Changes in insulin-receptor tyrosine, serine and threonine phosphorylation as a result of substitution of tyrosine-1162 with phenylalanine

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Previous studies, by ourselves and others, have shown that tyrosine residues 1158, 1162 and 1163 are very rapidly autophosphorylated on the human insulin receptor after insulin binding and that this is followed by the autophosphorylation of tyrosine residues 1328 and 1334. The autophosphorylation of these tyrosine residues, and their role in transmembrane signalling, were examined by using Chinese-hamster ovary cells transfected with either normal intact insulin receptors or receptors in which tyrosine residues 1162 or 1162/1163 were substituted with phenylalanine. These studies show the following. (1) Tyrosine-1158 could still be autophosphorylated when tyrosine-1162 and -1163 were substituted with phenylalanine. (2) Insulin-stimulated insulin-receptor tyrosine phosphorylation in intact cells was complete within 30 s and was accompanied, after a lag of 2–5 min, by a rise in serine and threonine phosphorylation of the β -subunit. (3) Replacement of tyrosine-1162 with phenylalanine blocked insulin-stimulated threonine phosphorylation of the insulin receptor in intact cells. (4) Insulin-stimulated serine phosphorylation of the β -subunit was found in both intact cells and partially purified receptor preparations incubated with [γ - 32 P]ATP and was still apparent after the replacement of tyrosine-1162 with phenylalanine. (5) Our data strongly suggest that insulin-stimulated insulin-receptor serine and threonine phosphorylations are initiated through two distinct pathways, with only the latter showing a strict dependence on autophosphorylation of tyrosine-1162.

INTRODUCTION

To date, the most rapid post-insulin-binding event detectable in intact cells is an increased autophosphorylation of the cytoplasmic domain of the receptor β -subunit on at least five tyrosine residues (Tavaré *et al.*, 1988; Tornqvist *et al.*, 1988; White *et al.*, 1988).

The partially purified insulin receptor incubated with insulin and [γ - 32 P]ATP is rapidly phosphorylated on tyrosine residues 1158, 1162 and 1163. This is then followed by the phosphorylation of two tyrosine residues (1328 and 1334) at the C-terminus and, finally, by that of at least two other tyrosine residues near the transmembrane sequence, which could correspond to any of the tyrosine residues 965, 972 and 984 (Tornqvist *et al.*, 1987; Tavaré & Denton, 1988; White *et al.*, 1988). Many partially purified preparations of the insulin receptor also exhibit insulin-stimulated serine phosphorylation of the β -subunit by a kinase which appears to be distinct from the receptor itself (Ballotti *et al.*, 1986; Smith *et al.*, 1988; Lewis *et al.*, 1990).

In intact cells, the binding of insulin also results in the rapid autophosphorylation of tyrosine residues 1158, 1162, 1163, 1328 and 1334, although tyrosine residues 965, 972 and 984 appear to be rather poorly phosphorylated (Tavaré *et al.*, 1988; Tornqvist *et al.*, 1988; White *et al.*, 1988). There is also an increase in serine and threonine phosphorylation of the β -subunit by kinases that are, again, presumably distinct from the receptor itself (Pang *et al.*, 1985; White *et al.*, 1985; Jacobs & Cuatrecasas, 1986; Ballotti *et al.*, 1987; Tavaré *et al.*, 1988).

Many of these phosphorylation sites can be rapidly distinguished using a two-dimensional thin-layer tryptic-phosphopeptide mapping technique that is summarized in Fig. 1 (see Tavaré & Denton, 1988; Tavaré *et al.*, 1988). Trypsin treatment

of 32 P-labelled insulin-receptor β -subunits reveals that tyrosine (Y) residues 1158, 1162 and 1163 can be recovered as a family of five phosphopeptides (general sequence DIYETDYRRK) 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). Tyrosine residues 1328 and 1334 are recovered as a bisphosphorylated tryptic peptide (sequence SYEEHIPYTHMNGGK) that runs as peptide B1 (Fig. 1). Additional tyrosine autophosphorylation

Fig. 1. Key to the identification of insulin-receptor tryptic phosphopeptides separated by two-dimensional thin-layer electrophoresis

32 P-labelled tryptic phosphopeptides were applied to the plate at the origin and separated, first by electrophoresis at pH 3.5 and then by ascending chromatography, as described in the Materials and methods section. The dotted oval shows the position of a marker, dinitrophenyl-lysine. See the text for further details.

