Biochem. J. (1988) 252, 607–615 (Printed in Great Britain)

Studies on the autophosphorylation of the insulin receptor from human placenta

Analysis of the sites phosphorylated by two-dimensional peptide mapping

Jeremy M. TAVARÉ and Richard M. DENTON*
Department of Biochemistry, School of Medical Sciences, University of Bristol, University Walk, Bristol BS8 1TD, U.K.

1. A partially purified preparation of human placental insulin receptors was incubated with [γ-^{32}P]ATP in the presence or absence of insulin. The ^{32}P-labelled insulin-receptor β-subunits were then isolated, cleaved with trypsin followed by protease V8 and the [^{32}P]phosphopeptides generated were analysed by thin layer electrophoresis and chromatography. This approach revealed that insulin stimulates autophosphorylation of the insulin-receptor β-subunit *in vitro* on at least seven tyrosine residues distributed among three distinct domains. 2. One domain (domain 2), containing tyrosine residues 1146, 1150 and 1151 was the most rapidly phosphorylated and could be recovered as mono-, di- and triphosphorylated peptides cleaved by trypsin at Arg-1143 and either Lys-1153 or Lys-1156. Multiple phosphorylation of this domain appears to partially inhibit the cleavage at Lys-1153 by trypsin. 3. In a second domain (domain 3) containing two phosphorylated tyrosine residues at positions 1316 and 1322 the tyrosines were phosphorylated more slowly than those in domain 2. This domain is close to the *C*-terminus of the β-subunit polypeptide chain. 4. At least two further tyrosine residues appeared to be phosphorylated after those in domains 2 and 3. These residues probably residue within a domain lying in close proximity to the inner face of the plasma membrane containing tyrosines 953, 960 and 972, but conclusive evidence is still required. 5. The two-dimensional thin-layer analysis employed in this study to investigate insulin-receptor phosphorylation has several advantages over previous methods based on reverse-phase chromatography. It allows greater resolution of ^{32}P-labelled tryptic peptides and, when coupled to radioautography, is considerably more sensitive. The approach can be readily adapted to study phosphorylation of the insulin receptor within intact cells.

INTRODUCTION

The insulin receptor is a heterodimer composed of an extracellular ligand-binding α-subunit connected through disulphide bonds to a transmembrane β-subunit. The intracellular domain of the β-subunit contains an intrinsic protein tyrosine kinase activity which is capable of both autophosphorylation and phosphorylation of a number of exogenous substrates (Kahn *et al.*, 1985). Autophosphorylation appears to increase the tyrosine kinase activity towards these exogenous substrates and render the kinase insulin-independent (Rosen *et al.*, 1983; Yu & Czech, 1984). There is mounting evidence from the study of modified insulin receptors that the tyrosine kinase activity probably plays an important role in the action of insulin (Ellis *et al.*, 1986*a*; Chou *et al.*, 1987; Ebina *et al.*, 1987; Morgan & Roth, 1987). However, it is not clear which proteins are physiologically important substrates within intact cells exposed to insulin. Indeed, the receptor itself may be the only important substrate within cells. Autophosphorylation could promote a conformational change in the β-subunit structure such that the receptor can then interact with other cellular components involved in signal transduction (e.g. protein serine kinases and/or GTP-binding proteins) (Denton, 1986; Herrera & Rosen, 1986).

In order to understand the role that β-subunit autophosphorylation plays in insulin action, it is necessary to determine which tyrosine residues in the intracellular domain of the β-subunit are autophosphorylated both within purified preparations of the receptor and in intact cells. The combined use of synthetic peptide substrates, antipeptide antisera, site-directed mutagenesis, limited proteolysis and, more recently, direct amino acid sequencing has led to proposals that there are at least two major domains of autophosphorylation when insulin is added to purified preparations of receptors (Ellis *et al.*, 1986*b*; Herrera & Rosen, 1986; Stadtmauer & Rosen, 1986; Goren *et al.*, 1987; Tornqvist *et al.*, 1987). One domain mid-way between the transmembrane sequence and the *C*-terminus contains three tyrosine residues at positions 1146, 1150 and 1151 (Ebina *et al.*, 1985; Ullrich *et al.*, 1985) all of which may be phosphorylated. A second domain contains two tyrosines at positions 1316 and 1322 and is close to the *C*-terminus of the β-subunit; again both residues appear to be phosphorylated (Tornqvist *et al.*, 1987).

Reverse-phase chromatography has formed the basis of most previous separations of tryptic peptides derived from the insulin receptor. There are some reports of two-dimensional thin layer separations of tryptic phosphopeptides (Jacobs & Cuatrecasas, 1986; Stadtmauer & Rosen, 1986; Ponzio *et al.*, 1987) but these have not been well characterized. In this paper we describe the use of a

Abbreviations used: DNP-lysine, dinitrophenyl-lysine; TPCK, tosylphenylalanylchloromethane.

* To whom all correspondence and reprint requests should be addressed.

