Phosphorylation of the insulin receptor was studied in intact well differentiated hepatoma cells (Fao) and in a solubilized and partially purified receptor preparation obtained from these cells by affinity chromatography on wheat germ agglutinin agarose. Tryptic peptides containing the phosphorylation sites of the \( \beta \)-subunit of the insulin receptor were analyzed by reverse-phase high performance liquid chromatography. Phosphoamino acid content of these peptides was determined by acid hydrolysis and high voltage electrophoresis. Separation of the phosphopeptides from unstimulated Fao cells revealed one major and two minor phosphoserine-containing peptides and a single minor phosphothreonine-containing peptide. Insulin \( (10^{-7} \text{ M}) \) increased the phosphorylation of the \( \beta \)-subunit of the insulin receptor 3- to 4-fold in the intact Fao cell. After insulin stimulation, two phosphotyrosine-containing peptides were identified. Tyrosine phosphorylation reached a steady state within 20 s after the addition of insulin and remained nearly constant for 1 h. Under our experimental conditions, no significant change in the amount of \([^{32}\text{P}]\)phosphoserine or \([^{32}\text{P}]\)phosphothreonine associated with the \( \beta \)-subunit was found during the initial response of cells to insulin.

When the insulin receptor was extracted from the Fao cells and incubated in vitro with \([\gamma-^{32}\text{P}]\)ATP and Mn\(^{2+}\), very little phosphorylation occurred in the absence of insulin. In this preparation, insulin rapidly stimulated autophosphorylation of the receptor on tyrosine residues only and high performance liquid chromatography analysis of the \( \beta \)-subunit digested with trypsin revealed one minor and two major phosphopeptides. The elution position of the minor peptide corresponded to that of the major phosphotyrosine-containing peptide obtained from the \( \beta \)-subunit of the insulin-stimulated receptor labeled in vivo. In contrast, the elution position of one of the major phosphopeptides that occurred during in vitro phosphorylation corresponded to the minor phosphotyrosine-containing peptide phosphorylated in vivo. The other major in vitro phosphotyrosine-containing peptide was not detected in vivo. Our results indicate that: 1) tyrosine phosphorylation of the insulin receptor occurs rapidly following insulin binding to intact cells; 2) the level of tyrosine phosphorylation remains constant for up to 1 h; 3) the specificity of the receptor kinase or accessibility of the phosphorylation sites are different in vivo and in vitro. The data suggest that tyrosine phosphorylation is an early intracellular signal in insulin action, but that caution should be used when relating in vitro data to in vivo phosphorylation events.

Cellular metabolism and growth is regulated by hormones and growth factors which specifically bind to cell surface receptors and stimulate tyrosine phosphorylation on an intracellular portion of these transmembrane proteins (1). Receptors for epidermal growth factor (2, 3), insulin (4, 5), platelet-derived growth factor (6-8), and insulin-like growth factor-I (9, 10) possess this distinctive kinase activity. In the intact cell, these receptors often contain both phosphoserine and phosphothreonine in addition to phosphotyrosine, whereas in the broken cell experiments phosphotyrosine is usually the only phosphoamino acid detected after incubation with the peptide ligand and \([\gamma-^{32}\text{P}]\)ATP. During the past few years, several groups have begun to characterize the tyrosine kinase activity of the insulin receptor purified from various tissue types (11). In both crude detergent extracts (11, 17) and in highly purified preparations of receptor (12-14), insulin stimulates tyrosine phosphorylation of the \( \beta \)-subunit of the insulin receptor. Using these experimental systems, many enzymatic characteristics of the solubilized insulin receptor have been described including its hormone specificity (15), metal ion sensitivity (16-18), proteolytic sensitivity (19, 20), thermal stability (16), effects of dithiothreitol (14, 21), substrate specificity (13, 14, 22, 23), and regulation by autophosphorylation (24, 25). These results and the identification of an ATP binding site in the \( \beta \)-subunit (26-28) strongly support the notion that the insulin receptor is a tyrosine-specific protein kinase.

