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Analysis of insulin receptor phosphorylation sites in intact rat liver cells by two-dimensional phosphopeptide mapping

Predominance of the tris-phosphorylated form of the kinase domain after stimulation by insulin

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1. Insulin receptors were partially purified from rat liver by chromatography on wheat-germ-lectin–Sepharose. Incubation with [γ-^{32}P]ATP in the presence of insulin resulted in increased phosphorylation of the β-subunit on both tyrosine and serine residues. Two-dimensional mapping of tryptic peptides showed that, in agreement with previous studies using preparations of receptors from other sources, the tyrosine residues involved were the three tyrosines in the kinase domain (corresponding to tyrosines 1158, 1162 and 1163 of the human receptor) plus two tyrosines close to the *C*-terminus (corresponding to tyrosines 1328 and 1334). 2. The effects of insulin on the phosphorylation of receptors within intact rat liver cells were determined by incubating cells in the presence of [^{32}P]P_i for 50 min and then with or without insulin for a further 10 min. The labelled receptors were then rapidly isolated by sequential use of wheat-germ-lectin–Sepharose chromatography and immuno-isolation using a monoclonal antibody to the *C*-terminal end of the β-subunit. 3. Insulin was found to increase overall phosphorylation of the receptor nearly 3-fold. Two-dimensional mapping was then carried out in combination with phosphoamino acid analysis. This revealed that the pattern of phosphorylation of the receptors in cells incubated in the absence and presence of insulin exhibited a number of marked differences from that observed in previous studies on intact cells, which had been restricted to cells expressing very high levels of insulin receptors such as certain hepatoma-derived cells or cells transfected with insulin receptor cDNA. The differences in the effects of insulin included a larger increase in the proportion of receptors being phosphorylated on the three tyrosine residues of the kinase domain, no apparent phosphorylation of the two tyrosine residues close to the *C*-terminus and no increase in either threonine or overall serine phosphorylation. 4. The receptors appeared to be phosphorylated on a number of different serine residues in cells incubated in the absence of insulin. Evidence for both increases and decreases in the phosphorylation of specific serine residues on addition of insulin was obtained. 5. It is concluded that care should be taken when extrapolating findings on the phosphorylation of the insulin receptor within cultured cells to more physiological situations.

INTRODUCTION

It is generally agreed that the earliest event that can be detected after the exposure of intact cell preparations to insulin is the increased phosphorylation of the insulin receptor itself. The intracellular domain of the β-subunit of the receptor exhibits tyrosine protein kinase activity which is greatly stimulated following the binding of insulin to the extracellular α-subunit (Kasuga *et al.*, 1982*a*,*b*; Van Obberghen & Kowalski, 1982). This stimulation results in the rapid autophosphorylation of multiple tyrosine residues within the β-subunit, which in turn results in the activation of the tyrosine protein kinase activity towards other substrates. This mechanism probably initiates the sequence of events that results in the wide range of intracellular changes that are observed in cells exposed to insulin (for reviews see Denton, 1986; Rosen, 1987; Kahn & White, 1988; Zick, 1989). Much evidence in favour of this view has been obtained in recent years, including experiments which have demonstrated that the intracellular effects of insulin are impaired in cells in which the tyrosine kinase activity of the receptor is specifically altered, either by site-directed mutagenesis or by micro-injection of monoclonal antibodies (Morgan *et al.*, 1986; Ebina *et al.*, 1987; Chou *et al.*, 1987; McClain *et al.*, 1987; Morgan & Roth, 1987). There is also mounting evidence that the kinase activity of the insulin receptor extracted from cells of rodent and human tissue exhibiting insulin-resistance may be impaired (Le Marchand-Brustel *et al.*, 1985; Shargill *et al.*, 1986; Caro *et al.*, 1987; Reddy & Kahn, 1988).

Further understanding of the mechanism of activation of the receptor by insulin requires the detailed analysis of the phosphorylation sites of the insulin receptor. Using purified preparations of the insulin receptor incubated with [^{32}P]ATP in the presence of insulin, it has been shown that the tyrosine phosphorylation occurs on up to seven tyrosine residues arranged in three domains. Phosphorylation of tyrosines 1158, 1162 and 1163 in the kinase domain occurs first, and this is associated with the stimulation of tyrosine kinase activity towards other substrates, followed by the phosphorylation of tyrosines 1328 and 1334 near the *C*-terminal domain and finally by the rather slow phosphorylation of probably two further tyrosines located in the juxtamembrane domain (tyrosines 965, 972 and 984) (Tavaré & Denton, 1988; Tornqvist & Avruch, 1988; White *et al.*, 1988).

The analysis of the sites of phosphorylation of the insulin receptor within intact cells is technically difficult. To date, such studies have been restricted to cultured cells expressing very high levels of either human or rodent insulin receptors, such as certain cell lines derived from hepatomas or cells transfected with the human insulin receptor cDNA. These studies have clearly demonstrated that insulin initiates the rapid phosphorylation of tyrosines 1158, 1162, 1163 and tyrosines 1328 and 1334, but probably not the phosphorylation of tyrosines 965, 972 and 984.

