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Cascade of Autophosphorylation in the β-Subunit of the Insulin Receptor

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Insulin stimulated autophosphorylation of the β-subunit of the insulin receptor purified from Fao hepatoma cells or purified from Chinese hamster ovary (CHO/HIRC) or Swiss 3T3 (3T3/HIRC) cells transfected with the wild-type human insulin receptor cDNA. Autophosphorylation of the purified receptor occurred in at least two regions of the β-subunit: the regulatory region containing Tyr-1146, Tyr-1150, and Tyr-1151, and the C-terminus containing Tyr-1316 and Tyr-1322. In the presence of antiphosphotyrosine antibody (α-PY), autophosphorylation of the purified receptor was inhibited nearly 80% during insulin stimulation. Tryptic peptide mapping showed that α-PY inhibited autophosphorylation of both tyrosyl residues in the C-terminus and one tyrosyl residue in the regulatory region, either Tyr-1150 or Tyr-1151. Thus, a bis-phosphorylated form of the regulatory region accumulated in the presence of α-PY, which contained Tyr(P)-1146 and either Tyr(P)-1150 or 1151. In intact Fao, CHO/HIRC, and 3T3/HIRC cells, insulin stimulated tyrosyl phosphorylation of the β-subunit of the insulin receptor. Tryptic peptide mapping indicated that the regulatory region of the β-subunit was mainly (>80%) bis-phosphorylated; however, all three tyrosyl residues of the regulatory region were phosphorylated in about 20% of the receptors. As the phosphotransferase was activated by tris-phosphorylation but not bis-phosphorylation of the regulatory region of the β-subunit (White et al.:*Journal of Biological Chemistry* 263:2969–2980, 1988), the extent of autophosphorylation in the regulatory region may play an important regulatory role during signal transmission in the intact cell.

Key words: transmembrane signal, protein phosphorylation, tyrosine kinase, signal transmission, phosphorylation cascade

The initial molecular events following insulin binding involve autophosphorylation of several tyrosyl residues in the β-subunit of the insulin receptor [1–4]. The first phosphorylation sites include Tyr-1146,[1] Tyr-1150, and Tyr-1151 [2–4]. In addition, two tyrosyl residues in the C-terminus, Tyr-1316 and Tyr-1322, undergo autophosphorylation [2,6]. The phosphorylation of three tyrosyl residues in the juxtamembrane

[1]The numbering sequence used in this paper was described by Ullrich et al. [5] and is based on the position of the amino acids in the precursor of the human insulin receptor.

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region of the β-subunit, including Tyr-953, Tyr-960, and Tyr-967, is uncertain [2,4,7]. Autophosphorylation activates a phosphotransferase in the β-subunit, which catalyzes the phosphorylation of tyrosyl residues in various peptides and proteins [3,8]. Autophosphorylation of Tyr-1146, Tyr-1150, and Tyr-1151 appears to activate the phosphotransferase of the β-subunit [3], whereas phosphorylation in the C-terminus is not required for phosphotransferase activity [6]. Tyr-960 in the juxtamembrane region may be important for signal transmission, although it is probably not phosphorylated in the intact cell [4,7].

Autophosphorylation carried out at 0°C suggests that Tyr-1146 is the initial site of autophosphorylation in the β-subunit [4]. The second step appears to be the phosphorylation of Tyr-1150 or Tyr-1151. This bis-phosphorylated region can be trapped and immunopurified with antiphosphotyrosine antibody; bis-phosphorylation does not appear to activate the tyrosine kinase [3]. In contrast, tris-phosphorylation of this region may be necessary to activate the phosphotransferase fully [3]. Thus, we provisionally call the region of the insulin receptor including tyrosyl residues 1146, 1150, and 1151 the regulatory region of the β-subunit. Since tris-phosphorylation is barely detected during insulin stimulation of Fao hepatoma cells, the state of phosphorylation of Tyr-1146, Tyr-1150, and Tyr-1151 may be an important site of kinase regulation in vivo [3]. In this report, we extend these observations to the human insulin receptor. We confirm that antiphosphotyrosine antibody inhibits the autophosphorylation cascade of the purified human insulin receptor, and we show that the bis-phosphorylated regulatory region of the β-subunit predominates during insulin stimulation of Chinese hamster ovary cells and Swiss 3T3 cells expressing the human insulin receptor.

