencing of the intracellular domain of the human insulin receptor $\beta$ subunit (Table I) indicated that only tryptic peptide 8 derived from the C terminus of the $\beta$ subunit contains those residues at positions 6 and 7 from the N terminus. Thereupon, a further 700 pmol of $^{32}$P phosphotyrosine peptide was fractionated under the same conditions, resulting in the pattern seen in Fig. 2A. Three zones of $^{32}$P are separated: peak 1, barely adsorbed, ~30% of $^{32}$P; peak 2, ~40%; and peak 3, a broad clearly heterogenous zone. Approximately 135 pmol of $^{32}$P from Peak 2 was subjected to gas-phase sequencing, resulting in a very clear dual sequence (Table II). Thus Peak 2 contains, in addition to peptide 8 (identified tentatively on the initial degradation), peptide 5 as well, in a molar ratio of ~2:1 assuming equivalent initial yield. Gas-phase sequencing of peak 1 yielded glutamic acid (cycle 4) and threonine (cycle 5) (cycles 1 through 3 were lost for technical reasons); these two residues are present at these cycles only in peptide 5. The detection of peptide 5 in the earliest eluting Cl/C8 peak 1 as well as in C1/C8 peak 2 suggested that peptide 5 might be precariously adsorbed and easily overloaded; its appearance in peak 1 (as well as peak 2) might simply reflect breakthrough (Fig. 2A). Therefore, column conditions were altered so as to enhance adsorption of peptide 5; the gradient of acetonitrile was made more shallow, and the pH decreased from 5 to 3 to protonate side-chain carboxyls. Under these conditions, peak 1 eluted reproducibly within the acetonitrile gradient but remained distinct from peak 2. The latter now eluted as a broad, notched, or frankly double peak (Fig. 2B). Further sequencing of C1/C8 peak 1 confirmed the presence of peptide

**TABLE I**

Tyrosine-containing tryptic peptides of the intracellular domain of the human insulin receptor $\beta$ subunit

| Peptide No.* | Proreceptor residue no. | Amino acid sequence* | Tyrosine at residue no. from peptide NH$_2$ terminus |
|--------------|-------------------------|----------------------|-----------------------------------------------|
| 1            | 944–981                 | QPDGGLPLGLYASSNPEYLSADVFFCSVYVPDEWEVSR | 10, 17, 29                                   |
| 2            | 988–1004                | EGQGQFEGMVYEGNAR     | 11                                           |
| 3            | 1074–1078               | SYLR                 | 2                                            |
| 4            | 1096–1114               | PPPPLQEMIQMAEADGMYLNAK | 21                                          |
| 5            | 1144–1152               | DIYETDDYR            | 3, 7, 8                                      |
| 6            | 1171–1208               | DGVFTTSMDWFGVVLWEITSLAEQPYQGLSDEQVYLK | 28                                          |
| 7            | 1209–1225               | FVMDDGYLDQPDNCPER   | 7, 2, 8                                      |
| 8            | 1314–1329               | SYEEHIPYTHMNGGK     |                                              |

*The tryptic peptides are numbered from the end of the transmembrane region toward the C terminus.

*Residue number and sequence taken from Ullrich et al. (1).
tryptic peptides from the insulin receptor. 

PMol of 32P) from a different 32P-insulin receptor preparation were applied to a C1/C8 column (Pharmacia P-L Biochemicals ProRPC, HR 5/10) in 25 mM ammonium acetate, pH 5.0, at 0.5 ml/min (22°C) and eluted with a column (Pharmacia P-L Biochemicals ProRPC, HR 5/10) in 25 mM 0-60% acetonitrile 0.5 ml/min (22°C) and eluted with a biphasic gradient of acetonitrile shown in Fig. 1B.