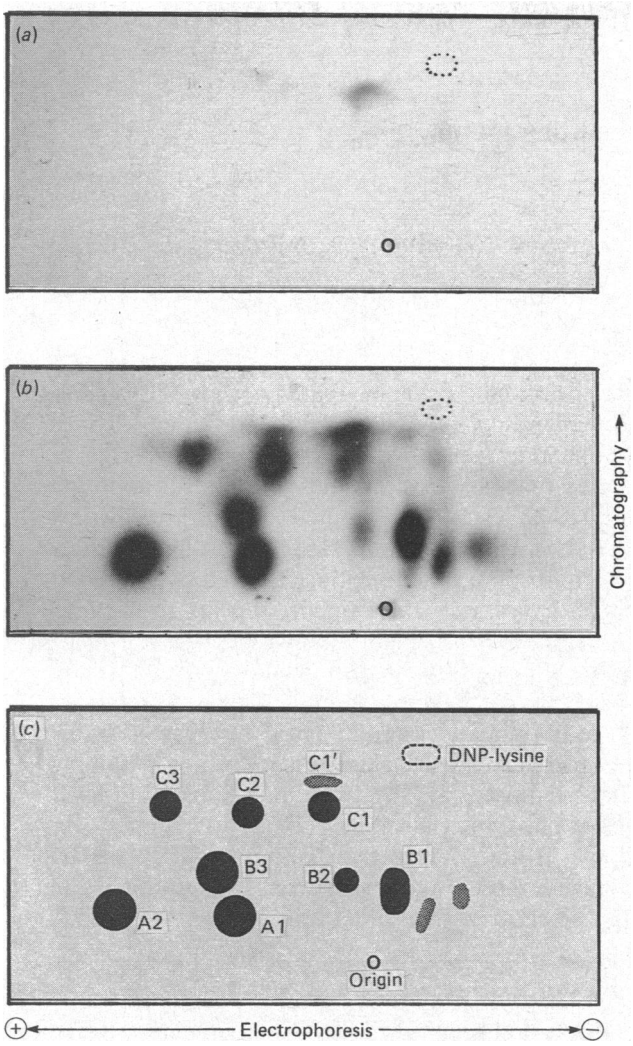

Fig. 1. Separation of insulin-receptor β-subunit tryptic phosphopeptides by two-dimensional thin layer analysis

Partially purified insulin receptors were preincubated in the absence (*a*) or presence (*b*) of 10^{-6} M-insulin and then with [γ-^{32}P]ATP for 60 min as described in the Materials and methods section. The reaction was terminated and ^{32}P-labelled β-subunits isolated by gel electrophoresis and digested with TPCK-treated trypsin. ^{32}P-labelled tryptic phosphopeptides were then separated on thin-layer cellulose plates by electrophoresis at pH 3.5 and ascending chromatography as described in the Materials and methods section. (*a*) and (*b*) show radioautographs of the resulting two-dimensional separations of ^{32}P-labelled insulin-receptor tryptic phosphopeptides. (*c*) is a key to the identification of the ^{32}P-labelled tryptic phosphopeptides relative to the mobility of an internal marker dye, DNP-lysine (broken open circle), and the origin of sample application (bold full circle).

two-dimensional thin layer procedure for the separation of insulin-receptor β-subunit tryptic phosphopeptides. The thin layer procedure has greater resolving power and, when coupled to radioautography, is more sensitive than reverse-phase chromatography. It is, therefore, particularly suitable for the study of phosphorylation of the insulin receptor in intact cells. In the present study, most of the major phosphopeptides resolved by this procedure have been identified largely from a consideration of their net charge. This has allowed us to confirm and considerably extend the findings of previous studies on the autophosphorylation of the purified receptor, including the identity of the tyrosines phosphorylated and their relative rates of phosphorylation.

MATERIALS AND METHODS

Materials

All materials used were as described by Tavaré *et al.* (1987) except for *Staphylococcus aureus* protease V8 which was from Sigma Chemical Co. (Poole, Dorset, U.K.) and h.p.l.c. reagents which were from BDH Chemicals (Poole, Dorset, U.K.).

Partial purification and autophosphorylation of the human placental insulin receptor

A Triton extract of human placental syncytiotrophoblast microvillous membranes (approx. 300 mg of protein) was prepared as described by Tavaré *et al.* (1987) and mixed gently with 2 ml of wheat-germ lectin–Sepharose 4B CL (approx. 9 mg of bound lectin) for 30 min at 4 °C. This mixture was poured into a column (12 mm diameter) and washed with 50 ml of column washing buffer [20 mM-Mops (pH 7.4)/250 mM-sucrose/0.1% (v/v) Triton X100/2.5 mM-benzamidine/1 μg/ml each of pepstatin, antipain and leupeptin] at a flow rate of 30 ml/h at 4 °C. The partially purified insulin receptors were eluted with 15 ml of column washing buffer supplemented with 0.3 M-*N*-acetylglucosamine and concentrated to 300 μl with a Centricon-30 microconcentrator (Amicon Corporation, Danvers, MA, U.S.A.).

Partially purified insulin receptors (30 μl) were preincubated for 30 min at 0 °C in a total volume of 60 μl containing 30 mM-Mops (pH 7.4)/0.25 mM-dithiothreitol/12 mM-$MgCl_2$/2 mM-$MnCl_2$ and $\pm 10^{-6}$ M-insulin. Following further incubation with [γ-^{32}P]ATP (100 μM; 2000 c.p.m./pmol) for the times indicated in the Figure legends, phosphorylation was terminated by the addition of SDS sample buffer [20% (w/v) sucrose/10% (w/v) SDS/125 mM-Tris/HCl (pH 6.8)/0.02% (w/v) Bromophenol Blue] and boiling for 5 min. ^{32}P-labelled insulin-receptor β-subunits were separated from the epidermal growth factor receptor by SDS/polyacrylamide-gel electrophoresis (6%) as described by Laemmli (1970). The β-subunit was located by radioautography of the untreated wet gel with Kodak X-Omat S pre-flashed film at room temperature for 15 min. The epidermal growth factor receptor is the only other ^{32}P-labelled protein in this fraction; few insulin-like growth factor I receptors appear to be present in this preparation as addition of 10^{-6} M-insulin-like growth factor I (in place of insulin) does not cause any appreciable increases in phosphorylation (Tavaré, 1985).

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

Polyacrylamide-gel pieces containing ^{32}P-labelled insulin-receptor β-subunits were thinly sliced and placed in a dialysis sac containing 500 μl of electroelution buffer [20 mM-Tris/HCl (pH 8.0)/2 mM-EDTA/0.1% (w/v) SDS/0.1% (v/v) 2-mercaptoethanol] which was, in turn, placed in a Bio-Rad transblot apparatus containing 2000 ml of electroelution buffer. Electroelution was

carried out for 16 h at room temperature and 50 mA. The eluant within each sac was collected, pooled with a 500 μl washing of the residual gel chips (with water) and concentrated to 200 μl in a Savant Speedvac rotary concentrator. Eluted protein was precipitated with 4 vol. of acetone at −80 °C for 60 min followed by centrifugation at room temperature for 10 min at 10000 *g*. The pellet was dried under N_2 and incubated with 100 μl of 50 mM-NH_4HCO_3 (pH 8.2) containing 10 μg of TPCK-treated trypsin (Worthington Diagnostic Systems, Freehold, NJ, U.S.A.) for 16 h at 30 °C. A further 10 μg of TPCK-treated trypsin was added, incubation continued for 6 h at 30 °C and the peptides freeze-dried, resuspended with water and re-freeze-dried at least three times to remove residual NH_4HCO_3. This procedure resulted in > 90% recovery of the ^{32}P-label present in the original gel piece.