Abbreviation used: Dnp, dinitrophenyl.

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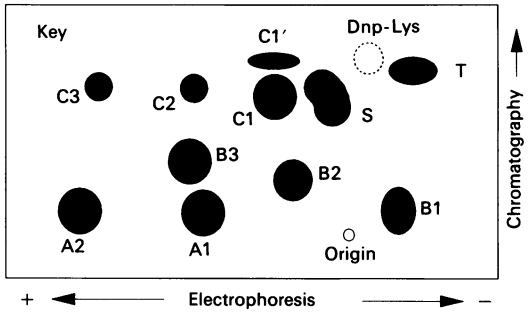


Fig. 1. Key to phosphopeptides derived from the human insulin receptor by digestion with trypsin and separated by two-dimensional t.l.c.

Taken from Tavaré *et al.* (1988). Autophosphorylation of tyrosines 1158, 1162 and 1163 in the kinase domain can give rise to a family of five phosphopeptides (general sequence Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys) which are mono- (C1), bis- (B2 and B3) or tris- (A1 and A2) phosphorylated and cleaved by trypsin at Arg-1155 and either Arg-1164 (C1, B3 and A2) or Lys-1165 (B2 and A1). Phosphorylated tyrosines 1328 and 1334 are recovered as a bis-phosphorylated peptide (sequence Ser-Tyr-Glu-Glu-His-Ile-Pro-Tyr-Thr-His-Met-Asn-Gly-Gly-Lys) that runs as peptide B1. Additional tyrosine phosphorylation sites (probably representing the phosphorylation of tyrosines 965, 972 and 984) run as peptides C1', C2 and C3. Within intact CHO and NIH-3T3 cells transfected with human insulin receptor cDNA, the major sites of serine and threonine phosphorylation appear to occur within two distinct peptides which run in positions S and T respectively.

In addition, increased phosphorylation of the receptor on both serine and threonine residues is observed in response to insulin (White *et al.*, 1985, 1988; Tornqvist *et al.*, 1988; Tavaré & Denton, 1988). These phosphorylations are presumably brought about by protein kinases that are distinct from the receptor itself, and probably play an important role in the signalling system involved in the action of insulin. The sites of threonine and serine phosphorylation are still areas of debate, but probably include serines 1305 and/or 1306 and threonine 1348 close to the *C*-terminus of the β-subunit (Koshio *et al.*, 1989; Lewis *et al.*, 1990*a*,*b*). It has been suggested that the increased phosphorylation of these sites may result in the diminution of kinase activity and hence be part of a feed-back inhibitory loop; it is also possible that increased phosphorylation of these sites may underlie the diminution of kinase activity associated with insulin-resistance (Kahn & White, 1988).

It is clearly important to explore the phosphorylation of the insulin receptor in physiologically relevant cells, since the cells used to date may have abnormal ratios of insulin receptors to other cell constituents, including phosphoprotein phosphatases and serine/threonine kinase. Moreover, the mechanisms underlying insulin-resistance need to be studied in cells derived directly from intact tissues. However, such studies are difficult because normal liver and fat cells contain only about 25000 receptors per cell, and thus a highly sensitive technique is required to explore changes in phosphorylation of all of the tyrosine, serine and threonine sites.

In this paper, we report the first study showing the effects of insulin on the phosphorylation sites of insulin receptors within normal fresh rat liver cells. We have used a purification procedure giving a high recovery of the receptors and have analysed the phosphorylation sites by using a two-dimensional phosphopeptide mapping technique which is capable of much greater resolution and sensitivity than separation of peptides by h.p.l.c. (Tavaré & Denton, 1988; Tavaré *et al.*, 1988; Fig. 1). Some major differences in phosphorylation patterns are found compared with those previously observed in transformed or transfected cell lines.

EXPERIMENTAL

Materials

These were as in Tavaré & Denton (1988) and Tavaré *et al.* (1988). In addition, collagenase (batches 11391821-84 and 11444923-85) was from Boehringer, Mannheim House, Lewes, East Sussex, U.K., and aprotinin was from Sigma Chemical Co., Poole, Dorset, U.K.

Animals

Male Wistar rats (120–150 g) had free access to water and standard laboratory chow (Biosure diet; CRM). They were housed in a room with the light on from 07:00 h to 19:00 h. All experiments began between 10:00 h and 12:00 h.

Partial purification and phosphorylation of rat liver insulin receptors *in vitro*

Livers from 8–10 rats were homogenized at 4 °C with a glass–Teflon homogenizer in a buffer (2 ml/g of liver) containing 250 mM-sucrose, 20 mM-Mops, 2.5 mM-benzamidine, 1 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride and 1 μg/ml of each of aprotinin, pepstatin, antipain and leupeptin. The homogenates were rapidly filtered through gauze, diluted 3-fold in the homogenization buffer and centrifuged for 10 min at 1000 ***g*** and then at 3000 ***g***. The supernatant was then centrifuged for 90 min at 27000 ***g***. The crude membrane pellet was resuspended in the homogenization buffer (to a protein concentration of 20 mg/ml), and Triton X-100 (1%, w/v) was added. After 45 min at 0 °C, the extract was centrifuged for 30 min at 61000 ***g*** and the supernatant was used for partial purification of insulin receptors by wheat-germ-lectin–Sepharose chromatography (Tavaré & Denton, 1988).