Anion exchange chromatography (Fig. 3, A and 5), whereas Cl/C8 peak 2 again exhibited both peptide 8 sequence and an isoleucine at Edman cycle 2, indicating the presence of peptide 5 as well (Table II). Reliable resolution of C1/C8 peak 2 was achieved on anion exchange (Mono Q) chromatography (Fig. 3, A and B). Two components are detected, 2a and 2b, containing 32P in a ratio of 2-3:1, eluting at 0.22 and 0.29 M NaCl, respectively. Gas-phase sequencing of peak 2a revealed peptide 5 (see also Fig. 4). C1/C8 peak 1, previously identified as peptide 5, elutes from Mono Q at 0.34 M NaCl, clearly more negatively charged than peak 2b (Fig. 3A). Thus, peptides 5 and 8 contain ~70% of 32P phosphorylase. Peptide 8 contains 28-30% of total 32P; peptide 5 is recovered in two forms which differ in charge. The more anionic form (peak 1) contains ~30% of 32P phosphorylase, whereas the less anionic form (peak 2b) contains 10-12% of 32P phosphorylase (Table III).

In order to determine which tyrosine residues are phosphorylated in vitro, a combination of solid-phase Edman degradation of P1, P2a, and P2b and subdigestion of these tryptic peptides with Staphylococcus aureus V8 protease was employed. Peaks 1 and 2b (Fig. 3B), the two charge variants of tryptic peptide 5, were subjected to a brief digestion with S. aureus V8 protease under identical conditions as for A. 32P recovery was 96 and 72%, respectively. C, aliquots of peak 1 (O- - - O) and peak 2b (O- - - O), the charge variants of tryptic peptide 5 (cf. Fig. 3B and Table III, step 6) were digested with S. aureus V8 protease (20 mg/ml) for 1 h at 37°C in 50 mM ammonium bicarbonate, pH 7.8. The reaction was terminated by 1 mM diisopropyl fluorophosphate; the digests were separately applied to the Mono Q column and eluted under the conditions described above. Note that both peaks 1 and 2b are each cleaved completely into two 32P-peptide fragments, which elute at 0.24 and 0.22 M NaCl (peak 1) and at 0.24 and 0.13 M NaCl (peak 2b), respectively. Overall 32P recovery is 81% (peak 1) and 77% (peak 2b). D, an aliquot of peak 2a (O- - - O) (peptide 8; see Fig. 3B and Table III, step 6) was digested with S. aureus V8 protease (50 mg/ml) for 24 h at 37°C. The digest was analyzed by Mono Q anion-exchange chromatography as above. Note that peak 2a, which elutes at 0.22 M NaCl, is completely cleaved into two 32P-peptide fragments, which elute at 0.11 and 0.25 M NaCl and contain equal amounts of 32P; overall 32P recovery is 81%.
V8 cleavage, this tyrosine residue must be phosphorylated in both peaks 1 and 2b. Note that peak 1 is cleaved into fragments that contain ^32P in a ratio of 2:1. Since Tyr-1146 is the only ^32P-phosphotyrosine in one V8 fragment, Tyr-1150 and -1151 must both be phosphorylated in the other V8 fragment of peak 1. This double phosphorylated V8 fragment, corresponding to residues 1148-1152, elutes at 0.22 M NaCl. Thus, all three tyrosines in peak 1 are phosphorylated. By contrast, peak 2b is cleaved by V8 protease into two fragments, eluting at 0.13 and 0.24 M NaCl, which contain equal amounts of ^32P; insignificant amounts of ^32P elute at 0.22 M NaCl, corresponding to the double phosphorylated V8 fragment (residues 1148-1152). These findings indicate that in peak 2b, either Tyr-1150 or Tyr-1151 is phosphorylated (or some mole fraction of both residues equaling a single site). The further elucidation of this problem was addressed by solid-phase manual Edman degradation of peak 1, peak 2b, and the V8 protease fragments of these tryptic peptides. Edman degradation of peak 1 itself permitted recovery of PTH-[^32P]phosphotyrosine at residues 1146, 1150, and 1151 (Fig. 4). Thus, phosphorylation of all three tyrosines in peak 1 is demonstrated directly and supports the conclusion derived from the profile of V8 cleavage fragments of peak 1. With regard to peak 2b, solid-phase Edman degradation directly demonstrates the release of PTH-[^32P]phosphotyrosine at Tyr-1146; three separate preparations of peak 2b gave PTH-[^32P]phosphotyrosine at both residues 1146, 1150, and 1151, but in each case, a substantially greater recovery of PTH-[^32P]phosphotyrosine was observed (by thin layer electrophoresis) at residue 1151, ranging from 2 to 3:1 over that seen at residue 1150 (Fig. 4). Manual Edman degradation of the S. aureus V8 protease cleavage fragments of peak 2b confirmed the distribution of ^32P observed with the intact tryptic peptides (not shown). Since the distribution of ^32P in the two S. aureus V8 digestion products of peak 2b indicates that Tyr-1150 and -1151 together contain an amount of ^32P equivalent to that in Tyr-1146 (Fig. 3C), we conclude that peptide 5 in peak 2b is double phosphorylated and is a mixture of Tyr-P-1146/1151 and Tyr-P-1146/1150 in a ratio of 2:3:1.