EXPERIMENTAL PROCEDURES

Cell Culture and Expression of the Human Insulin Receptor

Experiments were performed with Fao rat hepatoma cells [9] or with Chinese hamster ovary cells (CHO/HIRC) [10] or NIH/3T3 cells (3T3/HIRC) transfected with wild-type human insulin receptor cDNA [11]. The Fao cells are a well-differentiated and insulin-sensitive rat hepatoma cell line that possesses a high concentration of insulin receptors and many insulin-stimulated responses [9]. The 3T3/HIRC cells were a gift from J. Whittaker, Howard Hughes Medical Institute at The University of Chicago. These cells, grown in Dulbecco's minimum essential medium (MEM) containing 10% FBS were obtained by cotransfection of NIH/3T3 cells with pSVEneo and a bovine papilloma virus/insulin receptor cDNA construct [11]. CHO/HIRC cells, grown in F12 medium containing 10% FBS and 400 μg/ml of G418, were obtained by cotransfection of CHO cells with pSVEneo and an SV40/insulin receptor construct [10]. All cell lines were maintained at 37°C in a humidified atmosphere composed of 95% air and 5% CO_2.

Autophosphorylation of the Partially Purified Insulin Receptor

The insulin receptor was partially purified from confluent cells by wheat germ agglutinin affinity chromatography [12]. Ten 15-cm dishes of cells were each solubilized at 22°C with 3 ml of 50 mM HEPES (pH 7.4) containing 1% Triton X-100, 0.1 mg/ml aprotinin, and 2 mM phenylmethyl sulfonylfluoride (PMSF). Following centrifugation to remove the insoluble material, the cell extract was passed over a 2 ml

WGA[2]-agarose column, and the insulin receptor was eluted with 1–2 ml of 0.3 M N-acetylglucosamine in 50 mM HEPES (pH 7.4), 0.1% Triton X-100 as previously described [3].

WGA-purified insulin receptor (0.24 μg/μl) was incubated at 22°C for 10 min in a solution (100 μl) containing 50 mM HEPES and 5 mM $MnCl_2$ in the absence or presence of 100 nM insulin [13]. Phosphorylation was initiated by adding 25 μM [γ-^{32}P]ATP (2.5 mCi/ml) and was continued for the time intervals indicated in the figure legends. In certain experiments, 3 μg of α-PY was added to the reaction mixture before the addition of the [γ-^{32}P]ATP. The phosphorylation reaction was terminated by adding a 0.5-ml portion of 50 mM HEPES containing 0.1% Triton X-100, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 5 mM EDTA, and 2 mM sodium vanadate (Aldrich) at 4°C. The phosphorylated insulin receptor was immunoprecipitated from each reaction with α-PY, separated by SDS-PAGE using 7.5% resolving gels and identified by autoradiography [10,12].

[^{32}P]Phosphorylation of the Insulin Receptor in the Intact Cells

Confluent cells in 15-cm dishes were incubated at 37°C for 2 h with 5 ml of phosphate-free and serum-free RPMI 1640 medium (GIBCO) containing carrier-free [^{32}P]orthophosphate (0.5 mCi per ml) [10]. Insulin was added, and the incubation was continued at 37°C for 5 min and then stopped quickly by removing the incubation medium and freezing the cell monolayers with liquid nitrogen. The monolayers were thawed and homogenized immediately at 4°C with 2 ml of a solution containing 50 mM HEPES (pH 7.4), 1.0% Triton X-100, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium vanadate, 1 mg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride [14]. The phosphotyrosine-containing insulin receptor was immunoprecipitated from the eluate with α-PY and analyzed as described above.

HPLC Separation of Tryptic Phosphopeptides and Identification of the Phosphorylated Tyrosine Residues in the β-Subunit

The β-subunit, separated by SDS-PAGE, was digested with trypsin as previously described [3,13]. Tryptic phosphopeptides were separated with a Waters HPLC system equipped with a wide-pore C_{18} reverse-phase column (Bio-Rad, RP-318). The phosphopeptides were applied to the column, which was washed for 5 min at a flow rate of 1.1 ml/min with 0.05% trifluoroacetic acid, 95% water, and 5% acetonitrile. The peptides were eluted by a gradient of acetonitrile increasing linearly from 5% to 25% during 85 min. Greater than 90% of the radioactivity in the gel fragment was routinely recovered from the reverse-phase HPLC column.

The identity of the tryptic phosphopeptides was determined by immunoprecipitation with antiphosphotyrosine antibodies and antibodies that reacted with specific regions of the β-subunit: αPep-1 (residues 1314–1324), αPep-3 (residues 1143–1152), and αPep-4 (residues 952–962) [6]. The fractions containing the peptides were dried

[2]Abbreviations used: DTT, dithiothreitol; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electorphoresis; α-PY, antiphosphotyrosine antibody; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin.

in vacuo and the residue was dissolved in 0.5 ml of 50 mM HEPES containing 0.1% Triton X-100. Antibody (1 μg) was incubated with each sample for 12 h at 4°C, and then the antibody was precipitated by addition of 50 μl of a 10% suspension of Pansorbin [3]. The precipitate was washed three times with a solution containing HEPES (50 mM, pH 7.4), Triton X-100 (1%), SDS (0.1%), NaCl (150 mM), NaF (100 mM), and Na_3VO_4 (2 mM). The radioactivity in the precipitate was measured by Cerenkov counting.