Partially purified insulin receptors (50 μl) were preincubated for 15 min at 30 °C in a total volume of 160 μl containing 20 mM-Mops (pH 7.4), 0.25 mM-dithiothreitol, 1 mM-Na_3VO_4, 12 mM-$MgCl_2$ and 2 mM-$MnCl_2$ in the presence or absence of 1 μM-insulin. Autophosphorylation was initiated with [γ-^{32}P]ATP (100 μM, 500–5000 c.p.m./pmol) and carried out for 10 min at 30 °C. The reaction was terminated on ice by chelating divalent metals with 40 μl of a stock solution of EDTA (100 mM). The incubation mixture was then diluted by adding 500 μl of 50 mM-Hepes buffer (pH 7.6) containing 0.1% Triton X-100, 20 mM-EDTA, 10 mM-NaF, 30 mM-sodium pyrophosphate, 2 mM-benzamidine, 1 mM-Na_3VO_4, 1 mM-phenylmethanesulphonyl fluoride and 1 μg/ml of each of aprotinin, pepstatin, antipain and leupeptin.

Insulin receptor phosphorylation in intact hepatocytes

Hepatocytes were isolated by collagenase digestion of rat livers as described by Berry & Friend (1969) and modified by Thomas & Halestrap (1981), except that buffer containing collagenase (0.25 mg/ml) was not recycled but was continuously perfused through the liver. Cell viability was always higher than 90%, as judged by Trypan Blue exclusion. The cells (about 30×10^6 in 2 ml) were preincubated for 50 min at 37 °C with gentle shaking in stopped plastic containers, within lead shields, in bicarbonate-buffer medium (Krebs & Henseleit, 1932) containing 2.5 mM-$CaCl_2$, 3 mM-Hepes, 10 mM-glucose and [^{32}P]P_i (0.2 mM, 2 mCi/ml). The cells were then incubated for a further 10 min with or without added insulin (200 nM). The lead-shielded containers were transferred into liquid N_2 to rapidly cool the incubation to 2–4 °C. Simultaneously, the cells (and medium) were rapidly extracted in 2 ml of ice-cold Hepes buffer (100 mM, pH 7.6) containing 2% Triton X-100, 40 mM-EDTA, 20 mM-NaF, 60 mM-sodium pyrophosphate, 4 mM-benzamidine, 2 mM-

Na_3VO_4, 2 mM-phenylmethanesulphonyl fluoride and 2 μg/ml of each of aprotinin, pepstatin, antipain and leupeptin. After vigorous magnetic stirring at 2–4 °C for 10 min, wheat-germ-lectin–Sepharose was added (2 ml) and the incubation was continued for a further 40 min with gentle stirring. The mixture was poured into a column and washed with 50 ml of Hepes buffer (50 mM, pH 7.6) containing 0.1 % Triton X-100, 20 mM-EDTA, 10 mM-NaF, 30 mM-sodium pyrophosphate, 2 mM-benzamidine, 1 mM-Na_3VO_4, 1 mM-phenylmethanesulphonyl fluoride and 1 μg/ml of each of aprotinin, pepstatin, antipain and leupeptin (washing buffer). The partially purified insulin receptors were then eluted with 7 ml of the same buffer containing 0.3 M-*N*-acetylglucosamine and concentrated to 0.7 ml using a Centriprep-30 concentrator.

Immunopurification of partially purified insulin receptors

Receptors phosphorylated either *in vitro* or in intact cells were usually immunopurified using a monoclonal antibody (CT1 antibody). This antibody was kindly provided by Professor K. Siddle (University of Cambridge, Cambridge, U.K.) and was raised against a peptide (Tyr-Lys-Lys-Asn-Gly-Arg-Ile-Leu-Thr-Leu-Pro-Arg-Ser-Asn-Pro-Ser) corresponding to the *C*-terminus of the β-subunit of the human insulin receptor (R. Ganderton, M. Soos & K. Siddle, personal communication). The corresponding part of the rat insulin receptor only differs in one amino acid, the isoleucine being replaced by valine (Goldstein & Dudley, 1990). In some experiments, immunopurification was carried out with a polyclonal rabbit antibody (RIR) raised against purified intact insulin receptor from rat liver by Dr. K. Taylor and Professor K. Siddle. The antibodies were covalently linked to CNBr-activated Sepharose 4B CL as described by March *et al.* (1974) and stored at 4 °C, in a buffer containing 100 mM-Mops and 10 mM-NaN_3, for subsequent use. Partially purified receptor preparations (0.7 ml in washing buffer) were incubated with 0.12 ml of antibody–Sepharose for 2 h at 4 °C. The receptor–antibody–Sepharose complexes were isolated by centrifugation and washed three times with 1 ml of washing buffer and with 1 ml of this buffer diluted 10-fold in distilled water. The complexes were dissociated by boiling in SDS/PAGE sample buffer containing 1 % SDS and 200 mM-dithiothreitol.

