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Studies into the Identity of the Sites of Insulin-Stimulated Insulin Receptor Serine Phosphorylation. Characterization of Synthetic Peptide Substrates for the Insulin-Stimulated Insulin Receptor Serine Kinase†

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ABSTRACT: The identity of the sites of insulin-stimulated serine phosphorylation in the human insulin receptor was examined by synthesizing peptides that together encompassed all the serine residues of the cytosolic portion of the β-subunit and testing them as substrates for phosphorylation by a preparation of human insulin receptor copurified with insulin-stimulated insulin receptor serine kinase activity. Of the 14 peptides studied, only 4 (1071−1080, 1290−1298, 1253−1271, and 1313−1329) were phosphorylated on serine, with the serine phosphorylation stimulated 2−4-fold by insulin. Peptides 1071−1080 and 1290−1298 were 3−7-fold better substrates for the serine phosphorylation than the other serine-phosphorylated peptides. Peptides 1071−1080 and 1313−1329 also exhibited insulin-stimulated phosphorylation on tyrosine. Two-dimensional thin-layer tryptic mapping of the phosphorylated insulin receptor/insulin-stimulated insulin receptor serine kinase preparation or of insulin receptor phosphorylated in human Hep G2 cells yielded two major peptides, called S1 and S2, that ran as a pair of closely migrating spots, and other lesser peptides that contained phosphoserine. S1 and S2 also contained some phosphotyrosine and gave phosphoserine/phosphotyrosine ratios of ~6 and 0.96−1.50 for the *in vivo* and *in vitro* labeled receptor, respectively. S1 and S2 were not cleaved by V8. Of the serine-phosphorylated peptides, only 1290−1298 and 1071−1080 should be V8 resistant; 1290−1298 contains serine sites 1293/4 and migrated distinctly from S1 and S2 in tryptic maps. Peptide 1071−1080 mimicked the production of S1 and S2 in tryptic maps yielding a doublet of phosphopeptides, each containing phosphoserine and phosphotyrosine, which comigrated exactly with S1 and S2. Comigration was confirmed at a different pH and by mixing experiments. Radiosequenation showed that serine 1078 was phosphorylated. Tyrosine 1075 was also phosphorylated, but it was no more than a minor site *in vivo*. It is concluded that serine 1078 of the insulin receptor is a major site of insulin-stimulated phosphorylation *in vivo* and *in vitro*. The peptide sequences provide a range of substrates to facilitate the study, purification, and characterization of the insulin-stimulated insulin receptor serine kinase or kinases, and the identification of a major site of insulin-stimulated serine phosphorylation will help elucidate the function of the insulin receptor serine phosphorylation.

The insulin receptor is an insulin-stimulated tyrosine-specific protein kinase. The tyrosine kinase catalyzes the very rapid autophosphorylation of its own β-subunit on multiple tyrosines. At least seven tyrosines clustered in three domains appear to be phosphorylated both *in vivo* and *in vitro*. These include tyrosines 1316 and 1322 in the C-terminal domain; tyrosines 1146, 1150, and 1151 in the kinase domain; and tyrosine 960 and at least one other hitherto unidentified tyrosine in the juxtamembrane domain (Tornqvist *et al.*, 1987, 1988; White *et al.*, 1988a; Tavare & Denton, 1988; Tavare *et al.*, 1988; King & Sale, 1990; Feener *et al.*, 1993). A further unidentified site or sites of tyrosine phosphorylation representing ~15% of the phosphate incorporated during autophosphorylation have also been described (Tornqvist *et al.*, 1987, 1988). The autophosphorylation of tyrosines in the kinase domain functions to activate 10−20-fold the ability of the insulin receptor tyrosine kinase to phosphorylate other proteins, both *in vivo* and *in vitro*, such that the tyrosine kinase is constitutively active in the absence of insulin (Rosen *et al.*, 1983; Yu & Czech, 1984; Wilden *et al.*, 1992). Tyrosine 960 in the juxtamembrane domain appears to be required for the phosphorylation of IRS 1 (White & Kahn, 1994).

In intact cells, the tyrosine autophosphorylation is followed by an increase in phosphorylation of the β-subunit on serine and threonine residues (Gazzano *et al.*, 1983; Pang *et al.*, 1985; Stadtmauer & Rosen, 1986). The serine/threonine phosphorylation of the insulin receptor also occurs in response to treatment of cells with activators of either protein kinase C or cyclic AMP dependent protein kinase (Jacobs & Cuatrecasas, 1986; Stadtmauer & Rosen, 1986; Takayama *et al.*, 1988; Issad *et al.*, 1992). The insulin receptor has been shown *in vitro* to be a substrate for several serine/threonine kinases including an insulin-stimulated kinase or kinases that copurified with the insulin receptor (Smith *et al.*, 1988; Smith & Sale 1988, 1989) and a casein kinase 1-like enzyme (Rapuano & Rosen, 1991) as well as by cyclic

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AMP dependent protein kinase (Roth & Beaudoin, 1987) and protein kinase C (Ahn *et al.*, 1993).

The functional consequences of the serine/threonine phosphorylation of the insulin receptor are controversial. Basal phosphorylation of the insulin receptor in cells on serine has been reported to inhibit subsequent insulin-stimulated autophosphorylation on tyrosine (Pang *et al.*, 1985). In Fao hepatoma cells, phorbol ester-induced serine phosphorylation was reported to inhibit insulin receptor tyrosine kinase activity (Takayama *et al.*, 1984). However, studies overexpressing protein kinase C isoenzymes resulted in hyperphosphorylation of the insulin receptor on serine but no inhibition of tyrosine kinase activity (Chin *et al.*, 1993). Additionally, treatment of cells with okadaic acid, a serine/threonine protein phosphatase inhibitor, did not interfere with insulin-induced insulin receptor tyrosine autophosphorylation or with tyrosine kinase activity toward artificial substrates (Jullien *et al.*, 1993). In recent studies, it has been reported that the insulin-stimulated increase in anti-phosphotyrosine-precipitable phosphatidylinositol 3-kinase activity was decreased when the insulin receptor was extensively serine/threonine phosphorylated by activation of protein kinase C (Chin *et al.*, 1993; Coghlan & Siddle, 1993; Liu & Roth, 1994). Thus, serine/threonine phosphorylation of the insulin receptor in response to either insulin or antagonistic hormones may be important in limiting the lifetime of signal-competent insulin receptor. Moreover, protein kinase C activity has been reported to be increased in diabetes (Inoguchi *et al.*, 1992). Consequently, the identification of the sites of serine phosphorylation of the β-subunit as well as the insulin-stimulated insulin receptor serine kinase or kinases is important.

Serines 1293/4 and threonine 1336 in the C-terminal domain of the insulin receptor have been shown to be phosphorylated in intact cells transfected with the insulin receptor in response to both insulin and phorbol esters and by incubation of the insulin receptor *in vitro* with purified protein kinase C (Lewis *et al.*, 1990a,b; Tavare *et al.*, 1991; Ahn *et al.*, 1993). Phorbol ester treatment of H35 rat hepatoma cells has also been shown to stimulate phosphorylation of threonine 1336 (Koshio *et al.*, 1989). In cells transfected with the insulin receptor, serine 1315 of the insulin receptor has been reported as a phorbol ester-stimulated phosphorylation site with serines 955/6 of the insulin receptor acting as sites of insulin-stimulated and to a lesser extent phorbol ester-stimulated phosphorylation (Coghlan *et al.*, 1994; Feener *et al.*, 1993, 1994; Lewis *et al.*, 1994; Liu & Roth, 1994). Major sites of insulin-stimulated phosphorylation of the insulin receptor, however, remain unidentified even in cells transfected with the insulin receptor (Tavare *et al.*, 1991; Feener *et al.*, 1993, 1994; Liu & Roth, 1994). Moreover, the identity of the insulin receptor serines phosphorylated in response to insulin in normally insulin-responsive target cell types, in which the pattern of serine phosphorylation has been reported to be different from that in cells transfected with the insulin receptor (Issad *et al.*, 1991), has not been demonstrated.

In the present work, peptides that together encompassed all the serines of the intracellular part of the β-subunit of the insulin receptor were synthesized and compared as substrates for phosphorylation by a preparation of insulin receptor that had been copurified with insulin-stimulated insulin receptor serine kinase activity (Smith *et al.*, 1988). We have recently shown (Asamoah *et al.*, 1995) that this copurified insulin-stimulated insulin receptor serine kinase activity is distinct from the insulin receptor and is not attributable to a serine phosphorylating activity of the insulin receptor tyrosine kinase described by Baltensperger *et al.* (1992). Analysis of the peptides that acted as substrates by two-dimensional thin-layer tryptic peptide mapping and comparison with authentic insulin receptor derived peptides were used to study the identity of the sites of insulin-stimulated serine phosphorylation *in vitro* and in human liver Hep G2 cells. Additionally, the peptides described provide a spectrum of sequences to facilitate the study, purification, and identification of the insulin-stimulated insulin receptor serine kinase or kinases.