Insulin receptor phosphorylation was originally detected in \([^{32}\text{P}]\)orthophosphate-labeled cells (4). However, technical problems arising from the low number of insulin receptors in cultured cells have hampered progress in this area and only a few reports have explored the properties of receptor phosphorylation with intact cells (4, 29-31). Our previous experiments have shown that in contrast to the results with the soluble receptor, serine and threonine residues were the major sites of receptor phosphorylation in intact cells and tyrosine was a minor, although insulin-stimulated, phosphorylation site (5). In experiments with freshly isolated rat hepatocytes, phosphotyrosine was nearly undetectable (30). This distinction between the specificity of phosphorylation of insulin receptors in whole cells and the corresponding reaction in cell extracts suggests that the tyrosine kinase activity is intrinsic to the \( \beta \)-subunit of the insulin receptor and is stimulated directly by insulin binding. In contrast, the serine and threonine phospho-
phorylations probably occur through the action of other kinases. The low abundance of phosphotyrosine-containing proteins in the cell (32) and the association of tyrosine kinase activities with oncogene products (33, 34), growth factors receptors (1), and the insulin receptor (11) suggests an important role for tyrosine phosphorylation in cellular growth and metabolism.

In this report, we have studied insulin receptor phosphorylation in the intact hepatoma cell and have directly compared these results to the in vitro phosphorylation of the solubilized receptor. We find that tyrosine autophosphorylation of the insulin receptor in hepatoma cells reaches steady state within 20 s after the addition of insulin. This insulin-stimulated phosphorylation occurs initially in the absence of significant changes in [32P]phosphoserine and [32P]phosphothreonine. The major site of tyrosine phosphorylation in vivo corresponds to a minor site in vitro. Although a molecular mechanism has not yet been formulated to describe the relation between phosphotyrosine and cellular regulation, our data suggest that tyrosine autophosphorylation of the β-subunit is one of the first detectable cellular events that occurs after insulin binding and could provide a unique and rapid transmembrane signal which initiates the cellular insulin response.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the sources indicated: [γ-32P]ATP, [32P]orthophosphate, and Triton X-100 were from New England Nuclear; Hepes, α,α'-dipyridyl, phenylmethylsulfonyl fluoride, N-acetylglucosamine, bovine serum albumin, and phospho-nino acids were from Sigma; porcine insulin (Lot 1J858AN) was from Eliisa; reagents for SDS-PAGE were purchased from Bio-Rad; Panserin was from Calbiochem; wheat germ agglutinin-agarose was from Vector. The μBondapak C4 reverse-phase HPLC column was from Waters Associates. Phosphopeptides applied to the column were eluted at a flow rate of 1 ml/min with a mobile phase composed of water with 0.05% trifluoroacetic acid and a nonlinear, concave upward gradient of acetonitrile increasing between 0 and 40% during 85 min as shown in the inset of Fig. 3. The fractions (1 ml) were collected in polypropylene microfuge tubes and the radioactivity in each tube was measured as Cerenkov radiation using an LKB scintillation counter with an efficiency of 40%. The percentage of acetonitrile in each sample had no effect on the efficiency of the Cerenkov radiation. Trypsin digestion of the immunoprecipitated β-subunit from the polyacrylamide gel fragments ordinarily released 85% of the radioactivity. About 85% of the radioactivity in the trypsin digest was routinely recovered from the μBondapak C4 reverse-phase HPLC column. The phosphoamino acids were identified in fractionated or unfractionated trypsin peptides by a modification (31) of the method of Hunter and Setton (32).