Digestion of tryptic peptide peak 2a (Fig. 3, A and B) to completion with S. aureus V8 protease gave two new ^32P-peptides, each containing ~50% of the original ^32P in peak 2a (Fig. 3D). This indicates that both Tyr-1315 and -1322 are phosphorylated. This is supported by solid-phase Edman degradation of peak 2a; peaks of ^32P are recovered at cycles 2 and 8 from the N terminus, corresponding to Tyr-1316 and -1322 (Fig. 4).

The components of C1/C8 peak 3 (Fig. 2, A and B), which contains ~25% of ^32P from the C1/C8 fraction of the tryptic digest, were not identified. NaDodSO, urea gradient gel analysis revealed that the earliest eluting fractions in peak 3 contain [^32P]phosphotyrosine peptides of M, < 2000 only, whereas the later half of the peak contains the M, 4000-5000 [^32P]phosphotyrosine peptide present in the tryptic digest (Fig. 1B). Only tryptic peptides 1 and 6 achieve this size (Table I), but gas-phase Edman degradation of these C1/C8 fractions gave no clear sequence. Peak 3 was also heterogeneous on Mono Q anion exchange chromatography, where it eluted as two broad peaks at 0.25 and 0.27 M NaCl (Fig. 3B).

The overall purification of IR β subunit [^32P]phosphotyrosine peptides is shown in Table III. The use of anti-phosphotyrosine monoclonal antibody columns for initial purification of the tryptic digest is probably the step most essential to achieving purity sufficient for sequence analysis at picomole sensitivity. This step also gave high and nonselective recovery. The resolving power of the C1/C8 column was suboptimal,

### Table II

**Gas-phase microsequencing of ^32P-tyrosine tryptic peptides derived from insulin receptor autophosphorylated in vitro**

| Cycle No. | A. Amino acid sequence residue | B. Amino acid sequence deduced from cDNA sequence |
|-----------|--------------------------------|-----------------------------------------------|
|           | Peptide 5                     | Peptide 8                                    |
|           | Fig. 2A*                      | Fig. 2B*                                     | Fig. 3A |
| Peak 1    |                               |                                               |         |
| Peak 2    |                               |                                               |         |
| 1         | Tyr (5.0)                     | Glu                                           | Tyr     |
|           | Ile (20.2)                    | Ile (29.6)                                   |         |
| 2         | Thr (13)                      | His (14.3)                                   | Thr     |
|           | Ile (41.3)                    | Tyr (9.9)                                    |         |
| 3         | Glu (54.6)                    | Thr (29.4)                                   | Glu     |
|           | Tyr (10.9)                    | Thr (20.2)                                   | Tyr     |
| 4         | Glu (12.5)                    | Glu (7.7)                                    | Glu     |
|           | Ile (16)                      | Glu (7.7)                                    |         |
| 5         | Thr (4.3)                     | Tyr (2.0)                                    | Thr     |
|           | Tyr (6.5)                     | Tyr (2.0)                                    | Tyr     |
| 6         | Thr (2.5)                     | Tyr (2.0)                                    |         |
|           | Tyr (6.5)                     | Tyr (2.0)                                    |         |
| 7         | Tyr (2.5)                     | Tyr (2.0)                                    | Arg     |
|           | Tyr (2.0)                     | Tyr (2.0)                                    | Thr     |
| 8         | Ile (2.7)                     | Ile (2.0)                                    | Tyr     |
|           | Asp (2.0)                     | Asp (2.0)                                    | Thr     |
| 9         | His (2.7)                     | His (2.0)                                    |         |
|           | Asp (2.0)                     | Asp (2.0)                                    |         |
| 10        | Tyr (2.0)                     | Tyr (2.0)                                    |         |