EXPERIMENTAL PROCEDURES

Materials. Triton X-100, *N*-acetylglucosamine, phosphoamino acids, protease inhibitors, Hepes, bovine serum albumin, pig insulin, trypsin (treated with tosylphenylalanylchloromethane), *Staphylococcus aureus* protease V8, and reagents for SDS/PAGE were from Sigma Chemical Co. Arg C was from Boehringer. The Bradford protein assay kit and AG1-X2 acetate anion exchange resin were obtained from Bio-Rad. Wheat germ agglutinin−agarose came from Vector Laboratories. [γ-^{32}P]ATP, [^{32}P]P_i, and Hyperfilm-MP were purchased from Amersham International. Cellulose thin-layer plates (20 × 20 cm) were from Kodak. Tissue culture supplies were from GIBCO. Peptides were synthesized manually using a solid phase method (Merrifield, 1986). Amino acids and peptidyl resins were obtained from either Bachem, Peninsula, or Novabiochem. Peptides were purified by reverse phase HPLC on a C_{18} preparative column (Vydac). Structures of synthesized peptides were confirmed by mass spectrometry (fast atom bombardment or laser desorption as appropriate) or by sequencing. Other reagents came from BDH, Aldrich, Sigma, or Interchem.

Purification of Insulin Receptors from Human Placentae. Two human placentae were obtained within 1 h of delivery. All procedures were carried out at 4 °C. Placentae were trimmed of amnion and chorion, washed with 0.25 M sucrose, and cut into small pieces. The pieces were then homogenized for 3 × 1 min in a Waring blender in 1−2 volumes of 50 mM Tris/HCl (pH 7.4) containing 0.25 M sucrose and 1 mM phenylmethanesulfonyl fluoride. The homogenate (~1.4 L) was centrifuged at 10000*g* for 20 min, and the supernatants were collected and centrifuged at 100000*g* for 1 h. The pellets were suspended in 10 volumes of 50 mM Tris/HCl (pH 7.4) containing 1 mM phenylmethanesulfonyl fluoride using a hand-held glass/Teflon homogenizer and centrifuged at 100000*g* for 1 h. The sedimented membranes were resuspended using a hand-held glass/teflon homogenizer in 2 volumes of 50 mM Tris/HCl (pH 7.4)/1 mM-phenylmethanesulfonyl fluoride, separated into 2 × 15 mL batches, and stored at −70 °C. One of the 15 mL membrane aliquots (300−400 mg of protein) was solubilized in a final volume of 30 mL of 50 mM Tris/HCl (pH 7.4) containing 2% Triton X-100, 0.1 mg of aprotinin/mL, 0.34 mg of phenylmethanesulfonyl fluoride/mL, and 1 mM benzamidine with a hand-held glass/Teflon homogenizer, followed by stirring for 2 h. The mixture was then centrifuged at 200000*g* for 60 min and the supernatant collected. Insulin receptors were isolated from the solubilized membranes essentially as described previously (Smith

et al., 1988; Asamoah *et al.*, 1995). Solubilized membranes were recycled 3 times through a 5 mL wheat germ agglutinin−agarose column which had been equilibrated with 50 mM Tris/HCl (pH 7.4)/0.1% Triton X-100/0.1 mM phenylmethanesulfonyl fluoride. Columns were then washed with 150 mL of the equilibration buffer followed by elution of insulin receptors with 15 mL of the buffer containing 0.3 M *N*-acetylglucosamine. The protein-containing fractions, determined with the Bradford assay (standardized with bovine serum albumin), were pooled, separated into 200 μL batches, and stored at −70 °C. The protein concentration of the pooled fractions was 0.75−1.25 mg/mL.

Phosphorylations in Vitro. Insulin receptors (~1 mg/mL) were preincubated for 15 min at 22 °C with or without 150 nM insulin and with or without peptides in 50 mM Tris/HCl (pH 7.4)/0.1% Triton X-100 in the presence of 10 mM $MgCl_2$, 2 mM $MnCl_2$, 5 mM dithiothreitol and 20 μM sodium vanadate. [γ-^{32}P]ATP (250 μM, 5−10 cpm/fmol) was then added, and the samples were incubated for 30 min at 22 °C. For analysis of insulin receptor phosphorylation, incubations were terminated by adding 0.25 volume of 312.5 mM Tris/HCl, pH 7.4, containing 10% (w/v) SDS, 76 mg/mL dithiothreitol, and 0.1% (w/v) bromophenol blue. After addition of sucrose to 20% (w/v), samples were boiled for 5 min. Separation of insulin receptor β-subunits on 4% acrylamide stacking/7.5% acrylamide resolving gels and autoradiography were as described in Smith *et al.* (1988). For analysis of peptide phosphorylation, an equal volume of 1 M acetic acid was added. The reaction mixture was then applied at a flow rate of 1 mL/min to a 1−8 g column (1−9 cm x 1 cm) of AG1-X2 acetate anion exchange resin (1 g of resin/100 μL of incubation volume) which had been equilibrated with 10 bed volumes of 1 M acetic acid. The reaction mixture was washed through the column with 1 M acetic acid. One milliliter fractions were collected. Fractions were counted for ^{32}P and the peak fractions pooled. The material was lyophilized and electrophoresed at 400 V for 2.5 h on a cellulose thin-layer plate at pH 3.5 (pyridine/acetic acid/water, 1:10:189 by volume). Autoradiograms were obtained at −70 °C for 18 h. ^{32}P associated with peptides was quantified by liquid scintillation counting of radioactivity. To obtain higher levels of peptide phosphorylation, insulin receptors were preincubated in the absence of peptide as described above followed by addition of 250 μM ATP. After 30 min at 22 °C, [γ-^{32}P]ATP was added to give a final ATP concentration of 227 μM and a specific radioactivity of 5.2 cpm/fmol. Peptide was added to a final concentration of 3 mM at the same time as the [γ-^{32}P]ATP. Samples were incubated for 2 h at 22 °C and then processed as described above.

Cell Culture and in Vivo Phosphorylation. Hep G2 cells were cultured at 37 °C in Eagle's minimal essential medium containing 10% fetal calf serum. Confluent monolayers of cells were serum-starved for 18 h. Cells were then incubated for 2 h in phosphate-free Eagle's minimum essential medium containing 0.5 mCi/mL [^{32}P]P_i with or without 20 nM insulin for the last 10 min. Labeling was terminated with liquid N_2, and the cells were thawed and scraped from the dish with 50 mM HEPES (pH 7.4), 1% Triton X-100, 100 mM NaF, 2 mM sodium vanadate, 10 mM sodium pyrophosphate, 1 mg/mL aprotinin, 4 mM EDTA, and 2 mM phenylmethanesulfonyl fluoride. The following procedures were performed at 4 °C. Cell debris was pelleted at 100000*g* for 30 min, and insulin receptors were isolated from the supernatant by adsorption to wheat germ agglutinin−agarose followed by elution with 0.3 M *N*-acetylglucosamine. Insulin receptors were immunoprecipitated from the eluate (Smith *et al.*, 1988) and then subjected to SDS/PAGE as described above.

Two-Dimensional Thin-Layer Peptide Mapping. In the case of the insulin receptor, the region of the gel containing the β-subunit was excised, incubated with 10 mL of 20% (v/v) methanol for 18 h at 37 °C, and dried at 70 °C for 2 h in an oven. Then 2 mL of 50 mM NH_4HCO_3 containing 100 μg of trypsin (treated with tosylphenylalanylchloromethane) was added. The mixture was incubated at 37 °C for 6 h. The samples were freeze-dried. In the case of phosphorylated synthetic peptides, these were eluted from TLC plates by scraping off the spots and vortexing with 3 × 1 mL aliquots of water followed by centrifugation at 10000*g* for 10 s. The supernatants were lyophilized and subjected to trypsinolysis as described above. The ^{32}P-labeled phosphopeptides were dissolved in 30 μL of water and separated on cellulose thin-layer plates by electrophoresis at 400 V for 7 h at pH 3.5 (pyridine/acetic acid/water, 1:10:189 by volume) or for 2.5 h at pH 1.9 (formic acid/acetic acid/water, 3.6:14.6:181.8 by volume) in the first dimension and ascending chromatography (pyridine/acetic acid/butanol/water, 10:3:15:12 by volume, pH 5.2) in the second dimension. Autoradiograms were obtained at −70 °C for 3−56 days. ^{32}P associated with peptides was quantified by densitometric scanning or by excision of the spots and liquid scintillation counting of radioactivity. Both methods gave similar answers. In some experiments, trypsinolysis was followed by proteolysis using 10 μg of Arg C or 10μg/mL of V8 in 210 μL of 50 mM NH_4HCO_3 (pH 7.8) for 6 h at 25 °C and 16 h at 30 °C, respectively.