RESULTS

Vanadate Inhibits Dephosphorylation of the Insulin Receptor during Purification.—Previous attempts (4, 5) to study insulin receptor phosphorylation in vivo have been hampered by a low recovery of phosphotyrosine in the receptor. In these experiments, however, the receptor was isolated and purified in a solution which did not contain an effective inhibitor of phosphotyrosine phosphatases such as vanadate (39). Vanadate (2 mM) was included during solubilization and purification of the insulin receptor to determine if it would inhibit dephosphorylation of the insulin receptor. Fao hepatoma cells labeled for 2 h with [32P]orthophosphate were incubated at 37 °C with 100 nM insulin for 60 s. Each incubation was quenched rapidly by freezing the cells with liquid nitrogen after which the cells were solubilized in a solution containing sodium fluoride (100 mM), pyrophosphate (10 mM), EDTA (4 mM), and with or without sodium vanadate (2 mM). The autoradiograph in Fig. 1 shows the SDS-PAGE separation of the [32P]phosphoproteins purified from these extracts by affinity chromatography on immobilized wheat germ agglutinin followed by immunoprecipitation with anti-insulin receptor antiserum (B-9). Sodium vanadate (2 mM) increased the recovery of the phosphorylated β-subunit (M, 95,000), as well as some other proteins recovered in the immunoprecipitate due...
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FIG. 1. The effect of sodium vanadate on the phosphorylation of the insulin receptor in Fao cells. Fao cells (lanes a, b, d, and e) were labeled for 2 h with [32P]orthophosphate and then incubated for one additional minute without (lanes a and d) or with (lanes b and e) 100 nM insulin. The cells were frozen with liquid nitrogen and thawed into 2 ml of solubilization solution containing no vanadate (lanes a and b) or 2 mM vanadate (lanes d and e). For in vitro labeling with [γ-32P]ATP, the unlabeled cells were treated without (lane c) or with (lane f) 100 nM insulin and then frozen. These cells were solubilized in 2 ml of solubilization solution containing 1 mCi of [γ-32P]ATP with or without 2 mM vanadate. The insulin receptor from each sample was purified by chromatography on WGA agarose, immunoprecipitated, reduced with DTT, and separated by SDS-PAGE. An autoradiogram obtained following a 12-h exposure of the fixed and dried gel is shown in the figure.

to nonspecific binding to Pansorbin (Fig. 1, lanes a, b, d, and e).

To determine whether vanadate increased recovery of [32P]orthophosphate-labeled proteins by inhibiting dephosphorylation or by somehow enhancing phosphorylation of proteins during purification of the receptor, [γ-32P]ATP was added to crude cell extracts prepared without or with vanadate from unlabeled Fao cells. No phosphorylation of proteins immunoprecipitated by the anti-insulin receptor serum occurred during purification of this extract (Fig. 1, lanes c and f). Thus, as suggested previously, sodium vanadate appears to inhibit dephosphorylation of phosphoproteins (30) and results in the increased recovery of the phosphorylated receptor. Therefore, vanadate (2 mM) was used during cell lysis and receptor purification in all of the subsequent experiments.

Phosphorylation of the Insulin Receptor Is One of the First Cellular Responses to Insulin Binding—Hepatoma cells were labeled for 2 h with [32P]orthophosphate and then incubated without insulin or with 100 nM insulin for 20 s, 40 s, or 60 s. Before the addition of insulin, the β-subunit of the insulin receptor (Mr = 95,000) which was purified by WGA chromatography and immunoprecipitation with anti-insulin receptor serum was phosphorylated (Fig. 2A, lane a). Within 20 s, insulin caused a 3.5-fold increase in the phosphorylation of the β-subunit (Fig. 2A, compare lanes a and b). No additional insulin-stimulated phosphorylation was detected after 40 s or 60 s of incubation with insulin (Fig. 2A, lanes c and d) and, in fact, the steady state level of insulin receptor phosphorylation remained elevated for at least 1 h after the addition of insulin (Fig. 2B, lane d). At no time during these experiments was any phosphorylation of the α-subunit (Mr = 135,000) detected, consistent with the finding that tyrosine residues of the α-subunit are probably located entirely at the external face of the plasma membrane (40) and inaccessible to the catalytic site of the β-subunit which is inside of the cell.

Phosphoamino acid analysis of the β-subunit extracted from the Mr = 95,000 region of the SDS polyacrylamide gels revealed that the basal state phosphorylation was due entirely to phosphoserine and phosphothreonine (Fig. 3). Incubation of Fao cells with insulin for 1 min, 5 min, or 10 min stimulated mainly tyrosine phosphorylation of the β-subunit to a level approximately equal to that of phosphoserine. Exact quantitative analysis of these results is unreliable, however, because acid hydrolysis of the peptide linkages is not complete within 2 h and the phosphate bonds of serine, threonine, and tyrosine are hydrolyzed partially and unequally during this procedure (41). Thus, insulin-stimulated phosphorylation of the β-subunit quickly reached a steady state in Fao cells and the earliest new sites of phosphorylation were tyrosine residues.