Abbreviation used: CHO, Chinese-hamster ovary.

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sites (probably residues 965, 972 and 984) run as peptides C1', C2 and C3 (Fig. 1). Serine and threonine phosphopeptides from insulin-receptor β -subunits phosphorylated in intact cells appear to run in positions 'S' and 'T' respectively (Fig. 1; Tavaré *et al.*, 1988).

The availability of the human-insulin-receptor cDNA has allowed the expression of the normal intact (hereinafter referred to as 'wild-type') insulin receptor in a variety of mammalian cell lines and the detailed analysis of the effects of introducing point and deletion mutations into this molecule. Tyrosine residues 1162 (YF1 mutant) or 1162 and 1163 (YF3 mutant) have been substituted with phenylalanine (Ellis *et al.*, 1986). When over-expressed in Chinese-hamster ovary (CHO) cells, these mutations result in decreased insulin-stimulated autophosphorylation of the β -subunit both *in vitro* and in intact cells, and decreased kinase activity towards exogenous substrates *in vitro* (Ellis *et al.*, 1986). This, in turn, appears to result in the resistance of such cells to insulin with respect to glucose uptake and glycogen synthesis but, intriguingly, has no apparent effect on insulin-stimulated thymidine uptake or ribosomal-protein-S₆ phosphorylation (Ellis *et al.*, 1986; Debant *et al.*, 1988).

Trisphosphorylation of tyrosine residues 1158, 1162 and 1163 appears to correlate well with activation of the kinase towards exogenous substrates (White *et al.*, 1988; Flores-Riveros *et al.*, 1989). Furthermore, autophosphorylation of the receptor appears to promote a conformational change in the β -subunit such that it may then be recognized by antibodies raised to a synthetic peptide corresponding to the amino acid sequence surrounding tyrosine residues 1158, 1162 and 1163 (Herrera & Rosen, 1986; Perlman *et al.*, 1989). Thus the autophosphorylation of these three tyrosine residues probably plays a critical role in signal transduction by the insulin receptor.

In the present study we examine the possible order of phosphorylation of tyrosine residues 1158, 1162 and 1163 on the intact insulin receptor and investigate the effect of a tyrosine-1162 $\rightarrow$ phenylalanine substitution on receptor serine and threonine phosphorylation, both *in vitro* and in intact cells. These two problems are addressed using the two-dimensional t.l.c. technique previously developed by Tavaré & Denton (1988) to map the tyrosine, serine and threonine phosphorylation sites on the 'wild-type' insulin receptor.

MATERIALS AND METHODS

Materials

All materials were as described by Tavaré *et al.* (1988). CHO.T (transfected with intact 'wild-type' human insulin receptors) and the CHO.YF1 and CHO.YF3 cell lines were maintained in Ham's F12 medium (Flow Laboratories) with 10% (v/v) foetal-calf serum and 400 μ g of G418 (Geneticin)/ml (Gibco) at 37 °C in a CO₂/air (1:19) atmosphere. Monoclonal antibody 83.14 recognizes the α -subunit of the human insulin receptor and is unreactive towards rodent insulin receptors (Soos *et al.*, 1986).

Partial purification of the insulin receptor from transfected cells

Approx. 2×10^8 cells at confluence were extracted into buffer containing 1% (w/v) Triton X-100 with proteinase inhibitors and insulin receptors partially purified by wheat-germ-lectin-Sepharose chromatography as described in Tavaré *et al.* (1988). The eluate from the wheat-germ-lectin-Sepharose column was concentrated by using Centricon microconcentrators (Amicon) to allow sufficient ³²P incorporation into receptor β -subunits for rapid-examination by two-dimensional phosphopeptide mapping and phosphoamino acid analysis. Eluates from the three cell lines were adjusted to similar protein concentrations (approx. 1 mg/ml) and stored at -70 °C.