Phosphoamino Acid Analysis and Radiosequenation. Phosphopeptides were eluted from TLC plates by scraping off the spots and vortexing with 3 × 1 mL aliquots of water followed by centrifugation at 10000*g* for 10 s and lyophilization. For phosphoamino acid analysis, the ^{32}P-labeled tryptic phosphopeptides were hydrolyzed in 6 M HCl at 110 °C for 90 min. After addition of 1 mL of water and freeze-drying, the samples were dissolved in 20 μL of water containing phosphotyrosine, phosphoserine, and phosphothreonine, each at 1 mg/mL. Phosphoamino acids were separated by electrophoresis at 7 °C on cellulose thin-layer plates at pH 3.5 (pyridine/acetic acid/water; 1:10:189 by volume) for 70 min at 1 kV. Xylene cyanol (1%) was used as a tracking dye. The phosphoamino acid standards were identified by reaction with ninhydrin. Autoradiograms were obtained at −70 °C for 1−28 days. ^{32}P associated with phosphoamino acids was quantified by densitometric scanning or by excision of the spots and liquid scintillation counting of radioactivity. Both methods gave similar answers. For radiosequenation, the ^{32}P-labeled phosphopeptides were sequenced on an Applied Biosystems 477A sequencer.

RESULTS

Synthesis and Phosphorylation of Peptides. The intracellular portion of the insulin receptor β-subunit possesses 30 serine residues (Ullrich *et al.*, 1985). Fourteen peptides were synthesized that together contained all of these serines (Table 1). The peptides were synthesized either as the

Table 1: Phosphorylation of Synthetic Peptides by the Copurified Insulin Receptor/Insulin-Stimulated Insulin Receptor Serine Kinase Preparation[a]

peptide	sequence	^{32}P incorporation into serine or threonine (pmol)	relative phosphorylation on serine or threonine
944−981	QPDGPLGPLYASSNPEYLSASDVFPCSVYVPDEWEVSR		
989−1004	ELGQGSFGMVYEGNAR		
1019−1027	TVNESASLR		
1030−1040	IEFLNEASVMK		
1050−1056	LLGVVSK		
1071−1080	DLKSYLRSLR	76.8 ± 5.2 (Ser)	3.11 ± 0.21 (Ser)
1162−1172	RWMAPESLK		
1171−1208	DGVFTTSSDMWSFGVVLWEITSLAEQPYQGLSNEQVLK		
1253−1271	DDLHPSFPEVSFFHSEENK	24.7 ± 10.4 (Ser)	1.00 ± 0.42 (Ser)
1272−1292	APESEELQMEFEDMENVPLDR		
1290−1298	LDRSSHCQR	169.8 ± 45.7 (Ser)	6.88 ± 1.85 (Ser)
1305−1313	DGGSSLGFK		
1313−1329	KRSYEEHIPYTHMNGGK	27.9 ± 6.2 (Ser)	1.13 ± 0.25 (Ser)
1333−1343	RILTLPRSNPS	36.8 ± 18.0 (Thr)	1.49 ± 0.73 (Thr)

[a] The peptides are synthetic insulin receptor β-subunit sequences (tryptic or extended tryptic) that contain serine residues. The peptides were incubated with the insulin receptor copurified with the insulin-stimulated insulin receptor serine kinase activity. Incubations (400 μL) were for 2 h in the presence of insulin at a peptide concentration of 3 mM. Phosphorylation of the peptides was determined by AG1-X2 chromatography and electrophoresis at pH 3.5 on cellulose thin-layer plates followed by phosphoamino acid analysis. The absence of an entry in the ^{32}P incorporation column indicates that the ^{32}P incorporation into serine or threonine was zero.

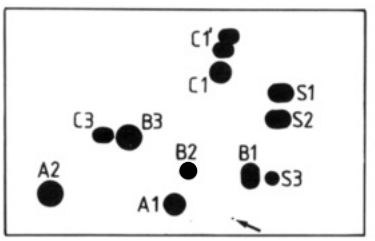

FIGURE 1: Key to the identification of the phosphopeptides resolved from the phosphorylated human insulin receptor by two-dimensional tryptic peptide mapping with an electrophoresis pH of 3.5. The phosphopeptides discussed in the text are shown in the key. The C1′ region comprised several poorly resolved phosphopeptides of which the two most dominant are shown in the key. The arrow marks the origin of sample application.

predicted tryptic sequence or as an extended version of the tryptic sequence if a serine was present near the end of the sequence. The ability of the peptides to act as substrates for phosphorylation by a preparation of human placental insulin receptor copurified with insulin-stimulated insulin receptor serine kinase activity was tested. Four of the peptides exhibited phosphorylation on serine (1071−1080, 1253−1271, 1290−1298, and 1313−1329), and one showed phosphorylation on threonine (1333−1343). The serine and threonine phosphorylation of these peptides was stimulated 2−4-fold by insulin. Peptides 1071−1080 and 1290−1298 were the best substrates for serine/threonine phosphorylation in a standard incubation of 2 h with 3 mM peptide in the presence of insulin (Table 1). Additionally, peptides 1071−1080 and 1313−1343 were phosphorylated on tyrosine (see Table 1 and below).

Two-Dimensional Mapping of Insulin Receptor Phosphorylated in Vitro with Electrophoresis at pH 3.5. The placental insulin receptor copurified with insulin-stimulated insulin receptor serine kinase activity was phosphorylated and subjected to SDS/PAGE. The β-subunits were isolated and digested with trypsin and the phosphopeptides resolved by two-dimensional mapping on cellulose thin-layer plates with an electrophoresis pH of 3.5 (Figures 1 and 2). The resolved phosphopeptides were subjected to phosphoamino acid analysis (Table 2). Insulin-stimulated sites of tyrosine autophosphorylation were present as follows (Tavare &

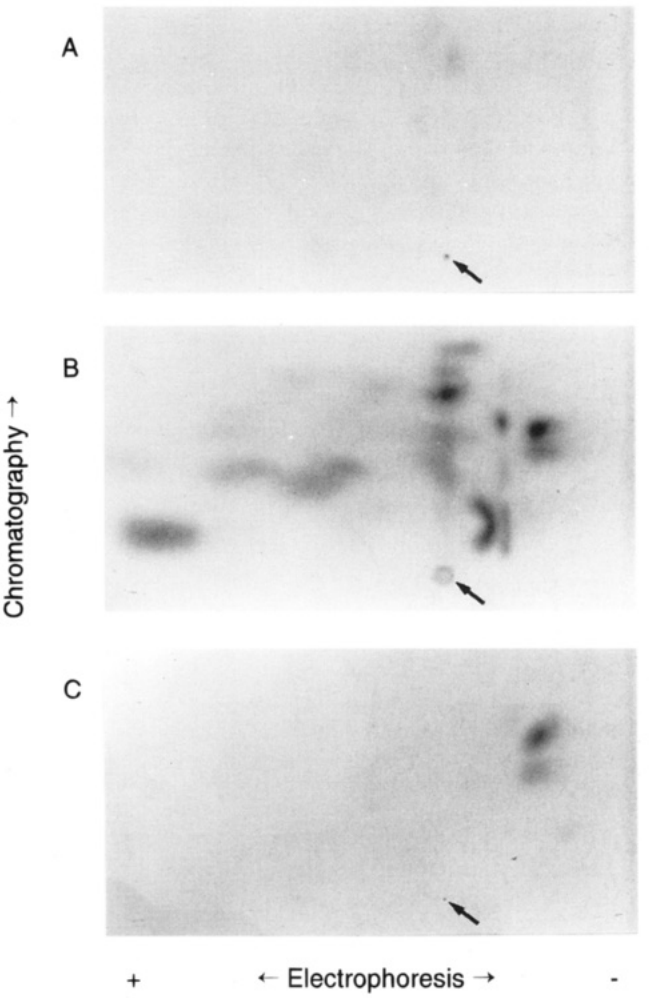

FIGURE 2: Two-dimensional tryptic peptide mapping with an electrophoresis pH of 3.5 of the human placental insulin receptor/insulin-stimulated insulin receptor serine kinase preparation phosphorylated *in vitro* in the absence (A) or presence of insulin (B) and of the phosphorylated peptide 1071−1080 (C). The peptide was phosphorylated using the insulin receptor copurified with the insulin-stimulated insulin receptor serine kinase activity by incubation for 2 h at 22 °C in the presence of insulin and digested with trypsin. The arrows mark the origins of sample application. The results are representative of four experiments.

