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Multisite serine phosphorylation of the insulin and IGF-I receptors in transfected cells

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Serine phosphorylation of insulin/IGF-I receptors in transfected fibroblasts was analysed by peptide mapping. PMA stimulated the phosphorylation of 5 distinct insulin receptor phosphopeptides: a single major phosphothreonine peptide containing Thr-1348, one major and 3 minor phosphoserine peptides. The major insulin-stimulated phosphoserine peptides were the same as those after PMA, with the exception of 2 minor phosphoserine peptides. PMA stimulated phosphorylation of a single major IGF-I receptor phosphoserine peptide which was phosphorylated to a lesser extent after IGF-I. We conclude that insulin/IGF-I and PMA stimulate phosphorylation of the same sites, but differ in the extents of phosphorylation.

Insulin; IGF-I; Protein kinase C; Receptor; Peptide mapping; Phorbol ester

1. INTRODUCTION

The binding of insulin to its receptor stimulates autophosphorylation and activation of the receptor's intrinsic tyrosine kinase, an event thought to be essential to signal transduction [1]. In intact cells (in situ) [2,10] and in some partially purified preparations (in vitro) [3,4,5], insulin-stimulated autophosphorylation on tyrosine residues of the β-subunit is followed by serine and threonine phosphorylation of the insulin receptor by poorly characterised kinase(s). Serine/threonine phosphorylation of the insulin receptor by protein kinase A or protein kinase C in response to various agonists [6,7,8] attenuates the tyrosine kinase activity of the receptor indicating a possible mechanism for modulating the receptor tyrosine kinase via other signalling systems.

Cell lines that over-express insulin receptor cDNA [9] have been utilised in a number of recent studies to investigate patterns of insulin receptor phosphorylation and identify phosphorylation sites [10,11,12]. Insulin receptor residue Threonine-1348* (1336) has been shown to be a major site of phosphorylation in response to PMA-stimulation in intact cells and protein Kinase C in vitro [11,13] while insulin-stimulated phosphorylation occurs on Serines-1305/1306 (1293/1294) and other sites [12]. In the present study, we sought to compare directly the patterns of serine/threonine phosphorylation of the insulin receptor and IGF-I receptors in response to phorbol ester and insulin or IGF-I using two-dimensional tryptic phosphopeptide mapping to determine whether there were unique sites associated with each agonist, or whether a common pathway might mediate the effects of these stimuli.

*Amino acid sequence numbers used are those of Ebina et al. (1986) Cell 40, 747, while the corresponding number in the sequence of Ullrich et al. (1986) Nature 313, 756 is shown in parentheses.

Abbreviations: PMA, 4β-phorbol 12β-myristate 13α-acetate; SDS-PAGE, Sodium Dodecyl sulphate-polyacrylamide gel electrophoresis; DMSO, Dimethylsulphoxide; IGF-I, insulin-like growth factor I; PKC, protein kinase C.

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2. MATERIALS AND METHODS

The NIH-3T3-HIR 3.5 cell line overexpressing human insulin receptor cDNA [9] and IGF-I-R/3T3 cell line overexpressing the human IGF-1 receptor cDNA [23] were as previously described. [^{32}P]Orthophosphate (PBS 41) was obtained from Amersham International, Amersham, Bucks, UK. TPCK-Trypsin (Worthington) was from Lorne Laboratories. Cellulose thin layer plates were from Eastman-Kodak, Liverpool, UK. Immobilon (PVDF) membranes were purchased from Millipore. 4β-phorbol 12β-myristate 13α-acetate (PMA), Protein-A sepharose CL-4B and *N*-ethyl morpholine were supplied by Sigma, Poole, Dorset, UK. Organic solvents (Analar grade) were purchased from BDH, Poole, Dorset, UK. All other reagents used were of the highest grade commercially available.

Metabolic labeling of receptors from intact cells was carried out as described [10] using 2 mCi/ml of [^{32}P]orthophosphate with subsequent exposure to insulin, IGF-I or PMA as indicated. Insulin receptors were immunoprecipitated with antibody 83-14 [14] and IGF-1 receptors with αIR3 [24] provided by Dr S. Jacobs as described [10]. Gel electrophoresis was performed on tricine-SDS 7.5% polyacryl-

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amide gels [15] and gels were subjected to autoradiography on Kodak X-omat XAR film. Incorporation of ^{32}P was quantified as described [14].

Peptide mapping of the insulin and IGF-I receptors was performed as described [16] except for the following modifications. Tryptic digestion was performed in 100 mM *N*-ethyl morpholine acetate pH 8.3. The lyophilised tryptic phosphopeptides were dissolved in 5 μl of 30% formic acid and spotted onto thin layer plates. High voltage electrophoresis was performed in 30% formic acid (pH 1.9) in the first dimension for 1500 V·h until the DNP-Lysine marker had migrated 10 cms. Plates were rapidly dried under an air stream and chromatography performed in the 2nd dimension [16] with isobutanol/acetic acid/water/pyridine (90:18:72:60; pH 3.5). Peptides from thin layer cellulose plates were eluted using 30% formic acid, evaporated, hydrolysed at 110°C for 1 h and analysed for phosphoamino acids [16].