Phosphorylation of the partially purified insulin receptor and two-dimensional phosphopeptide mapping

This was performed as described by Tavaré & Denton (1988). In essence, samples (20 μ l) of partially purified insulin receptor were preincubated, at the temperatures and for the times indicated in the Figure legends, in (final vol. 60 μ l) 20 mM-Mops (pH 7.4)/0.25 mM-dithiothreitol/1 mM-Na₃VO₄/12 mM-MgCl₂/2 mM-MnCl₂ ($\pm 1 \mu$ M-insulin). Autophosphorylation was initiated with [γ -³²P]ATP (100 μ M; 500–5000 c.p.m./pmol) and incubation continued at the temperatures and for the times indicated. The reaction was terminated with SDS/PAGE sample buffer, the phosphoproteins were separated by SDS/7%-(w/v)-PAGE using a Bio-Rad Minigel system and the ³²P-labelled insulin-receptor β -subunits were isolated from the gel by electroelution. Electroeluted proteins were precipitated with acetone and digested with 10 μ g of tosylphenylalanylchloromethane ('TPCK')-treated trypsin/ml in 100 μ l of 50 mM-NH₄HCO₃ for 16 h at 30 °C. A further 10 μ g of tosylphenylalanylchloromethane-treated trypsin was added for 5 h at 30 °C and the [³²P]phosphopeptides were separated on cellulose t.l.c. plates, first by electrophoresis at 400 V for 2 h at pH 3.5 (pyridine/acetic acid/water, 1:10:189, by vol.), then by ascending chromatography (pyridine/acetic acid/butanol/water, 10:3:15:12, by vol.) and finally detected by autoradiography using Kodak X-Omat pre-flashed film in cassettes with intensifying screens at -70 °C.

Phosphoamino acid analysis

Phosphoamino acid analysis was performed using a modification of the method of Kamps & Sefton (1989). Essentially, after separation of the insulin receptor by SDS/PAGE, the proteins were electrophoretically transferred to Immobilon membranes (Millipore). The region of Immobilon corresponding to the β -subunit was detected by autoradiography, cut out and incubated with 150 μ l of 6 M-HCl for 90 min at 110 °C under vacuum. Separation of the constituent phosphoamino acids by thin-layer electrophoresis at pH 3.5 was performed as described by Cooper *et al.* (1983).

Insulin-receptor phosphorylation in intact cells

This was performed using a modification of the method of Tavaré *et al.* (1988). Cells in 3.5 cm-diameter Petri dishes (approx. 5×10^6 cells/dish) were incubated for 3 h at 37 °C (CO₂/air, 1:19) in 1 ml of phosphate- and serum-free Dulbecco's modified Eagle's medium (Gibco) supplemented with 10 mM-Hepes, pH 7.5, and containing 0.2–1 mCi of [³²P]P_i (Amersham International). Insulin (1 μ M) was added as required and, after various times of incubation at 37 °C, the medium was aspirated and cells solubilized in 0.5 ml of ice-cold extraction buffer [50 mM-Hepes (pH 7.5)/1 mM-Na₃VO₄/10 mM-EDTA/10 mM-NaF/30 mM-sodium pyrophosphate/0.1 mM-phenylmethanesulphonyl fluoride/2.5 mM-benzamidine/pepstatin + antipain + leupeptin (1 μ g/ml each)] supplemented with 1% (w/v) Triton X-100. Extracts were centrifuged at 10000 g for 5 min at 4 °C and the supernatants tumbled end-over-end for 2 h at 4 °C with 5 μ l of anti-(insulin receptor) monoclonal antibody 83.14 and 5 mg of Protein A-Sepharose. The receptor-antibody-Sepharose complex was isolated by centrifugation and washed four times with 1 ml of ice-cold extraction buffer containing 0.1% (w/v) Triton X-100. The complex was dissociated with 25 μ l of SDS sample buffer and boiled for 5 min. The Sepharose was boiled with a further 200 μ l of water and the pooled eluates separated by SDS/PAGE as described above.

Expression of data

Designation of the amino acid residues is based on the sequence

described by Ebina *et al.* (1985). All data shown are representative of at least two separate experiments. Results are means $\pm$ S.E.M. for the number of experiments shown in parentheses.

RESULTS

Analysis of the phosphorylation of the partially purified insulin receptor and its mutants with [γ - 32 P]ATP

Insulin receptors were partially purified by wheat-germ-lectin-Sepharose chromatography from CHO.T, CHO.YF1 and CHO.YF3 cells. The preparations were adjusted to equal protein concentrations and possessed approximately equal amounts of insulin-receptor protein as judged by Western blotting with polyclonal antisera to both the α - and β -subunits of the insulin receptor (results not shown).