Table 2: Phosphoamino Acid Analysis[a]

	pH	species	P-Tyr (%)	P-Ser (%)	P-Thr (%)
insulin receptor	3.5	S1	51.0 ± 5.6	49.0 ± 4.7	0.0 ± 0.0
phosphorylated *in vitro*	3.5	S2	40.0 ± 1.9	60.0 ± 5.8	0.0 ± 0.0
	3.5	S3	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0
	3.5	B1	77.0 ± 5.2	23.0 ± 5.2	0.0 ± 0.0
	3.5	C1′	86.0 ± 14.0	2.0 ± 2.0	12.0 ± 12.0
	3.5	C3	61.0 ± 6.9	39.0 ± 6.9	0.0 ± 0.0
	1.9	S1	59.0	41.0	0.0
	1.9	S2	65.0	35.0	0.0
insulin receptor	3.5	S1	19.0 ± 3.4	81.0 ± 3.4	0.0 ± 0.0
phosphorylated *in vivo*	3.5	S2	14.0 ± 2.6	86.0 ± 2.6	0.0 ± 0.0
	3.5	S3	ND	ND	ND
	3.5	B1	78.6 ± 4.2	21.4 ± 4.2	0.0 ± 0.0
	3.5	C3	ND	ND	ND
	3.5	C1′	ND	ND	ND
phosphorylated and	3.5	upper	65.0 ± 7.0	35.0 ± 7.0	0.0 ± 0.0
trypsin-digested	3.5	lower	64.0 ± 5.5	36.0 ± 5.5	0.0 ± 0.0
peptide 1071−1080					

[a] The pH is that used in the electrophoresis dimension of the two-dimensional thin-layer peptide mapping. The insulin receptor-derived phosphopeptides A1, A2, B2, B3, and C1 yielded only phosphotyrosine (not illustrated). SEM values are based on at least three observations. ND, not determined; insufficient S3, C3 or C1′ was present.

Denton, 1988; King & Sale, 1990). The tyrosine 1150 domain, which contains tyrosines 1146, 1150, and 1151, was recovered as phosphopeptides that were mono (C1), bis (B2 and B3), and tris (A1 and A2) phosphorylated. The C-terminal domain, which contains tyrosines 1316 and 1322, was recovered as a bisphosphorylated phosphopeptide (B1). Phosphopeptides C1′ and C3 are believed to arise from the juxtamembrane domain, which contains tyrosines 953, 960, and 972, and to represent monophosphorylated and bisphosphorylated forms. C3 was often poorly resolved from B2. It was previously found (Smith & Sale, 1989) that the major phosphoseryl peptides, S1 and S2, migrated to a poorly resolved region that overlapped with C1 and that S1 and S2 both contained phosphotyrosine in addition to phosphoserine. Thus, it was unclear whether the presence of phosphotyrosine in S1 and S2 in the study of Smith and Sale (1989) was due to the incomplete resolution of S1 and S2 from C1. In the present work, the two-dimensional mapping conditions were refined to improve the resolution of this region. In particular, it was found that increasing the electrophoresis time in the first dimension to 7 h, instead of the more commonly used 2−4 h (Tavare & Denton, 1988; Smith & Sale, 1989), completely resolved S1 and S2 from C1 (Figure 2). Phosphotyrosine and phosphoserine were still, however, recovered from both S1 and S2 with the phosphoserine/phosphotyrosine ratios being 0.96 and 1.50 for S1 and S2, respectively (Table 2). S1 and S2 were barely evident in maps obtained for phosphorylations performed in the absence of insulin (Figure 2A). B1 and C3 were also found to contain phosphoserine (Table 2). Neither B1 nor C3 were detected when insulin were absent during the phosphorylation (Figure 2A). Pillay and Siddle (1991) have also observed the insulin-dependent presence of phosphoserine in B1. A variable amount of a phosphopeptide that contained exclusively phosphoserine and that migrated to the right of B1 was also obtained. This peptide is referred to as S3 in this paper. Preliminary experiments showed that S1 and S2 were not cleaved by protease V8 and were thus unlikely to originate from the juxtamembrane domain of the insulin receptor (Feener *et al.*, 1993). Of the serine-phosphorylated synthetic peptides, only 1290−1298 and 1071−1080 should be resistant to V8. One of these, 1290−1298, contains serines 1293/4 which are known serine phosphorylation sites. Phosphorylated and trypsinolyzed peptide 1290−1298 migrated markedly differently to S1 or S2 in two-dimensional maps (see below). Consequently, experiments were conducted with peptide 1071−1080.

Two-Dimensional Peptide Mapping of Phosphorylated Peptide 1071−1080 with Electrophoresis at pH 3.5. The peptide was phosphorylated by the copurified insulin receptor/insulin-stimulated insulin receptor serine kinase preparation for 2 h in the presence of insulin. The phosphorylated peptide was then digested with trypsin. Two-dimensional thin-layer phosphopeptide mapping, with electrophoresis at pH 3.5, of the phosphorylated and trypsinolyzed peptide 1071−1080 revealed two phosphopeptides that migrated analogously to authentic S1 and S2 generated from the insulin receptor phosphorylated *in vitro* (Figure 2B,C). Both the phosphopeptides produced from the synthetic peptide, like S1 and S2, contained phosphotyrosine and phosphoserine (Table 2).

Two-Dimensional Mapping of Insulin Receptor Phosphorylated in Vivo with Electrophoresis at pH 3.5. The pattern of phosphorylation, with an electrophoresis pH of 3.5, obtained for insulin receptor phosphorylated *in situ* within intact human Hep G2 hepatoma cells (Figure 3A,B) was broadly similar but not identical to that obtained for the human insulin receptor copurified with insulin-stimulated insulin receptor serine kinase activity and phosphorylated *in vitro* (Figure 2A,B). S1 and S2 were present as major phosphoseryl peptides derived from the *in vivo* phosphorylated insulin receptor. However, the phosphoserine content of S1 and S2 derived from insulin receptor phosphorylated *in vivo* was 81−86% of the total phosphoamino acids recovered, which was significantly higher than that obtained for the insulin receptor phosphorylated *in vitro* (Table 2). The serine phosphorylation of B1 observed *in vivo* was similar to that obtained *in vitro*; however, there was less phosphorylation of the C1′ region or of C3 observed with the insulin receptor phosphorylated in the Hep G2 cells compared with that obtained with the insulin receptor phosphorylated *in vitro*. Little insulin receptor phosphorylation was observed *in vivo* in the absence of insulin, in line with several other studies (Pillay & Siddle, 1991; Tavare *et*

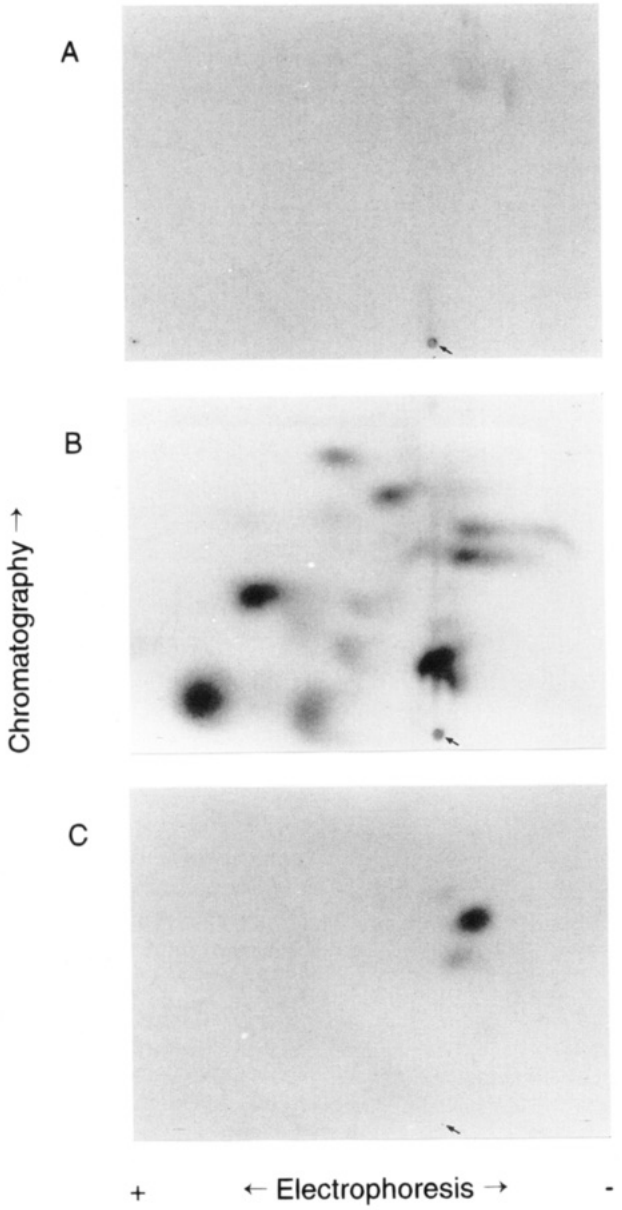

FIGURE 3: Two-dimensional tryptic peptide mapping with an electrophoresis pH of 3.5 of the insulin receptor phosphorylated in Hep G2 cells in the absence (A) or presence of insulin (B) and of the phosphorylated peptide 1071−1080 (C). The peptide was phosphorylated using the insulin receptor copurified with the insulin-stimulated insulin receptor serine kinase activity by incubation for 2 h at 22 °C in the presence of insulin and digested with trypsin. The arrows mark the origins of sample application. The results are representative of three experiments.

al., 1991). The positions of the phosphopeptides derived from the phosphorylated and trypsinolyzed peptide 1071−1080 again matched those of S1 and S2 (Figure 3B,C). The S2/S1 ratio for the insulin receptor phosphorylated *in vivo* in Figure 3B was higher than that obtained for the insulin receptor phosphorylated *in vitro* in Figure 2B and that obtained for the corresponding products of peptide 1071−1080 (Figures 2C and 3C). This appears to be due to different extents of trypsinolysis (see below). Both the receptor-derived S1/S2 and the phosphopeptides derived from the phosphorylated and trypsinolyzed peptide 1071−1080 had a tendency to streak horizontally in some maps, consistent with the receptor-derived and synthetic peptide-derived phosphopeptides arising from the same sequence.