RESULTS

Identification of the Autophosphorylation Sites in the β-Subunit of the Insulin Receptor

Insulin stimulates autophosphorylation of the β-subunit of the WGA-purified rat insulin receptor (data not shown). This reaction is rapid and occurs on at least five

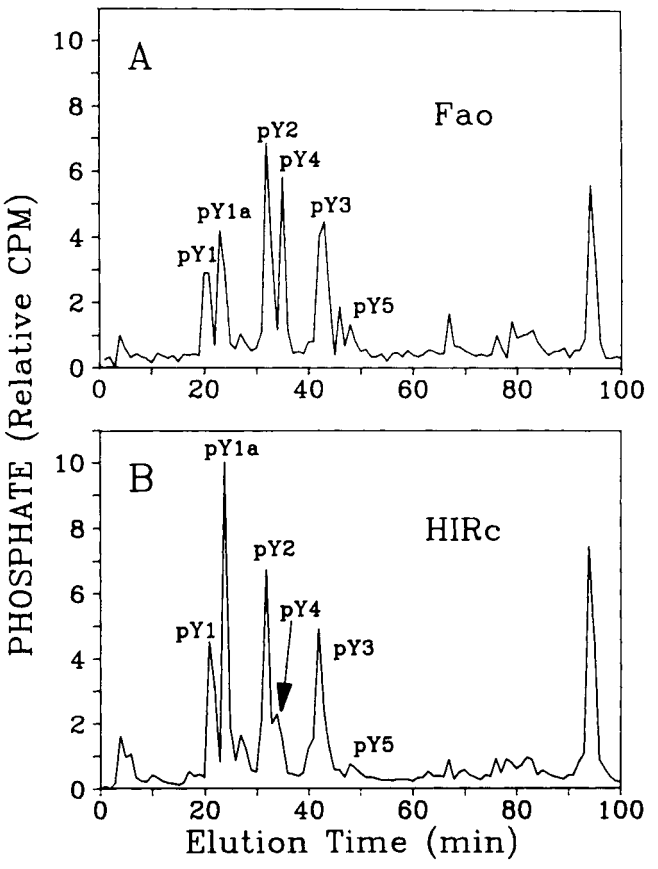

Fig. 1. Tryptic phosphopeptides of the β-subunit of the insulin receptor. Insulin receptor was solubilized from Fao cells (**A**) or CHO/HIRC cells (**B**) and partially purified on immobilized WGA. Insulin-stimulated autophosphorylation was carried out for 30 min, and the proteins were immunoprecipitated, reduced with DTT, separated by SDS-PAGE, digested exhaustively with trypsin, and the fragments were separated by HPLC.

tyrosyl residues [3]. A similar reaction occurs with the human insulin receptor [4,15]. Tryptic phosphopeptides were obtained from the β-subunit of the WGA-purified rat and human insulin receptor phosphorylated to steady state with [γ-^{32}P]ATP during insulin stimulation. Several phosphopeptides, labeled pY1, pY1a, pY2, pY3, pY4, and pY5 were obtained from the β-subunits (Fig. 1A,B). The HPLC elution profiles for each receptor were superimposable, suggesting that the phosphorylation sites in the β-subunit of the purified human and rat insulin receptor were identical.

The minimal composition of the tryptic peptides from the rat insulin receptor were deduced previously by a combination of techniques, and their identities are summarized in Table I. To determine whether the tryptic phosphopeptides obtained from the human insulin receptor purified from CHO/HIRC cells were correctly assigned, we immunoprecipitated the tryptic phosphopeptides with α-PY or with peptide-specific antibodies targeting presumed regions of autophosphorylation [3]. All of the tryptic phosphopeptides resolved from the human insulin receptor were immunoprecipitated with α-PY, indicating that each peptide contained phosphotyrosine. The pY1, pY1a, and pY4 were specifically immunoprecipitated with α-Pep3, the antibody that recognizes residues 1143–1152 of the β-subunit. This experiment verified that these peptides were derived from the regulatory region of the β-subunit, which contains tyrosyl residues 1146, 1150, and 1151. Previous studies indicated that both pY1 and pY1a contained three Tyr(P) residues but migrated differently on HPLC because of distinct tryptic cleavages at each C-terminus [3,4]. The pY4 was distinct because it contains only two Tyr(P) residues (Table I) [3]. In contrast, pY2 and pY3 were specifically precipitated with α-Pep1, the antibody directed against the C-terminus of the β-subunit, which includes Tyr(P)-1316 and Tyr(P)-1322 [6]. The α-Pep4, which recognizes amino acid residues 953–965 of the β-subunit, did not react with any of the major tryptic fragments, suggesting that none of them was derived from the Tyr-960 region of the receptor (Fig. 2); however, the final peak on our HPLC profile eluting at 95 min is composed of many minor components that have not been characterized and may contain peptides derived from the juxtamembrane region of the receptor. Thus, migration during reverse-phase HPLC and immunogenicity of the tryptic phosphopeptides obtained from the human insulin receptor indicated that the in vitro phosphorylated domains of the human insulin receptor were identical with those reported previously for the rat insulin receptor (Table I).