The three preparations were incubated in the absence or presence of insulin and then with [γ - 32 P]ATP at either 0 °C or 30 °C. Fig. 2 demonstrates that insulin had a substantially greater effect on wild-type insulin-receptor β -subunit phosphorylation after 30 min at 0 °C than after 10 min at 30 °C [15.08 ± 2.22 (4)- and 1.62 ± 0.33 (3)-fold respectively]. This was primarily due to an increased basal phosphorylation at 30 °C. A similar phenomenon has been noticed with the wheat-germ-lectin-Sepharose-purified human placental insulin receptor (J. M. Tavaré & M. Dickens, unpublished work).

In both of the tyrosine $\rightarrow$ phenylalanine mutants at 0 °C and 30 °C, insulin had only approx. 2-fold effects on receptor β -subunit phosphorylation (Fig. 2). In striking contrast with the wild-type receptor from CHO.T cells, the level of basal and insulin-stimulated 32 P incorporation into the two mutant receptors was lower at 30 °C than at 0 °C. Furthermore, the basal level of 32 P incorporation into the mutant insulin receptors at 0 °C,

Fig. 2. Effect of temperature on the phosphorylation of partially purified insulin receptors from CHO.T, CHO.YF1 and CHO.YF3 cells

Partially purified insulin receptors were preincubated without (–) or with (+) 1 μ M-insulin at either 30 °C (top panel) or 0 °C (lower panel) and then incubated with [γ - 32 P]ATP (100 μ M; 500 c.p.m./pmol) as described in the Materials and methods section. The reactions were terminated at the indicated times with sample buffer, and phosphoproteins were separated by SDS/7%-(w/v)-PAGE. The Figure shows autoradiographs (1 day) of the gels.

Fig. 3. Phosphoamino acid analysis of insulin receptors phosphorylated *in vitro* at 30 °C

Partially purified insulin receptor from CHO.YF3, CHO.YF1 or CHO.T cells were preincubated without (–) or with (+) 1 μ M-insulin at 30 °C for 15 min. [γ - 32 P]ATP (100 μ M; 5000 c.p.m./pmol) was added, and incubation was continued for 15 min at 30 °C. The reactions were terminated, the mixture separated by SDS/7%-PAGE and electrophoretically transferred to Immobilon for phosphoamino acid analysis as described in the Materials and methods section. The Figure shows autoradiographs (3 days) of the separations with the positions of carrier phosphotyrosine (P-Y), phosphothreonine (P-T) and phosphoserine (P-S), shown along with free [32 P] P_i (P_i) and the origin of sample application.

but not 30 °C, was higher than that into the wild-type insulin receptor. It should also be noted that, at 30 °C in particular, the maximal insulin-stimulated levels of 32 P incorporation into the YF1 and YF3 receptors were just 20.5 ± 3.4 % (3) and 10.6 ± 1.87 % (3) respectively, of that into the wild-type receptor [equivalent values at 0 °C were 47.5 ± 2.67 (3) and 26.7 ± 5.0 (3) respectively]. Receptor phosphorylation in all three preparations reached a maximum after approx. 30 min at 0 °C and 10 min at 30 °C.

Phosphoamino acid analysis revealed that insulin stimulated both tyrosine and serine phosphorylation of the β -subunit of the receptor from all three preparations at 30 °C (Fig. 3). The level of 32 P incorporation into serine residues, compared with that into tyrosine residues, of the two mutant receptors appeared to be lower than into the wild-type receptor (Fig. 3). At 0 °C there was little 32 P incorporation into β -subunit serine residues in any of the receptor preparations (results not shown). No threonine phosphorylation was detected with any of the partially purified insulin receptor preparations at either 0 °C or 30 °C.

Two-dimensional phosphopeptide mapping of the wild-type and mutant receptor preparations phosphorylated *in vitro*

Receptor preparations were preincubated with or without insulin and then with [γ - 32 P]ATP at 0 °C for 30 min. The β -subunits were isolated, digested exhaustively with trypsin, and the resulting phosphopeptides separated by two-dimensional t.l.c. as shown in Fig. 4.