* Aspartic acid and asparagine could not be determined in these runs because of coeluting contaminants derived from the phosphate buffer used in the elution of the C1/C8 reversed-phase column.

* Results lost due to misprogramming of Sequencer.

The resolving power of the C1/C8 column was suboptimal,
TABLE III
Purification of 32P-tyrosine-containing tryptic peptides from insulin receptor autophosphorylated in vitro

Insulin receptor, partially purified from two human placentas, was autophosphorylated as described under “Materials and Methods” for 40 min at 22 °C in the presence of 0.2 mM [γ-32P]ATP, 10 mM Mnan, and 0.1 μM insulin. Activation of H2b tyrosine kinase was calculated as 134% of maximal. The 32P-insulin receptor was immunoaffinity purified using immobilized antiphosphotyrosine monoclonal antibodies and subjected to NaDodSO4 gel electrophoresis. Steps 1–4 were at 4 °C; steps 5 and 6 at 22 °C; step 6 is depicted in Fig. 3B.

| Step No. | (overall recovery in this step) 32P pmol |
|----------|------------------------------------------|
| 1. IR M, 95,000 subunit in polyacrylamide gel | 495 |
| 2. IR M, 95,000 subunit eluted from gel | 470 (95%) |
| 3. 32P-peptides recovered from tryptic digest of eluted M, 95,000 subunit | 443 (94%) |
| 4. Immunoaffinity purification of 32P-peptides | |
| Applied | 443 |
| Eluted with 40 mM phenyl phosphate | 346 |
| Flow-through fraction | 46 |
| Retained on column | 3 |
| Recovered | 415 (94%) |
| 5. C1/C8 reversed-phase chromatography | |
| Applied (peaks 1 and 2 from step 5) | 337 |
| Peak 1 | 93 |
| Peak 2 | 116 |
| Peak 3 | 74 |
| Minor peaks | 9 |
| Recovered | 292 (87%) |
| 6. Anion exchange chromatography | |
| Applied (peaks 1 and 2 from step 5) | 209 |
| Peak 1 | 90 |
| Peak 2a | 57 |
| Peak 2b | 24 |
| Minor peaks | 30 |
| Recovered | 201 (96%) |

but the high recoveries achieved compensated. The major utility of the C1/C8 column was in permitting the components contained in “peak 3” to be fully separated from peaks 1 and 2, since peak 3 components overlapped extensively with peak 2 (A and B) on anion exchange chromatography (Fig. 3B).

DISCUSSION

The present studies directly identify 5 of the 13 residues of the insulin receptor β subunit as sites of autophosphorylation in vitro. These sites are located in tyrosines 1146, 1150, and 1151 in the “tyrosine kinase” region of the β subunit in a segment homologous to the major in vitro autophosphorylation sites of Rous sarcoma virus-transforming antigen, as well as tyrosines 1316 and 1322, located in the structurally unique C-terminal tail of the beta subunit intracellular domain (1, 2). Taken together, these tyrosines represent ~70% of the total in vitro autophosphorylation sites. A previous report, utilizing antipeptide antibodies and chemical degradation, also concluded that insulin receptor autophosphorylation in vitro occurred in part in these two domains (17). Although the remaining 30% of β subunit [32P]phosphotyrosine was not identified in the present study, a significant proportion (~50%) of the unidentified [32P]phosphotyrosine reside on a tryptic peptide of apparent M, ~4000–5000, probably corresponding to peptide 1 or 6 (Table I). Thus, at least six of the 13 IR β subunit tyrosines located in the intracellular portion of the β subunit undergo autophosphorylation in vitro in the presence of insulin.