TABLE I. The Structure of the Tryptic Phosphopeptides Obtained From the β-Subunit of the Insulin Receptor*

Peptide	Amino acid residues	Structure
pY1	1143–1153	AspIleTyr(P)GluThrAspTyr(P)Tyr(P)ArgLys
pY1a	1143–1152	AspIleTyr(P)GluThrAspTyr(P)Tyr(P)Arg
pY2/pY3	1312–1330	ArgSerTyr(P)GluGluHisIleProTyr(P)ThrHisMetAsnGlyGlyLysLys
pY4	1143–1152	AspIleTyr(P)GluThrAsp[TyrTyr](P)Arg
pY5	1143–1153	AspIleTyr(P)GluThrAsp[TyrTyr](P)ArgLys

*The identity of these tryptic fragments was determined previously [3]. Amino acids are numbered according to Ullrich et al. [5]. The exact length of the pY2 and pY3 peptide has not been determined. The amino acids in italics indicate variable trypsin cleavage sites that give rise to the various forms of these peptides.

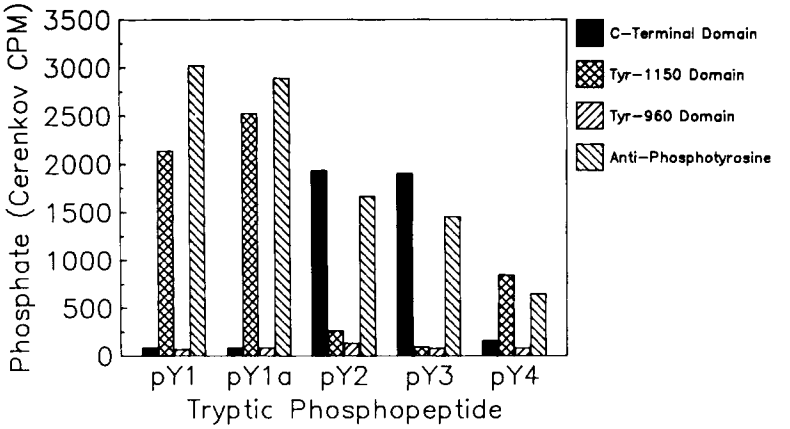

Fig. 2. Identification of tryptic phosphopeptides of the human insulin receptor. The insulin receptor was phosphorylated for 30 min, and then immunoprecipitated with α-PY, reduced with DTT, separated by SDS-PAGE, and digested exhaustively with trypsin. Tryptic phosphopeptides were separated by reverse-phase HPLC and identified by the elution position. Each phosphopeptide was immunoprecipitated with the indicated domain-specific antibody as described in "Material and Methods." pY5 was not tested.

The phosphorylation of the various sites in the rat and human insulin receptor follow distinct time courses [1,3,4,15]. After 1 min of insulin-stimulated autophosphorylation of the WGA-purified human insulin receptor, bis-phosphorylation of the regulatory region (pY4 + pY5) was near its maximal level (Fig. 3A). However, phosphorylation of the C-terminus (pY2 + pY3) and accumulation of the tris-phosphorylated form of the regulatory region (pY1 + pY1a) reached less than 25% of their steady-state level. After 30 min of autophosphorylation the aggregate intensity of pY4 and pY5 increased only 1.4-fold suggesting that the bis-phosphorylation of the regulatory region did not increase substantially (Fig. 3B). In contrast, tris-phosphorylation of the regulatory region and phosphorylation of the C-terminal sites increased five- and eight-fold, respectively (Fig. 3B). Thus, bis-phosphorylation of the regulatory region in the human insulin receptor was rapid, as previously shown for the rat receptor [3], and was the earliest detectable autophosphorylated form of the β-subunit, whereas tris-phosphorylation of this region and phosphorylation of the C-terminus lag behind.

Inhibition of Autophosphorylation of the WGA-Purified Human Insulin Receptor With Antiphosphotyrosine Antibody

Anti-phosphotyrosine antibody partially inhibits autophosphorylation of the β-subunit of the purified rat insulin receptor by binding to the initial sites of autophosphorylation; this association blocks the cascade of subsequent phosphorylations [3]. The α-PY has a similar effect on the human insulin receptor purified on immobilized WGA from CHO/HIRC cells (data not shown). In the presence of 1 μg/ml α-PY, autophosphorylation decreased 50–60% owing to the inhibition of phosphorylation of pY1, pY1a, pY2, and pY3 (Fig. 4). The pY4 (and sometimes pY5)[3] was the major

[3]The pY5 was not always detected; however, our previous report indicates that it is pY4 + Lys (see Table I).