Identification of the Basal and Insulin-stimulated Phosphorylation Sites in the β-Subunit of the Insulin Receptor—Trypsin digestion of the phosphorylated β-subunit purified from Fao cells yielded several phosphopeptides which were separated by reverse-phase HPLC. In the absence of insulin (Fig. 4A), a single major and several smaller [32P]-phosphopeptides were observed. Identification of the phosphoamino acids in these fractions (Fig. 5) by partial acid hydrolysis and high voltage electrophoresis confirmed that the major tryptic phosphopeptide in the unstimulated insulin receptor contained phosphoserine only (Fig. 4A, pS). Two minor tryptic fragments (Fig. 4A, Fractions 24 and 41) also contained phosphoserine. Phosphothreonine was detected in one minor site (Fig. 4A, pT). Although, the minor phosphoserine sites were somewhat variable in appearance, the major phosphoserine and minor phosphothreonine peptides were reproducibly detected. The predominance of phosphoserine in the insulin-free receptor was consistent with our previous studies of the total phosphoamino acid content of the β-subunit (5). Furthermore, as expected, no photophotyrosine was detected in the β-subunit before insulin stimulation.

After incubation with insulin for only 1 min, two additional phosphopeptides obtained by trypsin digestion of the β-subunit were identified (Fig. 4B, Fractions 49 and 62). These two new insulin-stimulated [32P]-phosphopeptides appeared to be entirely responsible for the observed insulin stimulation during this time interval. Phosphotyrosine, but not phosphoserine or phosphothreonine, was detected by phosphoamino acid analysis in both the new minor (pY2) and the major (pY3) peptides. The phosphoamino acid composition of the other fractions was identical with those obtained in the absence of insulin (Fig. 5). Therefore, within a 1-min time interval, insulin stimulated phosphorylation at one major and one minor tyrosine residue in the β-subunit of the insulin receptor.

Contrasts between the Sites of Phosphorylation in Vivo and in Vitro—When the insulin receptor extracted from Fao cells and partially purified by affinity chromatography on wheat germ agglutinin agarose was incubated with [γ-32P]ATP and Mn²⁺, little or no phosphorylation was observed in the absence of insulin (Fig. 6). This finding is in contrast to the substantial basal phosphorylation of the β-subunit which occurred in [32P]orthophosphate-labeled cells (Fig. 2, A and B, lane a). This disparity arises because the basal phosphorylation in the...
The insulin receptor was purified, and the cells were treated with 100 nM insulin for the indicated time intervals. The insulin receptor was purified, and the phosphoproteins were reduced with DTT and separated by SDS-PAGE with a 7.5% resolving gel. An autoradiogram of the separated phosphoproteins is shown for two experiments. The radioactivity in the β-subunit was quantified by Cerenkov radiation:

\[ \text{Cerenkov cpm} \]

60 min, 1740.

The phosphoproteins were reduced with 6 M HCl and separated by high voltage electrophoresis. The separation of the tryptic phosphopeptides obtained from the insulin-stimulated β-subunit revealed compared to cells without insulin (Fig. 7). When this elution profile was compared directly to the profile obtained from the β-subunit purified after insulin stimulation from \(^{32}\text{P}\)orthophosphate-labeled Fao cells (Fig. 4, panels B and C), two phosphopeptides (pY2, pY3) were found to elute at identical positions. However, the major phosphotyrosine-containing peptide (pY3) from the β-subunit labeled in vivo corresponded to a minor peptide from the receptor phosphorylated in vitro and the minor in vivo phosphotyrosine site (pY2) was prominent in the in vitro system (Fig. 4, B and C). An additional major phosphorylation site (pY1) was prominent in the in vitro phosphorylation that eluted at 41 min. This peptide migrated in the same position as the phosphoserine-containing peptide obtained from \(^{32}\text{P}\)orthophosphate-labeled cells, but in the in vitro system contained phosphotyrosines.