The ^{32}P-labelled tryptic peptides were then resuspended in 10 μl of water and loaded, with 5 μg of dinitrophenyl (DNP)-lysine, on to a 20 cm × 20 cm cellulose t.l.c. plate (Eastman Kodak Company, Rochester, NY, U.S.A.) for separation first by electrophoresis at 400 V for 2 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 finally detection by radioautography.

Alternatively, the ^{32}P-labelled tryptic peptides were separated by h.p.l.c. using a C_{18} reverse-phase column (Lichrosorb 5RP18) pre-equilibrated in buffer A [0.1% (v/v) triflouroacetic acid]. All procedures were performed at a flow rate of 1 ml/min. The sample was applied to the column in 100 μl of buffer A and peptides eluted isocratically at 100% buffer A for 5 min and then with a linear gradient to 40% buffer B [100% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid] over 40 min. Fractions (1 ml) were collected throughout the run and ^{32}P label was monitored by Čerenkov counting.

In some studies, peptides separated by two-dimensional thin layer analysis were scraped off the plates and eluted by resuspension of the cellulose in either 1 ml of water (for peptides, A1, A2, B1, B2 and B3) or 1 ml of 33% (v/v) acetonitrile (for other peptides) followed by centrifugation at 10000 *g* for 30 s. The supernatant was freeze-dried and peptides digested with *S. aureus* protease V8 (50 μl of 10 μg/ml for 16 h at 30 °C in 50 mM-NH_4CO_2, pH 7.8, followed by a further 50 μl of 10 μg/ml for 5 h at 30 °C). The reaction was terminated by boiling and the sample was freeze-dried and V8-peptides separated by electrophoresis on cellulose chromatography plates at 400 V and pH 3.5 for 2 h (pyridine/acetic acid/water, 1:10:189, by vol.).

Samples of tryptic-digested insulin-receptor β-subunits or tryptic peptides purified by two-dimensional thin layer analysis were resuspended in 100 μl of 6 M-HCl for determination of constituent phosphoamino acids by electrophoretic separation at pH 3.5 as described previously (Tavaré *et al.*, 1987).

Radioautography of thin layer plates were performed for 5–7 days at −80 °C using preflashed Kodak X-Omat S film in cassettes with intensifying screens.

Incorporation of ^{32}P into tryptic and V8 phosphopeptides

This was estimated by both densitometric scanning of the radioautographs using the 'Rasterscan' programme of a Chromoscan 3 (Joyce Loebl, Gateshead, U.K.) and scintillation counting of the cellulose in the region of the thin layer plates corresponding to the peptides resuspended in toluene-based scintillant.

Expression of results

Designation of phosphorylation sites and tryptic- and V8-cleavage sites is based on the sequence of Ullrich *et al.* (1985).

RESULTS

Separation of insulin-receptor β-subunit tryptic phosphopeptides by two-dimensional thin layer analysis

Insulin receptors purified from human placenta by wheat-germ lectin–Sepharose chromatography were

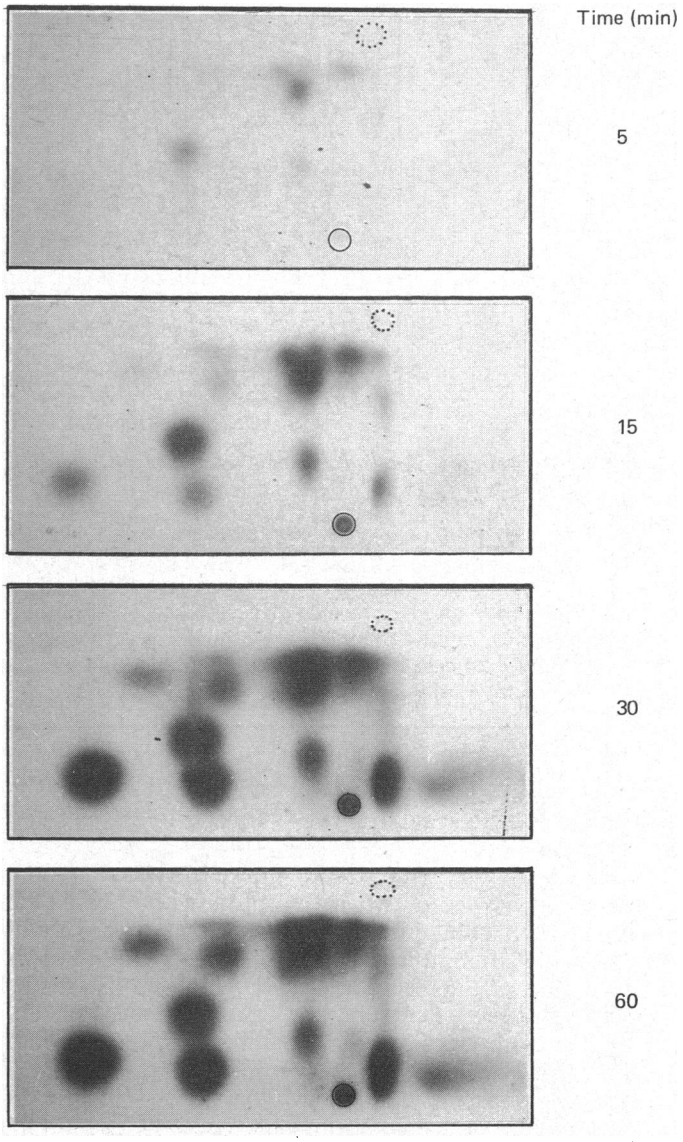

Fig. 2. Time course of phosphorylation of insulin receptor β-subunit tryptic phosphopeptides as determined by two-dimensional thin layer analysis

Partially purified insulin receptors were preincubated with 10^{-6} M-insulin for 30 min at 0 °C and then with [γ-^{32}P]ATP at 0 °C and the reaction terminated at 5, 15, 30 and 60 min by the addition of SDS sample buffer. ^{32}P-labelled insulin-receptor β-subunits were isolated, digested with TPCK-treated trypsin, separated by two-dimensional thin layer analysis and subjected to radioautography as in Fig. 1.