Comigration of Phosphopeptides from Phosphorylated and Trypsinolyzed Peptide 1071−1080 with S1 and S2 and Two-Dimensional Mapping with Electrophoresis at pH 1.9. In order to further establish the identity of S1 and S2, the two-dimensional tryptic peptide mapping conditions were changed such that the electrophoresis dimension was performed at pH 1.9 instead of pH 3.5. A doublet of phosphopeptides that contained both phosphoserine and phosphotyrosine was again produced from the insulin receptor phosphorylated *in vitro*, and these were located in the position anticipated if they were S1 and S2 (Figure 4A,B, Table 2). Analysis of the phosphorylated and trypsinolyzed peptide 1071−1080 by two-dimensional mapping with electrophoresis at pH 1.9 again revealed two phosphopeptides whose mobilities appeared identical to those of S1 and S2 (Figure 4C). Mixing phosphorylated and trypsinolyzed insulin receptor with phosphorylated and trypsinolyzed peptide 1071−1080 such that the amounts of receptor- and peptide-derived S1/S2 ^{32}P were approximately equal revealed in maps performed with an electrophoresis pH of both 1.9 (Figure 4D) and 3.5 (not illustrated) that the receptor-derived and synthetic peptide-derived phosphopeptides comigrated exactly. Use of other mix ratios in the range 1:0.2 to 0.2:1 (insulin receptor S1/S2 ^{32}P-peptide S1/S2 ^{32}P) confirmed the comigration.

Radiosequenation. Phosphorylated peptide 1071−1080 was phosphorylated on both tyrosine and serine. Tyrosine is present in peptide 1071−1080 only at position 1075 which thus has to be the site of tyrosine phosphorylation of the peptide. Serine is present in peptide 1071−1080 at two positions, 1074 and 1078. To distinguish which serine was phosphorylated, the phosphorylated peptide was subjected to radiosequenation. ^{32}P was released at cycles 5 and 8 (Figure 5A). Thus, serine 1078 in addition to tyrosine 1075 was phosphorylated.

The two-dimensional mapping experiments presented above showed that trypsinolysis of phosphorylated peptide 1071−1080 yielded two ^{32}P-labeled spots, each containing phosphoserine and phosphotyrosine, which comigrated exactly with S1 and S2 at two pHs. To confirm that serine 1078 was a site of phosphorylation in the insulin receptor and that S1 and S2 were derived from the 1074−1080 region of the insulin receptor, S1 and S2 prepared by *in situ* labeling of the insulin receptor and isolation by two-dimensional tryptic peptide mapping were subjected to radiosequenation. If phosphorylation occurred on serine 1078 and tyrosine 1075, the 1074−1080 region of the β-subunit potentially may yield the following tryptic phosphopeptides: 1074−1077 [SY(P)LR], 1078−1080 [S(P)LR], and if trypsinolysis was inhibited by the nearby phosphorylation 1074−1080 [SY(P)LRSLR or SYLRS(P)LR or SY(P)LRS(P)LR]. Radiosequenation of S2 showed release of ^{32}P at cycles 2 and 5, entirely consistent with S2 having the sequence 1074−1080 and being phosphorylated on serine 1078 and/or tyrosine 1075 (Figure 5C). There are no other β-subunit serine-containing tryptic sequences which upon radiosequenation would be expected to release ^{32}P at both these cycles. As the release of ^{32}P at the position corresponding to serine 1078 was 6.1-fold greater than at the position corresponding to tyrosine 1075, it was unlikely that S2 was bisphosphorylated on both serine 1078 and tyrosine 1075. This is supported by the results of phosphoamino acid analysis of S2 (Table 2). Thus, S2 is a mixture of SYLRS(P)LR and SY(P)LRSLR.

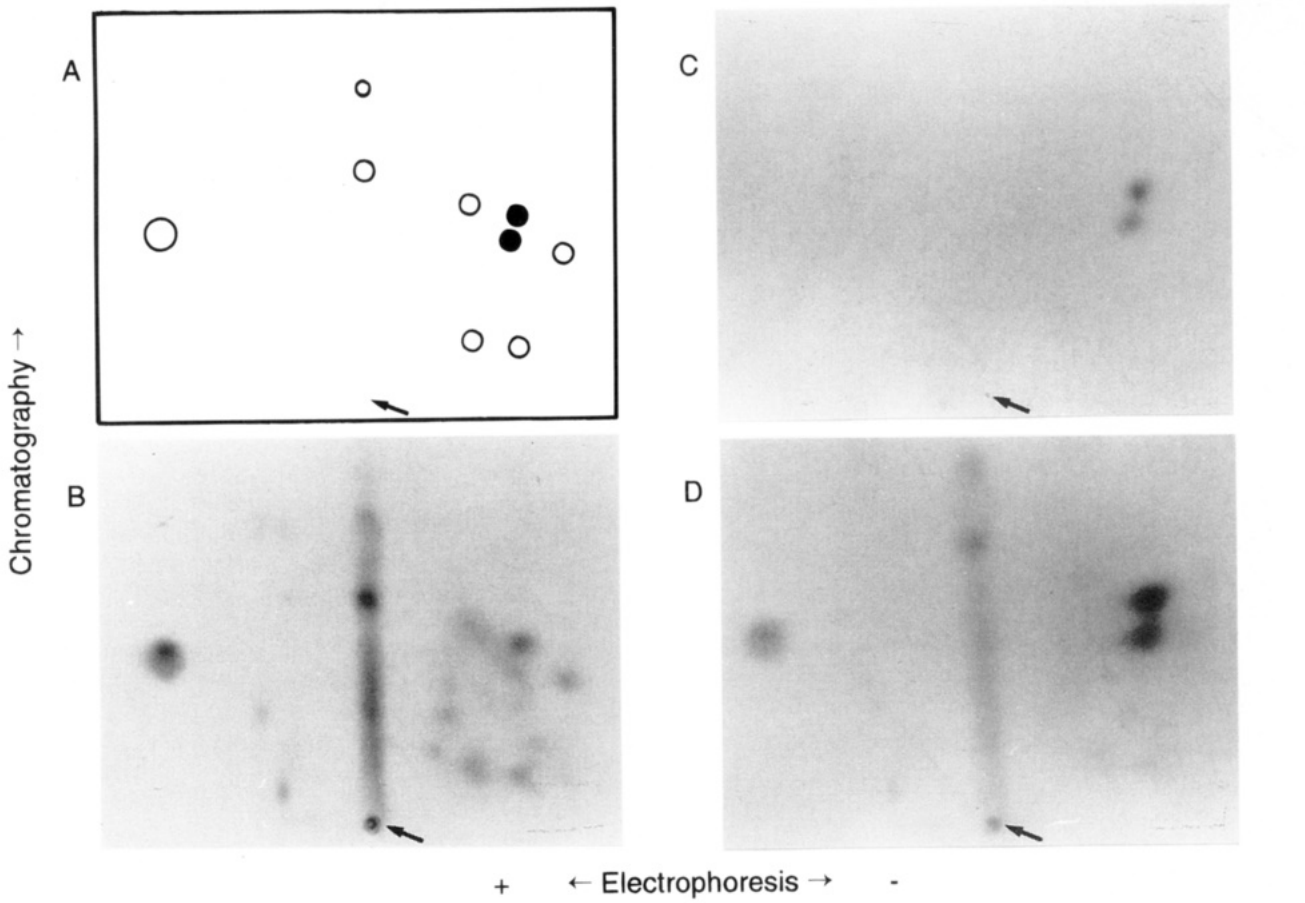

FIGURE 4: Two-dimensional tryptic peptide mapping with an electrophoresis pH of 1.9. (A) Key showing the positions of S1 and S2 (indicated by the black spots) under these mapping conditions. (B) The human insulin receptor/insulin-stimulated insulin receptor serine kinase preparation phosphorylated *in vitro* in the presence of insulin. (C) Phosphorylated peptide 1071−1080. The peptide was phosphorylated using the insulin receptor copurified with the insulin-stimulated insulin receptor serine kinase activity by incubation for 2 h at 22 °C in the presence of insulin and digested with trypsin. (D) Mixture of the trypsinolyzed human insulin receptor/insulin-stimulated insulin receptor serine kinase preparation phosphorylated *in vitro* in the presence of insulin and the trypsinolyzed phosphorylated peptide 1071−1080 (prepared as in panel C). The mixture contained approximately equal amounts of receptor- and peptide-derived S1/S2 ^{32}P. The arrows mark the origins of sample application. The results are representative of three experiments.