The Time Course of Insulin Receptor Phosphorylation Sites in Vivo—It has been difficult to identify the amino acids phosphorylated initially following the binding of insulin to its receptor in vivo because phosphoamino acid analysis by partial acid hydrolysis produces disproportionate losses of phosphotyrosine when compared to phosphoserine (41). Measurement of the tryptic \(^{32}\text{P}\)-phosphopeptides is more likely to provide an accurate estimate of the phosphorylation because it involves complete and reproducible digestion of proteins under mild conditions that do not hydrolyze phosphate bonds. Separation of the tryptic phosphopeptides obtained from the β-subunit by HPLC indicated that tyrosine phosphorylation was the initial and major phosphorylation reaction that occurred in vivo in response to insulin binding (Fig. 8). Within 20 s after the addition of insulin to the Fao cells, the phosphorylation of pY2 and pY3 reached steady state. The level of phosphorylation in these peptides remained constant during the next 40 s (Fig. 8, C and D) and, in fact, remained constant during a 1-h incubation with insulin (Fig. 9). The basal set of phosphopeptides was unchanged both during the initial response of Fao cells to insulin (Fig. 8) and after 1 h of insulin stimulation (Fig. 9). However, three new phosphopeptides were detected after 1 h of incubation with insulin when compared to cells without insulin (Fig. 9A) or insulin stimulation for 1 min (Fig. 9B). We have not yet determined the identity of the phosphoamino acids in these peptides, but they are probably phosphoserine or phosphothreonine. Thus, al-

**Fig. 2. Time course of insulin-stimulated phosphorylation of the insulin receptor.** Fao cells were incubated for 2 h with \(^{32}\text{P}\)orthophosphate and the cells were treated with 100 nM insulin for the indicated time intervals. The insulin receptor was purified, and the phosphoproteins were reduced with DTT and separated by SDS-PAGE with a 7.5% resolving gel. An autoradiogram of the separated phosphoproteins is shown for two experiments. The radioactivity in the β-subunit was quantified by Cerenkov radiation: Panel A (Cerenkov cpm), 0 s, 250; 20 s, 740; 40 s, 690; 60 s, 790; Panel B (Cerenkov cpm), 0 min, 560; 1 min, 1690; 30 min, 1688; 60 min, 1740.

**Fig. 3. Phosphoamino acid analysis of the β-subunit of the insulin receptor.** The β-subunit obtained from \(^{32}\text{P}\)orthophosphate-labeled Fao cells incubated with insulin for the indicated time intervals was purified by immunoprecipitation and PAGE. The β-subunit was identified by autoradiography, excised from the gel, and digested with trypsin. The tryptic peptides were partially hydrolyzed with 6 M HCl and separated by high voltage electrophoresis. The radioactive amino acids were identified by the migration of known standards.

**Fig. 4.** Phosphopeptides containing phosphotyrosines (Fig. 4A) and phosphoserines (Fig. 4B) were detected by reverse-phase HPLC of the tryptic phosphopeptides obtained from the insulin-stimulated β-subunit after phosphorylation in vitro or phosphorylation in vivo revealed a distinctly different set of peptides. After insulin stimulation for 1 h and in vitro phosphorylation for 1 min, three chromatographically distinct phosphopeptides were detected (peptides pY1, pY2, and pY3 in Fig. 4C), all of which contained only phosphotyrosine (Fig. 7). When this elution profile was compared directly to the profile obtained from the β-subunit purified after insulin stimulation from \(^{32}\text{P}\)orthophosphate-labeled Fao cells (Fig. 4, panels B and C), two phosphopeptides (pY2, pY3) were found to elute at identical positions. However, the major phosphotyrosine-containing peptide (pY3) from the β-subunit labeled in vivo corresponded to a minor peptide from the receptor phosphorylated in vitro and the minor in vivo phosphotyrosine site (pY2) was prominent in the in vitro system (Fig. 4, B and C). An additional major phosphorylation site (pY1) was obtained during in vitro phosphorylation that eluted at 41 min. This peptide migrated in the same position as the phosphoserine-containing peptide obtained from \(^{32}\text{P}\)orthophosphate-labeled cells, but in the in vitro system contained phosphotyrosines.
Peptides were separated by reverse-phase HPLC. The phosphorylated subunit, excised from the gel, and digested with trypsin and the phosphate were incubated without insulin or with insulin (100 nM) for 1 h. Phosphorylation was obtained from trypsin digestion of the insulin receptor phosphorylated in vivo or in vitro. The phosphopeptides in the indicated fractions obtained from the HPLC separation shown in Fig. 4, A and B were partially hydrolyzed with 6 M HCl. The 32P-phosphoamino acids were separated by high voltage electrophoresis and identified by the migration of known standards: P-Tyr, phosphotyrosine; P-Thr, phosphothreonine; P-Ser, phosphoserine. The autoradiogram was obtained by exposure to the film for 7 days. In the original autoradiogram, phosphotyrosine was detected in fraction 49 only after insulin stimulation, but the image was faint and did not reproduce in this photograph.