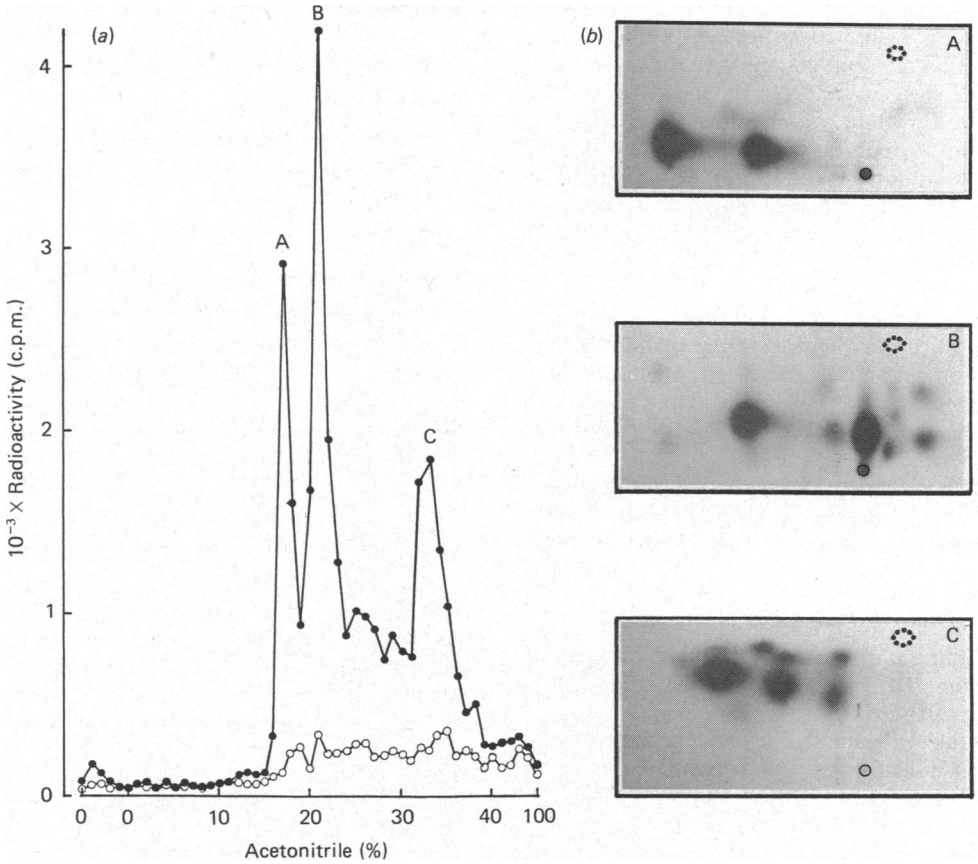

Fig. 3. Separation of insulin-receptor β-subunit tryptic phosphopeptides by reverse-phase h.p.l.c. followed by two-dimensional thin layer analysis

(*a*) Partially purified insulin receptors were incubated with [γ-^{32}P]ATP for 60 min at 0 °C in the absence (○) or presence (●) of 10^{-6} M-insulin, and ^{32}P-labelled insulin-receptor β-subunits were isolated by gel electrophoresis and digested with TPCK-treated trypsin as described in the Materials and methods section. Tryptic digests were resuspended in 0.1% trifluoroacetic acid and separated on a C_{18} reverse-phase h.p.l.c. column using a linear acetonitrile gradient with the radioactivity in 1 ml fractions being determined by Čerenkov counting as described in the Materials and methods section. (*b*) Fractions making up peaks A, B and C were freeze-dried, applied to thin-layer cellulose plates, separated by two-dimensional thin layer analysis and subjected to radioautography as in Fig. 1.

incubated with insulin and [γ-^{32}P]ATP in the presence of 2 mM-$MnCl_2$/12 mM-$MgCl_2$/1 mM-Na_3VO_4; conditions that preliminary studies showed led to maximum autophosphorylation of the receptors within 30–60 min at 0 °C. Insulin increased the extent of β-subunit phosphorylation at least 15-fold. After separation of the β-subunits of the receptors by SDS/polyacrylamide-gel electrophoresis, the subunits were electroeluted and digested with trypsin. Phosphoamino acid analysis (not shown) revealed that insulin promoted a large increase in tyrosine phosphorylation of the insulin receptor β-subunit under these conditions in agreement with other reports (Avruch *et al.*, 1982; Kasuga *et al.*, 1983). Overexposure of the radioautographs did reveal the presence of a trace of phosphoserine, although the effect of insulin on serine phosphorylation was variable (not shown).

Two-dimensional analysis of the phosphopeptides resulting from trypsin digestion clearly resolved at least nine major ^{32}P-labelled phosphopeptides (Fig. 1). The pattern of phosphopeptides was very consistent with some ten different preparations of receptor and two different batches of trypsin; longer times of digestion did not alter the pattern of phosphopeptides. In this study, we will refer to the phosphopeptides by the labels indicated in Fig. 1(*c*). The letters A, B and C denote the behaviour of the peptides on reverse-phase h.p.l.c. (see later). It should be noted that only one of the nine major phosphopeptides has a net positive charge at pH 3.5 (peptide B1). Phosphoamino acid analysis was carried out on each phosphopeptide, revealing that all were apparently phosphorylated exclusively on tyrosines except B1, which was found to contain a trace (< 10%) of phosphoserine (results not shown).

A study of the time course of autophosphorylation at 0 °C (Fig. 2), showed that ^{32}P was incorporated first into C1, B2 and B3, then into B1, A1 and A2 and finally into C2 and C3.

