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-

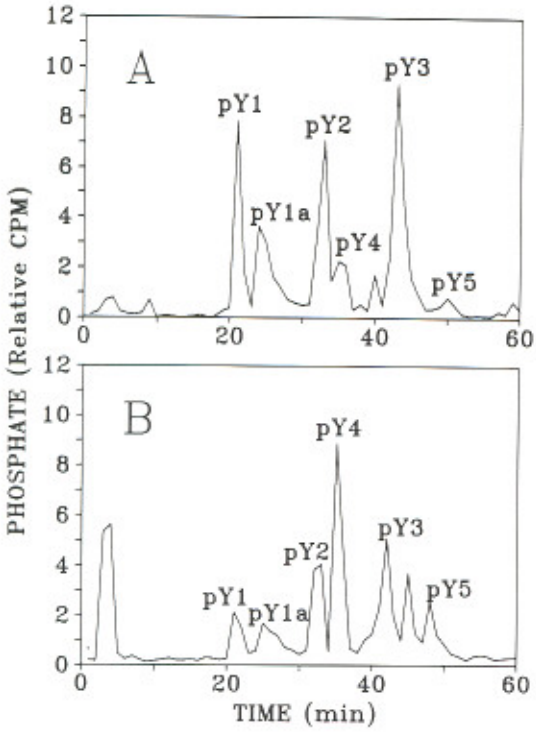

Fig. 5. A comparison of the tryptic phosphopeptides obtained from the human insulin receptor of 3T3/HIRC cells labeled in the intact cell with [^{32}P]orthophosphate (**B**) or labeled in vitro with [γ-^{32}P]ATP after WGA purification (**A**). The proteins were immunoprecipitated, reduced with DTT,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