Elution of ^{32}P-labelled insulin receptor β-subunit from polyacrylamide gels, separation of ^{32}P-labelled tryptic peptides and phosphoamino acid analysis

These procedures have been fully detailed in Tavaré & Denton (1988). Briefly, after SDS/PAGE (7 % gels), the phosphorylated β-subunit was located by radioautography, electroeluted, precipitated with acetone and digested with trypsin. The [^{32}P]phosphopeptides were separated on thin layer cellulose chromatography plates first by electrophoresis at 400 V for 2.5 h at pH 3.5 (pyridine/acetic acid/water, 1:10:189, by vol.) and then by ascending chromatography (pyridine/acetic acid/butanol/water, 10:3:15:12, by vol.), and were finally detected by radioautography using Kodak X-Omat pre-flashed film in cassettes with intensifying screens at −70 °C. Densitometric scanning of the radioautographs was carried out using a Chromoscan 3 (Joyce Loebl Ltd., U.K.) linked to a Hewlett–Packard series 300 computer.

Alternatively the [^{32}P]phosphopeptides were hydrolysed at 110 °C under vacuum in 6 M-HCl and separated by thin layer electrophoresis at pH 3.5 as described by Cooper *et al.* (1983), and phosphoamino acids were located by radioautography. Phosphoamino acid markers (phosphoserine, phosphothreonine and phosphotyrosine; 3 μg of each) were added to each sample and located by spraying the plates with ninhydrin solution and heating.

Expression of results

For ease of comparison of our data with that of the human insulin receptor, we have numbered the tyrosine residues and other amino acids in the β-subunit of the insulin receptor according to the human sequence described by Ebina *et al.* (1985). The amino acid sequence of the β-subunit of the rat receptor has been determined (Goldstein & Dudley, 1990) and is very similar to the human sequence. There is an insert of two amino acids in the α-chain. The behaviour on the two-dimensional mapping system of peptides containing the likely phosphorylation sites would be expected to be essentially unaltered. The sequence adjacent to the three phosphorylated tyrosine residues of the kinase domain is identical, whereas that adjacent to the two tyrosines in the *C*-terminal domain contains threonine and aspartate in place of serine and glutamate.

RESULTS AND DISCUSSION

***In vitro* phosphorylation of partially purified insulin receptors**

Partially purified insulin receptor preparations from rat liver membranes were incubated with [γ-^{32}P]ATP in the presence or absence of insulin for 10 min at 30 °C. Under these conditions, near-maximum phosphorylation of the receptors occurs in the presence of insulin. The phosphorylated receptors were then isolated by immunopurification and SDS/PAGE and located by radioautography. Fig. 2 shows results of a representative experiment. The efficiency of the immunopurification was estimated by running on SDS/PAGE a small fraction of the original incubation mixture before and after removal of the insulin receptor with the antibody–Sepharose complex (Fig. 2*a*). Scanning of the radioautographs showed that more than 80 % of the receptors were routinely recovered by this procedure.

An appreciable amount of phosphorylation of the β-subunit of the receptor was observed in the absence of insulin (Fig. 2*a*, lane 5). When insulin was present (Fig. 2*a*, lane 6), ^{32}P incorporation into the β-subunit was stimulated by 4.8 ± 1.1-fold (mean ± S.E.M. of results in three separate experiments).

Phosphoamino acid analysis (Fig. 2*b*) revealed that, in the absence of insulin, the receptors were phosphorylated predominantly on tyrosine residues. In the presence of insulin, phosphorylation of tyrosine residues was considerably increased and phosphorylation of serine residues also became clearly evident.

Two-dimensional thin layer analysis of ^{32}P-labelled tryptic phosphopeptides is shown in Figs. 2(*c*) and 2(*d*). In the absence of insulin (Fig. 2*c*), about 40 % of overall ^{32}P incorporation occurred in a peptide corresponding to C1 (the mono-phosphorylated form of the kinase domain containing tyrosines corresponding to residues 1158, 1162 and 1163 of the human receptor). The remaining incorporation occurred in a number of phosphopeptides, including B2, B3, A1 and A2 (the bis- and tris-phosphorylated forms of the same domain). In the presence of insulin (Fig. 2*d*), the amount of labelled C1 was essentially unaltered, but there was a 5–20-fold increase in the amount of labelled B2 and B3 and of A1 and A2. In three separate experiments, in the absence of insulin, 86 ± 4 % of β-subunits phosphorylated in this domain were only phosphorylated on one tyrosine residue, with 7 ± 3 % and 6 ± 2 % being phosphorylated on two or all three tyrosines respectively. In contrast, the corresponding values in the presence of insulin were 33 ± 6, 33 ± 4 and 34 ± 3 in mono-, bis- and tris-phosphorylated forms respectively. (Results are means ± S.E.M., with allowance being made for the number of tyrosines being phosphorylated). Insulin also resulted in a substantial increase (7.7 ± 2.2-fold; mean ± S.E.M. for three observations) in the phosphorylation of a