Separation of insulin-receptor β-subunit tryptic phosphopeptides by reverse-phase h.p.l.c.

Three major peaks of radioactivity were separated by reverse-phase h.p.l.c. from the tryptic digest of ^{32}P-labelled β-subunit of the insulin receptor. Denoted A, B

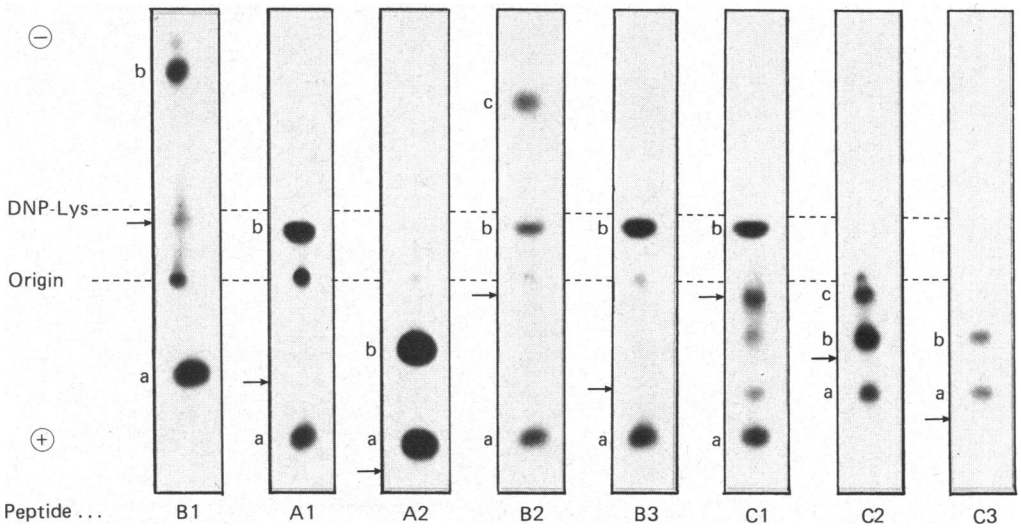

Fig. 4. Digestion of insulin-receptor β-subunit tryptic phosphopeptides by *S. aureus* protease V8 and analysis by electrophoresis

^{32}P-labelled tryptic phosphopeptides were generated and separated by two-dimensional thin layer analysis as described in the legend to Fig. 1(*b*). The individual tryptic peptides were isolated and digested with protease V8. The Figure shows a radioautograph of the resulting V8 peptides separated by thin-layer electrophoresis at pH 3.5 as described in the Materials and methods section. The positions of the origin and DNP-lysine are shown. Also marked to the left of the peptides are the relative mobilities of the original tryptic peptides (arrows) and letters referring to the identity of the V8 peptides (see the text). The results shown are from a single experiment in which peptides were separated under identical conditions. Similar results were obtained in a separate experiment.

and C, these eluted at 18, 21 and approx. 33% acetonitrile respectively (Fig. 3*a*). When each peak was collected, freeze-dried and separated by two-dimensional thin layer analysis, it was evident (Fig. 3*b*) that peak A contained two phosphopeptides (A1 and A2) and peak B contained three phosphopeptides (B1, B2 and B3) plus a number of minor phosphopeptides. Peak C contained at least four phosphopeptides (C1, C2, C3 and C1′). It should be noted that C1′ was found in rather more variable amounts than the other phosphopeptides and separate studies showed that it probably corresponds on reverse-phase h.p.l.c. to the small shoulder of peak C eluting at about 36% acetonitrile.

Our observations are in good agreement with the studies of others who have reported previously that three major ^{32}P-labelled peaks of tryptic phosphopeptides are separated by reverse-phase chromatography. In order of elution with increasing acetonitrile concentrations these peaks were denoted P1, P2 and P3 by Tornqvist *et al.* (1987) and PY1, PY2 and PY3 by White *et al.* (1985) and presumably correspond to the peaks denoted A, B and C in the present study.

Further analysis of the tryptic phosphopeptides by cleavage with *S. aureus* protease V8

The nine major tryptic phosphopeptides separated by two-dimensional thin layer analysis were eluted and digested with protease V8 which under the conditions used cleaves, rather specifically, at the *C*-terminal side of glutamate (Houmard & Drapeau, 1972). The resulting peptides were then separated by electrophoresis at pH 3.5 (Fig. 4). In every case, multiple phosphopeptides were evident indicating the original tryptic peptide was phosphorylated on more than one tyrosine.

In the case of B1, digestion of the original trytic peptide (which appears itself to have a small net positive charge at pH 3.5) with protease V8 leads to two peptides, one with a marked net positive charge (B1b) while the other appears to have a nearly comparable net negative charge (B1a). The ratio of ^{32}P in the two protease V8 peptides B1a/B1b was about 1:0.6 as determined by both direct scintillation counting or Raster densitometric scanning of radioautographs.

Digestion of the tryptic phosphopeptides A1, A2, B2, B3 and C1 suggests that they may all be related as each gave rise to a phosphopeptide which had a marked net negative charge and migrated to the same extent in each case (A1a, A2a, B2a, B3a and C1a). In addition, A1, B2, B3 and C1 all gave rise to peptides with a small net positive charge that migrated slightly less than DNP-lysine (A1b, B2b, B3b, C1b). However, A2 and B2 on digestion with protease V8 also gave rise to phosphopeptides A2b and B2c respectively which were not generated from the other tryptic phosphopeptides in this group. The approximate ratios of incorporation of ^{32}P were: A1a/A1b and A2a/A2b, 1:1.7; B2a/B2b/B2c, 1:0.6:1; B3a/B3b, 1:1.3 (means of two separate experiments).

Digestion of C2 by protease V8 gave three products (C2a, C2b and C2c), all with a net negative charge, of which the two with the greatest negative charge appeared to co-migrate with the two V8 peptides formed from C3 (Fig. 4). Digestion of C1′ by protease V8 also gave rise to peptides which appeared to migrate in a similar manner to those generated from C2 (results not shown). These three peptides were also evident in the digestion of C1 (see Fig. 4), presumably because C1 and C1′ were not fully resolved.