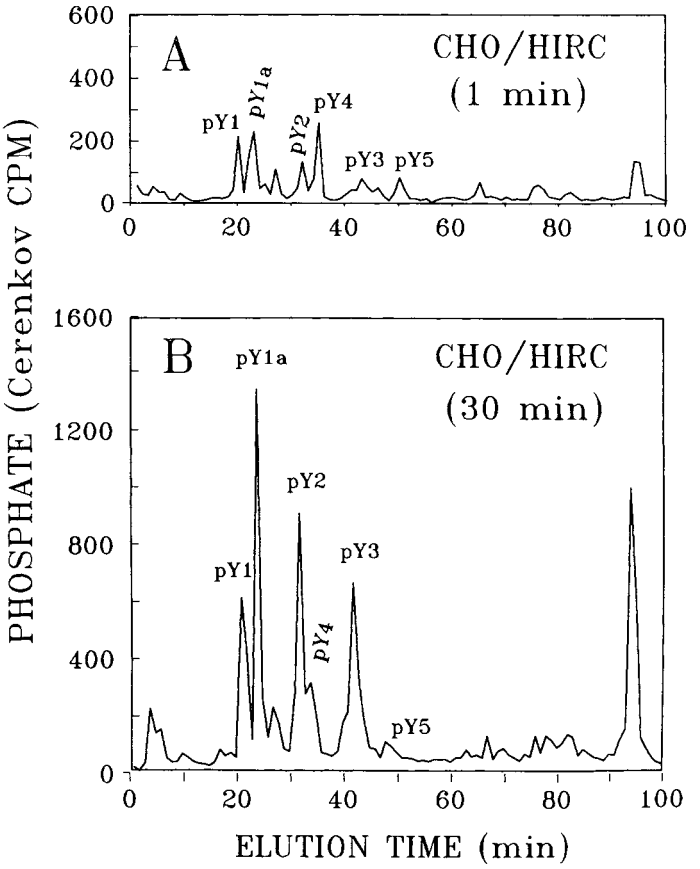

Fig. 3. The effect of time on the tryptic phosphopeptides of the β-subunit of the human insulin receptor. The WGA-purified human insulin receptor from CHO/HIRC cells was phosphorylated for 1 min (**A**) or 30 min (**B**) in the presence of 100 nM insulin. Insulin-stimulated autophosphorylation was carried out for 30 min, and the proteins were immunoprecipitated, separated by SDS-PAGE, digested exhaustively with trypsin, and separated by HPLC.

phosphopeptide detected by HPLC during insulin-stimulated autophosphorylation in the presence of α-PY. The pY4 corresponded exactly to pY4, which was obtained during phosphorylation in the absence of α-PY [3]. These data suggest that α-PY binds to the initial tyrosine phosphorylation sites in the β-subunit, which are found in pY4 (Tyr-1146 and either Tyr-1150 or Tyr-1151), and inhibits autophosphorylation at the other tyrosine residues. Thus, consistent with the time course of autophosphorylation, bis-phosphorylation of the regulatory region preceded the other phosphorylation events.

Based on our identification of the tryptic phosphopeptides from the regulatory region (i.e., pY1 and pY1a are tris-phosphorylated peptides, and pY4 and pY5 are bis-phosphorylated peptides) [3], the relative stoichiometry of autophosphorylation in this region was calculated from HPLC profiles (Table II). In the absence of α-PY, about 77% of the phosphorylated regulatory regions were tris-phosphorylated, whereas the remaining 23% were bis-phosphorylated. In contrast, during inhibition