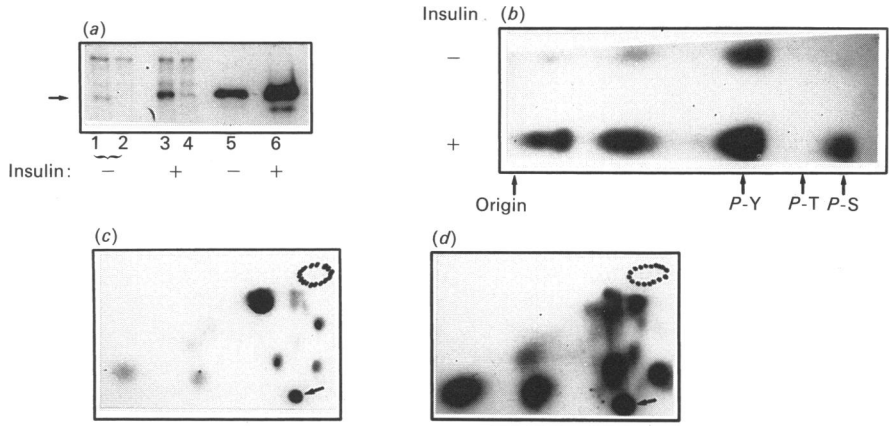


Fig. 2. Effects of insulin on the phosphorylation of the rat liver insulin receptor *in vitro*

Partially purified receptors were incubated at 30 °C in the presence or absence of 1 μM-insulin for 15 min and then with [γ-^{32}P]ATP for a further 10 min. (*a*) Radioautograph showing the SDS/PAGE separation of ^{32}P-labelled proteins precipitated by treatment with CT1-antibody–Sepharose (tracks 5 and 6). The efficiency of precipitation was checked by running on the same gel 5% of the original incubation mixture before (tracks 1 and 3) and after (tracks 2 and 4) antibody treatment. The position of the β-subunit of the insulin receptor is indicated by an arrow. (*b*) Phosphoamino acid analysis of the β-subunit of the receptor after incubation in the absence or presence of insulin. The migration of phosphotyrosine, phosphothreonine and phosphoserine is indicated by *P*-Y, *P*-T and *P*-S. (*c*, *d*) Two-dimensional t.l.c. analysis of tryptic phosphopeptides derived from the β-subunit of the receptor after incubation in the absence (*c*) or the presence (*d*) of insulin. Further details are given in the Experimental section. Results are typical of at least three separate experiments. The positions of an internal marker, Dnp-lysine (dotted oval) and the origin of sample application (arrow) are shown.

peptide corresponding to B1 (a peptide derived from near the *C*-terminus containing tyrosines corresponding to residues 1328 and 1334). These results are broadly similar to those found previously for the human insulin receptor phosphorylated *in vitro* (Tavaré & Denton, 1988; J. M. Tavaré & M. Dickens, unpublished work). Nevertheless, a marked difference between these and previous results is the lack of any formation of phosphopeptides corresponding to C2 and C3 with the rat liver receptor.

Since the phosphoamino acid analysis showed that insulin also markedly increases the phosphorylation of serine residues within the β-subunit of the receptor, it would appear that a receptor serine kinase has co-purified with the receptor through wheat-germ lectin chromatography. A similar phenomenon has previously been observed with partially purified receptor preparations from human placenta (Smith *et al.*, 1988; Lewis *et al.*, 1990*b*) and human and rat hepatoma cells (Zick *et al.*, 1983; Smith *et al.*, 1988). Smith & Sale (1989) have presented evidence that in receptors prepared from human placenta the major phosphoserine-containing peptide appears to be the same as peptide S in Fig. 1. In agreement with this, insulin was found to increase the phosphorylation of a peptide from the rat liver receptor with similar behaviour in the two-dimensional t.l.c. system by 175 ± 34.2% (mean ± S.E.M. for three observations). The variability in this stimulation might be the result of differences in the amounts of protein serine kinase activity co-purifying with the receptor. Phosphoamino acid analysis of the peptide confirmed that phosphorylation occurred predominantly on serine residues, whereas the phosphorylation of peptides corresponding to C1, B1, B2, B3, A1 and A2 was restricted to tyrosine residues (results not shown).