The approximate size of the major tryptic phosphopeptides was determined by the use of SDS/polyacrylamide-gel electrophoresis (20% polyacrylamide) with insulin B-chain (molecular mass 3000 Da), lysozyme

Table 1. Approximate net charges at pH 3.5 of possible phosphopeptides derived from the β-chain of the insulin receptor by sequential digestion with trypsin and protease V8

Charges have been calculated assuming that at pH 3.5 the net charge of the various groups is as follows: +1 (*N*-terminal NH_3^+ group and R groups of lysine, arginine and histidine); −0.2 (R group of glutamate); −0.5 (R group of aspartate); −1.0 (*C*-terminal CO_2^- group and R group of tyrosine phosphate). The Table gives the approximate net charges of the indicated tryptic phosphopeptides when phosphorylated on 1, 2 or 3 tyrosines as well as the net charges of the phosphopeptides formed by subsequent digestion with V8 protease. The protease V8 cleavage sites on the tryptic peptides are indicated by arrows.

Domain possible tryptic peptides derived from	Number of phosphotyrosines...	Approximate charge at pH 3.5 of: Tryptic phosphopeptides 1	2	3	Corresponding *N*-terminal protease V8 phosphopeptide 1	Corresponding *C*-terminal protease V8 phosphopeptides 1	2	3
Domain 1 (containing tyrosines 953, 960 and 972)								
KRQPDGPLGPLYASSNPEYLSASDVFPCSVYVPDEWEVSR		+0.1	−0.9	−1.9	+0.3	−2.2	−3.2	–
RQPDGPLGPLYASSNPEYLSASDVFPCSVYVPDEWEVSR		−0.9	−1.9	−2.9	−0.7	−2.2	−3.2	–
QPDGPLGPLYASSNPEYLSASDVFPCSVYVPDEWEVSR		−1.9	−2.9	−3.9	−1.7	−2.2	−3.2	–
Domain 2 (containing tyrosines 1146, 1150 and 1151)								
DIYETDYYRKGGK		+0.8	−0.2	−1.2	−1.7	+1.5	+0.5	–
DIYETDYYRK		−0.2	−1.2	−2.2	−1.7	+0.5	−0.5	–
DIYETDYYR		−1.2	−2.2	−3.2	−1.7	−0.5	−1.5	–
Domain 3 (containing tyrosines 1316 and 1322)								
SYEEHIPYTHMNGGK		+1.6	+0.6	–	−1.4	+2.0	–	–
SYEEHIPYTHMNGGKK		+2.6	+1.6	–	−1.4	+3.0	–	–
RSYEEHIPYTHMNGGK		+2.6	+1.6	–	−0.4	+2.0	–	–
RSYEEHIPYTHMNGGKK		+3.6	+2.6	–	−0.4	+3.0	–	–
Domain 4 (containing tyrosines 902, 904 and 908)								
ATSLAGNGSWTEPTYFYVTDYLDVPSNIAK		−1.2	−2.2	−3.2	–	−1.0	−2.0	−3.0

Table 2. Assignment of tryptic phosphopeptides separated by two-dimensional thin layer analysis

Numbering of phosphopeptides is as in Fig. 1(*c*). Definition of domains is as in Table 1.

Phospho-peptide	Structure
C1	DIYETDYYRK (mixture of monophosphorylated forms)
B2	DIYETDYYRKGGK (mixture of bisphosphorylated forms)
B3	DIY(P)ETDY(P)YRK and DIY(P)ETDYY(P)RK
A1	DIY(P)ETDY(P)Y(P)RKGGK
A2	DIY(P)ETDY(P)Y(P)RK
B1	SY(P)EEHIPY(P)THMNGGK
C1′, C2, C3	Probably derived from domain 1 (containing tyrosines 953, 960 and 972)

(molecular mass 14300 Da) and soya-bean trypsin inhibitor (molecular mass 20500 Da) as standards. The molecular masses of A1, A2, B1, B2, B3 and C1 were all less than 2000, whereas those of C2 and C3 were close to 3500 (results not shown).

DISCUSSION

Identity of the major tryptic phosphopeptides separated by two-dimensional thin layer analysis

Inspection of the amino acid sequence deduced by Ullrich *et al.* (1985) and Ebina *et al.* (1985) from the cDNA of the human insulin receptor shows that there are a total of 13 tyrosines in the intracellular portion of the β-subunit. Eight of these tyrosines occur in three domains each of which would give rise to tryptic peptides containing two or three tyrosines. These are the only plausible sites of phosphorylation as cleavage of all the tryptic peptides with protease V8 gave rise to at least two distinct phosphorylated products (Fig. 4). The sequence of these domains are given in Table 1 together with the calculated net charges at pH 3.5 of the various possible tryptic peptides and their corresponding protease V8 peptides. In addition, the sequence of one extracellular domain which also contains three tyrosines is given as, theoretically, this might also be a possible domain of phosphorylation in the isolated receptor.

The identity of at least six of the phosphopeptides can be deduced with some confidence from inspection of the predicted net charges of the various peptides taken together with the ratio of ^{32}P incorporation into the V8 products, the approximate size of the tryptic phosphopeptides, their behaviour on reverse-phase chromatography and the reported results of Goren *et al.* (1987) and Tornqvist *et al.* (1987).

Phosphopeptide B1. Inspection of Table 1 shows that this phosphopeptide is derived from domain 3 which occurs near the *C*-terminal end of the β-chain since it is the only domain that, when multiply phosphorylated, can give rise to a positively charged tryptic phosphopeptide. The migration of this peptide relative to DNP-lysine suggests that its net charge lies between 0 and +1. The behaviour of the V8 products on electrophoresis relative to B1 itself indicates that the peptide is the minimum length tryptic peptide (Ser-1315 to Lys-1329) phosphorylated on both tyrosines 1316 and 1322 (Table 2).

Partial tryptic digestion of the insulin receptor results in the cleavage of a fragment (molecular mass about 10000) apparently from the *C*-terminal end of the β-subunit (Goren *et al.*, 1987). This clipped receptor is still capable of autophosphorylation, but upon two-dimensional mapping, phosphopeptide B1 is no longer evident (M. Dickens & J. M. Tavaré, unpublished results) further confirming the identity of this peptide.