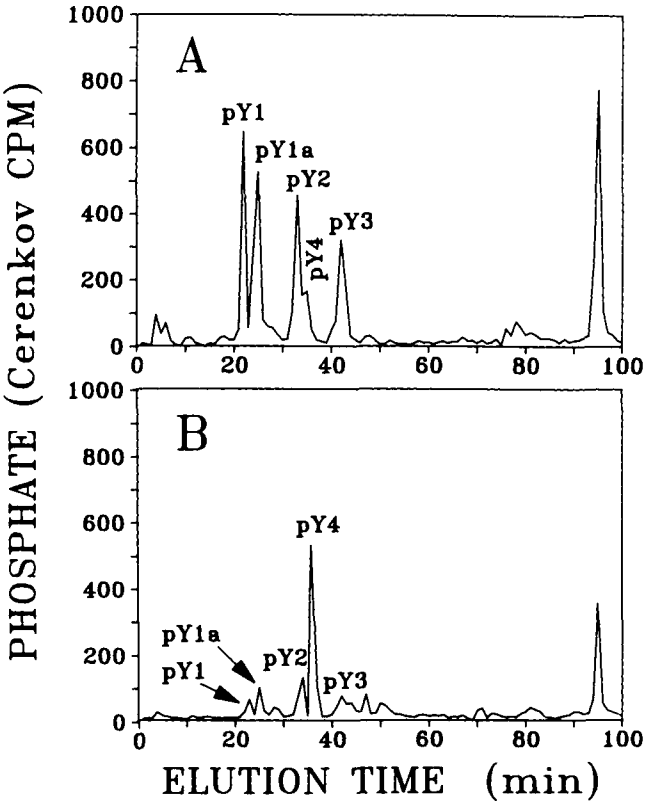

Fig. 4. The effect of antiphosphotyrosine antibody on the tryptic phosphopeptides of the β-subunit of the human insulin receptor. The WGA-purified insulin receptor from CHO/HIRC cells was stimulated with 100 nM insulin and phosphorylated for 30 min in the absence (**A**) or presence (**B**) of 0.03 μg/μl α-PY. The proteins were immunoprecipitated, reduced with DTT, and separated by SDS-PAGE, digested exhaustively with trypsin, and the phosphopeptides were separated by HPLC.

with α-PY, 83% of the regulatory regions were bis-phosphorylated, and only 17% reach the trisphosphorylated state. Moreover, during α-PY inhibition about one-half of the Tyr-1150 regions were bis-phosphorylated compared to the total phosphorylated in the absence of α-PY (Table II, compare the normalized regulatory region (Total) in the absence of α-PY to the bis-phosphorylated regulatory region during α-PY inhibition). These results suggest that only one β-subunit in the insulin-stimulated heterotetramer undergoes autophosphorylation in the presence of α-PY.

Bis-Phosphorylation Around Tyr-1150 Predominated During Insulin Stimulation of Intact Cells

Insulin-stimulated tyrosine autophosphorylation of the human insulin receptor in intact cells is quantitatively different from the in vitro reaction (Fig. 5). Tris-phosphorylation of the regulatory region was barely detected, as the recovery pY1 and pY1a was uniformly low (Fig. 5B). In contrast to in vitro autophosphorylation, only

TABLE II. The Stoichiometry of β-Subunit Phosphorylation in the Absence and Presence of Antiphosphotyrosine Antibody *

	No α-PY		α-PY Inhibited	
	CPM	Normalized CPM	CPM	Normalized CPM
Regulatory region				
Tris-phosphorylated				
pY1	743	248	96	32
pY1a	889	296	132	44
Total (pY1 + pY1a)	1,632	544 (77%)	228	76 (17%)
Bis-phosphorylated				
pY4	269	134	668	334
pY5	56	28	84	42
Total (pY4 + pY5)	325	162 (23%)	752	376 (83%)
Regulatory region total	1,957	707	980	452
C-terminal region				
pY2	478	239	217	108
pY3	574	287	167	83
C-terminal region total	1,052	526	384	191

*The WGA-purified insulin receptor from CHO/HIRC cells was stimulated with 100 nM insulin and incubated for 30 min in the absence (No α-PY) or presence (α-PY Inhibition) of 0.03 μg/μl α-PY. The β-subunit was immunopurified, separated by SDS-PAGE, and digested exhaustively with trypsin. The radioactivity (cpm) in each tryptic phosphopeptide was determined. Based on the number of Tyr(P) residues in each peptide, the normalized cpms were also calculated assuming 3 Tyr(P) residues in pY1 and pY1a (tris-phosphorylated), 2 Tyr(P) residues in pY4 and pY5 (bis-phosphorylated), and 2 Tyr(P) residues in pY2 and pY3 (C-terminus). The parenthetical values are the percentages of the normalized cpm's in the bis- or tris-phosphorylated Tyr-1150 region relative to the total.

19% of the regulatory regions of the β-subunits in intact Fao, CHO/HIRC, and 3T3/HIRC cells were tris-phosphorylated during insulin stimulation (Table III). This result was consistently observed in Fao, CHO/HIRC, and 3T3/HIRC cells (Table III). However, insulin stimulated the phosphorylation of tyrosine residues in a major phosphotyrosine-containing tryptic peptide, which migrated at the same position as pY4, suggesting that bis-phosphorylation of the regulatory region predominated in vivo (Fig. 5B). Moreover, phosphorylation of the C-terminus was also relatively low in vivo (Fig. 5). Thus, the in vivo milieu inhibited the complete autophosphorylation of the insulin-stimulated β-subunit in a way that was nearly identical with antiphosphotyrosine antibody inhibition of in vitro autophosphorylation.