Fig. 4. Two-dimensional phosphopeptide mapping of insulin receptors phosphorylated *in vitro* at 0 °C

Partially purified insulin receptors from CHO.YF3 (a and d), CHO.YF1 (b and e) and CHO.T (c and f) cells were preincubated at 0 °C for 30 min without (a, b and c) or with (d, e and f) 1 μ M-insulin. [γ - 32 P]ATP (100 μ M; 5000 c.p.m./pmol) was added, and the reactions were terminated with sample buffer after 30 min at 0 °C. 32 P-labelled β -subunits were isolated, digested with trypsin, and the phosphopeptides were separated by electrophoresis at pH 3.5 (horizontal dimension; anode to the left) and then by ascending chromatography. The Figure shows autoradiographs (3 days) of the separations, with the origin of sample application (arrow) and dinitrophenyl-lysine marker (dotted oval) indicated.

Consistent with our previous studies (Tavaré & Denton, 1988; Tavaré *et al.*, 1988), insulin stimulated the appearance of phosphopeptides A1, A2, B1, B2, B3, C1 and C2 in the receptor from CHO.T cells (Fig. 4c versus Fig. 4f). Very little 32 P incorporation was observed in the basal state (Fig. 4c).

Examination of the YF1 mutant showed that insulin stimulated 32 P incorporation into peptides B1, B2, B3, C1 and C2, with only a low level of incorporation into peptides A1 and A2 apparent (Fig. 4b versus Fig. 4e). Increased basal 32 P incorporation into peptides C1 and B1 was also noted when compared with the receptor from CHO.T cells (Fig. 4b versus Fig. 4c).

In the YF3 mutant, insulin-stimulated 32 P incorporation was restricted primarily to peptide C1, with considerably lower incorporation into B1 (Fig. 4a versus Fig. 4d). As with the YF1 receptor, a higher level of incorporation into peptide C1 was evident in the basal state when compared with the wild-type receptor (Fig. 4a versus Fig. 4c). This peptide was confirmed as C1, as it was found to co-migrate with a synthetic monophosphopeptide of sequence DIYETDY(P)YR (results not shown). Very low levels of 32 P incorporation into peptides A1, A2 and B3 were also evident after insulin stimulation (Fig. 4d).

Analysis of the phosphorylation of the wild-type and mutant insulin receptors in intact CHO cells

CHO cell lines transfected with the wild-type, YF1 or YF3 insulin-receptor variants were incubated with [32 P] P_i and then with or without insulin. Receptors were immunoprecipitated with an antibody that only precipitates human receptors and separated by SDS/PAGE. Even 30 s after the addition of insulin, receptor phosphorylation was found to be almost maximally stimulated in all three cell lines. At 15 min, insulin had 2.21 ± 0.44

Fig. 5. Phosphoamino acid analysis of insulin receptors phosphorylated in intact cells

Approx. 5×10^5 CHO.YF1 or CHO.T cells were preincubated with 200 μ Ci of [32 P] P_i and then without (0) or with 1 μ M-insulin for the times indicated. Cells were extracted rapidly and the 32 P-labelled β -subunits isolated as described in the Materials and methods section. Phosphoamino acid analysis was performed as described in the legend to Fig. 3. The Figure is an autoradiograph (14 days) of the resulting thin-layer plate.

Fig. 6. Analysis of the time course of ^{32}P -labelling of insulin-receptor phosphorylation sites in intact CHO.T cells by two-dimensional t.l.c.

Approx. 10^6 CHO.T cells were preincubated with 2 mCi of ^{32}P and then without (0) or with $1\ \mu\text{M}$ -insulin for 2, 5, 10 or 15 min. Cells were solubilized and then ^{32}P -labelled β -subunits were isolated and digested with trypsin for analysis by two-dimensional t.l.c. as described in the Materials and methods section. The Figure shows autoradiographs (5 days) of the separations.

(3)-, 2.70 ± 0.15 (3)- and 6.85 ± 0.52 (3)-fold effects on receptor phosphorylation in the CHO.YF3, CHO.YF1 and CHO.T cells respectively. The maximal insulin-stimulated levels of ^{32}P incorporation into insulin receptors in intact CHO.YF1 and CHO.YF3 cells were $27.9 \pm 5.9\%$ (3) and $6.96 \pm 0.57\%$ (3) respectively of that into CHO.T-cell insulin receptors.

Phosphoamino acid analysis (Fig. 5) revealed that the initial and rapid onset of receptor phosphorylation in both the CHO.T and CHO.YF1 cells was almost entirely due to increased tyrosine autophosphorylation. This was accompanied, in both cell lines, by a slower rise in serine phosphorylation that only became significant at 2–5 min (Fig. 5). In the CHO.T but not the CHO.YF1 cells there was also an insulin-stimulated rise in threonine phosphorylation; however, this effect was again only clearly evident after some 2–5 min of incubation. ^{32}P incorporation into the receptor in the CHO.YF3 cells was insufficient to obtain a similar and interpretable time course of phosphorylation of the individual amino acids.