As S2 represented the peptide sequence in which trypsinolysis was inhibited after arginine 1077, it seemed reasonable to anticipate that S1 would be the fully trypsinolyzed version of the peptide. The likelihood of this possibility was reinforced by time course studies which showed that S1 was generated from S2; for example, exhaustive trypsinolysis (18 h including a further addition of 100 μg of trypsin after 6 h) converted nearly all the S2 into S1 such that S2 was reduced to <10% of the total of the two spots (not illustrated). If S1 contained S(P)LR (1078−1080), ^{32}P should be released at cycle 1; this was found (Figure 5B). S1 also contained some phosphotyrosine (Table 2). As S2 was phosphorylated on tyrosine 1075, complete trypsinolysis would also yield SY(P)LR (1074−1077). SY(P)LR is of similar sequence and charge to S(P)LR, and these two phosphopeptides appeared to comigrate under our mapping conditions (see the Discussion). S1 being a mixture of SY(P)LR and S(P)-LR was entirely consistent with the radiosequenation results for S1 as ^{32}P was also released at cycle 2. It seems less likely that all the release of ^{32}P at cycle 2 was due to a trail from phosphorylated serine 1078 as only a minor trail was evident from phosphorylated serine 1078 during radiosequenation of S2 (Figure 5C).

Two-Dimensional Mapping of Other Serine/Threonine Phosphorylated Synthetic Peptides with Electrophoresis at pH 3.5. The phosphopeptides 1253−1271, 1290−1298, 1313−1329, and 1333−1343 were phosphorylated by incubation with the insulin receptor copurified with insulin-stimulated insulin receptor serine kinase activity and insulin for 2 h. The phosphorylated peptides were digested with trypsin prior to further analysis. Peptide 1333−1343 contains arginine 1333 which was included to facilitate separation of the phosphorylated peptide from [γ-^{32}P]ATP. As trypsin has relatively weak exopeptidase action, this peptide was also treated with Arg C to cleave off this arginine. Figure 6 illustrates the two-dimensional mapping of the resulting phosphopeptides and shows that none of the phosphopeptide products derived from peptides 1253−1271, 1290−1298, 1313−1329, or 1333−1343 migrated analogously to S1 or S2.

Peptide 1290−1298 contains serines 1293/4. These serines have previously been identified as sites of both insulin- and phorbol ester-stimulated phosphorylation (Lewis *et al.*, 1990a,b; Tavare *et al.*, 1991; Ahn *et al.*, 1993). Two-dimensional mapping of phosphorylated and trypsinolyzed peptide 1290−1298 yielded a phosphopeptide that migrated analogously to S3, in line with the synthetic peptide studies of Lewis *et al.* (1990a). Comigration was confirmed by mixing experiments (not illustrated). Similarly to the study of Tavare *et al.* (1991), S3 was a relatively minor phosphopeptide with the *in vivo* labeled insulin receptor.

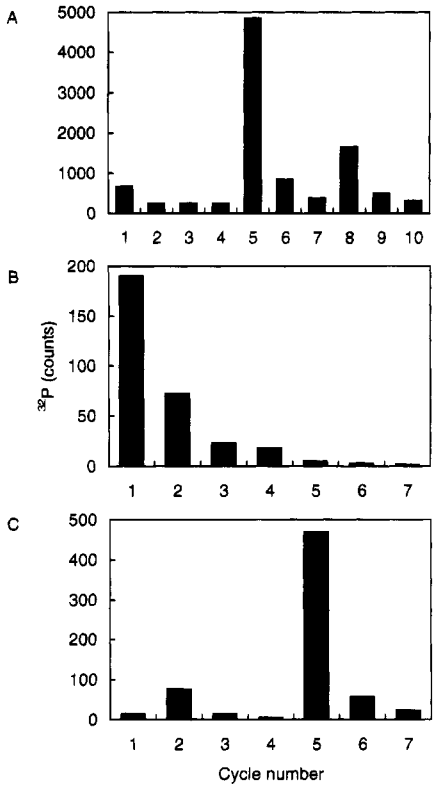

FIGURE 5: Radiosequenation of phosphorylated peptide 1071−1080 (A), S1 (B), and S2 (C). The peptide was phosphorylated using the insulin receptor copurified with the insulin-stimulated insulin receptor serine kinase activity by incubation for 2 h at 22 °C in the presence of insulin and isolated by AG1-X2 chromatography and electrophoresis at pH 3.5 on a cellulose thin-layer plate. Release of ^{32}P at cycles 5 and 8 shows phosphorylation on tyrosine 1075 and serine 1078. Phosphoamino acid analysis of the phosphorylated peptide used in this experiment gave a phosphoserine/phosphotyrosine ratio of 0.28, which agrees well with the ratio of ^{32}P released at cycles 8 and 5 of 0.37. S1 and S2 were prepared by *in situ* phosphorylation of the human insulin receptor in the presence of insulin and isolated by two-dimensional tryptic peptide mapping.

Phosphoamino acid analysis of phosphorylated peptide 1313−1329 showed the presence of both phosphotyrosine and phosphoserine with 89.9 ± 2.8% and 10.1 ± 2.8% of the ^{32}P recovered in phosphoamino acids present as phosphotyrosine and phosphoserine, respectively (values are means ± SEM for three observations). The presence of both phosphotyrosine and phosphoserine in the phosphorylated peptide was in line with the presence of both these phosphoamino acids in B1 derived from phosphorylated insulin receptor (Table 2). Both the peptide and B1 contain tyrosines 1316 and 1322, which are major tyrosine autophosphorylation sites in the insulin receptor. The only serine present in the peptide is serine 1315, which must be the site of serine phosphorylation. B1 also contains serine 1315. Thus, serine 1315 appears to be a site of insulin-stimulated phosphorylation in the insulin receptor. Two-dimensional mapping of the phosphorylated and trypsinolyzed peptide 1313−1329 revealed three phosphopeptides, none of which migrated near B1. The major phosphopeptide, which contained both phosphoserine and phosphotyrosine (not illustrated), migrated toward the cathode and appeared analogous to NP1 (King & Sale, 1990). NP1 represents a singly phosphorylated product of the C-terminal domain which is phosphorylated on tyrosine 1322 and which has only been detected during the dephosphorylation of autophosphorylated insulin receptor. Thus, it appears that the inability to reproduce the production of B1 by *in vitro* phosphorylation of peptide 1313−1329 was because of a difficulty of phosphorylating the peptide sufficiently. Alternate tryptic cleavage may be responsible for the production of the other tryptic phosphopeptides from the phosphorylated peptide 1313−1329.

Peptide 1333−1343 was only phosphorylated on threonine. This peptide contains threonine 1336 which has been identified as a site of both insulin- and phorbol ester-stimulated phosphorylation in the insulin receptor (Koshio *et al.*, 1989; Lewis *et al.*, 1990b). On two-dimensional peptide mapping, the phosphorylated and digested peptide migrated to the C1′ region of the insulin receptor map, a region that was sometimes observed to contain threonine when the insulin receptor phosphorylated *in vitro* was used (Table 2). Mixing experiments confirmed comigration with the phosphothreonine region of C1′ (not illustrated). The threonine phosphorylation was not observed for the insulin receptor phosphorylated *in vivo*. Variable phosphorylation of the threonine has previously been reported both *in vitro* and in various cell types (Smith & Sale, 1989; Pillay & Siddle, 1991; Issad *et al.*, 1991; Feener *et al.*, 1993).

Phosphorylated peptide 1253−1271, which was phosphorylated on serine *in vitro*, yielded a phosphopeptide that again migrated to the C1′ region. However, only minor phosphorylation of the C1′ region on serine was detected *in vitro*, and none was seen *in vivo*.

DISCUSSION

In the present work, a synthetic peptide approach was used to study the identity of the sites of insulin-stimulated serine phosphorylation in the insulin receptor. This approach had the advantage that peptide substrates identified could be used in the purification and characterization of the insulin-stimulated insulin receptor serine kinase. Peptides were synthesized that together contained all the serine residues in the cytosolic portion of the β-subunit of the insulin receptor. The peptides were synthesized as tryptic or extended tryptic sequences. The ability of the peptides to be serine phosphorylated by a preparation of insulin receptor copurified with insulin-stimulated insulin receptor serine kinase activity was tested. Of the 30 serine residues present within the peptides, serines in only 4 peptides were phosphorylated (Table 1). The peptide phosphorylation on serine was stimulated 2−4-fold by insulin. The phosphorylated peptides were analyzed by two-dimensional tryptic peptide mapping and compared with two dimensional tryptic peptide maps of the human insulin receptor phosphorylated *in vitro* or *in vivo* in Hep G2 cells.