![Graph showing phosphorylation of insulin receptor](image)

**Fig. 5.** Phosphoamino acid analysis of tryptic phosphopeptides obtained from the β-subunit of the insulin receptor phosphorylated in vivo. The phosphopeptides in the indicated fractions obtained from the HPLC separation shown in Fig. 4, A and B were partially hydrolyzed with 6 M HCl. The 32P-phosphoamino acids were separated by high voltage electrophoresis and identified by the migration of known standards: P-Tyr, phosphotyrosine; P-Thr, phosphothreonine; P-Ser, phosphoserine. The autoradiogram was obtained by exposure to the film for 7 days. In the original autoradiogram, phosphotyrosine was detected in fraction 49 only after insulin stimulation, but the image was faint and did not reproduce in this photograph.

**DISCUSSION**

It is now widely accepted that the insulin receptor undergoes phosphorylation during its initial response to insulin binding. After solubilization and purification by wheat germ agglutinin agarose or insulin-Sepharose affinity chromatography, this receptor retains insulin-stimulated tyrosine phosphorylation.

![Graph showing time course of phosphorylation](image)

**Fig. 6.** Time course of in vitro phosphorylation of the β-subunit. The WGA-purified receptor (4 μg) was incubated with Mn2+ (5 mM) and without or with insulin (100 nM) for 1 h. Phosphorylation was initiated by addition of 50 μM [γ-32P]ATP for the indicated time intervals. The insulin receptor was immunoprecipitated, reduced with DTT, and separated by SDS-PAGE. The radioactivity in the gel fragment containing the β-subunit was quantified by Cerenkov counting.

though there is an apparent steady state of phosphorylation of the insulin receptor over a 1-h incubation interval with insulin (Fig. 2), the pattern of trypsin digestion, or probably, the relative phosphoamino acid content of the β-subunit, is changing.
Fig. 7. Phosphoamino acid analysis of tryptic phosphopeptides obtained from the β-subunit phosphorylated in vitro. The phosphopeptides in the indicated fractions obtained from the HPLC separation shown in Fig. 4C were partially hydrolyzed. The 32P-phosphoamino acids were separated by high voltage electrophoresis and identified by the migration of known standards: P-TYR, phosphotyrosine; P-THR, phosphothreonine; P-SER, phosphoserine. The autoradiogram was obtained by exposure for 7 days.

Phosphorylation but loses substantially the ability to undergo phosphorylation at serine and threonine residues. The separation of the kinase activities suggests that the tyrosine kinase, but not serine/threonine kinase activity, is intrinsic to the insulin receptor (12, 14, 42). The role of tyrosine phosphorylation of the receptor during the response of cells to insulin, however, has not yet been clearly established. Several observations support the notion that tyrosine phosphorylation is important for insulin action. First, dissociation of insulin from its receptor results in a loss of the insulin-stimulated receptor, whereas serine and threonine phosphorylation is consistent with the notion that tyrosine phosphorylation is an expression of the insulin-stimulated receptor, whereas serine and threonine phosphorylation probably result from a complex sequence of other biochemical events. Phorbol esters stimulate serine and threonine phosphorylation of the β-subunit (51), possibly through activation of calcium-dependent and phospholipid-stimulated protein kinase C (53-55). These results suggest that phosphorylation of the β-subunit at serine and threonine residues may be due to an interaction with protein kinase C or another kinase that is activated by a protein kinase C-dependent pathway (53), but further studies are necessary to establish this relation.