DISCUSSION

Insulin-stimulated autophosphorylation is identical for the WGA-purified insulin receptor of rat or human origin. This relation was shown by an exact correspondence between the HPLC profiles of tryptic phosphopeptides obtained from β-subunit of each species phosphorylated to steady state. Moreover, the identity of each tryptic phosphopeptide determined by specific immunoprecipitation was the same; pY1, pY1a, and pY4 were derived from the regulatory region between Asp-1144 to Arg-

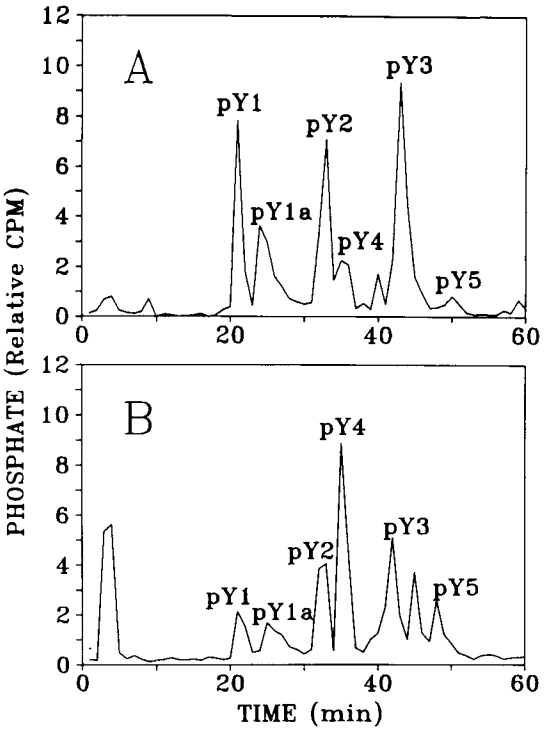

Fig. 5. A comparison of the tryptic phosphopeptides obtained from the human insulin receptor of 3T3/HIRC cells labeled in the intact cell with [^{32}P]orthophosphate (**B**) or labeled in vitro with [γ-^{32}P]ATP after WGA purification (**A**). The proteins were immunoprecipitated, reduced with DTT, and separated by SDS-PAGE, digested exhaustively with trypsin, and the phosphopeptides were separated by HPLC.

1152/Lys-1153, and pY2 and pY3 were derived from the C-terminal region between Lys-1313/Arg-1314 to Lys-1329/Lys-1330. The exact tryptic cleavage sites are quite variable, as previously discussed [3,4]. By analogy to our previous work, five tyrosyl residues in these two regions of the β-subunit undergo autophosphorylation: 1) Tyr-1146, Tyr-1150, and Tyr-1151, and 2) Tyr-1316 and Tyr-1322. These results agree with the findings of others [2,4], and are consistent with the report that the primary amino acid sequence around these tyrosyl residues is the same for the human and rat insulin receptor (R. Lewis, M. Tepper, and M.P. Czech, personal communication). However, we do not find evidence for phosphorylation of Tyr-960 or the adjacent residues Tyr-953 or Tyr-967, in contrast to previous reports [4,16,17].

As shown previously for the rat insulin receptor [3], autophosphorylation of the human insulin receptor is an ordered reaction that begins in the regulatory region at Tyr-1146 and either Tyr-1150 or Tyr-1151. Phosphorylation of two (bis-phosphorylation) of these three (tris-phosphorylation) tyrosyl residues is detected immediately after incubation of the WGA-purified insulin receptor with [γ-^{32}P]ATP and insulin. A distinct initial step in the cascade of autophosphorylation is confirmed by the fact that antiphosphotyrosine antibody (α-PY) traps the bis-phosphorylated form of the insulin receptor (found in pY4 and pY5) and actually causes its accumulation, whereas α-PY inhibits autophosphorylation of additional tyrosyl residues in the β-subunit. Thus, α-

TABLE III. Recovery of Bis- and Tris-Phosphorylated Tyr-1150 Region Phosphorylated In Vivo and In Vitro*

Cell line	Tris-phosphorylated regulatory region		Bis-phosphorylated regulatory region	
	In vitro (%)	In vivo (%)	In vitro (%)	In vivo (%)
Fao	72	12	28	88
CHO/HIRC	78	23	22	77
3T3/HIRC	74	23	26	77
Average	75	19	25	81

*The insulin receptor was phosphorylated in the intact Fao, CHO/HIRC, or 3T3/HIRC cells (in vivo), or after solubilization and WGA purification of the receptor (in vitro). The tryptic phosphopeptides were separated by reverse-phase HPLC, and pY1, pY1a, and pY4 were identified. Phosphate-labeled cells were stimulated with insulin (100 nM) for 5 min, whereas the purified receptor was incubated with insulin (100 nM) and 25 μM [γ-^{32}P]ATP for 30 min; steady-state labeling was achieved in each case. The phosphorylated receptor was immunoprecipitated with α-PY, separated by SDS-PAGE, and digested exhaustively with trypsin. The phosphopeptides were separated by HPLC, and the radioactivity in pY1 + pY1a and pY4 + pY5 was measured. The normalized amount of the tris-phosphorylated regulatory region was calculated as (pY1 + pY1a)/3, and the amount of the bis-phosphorylated regulatory region was calculated as (pY4 + pY5)/2; the percent of the Tyr-1150 region in the bis- and tris-phosphorylated forms was calculated from these values.