Two-dimensional t.l.c. of insulin receptors from ^{32}P -labelled CHO.T cells confirmed the rapid insulin-stimulated rise in

tyrosine phosphorylation on peptides A1, A2, B1, B2 and B3, with increased T-peptide phosphorylation only apparent after approx. 2–5 min. (Fig. 6). Furthermore, the pattern of ^{32}P incorporation into the phosphotyrosine-containing peptides did not change between 2 and 15 min (Fig. 6).

Little ^{32}P incorporation into the phosphotyrosine-containing peptides was evident, in the absence of insulin, in any of the cell lines (Figs. 7a, 7b and 7c). After insulin stimulation, peptides C1 and B3 were evident in insulin receptors from CHO.YF1 cells (Fig. 7e), but only peptide C1 was evident in receptors from the CHO.YF3 cells (Fig. 7d). In contrast with CHO.T cells, no significant ^{32}P incorporation into peptide B1 was observed in either CHO.YF1 or CHO.YF3 cells (Figs. 7d and 7e versus Fig. 7f).

Two-dimensional t.l.c. demonstrated that a 15 min incubation of CHO.T cells with insulin had a substantial effect (approx. 10-fold) on the phosphorylation of the phosphothreonine-containing T-peptide of the β -subunit (Fig. 6 and Fig. 7c versus Fig. 7f). By contrast, there was no apparent effect of insulin on threonine phosphorylation of the receptor in the CHO.YF1 (Fig. 7b versus Fig. 7e) or CHO.YF3 (Fig. 7a versus Fig. 7d) cells. The basal level of ^{32}P incorporation into the T-peptide was broadly similar in all three cell lines (Fig. 7, compare a, b and c).

The phosphoserine-containing peptides were poorly resolved and migrated close to peptide C1 (Tavaré *et al.*, 1988). The phosphothreonine-containing peptide was estimated to have approx. 0.6 ± 0.1 mol of phosphate/mol of β -subunit (three separate experiments) after 5 min treatment of CHO.T cells with insulin. This was estimated by dividing ^{32}P radioactivity incorporated into the T-peptide by the amount of β -subunit estimated from ^{32}P incorporation into peptides A1, A2, B2, B3 and C1, assuming that all receptors become phosphorylated on tyrosine residues 1158, 1162 and 1163.

DISCUSSION

Phosphorylation of tyrosine residues 1158, 1162 and 1163

To date, no detailed studies on the order of autophosphorylation of insulin-receptor tyrosine residues 1158, 1162 and 1163 in intact cells have been reported, although these residues are probably the most rapidly phosphorylated in the partially purified insulin receptor *in vitro* (Tavaré & Denton, 1988).

The presence of peptides C1 (monophosphorylated), B2 and B3 (both bisphosphorylated) in the peptide maps of YF1 receptor phosphorylated both *in vitro* (Figs. 4b and 4e) and in intact cells (Figs. 7b and 7e) suggests that substantial phosphorylation of tyrosine residues 1158 and 1163 can still occur when tyrosine-1162 is substituted with phenylalanine. When tyrosine residues 1162 and 1163 were both substituted with phenylalanine (YF3 mutant), tyrosine-1158 still appeared to be phosphorylated, as evidenced by the presence of peptide C1 (Figs. 4a and 4d, and Figs. 7a and 7d).

The present data therefore suggest that autophosphorylation of the wild-type insulin receptor might be initiated at tyrosine-1158. However, in a recent study (Zhang *et al.*, 1991), we have shown that tyrosine-1162 and -1163 are rapidly phosphorylated in a mutant insulin receptor in which tyrosine-1158 was substituted with phenylalanine. This raises the possibility that phosphorylation of tyrosine residues 1158, 1162 and 1163 may occur in a random order. Precise determination of the order of phosphorylation of these tyrosine residues in the wild-type receptor awaits the development of methods that can clearly resolve each individual tyrosine residue in this complex molecule. On the other hand, the fact that substitution of tyrosine-1162 results in quite substantial reductions in the maximal level of insulin-stimulated ^{32}P incorporation attained, both *in vitro* and