This conclusion is consistent with the results of Tornqvist *et al.* (1987), who demonstrated by direct amino acid sequencing that the least anionic component from their reverse-phase fraction P2 was probably this diphosphorylated peptide, although they did not formally have evidence that it was the diphosphorylated form rather than a near equal mixture of monophosphorylated forms.

Phosphopeptides A1, A2, B2, B3, C1. It has already been pointed out from the behaviour of their protease V8 products that these phosphopeptides are probably related. Inspection of Table 1 shows that they are all derived from domain 2 containing tyrosines 1146, 1150 and 1151. Previous studies have indicated that this domain may be an important site of autophosphorylation. In particular Tornqvist *et al.* (1987) found that the triphosphorylated tryptic peptide from this domain elutes first during reverse-phase chromatography (their peak P1). We found two distinct phosphopeptides in our corresponding reverse-phase h.p.l.c. fraction (peak A). Both phosphopeptides appeared to be triphosphorylated forms; in one case (A2) it can be deduced that tryptic cleavage has occurred at Lys-1153 whereas in the other (A1) it has occurred at Lys-1156 (Table 2). Thus the behaviour of A2 on electrophoresis corresponds to a net charge of −2.2 whereas that of its two V8 products corresponds to net charges of −1.7 (A2a) and −0.5 (A2b). In contrast, A1 has a net charge of −1.2 giving rise to one identical V8 product derived from the *N*-terminus (Ala) with a net charge of −1.7 and a quite distinct diphosphorylated product from the *C*-terminus (A1b) with a net charge of +0.5. The relative amounts of A1 and A2 were quite similar in a series of experiments. The mean ratio (A1/A2 ± S.E.M.) in ten separate experiments was 1.02 ± 0.11. Thus it appears that, when both the adjacent tyrosines 1150 and 1151 are phosphorylated, cleavage by trypsin can occur at either Lys-1153 or Lys-1156 at near equal rates. Cleavage at Arg-1152 does not appear to occur. The relative ^{32}P incorporation into the V8 products of both A1 and A2 were close to 1:1.7. The fact that these ratios are not exactly 1:2 suggests that a proportion of tyrosines 1150 and/or 1151 were phosphorylated in the insulin-receptor preparations before treatment with insulin and [γ-^{32}P]ATP.

Further inspection of Table 1 and the behaviour of the V8 products shown in Fig. 4 leads to the conclusion that B2 and B3 are diphosphorylated peptides derived from domain 2, whereas C1 is largely made up of a mixture of monophosphorylated peptides from the same domain (Table 2). B3 is the result of cleavage at Lys-1153 and is phosphorylated on Tyr-1146 and either Tyr-1150 or Tyr-1151, hence its V8 products have apparent net charges of

−1.7 and +0.5 (Table 1). On the other hand, B2, which was always present in smaller amounts than B3, is the result of cleavage at Lys-1156. The V8 products of B2 are compatible with it being a mixture of diphosphorylated forms including those phosphorylated at Tyr-1146 and either Tyr-1150 or Tyr-1151 (giving rise to V8 products B2a and B2c respectively) and those phosphorylated at tyrosines 1150 and 1151 (giving rise to V8 product B2b). The amount of this latter product suggests that only 10–15% of the diphosphorylated forms are phosphorylated on both Tyr-1150 and Tyr-1151. The ratios of incorporation of ^{32}P into B3a/B3b and B2a/B2c were both about 1:1.

Finally, C1 represents a mixture of monophosphorylated forms of domain 2 cleaved at Lys-1153 and hence its major V8 products (C1a and C1b) migrate with net charges of −1.7 and +0.5. C1a is derived from the form phosphorylated on Tyr-1146 and C1b from a mixture of the forms phosphorylated on either Tyr-1150 or Tyr-1151 (Table 2).

It should be noted that A2, B3 and C1, which are the tri-, di- and monophosphorylated forms of domain 2 cleaved at Lys-1153, lie close to a diagonal line in the two-dimensional maps (Figs. 1 and 2). Thus the introduction of successive phosphates affects migration in both the electrophoretic and chromatographic dimensions and also its elution from the C_{18} reverse-phase h.p.l.c. column. The corresponding forms cleaved at Lys-1156 all carry an extra positive charge and would appear to lie on an approximately parallel line shifted to the cathode. However, the diphosphorylated form (B2) was only found in relatively small amounts and we have not been able to detect any monophosphorylated form cleaved at Lys-1156.

Phosphopeptides C1′, C2 and C3. The identity of these apparently related peptides cannot be assigned with confidence without further information such as direct sequencing data. It seems most likely that they are derived from domain 1 which would give tryptic peptides of the appropriate size and a net negative charge at pH 3.5 which are also susceptible to cleavage by protease V8 at Glu-959 and Glu-976 to give two products each containing tyrosine residues. However, the actual migration of tryptic peptides C1′, C2 and C3 and their V8 products on thin layer analysis does not correspond to the expected net charges given in Table 1, if their behaviour is related to that of tryptic phosphopeptides derived from domains 2 and 3. A most likely explanation is that the increased size and hydrophobicity of peptides C1′, C2 and C3 and their V8 products, results in relatively slower rates of migration upon electrophoresis. Tentatively, we suggest that C3 is derived from domain 1 by tryptic cleavage after Arg-943 and Arg-981 and is phosphorylated on Tyr-953 and either Tyr-960 or Tyr-972; this peptide would have an expected net negative charge of −2.9 (Table 1). The net negative charges of C2 and C1′ may then be −1.9 and −0.9 respectively and thus these phosphopeptides may represent mono- or diphosphorylated forms of domain 1 in which cleavage has occurred either at Arg-943 or Lys-942. These, on treatment with protease V8, would be expected to give products of net charges −0.7, −1.7 and −2.2 (see Table 1).