The present data also permit some qualified conclusions concerning the quantitative relationships among the two major autophosphorylated domains. An estimate as to the molar ratios of peptides 5 and 8 can be obtained from the sequence analysis, if initial yields are equivalent. When sequenced as a mixture (i.e. C1/C8 peak 2) at reliably quantifiable levels (Table II), the PTH-derivatives corresponding to peptide 8 (peak 2a) were recovered at approximately 60 pmol/cycle, and those corresponding to peptide 5 (peak 2b) at approximately 40 pmol/cycle. Sequence analysis of a proportionate amount of C1/C8 peak 1 (the more negatively charged variant of peptide 5) from the same tryptic digest gave about 12–13 pmol of PTH-Glu/PTH-Thr. Thus, to a first approximation, peptide 8 (C1/C8/Mono Q peak 2a) and peptide 5 (C1/C8/Mono Q peaks 1 plus 2b) are recovered in nearly equimolar quantities. The receptor preparation employed for this estimate had been autophosphorylated to ~90% activation of maximal attainable histone 2b tyrosine kinase; however, a very similar distribution of [32P]phosphotyrosine among peak 1 plus 2b (peptide 5) and peak 2a (peptide 8), and peak 3 (unidentified) is observed with IR preparations autophosphorylated so as to achieve between 60 and 100% maximal activation of histone...
phosphorylation. The asymmetry in the phosphorylation of peak 1. To the contrary, one proceeds so as to achieve essentially maximal kinase activation presumably the C-terminal fragment of peptide 5 (residues 1148-1152) in addition to containing relatively less 32P, is less negatively charged than the same segment derived from V8 digestion of C1/C8 peak 1. Thus, the lack of 32P in a portion of Tyr-1150/1151 remains unphosphorylated in the setting of maximal kinase activation.

A further conclusion of these studies is that one of the domains bearing autophosphorylation sites, peptide 5 located in the "tyrosine kinase" region of the β subunit, is recovered in two charge forms which differ in their extent of phosphorylation. Thus, the more anionic form of peptide 5 (C1/C8 peak 1) contains 32PO4 on tyrosines 1146, 1150, and 1151, whereas the less anionic form (C1/C8 Mono Q peak 2b) contains 32P on tyrosine 1146 and 1150, predominantly Tyr-1151. The lower amount of 32P detected in peak 2b as compared to peak 1 is not due to prior occupancy by nonradioactive PO4; this would result in a triple phosphorylated form of peptide 5, which should then comigrate with C1/C8 peak 1 on ion-exchange chromatography and whose S. aureus V8 cleavage products would be identical to those of peak 1. To the contrary, one V8 cleavage product of peak 2b, presumably the C-terminal fragment of peptide 5 (residues 1148-1152) in addition to containing relatively less 32P, is less negatively charged than the same segment derived from V8 digestion of C1/C8 peak 1. Thus, the lack of 32P in a portion of Tyr-1150/1151 in peak 2b accurately reflects an absence of phosphorylation. The asymmetry in the phosphorylation of peptide 5 is not simply reflective of an intermediate stage in the progress of β subunit autophosphorylation, since the distribution of 32P observed in peaks 1, 2a, and 2b has been quite similar over a range of β subunit autophosphorylation corresponding to 60-100% activation of insulin receptor (histone 2b) tyrosine kinase. Selective dephosphorylation of Tyr-1150/1151 is unlikely to account for the two forms of peptide 5 because identical C1/C8/Mono Q tryptic maps are observed if immunoaffinity purification is omitted and autophosphorylation is terminated by direct addition of NaDodSO4. Thus, we conclude that insulin receptor autophosphorylation can proceed so as to achieve essentially maximal kinase activation with a significant portion (20-25%) of tyrosine 1150 remaining unphosphorylated.