The two-dimensional maps obtained for the insulin receptor phosphorylated *in vitro* (Figure 2A,B) and *in vivo* (Figure 3A,B) were similar but not identical. Both showed the presence of phosphotyrosine-containing peptides derived from the tyrosine 1150 and C-terminal domains of the β-subunit. The phosphotyrosine-containing peptides that are believed to originate from the juxtamembrane domain region of the β-subunit were less evident for the receptor phosphorylated *in vivo* compared with the receptor phosphorylated *in vitro*, in agreement with studies of Tavare *et al.* (1988), White *et al.* (1988a,b), and Issad *et al.* (1991). The major phosphoserine-containing peptides were present as a pair of closely migrating phosphopeptides, S1 and S2, for the insulin

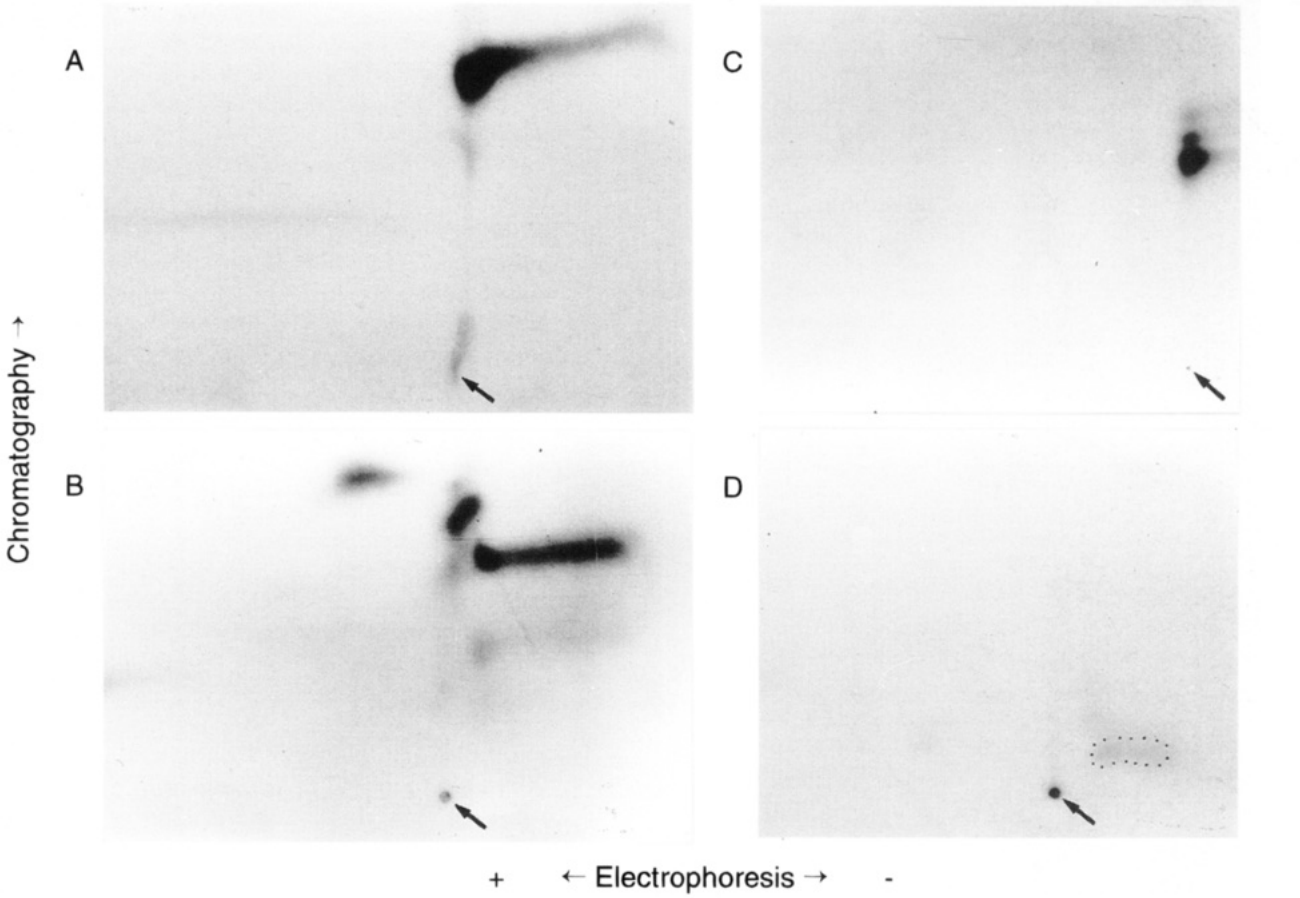

FIGURE 6: Two-dimensional peptide mapping with an electrophoresis pH of 3.5 of phosphorylated peptides 1333−1343 (A), 1313−1329 (B), 1253−1271 (C), and 1290−1298 (D). The peptides were phosphorylated using the insulin receptor copurified with the insulin-stimulated insulin receptor serine kinase activity by incubation for 2 h at 22 °C in the presence of insulin. The peptides were digested with trypsin. Peptide 1333−1343 was further digested with Arg C. The peptides were then subjected to two-dimensional mapping. In panel D, the position of the phosphorylated and trypsinolyzed peptide 1290−1298 is clarified by the dots. The arrows mark the origins of sample application. The results are representative of three experiments.

receptor phosphorylated both *in vitro* and *in vivo*. S1 and S2 also contained phosphotyrosine (Table 2). We were unable to remove the phosphotyrosine from S1 and S2 by increasing the electrophoresis time at pH 3.5 to 7 h compared with a normal 2−4 h (Tavare & Denton, 1988; Smith & Sale, 1989) or by altering the electrophoresis pH to 1.9. Whereas S1 and S2 derived from the insulin receptor phosphorylated *in vivo* yielded 81−86% of the recovered phosphoamino acids as phosphoserine, significantly more phosphotyrosine was obtained for the peptides derived from the insulin receptor phosphorylated *in vitro*. S1 and S2 were not digested by protease V8, indicating that they did not arise from the juxtamembrane domain of the insulin receptor (Feener *et al.*, 1993). Phosphoserine was also present in the C-terminal phosphopeptide, B1, and in a peptide called S3 (Table 2). With maps of the insulin receptor phosphorylated *in vitro*, C3 also contained phosphoserine, and the C1′ region, which comprised a cluster of poorly resolved phosphopeptides, contained a small amount of phosphoserine and with some preparations of the insulin receptor phosphothreonine as well (Table 2). As noted above, C3 and C1′ were not prominent with the insulin receptor phosphorylated *in vivo*. The variable threonine phosphorylation is in line with the observed threonine phosphorylation of the insulin receptor in a number of cell lines which have been transfected with the insulin receptor (Tavare *et al.*, 1988), the lack of the threonine phosphorylation of the insulin receptor in hepatocytes (Issad *et al.*, 1991), and the variable recovery of insulin-stimulated insulin receptor threonine kinase activity during the isolation of the insulin receptor (Smith & Sale, 1989; Pillay & Siddle, 1991; present work).

The two peptides which acted as the best substrates for serine phosphorylation *in vitro* by the insulin receptor copurified with insulin-stimulated insulin receptor serine kinase activity were peptides 1290−1298 and 1071−1080. The former peptide contained known serine phosphorylation sites of the insulin receptor, serines 1293/4 (Lewis *et al.*, 1990a; Tavare *et al.*, 1991; Ahn *et al.*, 1993), and the phosphorylated and trypsinolyzed peptide migrated to the same position as phosphopeptide S3. However, as found by Tavare *et al.* (1991), serines 1293/4 were relatively minor sites of insulin receptor serine phosphorylation *in vivo*.

Phosphorylated and trypsinolyzed peptide 1071−1080 generated two phosphorylated spots on two-dimensional maps which comigrated with the authentic insulin receptor-derived phosphopeptides, S1 and S2, at two pHs. Importantly, the two synthetic peptide-derived spots, like S1 and S2, contained both phosphoserine and phosphotyrosine. Thus, the production of S1 and S2 was mimicked exactly by phosphorylation and trypsinolysis of peptide 1071−1080. Peptide 1071−1080 contains serine at positions 1074 and 1078 and tyrosine at position 1075. Radiosequenation of the phosphorylated peptide showed phosphorylation at positions 1075 and 1078. Additionally, phosphorylation at just residues 1074 and 1075 was not consistent with the production of both S1 and S2 and the results of the phosphoamino