In this report, we describe insulin receptor phosphorylation in the well differentiated, insulin-sensitive hepatoma cell, Fao. Consistent with our previous reports, only the β-subunit of the insulin receptor is phosphorylated in intact cells (4, 5, 29, 31). This phosphorylation occurs very rapidly, reaching a maximum within 20 s after insulin addition. This time course is much faster than we reported previously where nearly 30 min was required to reach steady state (31). Our ability to detect significant tyrosine phosphorylation of the β-subunit after short time intervals of insulin incubation arises presumably from two procedural changes employed during this study which were adopted to inhibit dephosphorylation of phosphotyrosine: 1) liquid nitrogen was used to freeze the cell monolayers to stop the reaction and minimize enzymatic activity during the initial phases of cell solubilization; 2) thawing the cells into a detergent solution containing phosphatase and protease inhibitors, including sodium vanadate (2 mM), ensured exposure of these agents to the cellular components before the temperature of the extract increased to a level that permitted dephosphorylation. Sodium vanadate, a potent inhibitor of phosphotyrosine-specific phosphatases (39), was also added to all solutions during receptor purification. This inhibited the loss of phosphotyrosine and did not stimulate phosphorylation of immunoprecipitable proteins during purification. Thus, our results now establish that insulin binding and tyrosine phosphorylation probably occur almost simultaneously in the intact cell which places the tyrosine autophosphorylation reaction in the position to act as an initial transmembrane signal.

This difference in methodology also explains the difference in the identity of the phosphoamino acids contributing to the observed insulin effect in this versus previous studies. In our earlier studies, the phosphoserine content of the β-subunit purified from insulin-stimulated Fao cells always predominated significantly over the amount of phosphotyrosine (5). In addition, studies with freshly isolated hepatocytes suggested that only phosphoserine was stimulated by the incubation of 32P-orthophosphate-labeled cells with insulin for 15 min (30). We suspect that the apparently sluggish rate of insulin-stimulated phosphorylation of the receptor reported earlier was due to the underestimation of tyrosine phosphorylation due to significant dephosphorylation of phosphotyrosine residues during receptor extraction and purification.

In the β-subunit of the insulin receptor from cells labeled with 32P-orthophosphate in the absence of insulin, we have identified one major and three minor phosphopeptides, two containing phosphoserine and one containing phosphothreonine. Within seconds after the addition of insulin, the level of phosphotyrosine in the β-subunit rose rapidly and reached steady state with no significant changes in the content of phosphothreonine and phosphoserine. This relation between serine, threonine, and tyrosine phosphorylation is consistent with the notion that tyrosine phosphorylation is an expression of the insulin-stimulated receptor, whereas serine and threonine phosphorylation probably result from a complex sequence of other biochemical events. Phorbol esters stimulate serine and threonine phosphorylation of the β-subunit (51), possibly through activation of calcium-dependent and phospholipid-stimulated protein kinase C (53-55). These results suggest that phosphorylation of the β-subunit at serine and threonine residues may be due to an interaction with protein kinase C or another kinase that is activated by a protein kinase C-dependent pathway (53), but further studies are necessary to establish this relation.

Although some reports (52), including our own (17, 42), have suggested that the α-subunit may also be phosphorylated in response to insulin, this reaction has only been shown to
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FIG. 8. Reverse-phase HPLC separation of phosphopeptides obtained by trypsin digestion of the β-subunit phosphorylated in vivo. Fao cells were incubated for 2 h with [32P]orthophosphate and the cells were treated with 100 nM insulin for the indicated time intervals. The insulin receptor was purified, and the phosphoproteins were reduced with DTT and separated by SDS-PAGE. The phosphorylated β-subunit was digested with trypsin and separated by reverse-phase HPLC. The shaded areas (pY2 and pY3) were determined by phosphoamino acid analysis to contain phosphotyrosine; pS and pT were shown to contain phosphoserine and phosphothreonine, respectively.