The possibility must be considered that phosphopeptides C1′, C2 and C3 might be derived from an extracellular region of the β-subunit. In this region only domain 4 could yield a tryptic peptide (Ala-888 to Lys-917) containing more than one tyrosine and having an appropriate molecular mass. However, although all the tryptic phosphopeptides from this domain would be negatively charged at pH 3.5, it is unlikely to give rise to C1′, C2 and C3 as protease V8 cleavage at Glu-899 would only result in a single labelled product. This domain is also a possible site of glycosylation (Ebina *et al.*, 1985; Ullrich *et al.*, 1985).

Time course of autophosphorylation of the partially purified insulin receptor from human placenta

Our studies indicate that the first sites to be phosphorylated upon insulin binding to the α-subunit lie within domain 2 containing tyrosines 1146, 1150 and 1151. Precise details of the order of phosphorylation cannot be deduced because we cannot discriminate between phosphorylation of Tyr-1150 and Tyr-1151; but it would seem that there may not be a strict order of phosphorylation and that eventually an appreciable proportion of insulin receptors become phosphorylated on all three tyrosines in this domain.

The rates of phosphorylation of the two sites in domain 3 appear to be lower than those in domain 2. However, we have been unable to detect a monophosphorylated peptide from domain 3, although with an expected net charge of +1.6, it would be well separated from all the other labelled phosphopeptides. This suggests that once one tyrosine in this domain is phosphorylated, the other is rapidly phosphorylated. Finally, the sites that are slowest at becoming phosphorylated are in peptides C1′, C2 and C3 which, as discussed above, may be derived from domain 1.

One plausible explanation of this sequential phosphorylation is that phosphorylation within domain 2 leads to a conformational change of the intracellular region of the insulin-receptor β-subunit which then allows phosphorylation of the tyrosines within domain 3 and possibly also domain 1.

In the present studies, it is clear that we have not achieved complete phosphorylation of all insulin receptors in the preparation. In particular, mono- and diphosphorylated peptides derived from domain 2 are still evident after maximum incorporation of ^{32}P has been reached. One possible explanation is that the preparation contains an appreciable amount of protein phosphatase activity even in the presence of vanadate.

Utility of the use of two-dimensional peptide mapping to study phosphorylation of the insulin receptor

The two-dimensional mapping approach employed in the present studies offers a means of resolving the major tryptic phosphopeptides by a relatively simple and rapid technique and to a higher degree of resolution than that achieved by h.p.l.c. (Figs. 1 and 2). The approach is, therefore, particularly suitable for the study of the phosphorylation of the insulin receptor within intact cells and we have successfully applied to it two different cell types transfected with human insulin receptor cDNA (J. M. Tavaré, R. M. O'Brien, K. Siddle & R. M. Denton, unpublished work). In fact, if radioautography is carried out for 2–3 weeks, then it is possible to obtain much useful information on the sites of phosphorylation when only approx. 500 c.p.m. of ^{32}P is incorporated into insulin receptors.

We thank the British Diabetic Association and Medical Research Council for long-term support of our research.

REFERENCES

Avruch, J., Nemenoff, R. A., Blackshear, P. J., Pierce, M. W. & Osathanondh, R. (1982) J. Biol. Chem. **257**, 15162–15166

Chou, C. K., Dull, T. J., Russell, D. S., Gherzi, R., Lebwohl, D., Ullrich, A. & Rosen, O. M. (1987) J. Biol. Chem. **262**, 1842–1847

Denton, R. M. (1986) Adv. Cyclic Nucleotide Protein Phosphorylation Res. **20**, 293–341

Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J., Masiarz, F., Yan, Y. W., Goldfine, I. D., Roth, R. A. & Rutter, W. J. (1985) Cell **40**, 747–758

Ebina, Y., Araki, E., Taira, M., Shimada, F., Mori, M., Craik, C. S., Siddle, K., Pierce, S. B., Roth, R. A. & Rutter, W. J. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 704–708

Ellis, L., Morgan, D. O., Clauser, E., Edery, M., Jong, S., Wang, L., Roth, R. A. & Rutter, W. J. (1986*a*) Cold Spring Harbor Symp. Quant. Biol. **51**, 773–784

Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A. & Rutter, W. J. (1986*b*) Cell **45**, 721–732

Goren, H. J., White, M. F. & Kahn, C. R. (1987) Biochemistry **26**, 2374–2382

Herrera, R. & Rosen, O. M. (1986) J. Biol. Chem. **261**, 11980–11985

Houmard, J. & Drapeau, G. R. (1972) Proc. Natl. Acad. Sci. U.S.A. **69**, 3506–3509

Jacobs, S. & Cuatrecasas, P. (1986) J. Biol. Chem. **261**, 934–939

Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L. & Kahn, C. R. (1983) J. Biol. Chem. **80**, 2137–2141

Kahn, C. R., White, M. F., Grigorescu, F., Takayama, S., Haring, H. U. & Cretaz, M. (1985) in Molecular Basis of Insulin Action (Czech, M. P., ed.), pp. 67–94, Plenum Press, New York

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Morgan, D. O. & Roth, R. A. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 41–45

Ponzio, G., Dolais-Kitabgi, J., Louvard, D., Gautier, N. & Rossi, B. (1987) EMBO J. **6**, 333–340

Rosen, O. M., Herrera, R., Olowe, Y., Petruzzelli, L. M. & Cobb, M. H. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 3237–3240

Stadtmauer, L. & Rosen, O. M. (1986) J. Biol. Chem. **261**, 10000–10005

Tavaré, J. M. (1985) Ph.D. Thesis, University of Bristol

Tavaré, J. M., Diggle, T. A. & Denton, R. M. (1987) Biochem. J. **244**, 769–774

Tornqvist, H. E., Pierce, M. W., Frackelton, A. R., Nemenoff, R. A. & Avruch, J. (1987) J. Biol. Chem. **262**, 10212–10219

Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M. & Ramachandran, J. (1985) Nature (London) **313**, 756–761

White, M. F., Takayama, S. & Kahn, C. R. (1985) J. Biol. Chem. **260**, 9470–9478

Yu, K. & Czech, M. P. (1984) J. Biol. Chem. **259**, 5277–5286

Received 22 October 1987/8 February 1988; accepted 16 February 1988