Why is peptide 5 phosphorylated in an asymmetric fashion? An obvious explanation is the contiguity of tyrosines 1150/1151; substitution of one of these tyrosines may hinder sterically subsequent phosphorylation at the nearby tyrosine. The extent of this hindrance may, however, be influenced by adjacent residues so as to give the asymmetric phosphorylation of 1150/1151 which we have detected; for example, studies with synthetic peptide substrates have shown that for several tyrosine kinases, acidic residues to the N-terminal side of the tyrosine enhance phosphorylation. Conceivably phosphorylation of Tyr-1150 may be less inhibitory to subsequent phosphorylation of 1151 than vice versa. Such factors could result in the mixture of 1150-P/1151-P, 1150/1151-P and 1150-P/1151 observed, roughly 6:2:1.

It is perhaps more remarkable that the majority of auto-phosphorylated β subunits exhibits phosphorylation of both vicinal tyrosines. Synthetic peptides corresponding to tryptic peptide 5, although phosphorylated by the activated insulin receptor tyrosine kinase, exhibit rather low affinity for the enzyme (19); we consequently infer that the binding of insulin to the receptor stabilizes the domain containing peptide 5 in a conformation that strongly favors phosphorylation at Tyr-1150/1151, with a free energy sufficient to overcome the presumably destabilizing effect of the neighboring large negatively charged substituents. Moreover, it is clear that insulin-induced autophosphorylation itself leads to yet a further structural transition, characterized functionally by a capacity to phosphorylate exogenous substrates and a loss of the requirement for continued insulin binding to maintain kinase activation, it is tempting to hypothesize that the vicinal phosphorytrosines, perhaps in a trans-orientation, contribute uniquely to the stabilization of this conformation. In support of this view are the data of Ellis et al. (19) which demonstrate that replacement of Tyr-1150 alone or both 1150/1151 by phenylalnine residues through mutagenesis leads to mutant insulin receptor with markedly or totally impaired substrate phosphorylation. This is so despite total β subunit autophosphorylation in vitro in the presence of insulin which is only slightly diminished as compared to the wild-type IR; the autophosphorylation which occurs in the mutant IRS in the absence of insulin actually increases so as to diminish (1150) and ultimately abolish (1150/1151) the increment due to insulin. These data argue that the attainment of the catalytically optimal conformation is obliatorly dependent on the availability of these two tyrosines for phosphorylation; thus, their function is more than just to contribute 10-15% each of the total free energy which stabilizes the active conformation.

The present observation that a significant fraction of Tyr-1150/1151 remains unphosphorylated in the setting of maximal kinase activation suggests that it may be more accurate to envision a concerted functional role for the tyrosine residues 1146, 1150, and 1151 on peptide 5 in kinase activation, providing a highly phosphorylable domain rather than an individual critical tyrosine residue. Moreover, potential functional differences between an IR which bears a triple phosphorylated peptide 5 versus one with a double phosphorylated peptide 5 are likely to be less evident if the catalytically competent form of the receptor is the disulfide cross-linked (αβ)20 rather than (α/β)21.

The emphasis so far on peptide 5 should not detract from consideration of the potential functional importance of the C-terminal domain defined by Tyr-P at 1316 and 1322. Our earlier data indicated that overall β subunit tyrosine autophosphorylation correlated closely with activation of the tyrosine kinase, and autophosphorylation of this C-terminal domain may be important in the initiation and/or maintenance of kinase activation. Alternatively, phosphorylation of this domain may be an incidental concomitant or serve some other function such as signal transduction not involving substrate phosphorylation, targeting intracellular traffic, receptor turnover, etc. Expression of appropriate insulin receptor mutants will clarify this question, in conjunction with mapping of the phosphotyrosine sites on IR autophosphorylated in intact cells containing normal and mutant receptors. In addition, detailed kinetic evaluation of site-specific autophosphorylation in vitro in the presence and absence of insulin will be required to further clarify the role of individual β subunit phosphotyrosine residues and β subunit domains in receptor kinase function and in transmembrane signaling. The present results serve to identify directly the major candidate sites.

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