PY prevents tris-phosphorylation of the regulatory region (pY1 and pY1a), and inhibits autophosphorylation of Tyr-1316 and Tyr-1322 (pY2 and pY3) in the C-terminus of the molecule. A monophosphorylated form of the Tyr-1150 region cannot be detected and must exist only briefly during the initiation of the cascade or be inaccessible to α-PY. At 0°C, Tyr-1146 appears to be the first site of autophosphorylation [4]. Based on the relative stoichiometry of autophosphorylation, one-half of the available Tyr-1150 regions become bis-phosphorylated in the presence of α-PY. These results suggest that autophosphorylation of both β-subunits in the $\alpha_2\beta_2$ oligomer is not a concerted reaction and must begin in one of the two β-subunits and propagate to the other.

The cascade of autophosphorylation in the β-subunit is significant as tris-phosphorylation of the regulatory region appears to be required to activate the tyrosyl-specific phosphotransferase [3]. Our previous report indicated that bis-phosphorylation of the regulatory region obtained in the presence of α-PY did not activate the receptor kinase eluted from the antibody, and assayed under conditions that inhibit additional autophosphorylation, it was inhibited [3]. In contrast, Tornqvist et al. suggested that autophosphorylation occurs randomly and that less than complete autophosphorylation of the regulatory region activates the kinase [16]. However, substitution of Tyr-1150 alone with phenylalanine inhibited almost completely the activation of the phosphotransferase [18]. Thus, we conclude provisionally that phosphorylation of all three tyrosyl residues is necessary to activate the kinase [3].

The physiological significance of the autophosphorylation cascade is emphasized by the fact that the bis-phosphorylated Tyr-1150 region predominates in the intact cell during insulin stimulation [3,19]. Only about 20% of the phosphorylated regulatory regions are tris-phosphorylated, whereas 80% are bis-phosphorylated. This stoichiometry is found in Fao cells, as well as the CHO/HIRC and 3T3/HIRC cells, which

express the human insulin receptor, suggesting that inhibition of tris-phosphorylation is a general characteristic in vivo. Although insulin binding initiates the autophosphorylation cascade in vivo [14], some other factors in the intact cell prevent the accumulation of the tris-phosphorylated β-subunit. The inhibition is removed during WGA-purification as tris-phosphorylation around Tyr-1150 predominates by 4 to 1 in the β-subunit during in vitro phosphorylation. Inhibition of in vivo autophosphorylation may be due to phosphatases present in the intact cell, which are removed during WGA chromatography. Whereas this explanation is likely for a hepatocyte-derived cell line (Fao), it is less likely for CHO or 3T3 cells, which should contain less Tyr(P) phosphatase activity [20]. Seryl phosphorylation of the β-subunit, presumably mediated through a protein kinase C pathway, may also regulate the cascade of autophosphorylation [21]; however, seryl phosphorylation appears to inhibit the cascade completely, rather than inhibit the phosphorylation of specific residues selectively [22].

It is interesting that in vivo tyrosine autophosphorylation of the β-subunit is very similar to in vitro autophosphorylation during inhibition with the α-PY. Partial autophosphorylation in vivo may result from the presence of binding proteins, which specifically interact with the bis-phosphorylated regulatory region. These binding proteins could inhibit additional autophosphorylation while simultaneously transducing the insulin signal. However, transmission of the insulin signal may occur in cells by tyrosyl phosphorylation of cellular substrates. In this case, inhibition of tris-phosphorylation could play a regulatory role in signal transmission in vivo.

In conclusion, the autophosphorylation cascade in the β-subunit demonstrated for the rat insulin receptor [3] also applies to the human insulin receptor. The first site of autophosphorylation is probably Tyr-1146, followed immediately by either Tyr-1150 or Tyr-1151. At this stage, bis-phosphorylation can be trapped by antiphosphotyrosine antibodies [3]. In the absence of α-PY, autophosphorylation of the Tyr-1150 region rapidly progresses to tris-phosphorylation and phosphorylation of Tyr-1316 and Tyr-1322 in the C-terminus of the β-subunit. Progression to the tris-phosphorylated form appears necessary for full activation of the phosphotransferase during in vitro assays and may play an important regulatory role in vivo [3].

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