Phosphorylation of insulin receptors within intact rat liver cells

Preliminary experiments indicated that maximum steady-state labelling of phosphoproteins within rat liver cells was achieved after incubation of the cells for 50 min in medium containing 0.2 mM-[^{32}P]P_i, as used in previous studies (Vargas *et al.*, 1982; Hopkirk & Denton, 1986). Cells were then incubated with or without insulin (200 nM) for a further 10 min before rapid extraction and isolation of the receptors at 2–4 °C. The extraction medium contained EDTA, phosphatase inhibitors and proteinase inhibitors to minimize changes in phosphorylation and any proteolysis. Isolation of the receptors involved the sequential use of wheat-germ-lectin–Sepharose chromatography, immunopurification with CT1-antibody–Sepharose and SDS/PAGE (see the Experimental section). Recovery of phosphorylated receptors by this procedure was checked by adding, at the time of extraction of unlabelled cells, a known quantity of partially purified rat liver insulin receptors pre-labelled by incubation *in vitro* with [γ-^{32}P]ATP and insulin. No evidence of any dephosphorylation (including the dephosphorylation of specific sites) or proteolysis of the β-subunit of the insulin receptor was found. Recovery through to the elution of the receptor from the wheat-germ lectin was greater than 80%. Essentially all of the phosphorylated receptors so eluted appeared to bind to the CT1 antibody, and more than 80% were recovered as a single protein band migrating with an apparent subunit molecular mass of 95000 kDa on SDS/PAGE. A high recovery is essential, as it is important that the isolated receptors are an accurate reflection of the receptors in the original cells. It also means that sufficient ^{32}P-labelled β-subunits can be isolated from 30×10^6 liver cells incubated in 2 ml of medium containing 4 mCi of [^{32}P]P_i to allow excellent phosphopeptide maps to be generated.

Insulin increased the overall incorporation of ^{32}P into the β-subunit (Fig. 3*a*) by 2.7-fold (2336 ± 396 d.p.m. incorporated in the absence and 6200 ± 1793 d.p.m. in the presence of insulin; means ± S.E.M. of three separate observations). Phosphoamino acid analysis (Fig. 3*b*) revealed that, in the absence of insulin, the receptors appeared to be essentially phosphorylated on serine residues, with little or no evidence of any labelled phosphotyrosine. In the presence of insulin there was little change in the overall amount of serine phosphorylation, but there was clearly a substantial increase in the amount of tyrosine phosphorylation. Similar results have been previously obtained by Ballotti *et al.* (1987) with insulin receptors phosphorylated in intact rat liver cells. In our experiments the insulin receptors were always co-purified with two other labelled phosphoproteins of subunit molecular masses of about 180 and 35 kDa, which were well

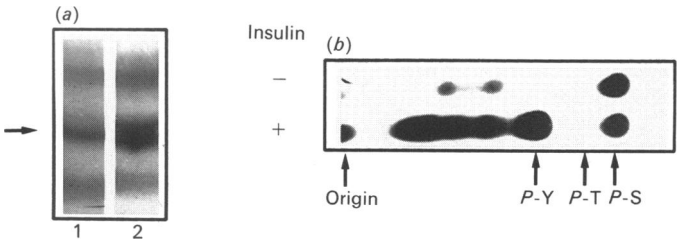


Fig. 3. Effects of insulin on the phosphorylation of insulin receptors within intact liver cells

Liver cells (30×10^6 in 2 ml) were preincubated in medium containing [^{32}P]P_i (0.2 mM; 2 mCi/ml) for 50 min and then with or without insulin (200 nM) for a further 10 min. Receptors were extracted and purified by sequential chromatography on wheat-germ-lectin–Sepharose and CT1-antibody–Sepharose. (*a*) Radioautograph showing labelled phosphoproteins eluted from CT1-antibody–Sepharose and separated by SDS/PAGE. The position of the β-subunit of the insulin receptor is indicated by an arrow. Lane 1, without insulin; lane 2, with insulin. (*b*) Phosphoamino acid analysis of the β-subunit of the receptor after purification and SDS/PAGE as in (*a*). The migration of phosphotyrosine, phosphothreonine and phosphoserine are indicated by *P*-Y, *P*-T and *P*-S. Further details are given in the Experimental section. Results are typical of three separate experiments.

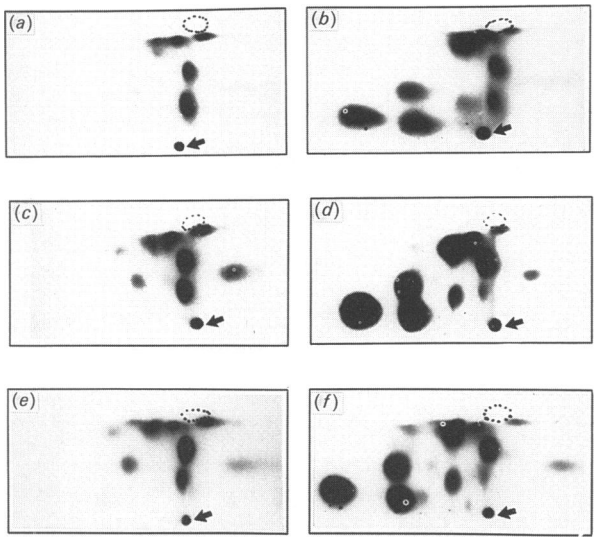


Fig. 4. Two-dimensional t.l.c. of the phosphopeptides derived from digestion of insulin receptors purified from liver cells incubated in the presence and absence of insulin

Liver cells were incubated and receptors isolated as in Fig. 3(*a*). After electroelution and digestion with trypsin, [^{32}P]phosphopeptides were separated by two-dimensional t.l.c. and located by radioautography. (*a*, *c* and *e*) Maps obtained from three separate preparations of cells incubated in the absence of insulin; (*b*, *d* and *f*) maps obtained from the three cell preparations incubated in the presence of insulin. The positions of an internal marker, Dnp-lysine (dotted oval) and the origin of sample application (arrow) are shown.

separated from the β-subunit of the insulin receptor by SDS/PAGE (Fig. 3*a*). Phosphoamino acid analysis indicated that these proteins contained predominantly phosphoserine, with a trace of phosphothreonine but no phosphotyrosine (results not shown). Insulin had no effect on the incorporation of ^{32}P into either of these proteins.