acid analyses of S1 and S2 (Table 2). To further prove that serine 1078 was a site of phosphorylation in the insulin receptor, S1 and S2 from *in situ* phosphorylated human insulin receptor were subjected to radiosequenation. With the S2 phosphopeptide, ^{32}P was released at cycles 2 and 5 in the ratio of 1:6.1. There are no other β-subunit serine-containing tryptic sequences which upon radiosequenation would be expected to release ^{32}P at both these cycles. The radiosequenation data together with the comigration experiments show that S2 comprises a mixture of SY(P)LRSLR and SYLRS(P)LR. The unequal release of ^{32}P at cycles 2 and 5 and the results of phosphoamino acid analysis of S2 (Table 2) show that the peptide was not bisphosphorylated on both serine 1078 and tyrosine 1075. These results show that serine 1078 is a major site of insulin-stimulated serine phosphorylation. S2 is an incomplete tryptic cleavage product in which trypsinolysis after arginine 1077 has been inhibited by the presence of phosphate at tyrosine 1075 or serine 1078. Inhibition of cleavage of peptides by trypsin as a consequence of phosphorylation is well-known. For example, trypsinolysis of the insulin receptor peptide 1144−1153 (DIYETDYYRK) between the R and K is inhibited by upstream phosphorylation of either tyrosine 1150 or tyrosine 1151 whereas the cleavage before residue 1144 is not inhibited by downstream phosphorylation of tyrosine 1146, analogously to that with the 1071−1080 sequence. Because S1 was shown to be generated from S2 by time course studies and because S2 was an incompletely cleaved tryptic phosphopeptide, it was anticipated that S1 would comprise the completely cleaved products, S(P)LR and SY(P)LR, the latter being most evident when the receptor was phosphorylated *in vitro*. In support of this, we observed that exhaustive trypsinolysis (18 h including a further addition of 100 μg of trypsin after 6 h) converted nearly all the S2 into S1 such that S2 was reduced to <10% of the total of the two spots, indicating that S(P)LR and SY(P)LR do indeed comigrate under our two-dimensional peptide mapping conditions (not illustrated). Radiosequenation of S1 gave release of ^{32}P at cycles 1 and 2, as expected if S1 comprised these phosphopeptides. The release of ^{32}P at cycle 2 was greater than that attributable to the trail from cycle 1 (see Results). Although serine 1074 was not phosphorylated in the synthetic peptide, it could be argued that the specificity of the insulin-stimulated insulin receptor serine kinase toward the intact β-subunit is different than that against the peptide and thus that it is possible that a fraction of S1 (i.e., part or all of any produced by direct trypsinolysis rather than via S2) was phosphorylated on serine 1074. As the S2 to S1 ratio obtained with the *in vivo* labeled insulin receptor was 3 ± 0.7 (mean ± SEM, n = 3) to 1 under normal trypsinolysis conditions, and S2 was clearly phosphorylated on serine 1078 and not serine 1074, the results show that serine 1078 was the major serine phosphorylation site *in vivo* even allowing for this possibility.

The identity of serine 1078 as a site of insulin-stimulated serine phosphorylation is supported by our studies with the rat liver insulin receptor copurified with insulin-stimulated insulin receptor serine kinase activity (W. G. Carter, K. A. Asamoah, A. Sullivan, and G. J. Sale, unpublished results). Peptide 1071−1080 of the rat insulin receptor has the sequence DLKSHLRSLR and thus has tyrosine 1075 replaced with a histidine. The rat insulin receptor yielded S1 but not S2, with the rat derived S1 being exclusively phosphorylated on serine, consistent with S1 containing S(P)LR. With the rat liver insulin receptor, a phosphopeptide that migrated to the position of S2 was not found. This is consistent with the replacement of tyrosine 1075 with a histidine in the rat insulin receptor yielding a more positively charged peptide that migrates further toward the cathode than S2. The identities of S1 and S2 also account for the different phosphoserine/phosphotyrosine ratios in S1 and S2 derived from the insulin receptor phosphorylated *in vitro* and *in vivo* and for the corresponding synthetic peptide-derived phosphopeptides, and for the varying ratios of S1/S2 and the synthetic phosphopeptide products observed as a consequence of differing extents of trypsinolysis.

The sequence SYLRSLR is conserved in the human IGF 1 receptor. The results also indicate that tyrosine 1075 of the human insulin receptor can act as a site of insulin-stimulated phosphorylation although it was rather minor in the case of the insulin receptor phosphorylated in Hep G2 cells. Phosphorylation of tyrosine 1075 could account for some of the unidentified tyrosine phosphorylation noted in earlier studies (Tornqvist *et al.*, 1987, 1988). Feener *et al.* (1993) have also observed the insulin-dependent presence of phosphoserine and phosphotyrosine in an insulin receptor-derived peptide they call I3, and analogously to the situation reported herein with Hep G2 cells, the dominant phosphorylation of I3 was on serine. I3 was obtained both from *in vivo* labeled insulin receptor using cells transfected with the receptor and from *in vitro* labeled insulin receptor (Feener *et al.*, 1993, 1994).

The insulin receptor is known to undergo insulin-stimulated serine phosphorylation in the juxtamembrane domain although the extent of phosphorylation reported for this region has been variable. Using intact cells transfected with the insulin receptor, Feener *et al.* (1993) reported that the juxtamembrane domain was the most heavily serine-phosphorylated domain of the insulin receptor and that the serine phosphorylation of the juxtamembrane domain was higher than that attributable to I3. The juxtamembrane serine phosphorylation was eliminated in a mutant insulin receptor lacking residues 954−965 and thus lacking serines 955, 956, 962, and 964 (Feener *et al.*, 1993). In contrast, another study in which serines 955 and 956 or 962, and 964 of the juxtamembrane region were mutated indicated that serines 955, 956, 962, and 964 together accounted for only ~20% of the insulin-stimulated serine phosphorylation of the insulin receptor in cells overexpressing the receptor (Liu & Roth, 1994). Additionally, in the present work, phosphopeptides C3 and C1′, which are believed to arise from the juxtamembrane domain, were not prominent with the insulin receptor phosphorylated in Hep G2 cells, in line with studies of Tavare *et al.* (1988) and White *et al.* (1988a,b).

Phosphopeptides corresponding to S1 and S2 are not obviously present in the two-dimensional tryptic peptide maps of Tavare *et al.* (1991) obtained using COS cells transfected with the insulin receptor. Phosphopeptides that may correspond to S1 and S2 are present in two-dimensional tryptic maps of the insulin receptor from ^{32}P-labeled and insulin-stimulated hepatocytes (Issad *et al.*, 1991).

The variations in the serine phosphorylation presumably reflect differences in the activities of the serine/threonine kinases and phosphatases in the different cellular backgrounds (Feener *et al.*, 1994). Cell types used for insulin receptor transfection have been chosen because they have

few native insulin receptors. Such cells may not always have sufficient amounts of the appropriate insulin-stimulated insulin receptor serine kinase to achieve phosphorylation of serine 1078; this may account for the apparent absence of S1/S2 in the two-dimensional maps of Tavare *et al.* (1991) in which COS cells transfected with the insulin receptor were used.

Some serine phosphorylation of C3 derived from *in vitro* labeled insulin receptor was found in the present work. We were unable to duplicate the serine phosphorylation of the juxtamembrane domain *in vitro* using a synthetic juxtamembrane peptide. This was tried using the tryptic sequence 944−981, but this peptide was not a substrate for either tyrosine or serine phosphorylation by the insulin receptor/insulin-stimulated insulin receptor serine kinase preparation. Studies with rhodopsin kinase substrate peptides have shown that the phosphorylation efficiency can markedly decrease as the peptide length increases (Brown *et al.*, 1992). Peptide 944−981 is a 38-mer and thus may not have adopted a suitable conformation for phosphorylation.

The insulin-stimulated phosphorylation of the insulin receptor on threonine 1336 (Koshio *et al.*, 1989; Lewis *et al.*, 1990b; Tavare *et al.*, 1991) was confirmed using synthetic peptide 1333−1343. A number of observations indicate that the insulin-stimulated insulin receptor serine kinase may not be responsible for the threonine phosphorylation. These include mutagenesis studies (Tavare & Dickens, 1991), the fact that some insulin receptor preparations undergo insulin-stimulated serine phosphorylation but not insulin-stimulated threonine phosphorylation (Pillay & Siddle, 1991), and the inconsistent recovery of the insulin-stimulated threonine kinase activity but the consistent recovery of the insulin-stimulated serine kinase activity in the present work.

It has previously been speculated that serine 1315 is a site of insulin-stimulated phosphorylation on the insulin receptor (Pillay & Siddle, 1991). However, as the C-terminal peptide region was heavily tyrosine-phosphorylated, it was unclear whether the presence of the serine was due to a comigrating peptide. The results herein add weight to the idea that serine 1315 is a site of insulin-stimulated phosphorylation by showing the serine phosphorylation of synthetic peptide 1313−1329 by the insulin receptor copurified with insulin-stimulated insulin receptor serine kinase activity. It was not, however, possible to duplicate the appearance of B1 using the phosphorylated and trypsinolyzed peptide, and this probably reflects an inability to more than singly phosphorylate the peptide *in vitro*. Nonetheless, it is noteworthy that one of the serine-phosphorylated phosphopeptides migrated to the same position as a singly phosphorylated product from the C-terminal region that has only been detected during dephosphorylation (King & Sale, 1990). Evidence that serine 1315 of the insulin receptor is phosphorylated in response to phorbol ester treatment has been obtained using cells transfected with the insulin receptor (Coghlan *et al.*, 1994; Feener *et al.*, 1994; Lewis *et al.*, 1994).

In summary, the results show that serine 1078 of the insulin receptor is a major site of insulin-stimulated serine phosphorylation. This is the first identification of a site of insulin-stimulated insulin receptor serine phosphorylation in a cell type that has not been transfected with the insulin receptor. The peptide substrates described will facilitate the purification and characterization of the insulin-stimulated insulin receptor serine kinase or kinases, and the identification of sites of insulin-stimulated serine phosphorylation will help elucidate the function of insulin-stimulated serine phosphorylation of the insulin receptor.

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