occur in vitro. Our reports confirm that the α-subunit is not phosphorylated in response to insulin in the intact cell. Since tyrosine residues of the α-subunit are not surface-iodinated on inside-out plasma membrane vesicles, it is likely that the tyrosine residues of the α-subunit and possibly the entire subunit, are exposed on the extracellular surface of intact cells (40). Therefore, the catalytic site of the β-subunit which is located presumably at the inner face of the plasma membrane may be inaccessible to phosphorylation domains of the α-subunit. Thus, while certain domains of the α-subunit may interact with the catalytic site of the β-subunit of the insulin receptor after solubilization, this reaction is probably not physiologically relevant.

The tryptic phosphopeptides separated from the β-subunit labeled in vivo eluted differently during reverse-phase HPLC than the peptides obtained from the insulin receptor labeled in vitro. After in vitro labeling, the β-subunit did not contain the phosphoserine or phosphothreonine peptides obtained during in vivo labeling. Furthermore, three phosphotyrosine-containing peptides, pY1, pY2, and pY3, were observed in vitro, but only two of these phosphopeptides (pY2 and pY3) were detected in the β-subunit purified from [32P]orthophosphate-labeled cells. The phosphorylation of pY3 was significant in vivo but minor in vitro, whereas pY2, a minor phosphopeptide in vivo, was prominent in vitro. Phosphopeptide pY1 was not detected in vivo. At least two major phosphotyrosine-containing peptides have also been identified after in vitro phosphorylation of the epidermal growth factor receptor purified from human placenta (14, 25). In one of these reports, the peptides also contained phosphoserine (25). In vitro phosphorylation of the epidermal growth factor receptor purified from A431 cells yielded three major phosphotyrosine-containing peptides, but only one of them occurred predominantly in vivo (56).

The exact reasons for the differences between the in vivo and in vitro phosphorylation sites are not clear. A likely possibility is that solubilization of the receptor alters the conformation or accessibility of the catalytic or phosphoryla-
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may be cleaved immediately from the receptor and therefore not recovered when the receptor is extracted. Whether this could be related to the generation of an intracellular peptide messenger is unknown (57, 58). Whatever the reason, our findings emphasize that caution should be used during the interpretation of results obtained with the purified receptors, since regulatory effects seen in vitro resulting from autophosphorylation (24, 25) may not reflect exactly those which occur in the intact cell.

Although the major sites of phosphorylation of the β-subunit remained constant during 60 min of incubation with 100 nM insulin, after 1 h of insulin incubation, 3 new sites of phosphorylation were observed. The identity of the phosphoamino acids in these peptides has not been established. Since the insulin receptor is internalized into Fao cells after insulin binding (59), these late phosphorylations may be linked to the process of internalization or reflect changes in the receptor structure subsequent to internalization and processing. Internalization is relatively rapid in freshly isolated hepatocytes; nearly half of the receptors labeled covalently with a photo-reactive insulin analog are internalized within 30 to 60 min (60). After several hours, most of these receptors eventually return to the cell surface. In rat adipocytes, both of the subunits are apparently internalized intact (40). Possibly, the phosphorylated receptor enters the Fao cells, undergoes additional phosphorylation, but during this process is protected from degradation and dephosphorylation. In this scenario, phosphorylase may provide both an immediate signal at the plasma membrane and a persistent signal at other sites inside of the cell. However, it is possible that the tyrosine-phosphorylated subset of receptors is not internalized and additional experiments will be necessary to clarify this point.

In summary, our experiments establish that the level of phosphotyrosine in the insulin receptor increases very rapidly after addition of insulin and is constant during 1 h of incubation with insulin. Tyrosine phosphorylation of the β-subunit occurs at two sites in the intact Fao cell and at three sites after receptor solubilization. One of the prominent phosphotyrosine-containing peptides detected in vitro is not found in vivo, whereas the predominant phosphotyrosine-containing peptides occurring in vivo correspond to a minor site in vitro. It is likely that further studies will provide additional information about the role of this multisite phosphorylation of the insulin receptor in insulin action.

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