Fig. 4 shows the effects of insulin on the labelling of the tryptic phosphopeptides resolved by the two-dimensional t.l.c. mapping procedure of Tavaré & Denton (1988). Results of three independent experiments are shown, involving three different preparations of cells using two batches of collagenase to indicate the good reproducibility obtained. Relative incorporation into the various phosphopeptides was determined by densitometric scanning and the results are summarized in Table 1. Whereas the peptides corresponding to C1, B2, B3, A1 and A2 derived from the kinase domain are easily recognized, a further seven peptides are less easy to relate to previous studies, and these have been denoted X1–X7 for the purposes of this paper. Phosphoamino acid analysis confirmed that the first set of peptides was only phosphorylated on tyrosines, whereas X1 and X2 contained only phosphoserine (results not shown). Incorporation in X3–X7 was too low to allow analysis to be carried out.

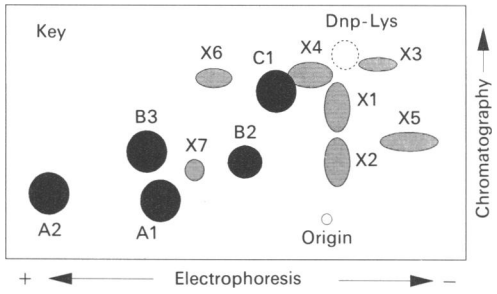
The major effect of insulin was to increase the phosphorylation of the kinase domain by more than 20-fold. In the absence of insulin only a small amount of monophosphorylated peptide (C1) was evident, whereas in the presence of insulin substantial amounts of mono-, bis- and tris-phosphorylated forms from the kinase domain were apparent. The exposure of liver cells to insulin increased the proportion of ^{32}P being incorporated into the domain from 8 to 72% of the total incorporation (Table 1). After insulin exposure the percentage of phosphorylated β-subunits in the mono-, bis- and tris-phosphorylated forms can be calculated to be 32 ± 1.2%, 22 ± 1.2% and 46 ± 1.3% respectively when allowance is made for the number of tyrosine residues phosphorylated (means $\pm$ S.E.M. for three observations).

There was little evidence of phosphopeptides corresponding to C2 and C3, suggesting that the tyrosine residues close to the transmembrane domain may not be phosphorylated, in agreement with the apparent lack of phosphorylation of these tyrosines in the rat liver receptor phosphorylated *in vitro* (Figs. 2*c* and 2*d*). However, in marked contrast with the studies with the partially purified rat liver receptor phosphorylated *in vitro*, there was no evidence of labelled phosphopeptide B1 (corresponding to the phosphorylation of tyrosines 1328 and 1334 of the human insulin receptor) occurring within intact liver cells in either the absence or the presence of insulin.

Consideration has to be given to the possibility that the lack of phosphorylation of this domain in the receptor within intact cells is due to partial proteolysis of the *C*-terminus, specific dephosphorylation of this domain or poor binding of the receptors phosphorylated in this domain to the monoclonal antibody CT1. None of these possibilities appears to be an explanation of the observation for the following reasons. (1) The use of CT1 antibody directed against the *C*-terminus ensures that all receptors recovered will contain the *C*-terminal tyrosines. (2) On addition of *in vitro* [^{32}P]phosphorylated rat liver receptors to an extract of unlabelled cells, we could find no evidence of any specific or general dephosphorylation occurring during the isolation of the receptors. (3) The tris-phosphorylated form of the kinase domain has been recently reported to be at least 4 times more sensitive to dephosphorylation by phosphotyrosyl protein phosphatases present in rat liver than the phosphorylated tyrosines in the *C*-terminus domain (King & Sale, 1990). (4) The epitope recognized by the antibody CT1 is Ile-Leu-Thr-Leu-Pro-Arg-Ser and thus is some distance from the *C*-terminal tyrosines, which precede this epitope by 12 and 18 amino acids (R. Ganderton, M. Soos & K. Siddle, personal communication). In any case, the antibody was capable of binding tightly to the rat liver receptor phosphorylated *in vitro* on these tyrosines (Fig. 2). Moreover, after treatment of the wheat-germ-lectin eluate with CT1-antibody–Sepharose, no detectable radiolabelled receptor remained that bound to RIR-antibody–Sepharose. The RIR antibody recognizes both the α- and β-chains of the rat liver

Table 1. Effects of insulin on the phosphorylation of the insulin receptor within intact liver cells

Results are taken from three experiments shown in Fig. 4. Relative phosphorylation was determined by scanning of the radioautographs (and is expressed as means ± S.E.M. of three observations). *$P < 0.05$ for effect of insulin compared with control. The key to the phosphopeptide positions is shown below. N.D., not detectable.