Fig. 7. Analysis of insulin-receptor phosphorylation sites in intact CHO.YF3, CHO.YF1 and CHO.T cells

Approx. 10^6 CHO.YF3 (a and d), CHO.YF1 (b and e) or CHO.T (c and f) cells were preincubated with 2 mCi of [32 P]P_i and then in the absence (a, b and c) or presence (d, e and f) of 1 μ M-insulin for 15 min. 32 P-labelled β -subunits were isolated and digested with trypsin for separation by two-dimensional t.l.c. as described in the Materials and methods section. The Figure shows autoradiographs (5 days) of the resulting separations.

in intact cells, strongly implicates an important role for tyrosine-1162 in regulating receptor autophosphorylation.

Low levels of 32 P incorporation into the triphosphorylated peptides A1 and A2 were evident in the YF1 receptor phosphorylated *in vitro* (Fig. 4e). These peptides were almost certainly derived from the endogenous rodent insulin receptors present in the CHO cells. Fairly recently it has been reported that insulin-receptor autophosphorylation may occur through an inter-molecular transphosphorylation mechanism (Ballotti *et al.*, 1989; Cobb *et al.*, 1989). This raises the possibility that the endogenous, fully functional, rodent insulin receptors could transphosphorylate the YF1 mutant receptors, leading to the appearance of peptides C1, B2 and B3 (Fig. 4e and Fig. 7e). This is unlikely, however, owing to the very low number of rodent insulin receptors compared with human YF1 receptors (at least a 100-fold excess of the latter; Ellis *et al.*, 1986) and the very rapid onset of maximal autophosphorylation of the YF1 receptor observed in intact cells. However, we cannot state conclusively that the YF1 receptor is itself capable of intra- or inter-molecular autophosphorylation of tyrosine residues 1158 and 1163 until it can be completely separated from the rodent receptors. Clearly, we now require a cell line completely lacking endogenous insulin receptors that is suitable for transfection studies.

Ellis *et al.* (1986) observed that the partially purified insulin receptor from CHO.YF1 and CHO.YF3 cells exhibited a higher basal level of autophosphorylation at 24 °C than that of the wild-type receptor from CHO.T cells. This was confirmed in the present study at 0 °C, but was not the case at 30 °C (Fig. 2). Two-dimensional phosphopeptide mapping revealed this to be the result of increased basal monophosphorylation of the peptide containing tyrosine residues 1158, 1162 and 1163 (C1, with a further increase in basal phosphorylation of peptide B1 con-

taining tyrosine residues 1328 and 1334; Figs. 4a and 4b versus Fig. 4c). As yet no explanation can be offered for this phenomenon, except, perhaps, that the introduction of these relatively conservative point mutations into the β -subunit induces a perturbation of its conformation and thus increased kinase activity towards the β -subunit itself.

Phosphorylation of tyrosine residues 1328 and 1334

Peptide B1 corresponds to the bisphosphopeptide containing tyrosine residues 1328 and 1334. There was no significant phosphorylation of this peptide in the YF3 and YF1 receptors phosphorylated in intact cells when compared with the receptor in CHO.T cells (Figs. 7d, 7e and 7f respectively). This suggests that autophosphorylation of tyrosine residues 1328 and 1334 can only occur after the initiation of phosphorylation of tyrosine residues 1158, 1162 and 1163. This would be consistent with our previous observations concerning the time course of phosphorylation of the human placental insulin receptor *in vitro* (Tavaré & Denton, 1988). However, partially purified receptor preparations from CHO.YF1 and CHO.YF3 cells phosphorylated *in vitro* exhibit substantial phosphorylation of peptide B1 (Figs. 4d, 4e and 4f).

Phosphorylation of the insulin receptor on serine and threonine residues

In CHO.T cells insulin promoted an almost immediate rise in tyrosine autophosphorylation which was accompanied, after a lag of approx. 2–5 min, by a rise in threonine phosphorylation (Fig. 6). The effect on threonine phosphorylation of the β -subunit is one of the largest effects of insulin (approx. 10-fold), apart from tyrosine autophosphorylation itself, described in cells transfected with the human-insulin-receptor cDNA. This makes

threonine phosphorylation a useful and highly sensitive monitor for insulin action in transfected cells.