Peptide	Effects of insulin (% of control)	Phosphorylation of receptor (% of overall phosphorylation)	
		Control	Insulin
Kinase domain			
C1	500±158*	8±1	10±0.4
B2	> 2000	N.D.	4±0.5
B3	> 2000	N.D.	11±1
A1	> 2000	N.D.	19±0.4
A2	> 2000	N.D.	27±4
C1+B2+B3+A1+A2	> 2000	8±1	72±6
Other phosphorylation sites			
X1	144±28	27±6	10±0.2
X2	69±9*	22±4	5±3
X3	63±10*	9±1	2±0.2
X4	165±21	10±1	4±1
X5–X7	95±6	22±3	6±1
X1–X7	105±7	92±1	28±6
Overall	377±65*	100	100

receptor (T. Issad, unpublished work) and binds more than 80% of phosphorylated receptors if added directly as the Sepharose complex to the wheat-germ eluate.

Overall incorporation into the phosphopeptides X1–X7 accounted for over 90% of the total incorporation into the receptors of cells incubated in the absence of insulin. Overall, insulin appeared to have no effect on the incorporation of ^{32}P into these peptides. However, insulin consistently caused a decrease in the amounts of X2 and X3, while possibly increasing X1 and X4.

General conclusion

These results represent the first study of the phosphorylation of the individual sites on the insulin receptor within freshly isolated cells. A number of marked differences are apparent compared with the pattern observed in previous studies, which had been restricted to cultured cells expressing very high levels of insulin receptors (Tavaré *et al.*, 1988; White *et al.*, 1988; Tornqvist *et al.*, 1988).

The predominant change in the phosphorylation of the insulin receptor after a brief exposure of rat liver cells to insulin is a large increase in the phosphorylation of the three tyrosine residues in the kinase domain of the β-subunit. The overall increase in the kinase domain is at least 20-fold, with nearly half of the phosphorylated β-subunits exhibiting phosphorylation on all three tyrosines. This further supports the widely held view that phosphorylation of this domain plays an important role in the mechanism whereby the insulin receptor acts to regulate intracellular functions (Tornqvist & Avruch, 1988; White *et al.*, 1988; Flores-Riveros *et al.*, 1989). Sustained increases in the phosphorylation of tyrosine residues in this domain have also been observed in rat hepatoma cell lines and in cells transfected with the human insulin receptor cDNA (Tavaré *et al.*, 1988; White *et al.*, 1988; Tornqvist *et al.*, 1988). However, in these cultured cells a greater proportion of the phosphorylated β-subunits appears to be phosphorylated on only two tyrosines in the kinase domain after exposure of the cells to insulin. Indeed, in rat hepatoma cells less than 15% of the receptors were in the tris-phosphorylated form (White *et al.*, 1988; White & Kahn, 1989), compared with nearly 50% in freshly isolated liver cells used in the present study.

An important and unexpected finding of these studies is the apparent lack of any phosphorylation of the tyrosine residues located near the *C*-terminus of the β-subunit (corresponding to tyrosines 1328 and 1334 of the human receptor) in intact rat liver cells. Increases in the phosphorylation of this domain in the presence of insulin have been observed in previous studies using both isolated receptors and intact cultured cells. In the present study, clear evidence for marked increases in the phosphorylation of this domain was obtained with rat liver receptors when incubated *in vitro* with [γ-^{32}P]ATP and insulin. Further studies are required to establish whether or not the lack of phosphorylation of the *C*-terminal tyrosines is restricted to intact rat liver cells or will be exhibited by freshly isolated cells from

other tissues such as fat. The relationship between the phosphorylation of these tyrosines and signal transduction has not be addressed directly by site-directed mutagenesis, but it should be noted that a truncated receptor lacking 43 amino acids from the *C*-terminus has been reported to exhibit normal kinase activity but impaired metabolic signalling when expressed in cultured fibroblasts (Maegawa *et al.*, 1988; Thies *et al.*, 1989).

The maps of phosphopeptides from the receptor phosphorylated within intact cells also show a number of further peptides that are difficult to relate to those seen in earlier studies (denoted X1–X7 in this study). Since all of these phosphopeptides are evident in the receptors from cells incubated in the absence of insulin where only phosphoserine was detected, it must be expected that most, if not all, of these peptides contain phosphoserine. This was confirmed in the case of X1 and X2. The high level of serine phosphorylation in the absence of insulin is in marked contrast with the rather low levels seen in previous studies with cultured cells under comparable conditions. It is possible that X1 and X3 are the peptides that we have denoted S and T (see Fig. 1), which show marked increases in the phosphorylation of serine and threonine residues respectively on exposure of cultured cells to insulin (Tavaré *et al.*, 1988). In freshly isolated liver cells there was only evidence of a modest increase in the phosphorylation of X1, whereas X3 phosphorylation appeared to be actually decreased. A decrease in the phosphorylation of X2 (a phosphoserine-containing peptide) was also evident. Insulin-induced decreases in the phosphorylation of sites on the insulin receptor have not been previously reported and further underline the need to study the phosphorylation of the insulin receptor in intact freshly isolated cells.

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