The most striking result of substituting tyrosine-1162 with phenylalanine was the apparent complete abolition of the insulin effect on threonine phosphorylation of the β -subunit in intact cells (Figs. 7b and 7e); there was also no effect of insulin on threonine phosphorylation in the CHO.YF3 mutant (Figs. 7a and 7d). Interestingly, although insulin was probably capable of stimulating rodent-insulin-receptor autophosphorylation in intact CHO.YF1 cells, the overexpression of the YF1 mutant was sufficient to block insulin-stimulated glucose transport (Ellis *et al.*, 1986), as well as threonine phosphorylation of the insulin receptor itself.

The effect of insulin on insulin-receptor serine phosphorylation in intact cells was approx. 3–4-fold (Fig. 5), but could only be conveniently analysed by using phosphoamino acid analysis, as the serine-containing tryptic phosphopeptides were poorly resolved by two-dimensional t.l.c. Insulin also stimulated receptor serine phosphorylation in partially purified receptor preparations (Fig. 3). In both intact cells and partially purified receptors, an effect of insulin on serine phosphorylation was still apparent although reduced, when tyrosine residues 1162 or 1162/1163 were substituted with phenylalanine (Figs. 3 and 5).

There is now a growing body of evidence to suggest that there are multiple diverging signalling pathways initiated by the insulin receptor. The substitution of tyrosine residues 1162 and 1163 with phenylalanine has been shown previously to block insulin-stimulated glucose transport and glycogen synthesis, but not thymidine uptake or S_6 phosphorylation (Ellis *et al.*, 1986; Debant *et al.*, 1988). Olefsky, Ullrich and co-workers (Maegawa *et al.*, 1988; McClain *et al.*, 1988; Theis *et al.*, 1989) have described a mutant receptor lacking the C-terminal 43 amino acids of the β -subunit which is capable of autophosphorylation, phosphorylation of exogenous substrates, internalizing normally, mediating an elevated level of thymidine uptake, but cannot mediate insulin-stimulated glucose transport or glycogen synthesis. More recently, Levitski and co-workers (Schechter *et al.*, 1989) have described inhibitors of the insulin-receptor tyrosine kinase (non-competitive with ATP) that block insulin-stimulated lipogenesis but have no effect on insulin-stimulated anti-lipolysis.

Our observations are also consistent with the hypothesis that insulin initiates diverging signalling pathways. Substitution of tyrosine-1162 with phenylalanine disrupted insulin-stimulated receptor threonine, but not serine, phosphorylation, although the latter was probably reduced in magnitude.

The mechanisms of insulin-stimulated insulin-receptor threonine and serine phosphorylation are not known. Insulin stimulates the activity of several, apparently distinct, serine/threonine kinase activities in intact cells (see Denton, 1986; Czech *et al.*, 1988). King & Sale (1988) have proposed that insulin-stimulated serine phosphorylation of the partially purified human placental insulin receptor occurred only if the receptor could be previously autophosphorylated.

Replacement of tyrosine-1162 with phenylalanine also prevented autophosphorylation of tyrosine residues 1328 and 1334 in intact cells (Fig. 7). Lack of phosphorylation of these three tyrosine residues appeared to correlate with the lack of insulin-stimulated insulin-receptor threonine phosphorylation. Removal of tyrosine-1162 would prevent the receptor from attaining trisphosphorylation of the domain containing tyrosine residues 1158, 1162 and 1163. Trisphosphorylation of this domain has been proposed by White *et al.* (1988) to result in maximal activation of the tyrosine kinase towards exogenous substrates *in vitro*. Activation of the insulin-receptor tyrosine kinase by

trisphosphorylation might, therefore, initiate a cascade of phosphorylation events resulting in the stimulation of the putative insulin-receptor-associated threonine kinase.

This work was supported by grants from the Medical Research Council (MRC) and British Diabetic Association to J.M.T. and Professor R. M. Denton. M.D. holds an MRC studentship. We are grateful to Professor R. M. Denton for his critical appraisal of the manuscript and for many useful discussions. We are also indebted to Dr. K. Siddle (Department of Clinical Biochemistry, University of Cambridge, Cambridge, U.K.) for generously providing antibody 83.14, Dr. L. Ellis (Howard Hughes Medical Institute, Dallas, TX, U.S.A.) for providing the CHO.T and CHO.YF1 cells, and Dr. R. A. Roth (Stanford University, Stanford, CA, U.S.A.) for providing the CHO.YF3 cells.

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