3. RESULTS

Treatment of the NIH-3T3-HIR 3.5 cells with PMA caused a dose- and time-dependent increase in insulin receptor β-subunit phosphorylation of up to 3-fold, with maximal effects after 30 min at micromolar concentrations of PMA (Fig. 1a). Similar increases were observed in IGF-I receptor phosphorylation in IGF-I-R/3T3 cells (Fig. 1b). Tryptic phosphopeptide mapping of the insulin receptor β-subunit revealed 5 distinct phosphopeptides after phorbol ester stimulation (Fig. 2b). Individual phosphopeptides were analysed for phosphoamino acid content (Fig. 3). The major phosphopeptides were a relatively hydrophobic phosphothreonine peptide (T) and a hydrophobic phosphoserine peptide (S1). In addition, three other less hydrophobic minor labeled tryptic peptides are seen (S2, S3, S4) (Fig. 2b). In unstimulated (basal) cells, insulin receptor phosphopeptides T and S1 (Fig. 2a) were barely detectable in comparison with cells stimulated by PMA.

Tryptic digestion of β-subunit from insulin-stimulated cells showed the major tyrosine phosphopeptides in addition to the serine and threonine phosphopeptides S1, S2 and T (Fig. 2c). Peptide S1 comigrated with Y6 as phosphoamino acid analysis of this spot (Y6/S1; Fig. 2c) revealed phosphotyrosine and a small amount of phosphoserine (Fig. 3). Peptides S3 and S4 were not observed with insulin and appeared to be specific for the phorbol ester-induced phosphorylation. Insulin also increased the phosphorylation of phosphopeptide T above the basal level but to a lesser extent than that seen with PMA.

In unstimulated IGF-I-R/3T3 cells (Fig. 2d), the IGF-I receptor showed 5 phosphopeptides designated SI, SII, SIII, SIV and SV. Addition of IGF-I (Fig. 2f) to intact cells resulted in the appearance of additional

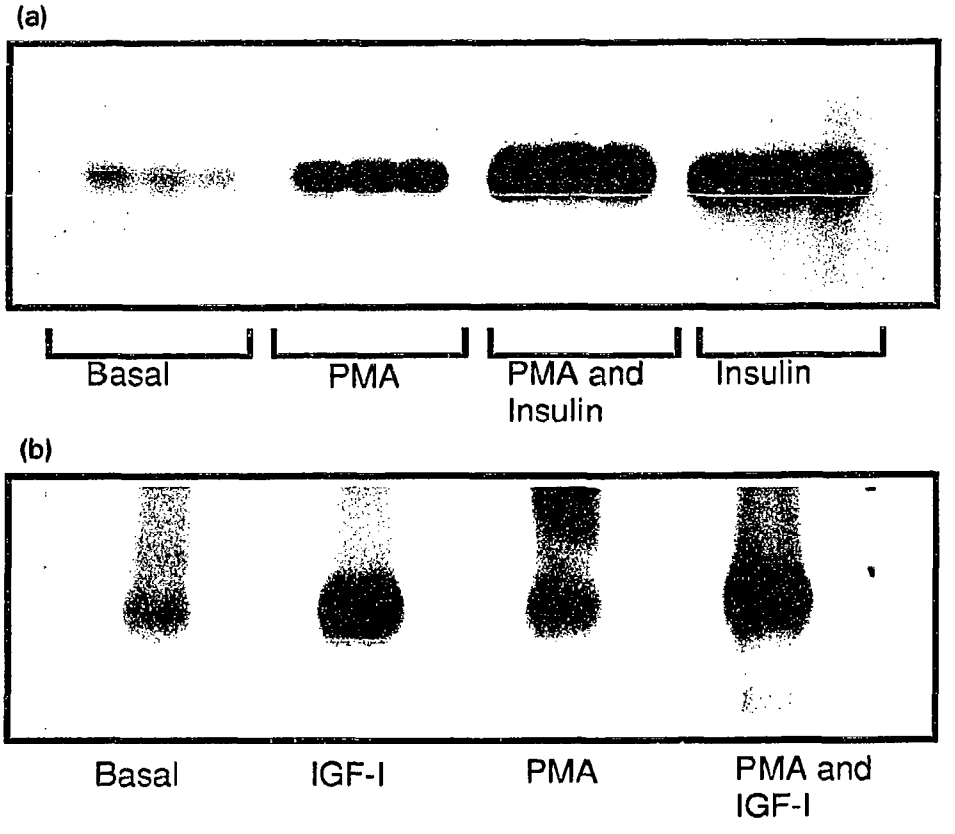

Fig. 1. NIH-3T3-HIR 3.5 cells (panel a) and IGF-I-R/3T3 cells (panel b) were labeled with ^{32}P and exposed to 1 μM PMA (30 min) and/or 1 μM insulin (NIH-3T3-HIR 3.5 cells) (5 min) and 0.1 μM IGF-I (IGF-I-R/3T3 cells) (5 min) as indicated. Insulin or IGF-I receptors were immunoprecipitated from solubilised cells and analysed by SDS-PAGE (7.5%) and autoradiography. Only the receptor β-subunit bands are shown. Panel a: insulin receptor from NIH-3T3-HIR 3.5 cells. Panel b: IGF-I receptor from IGF-I-R/3T3 cells.

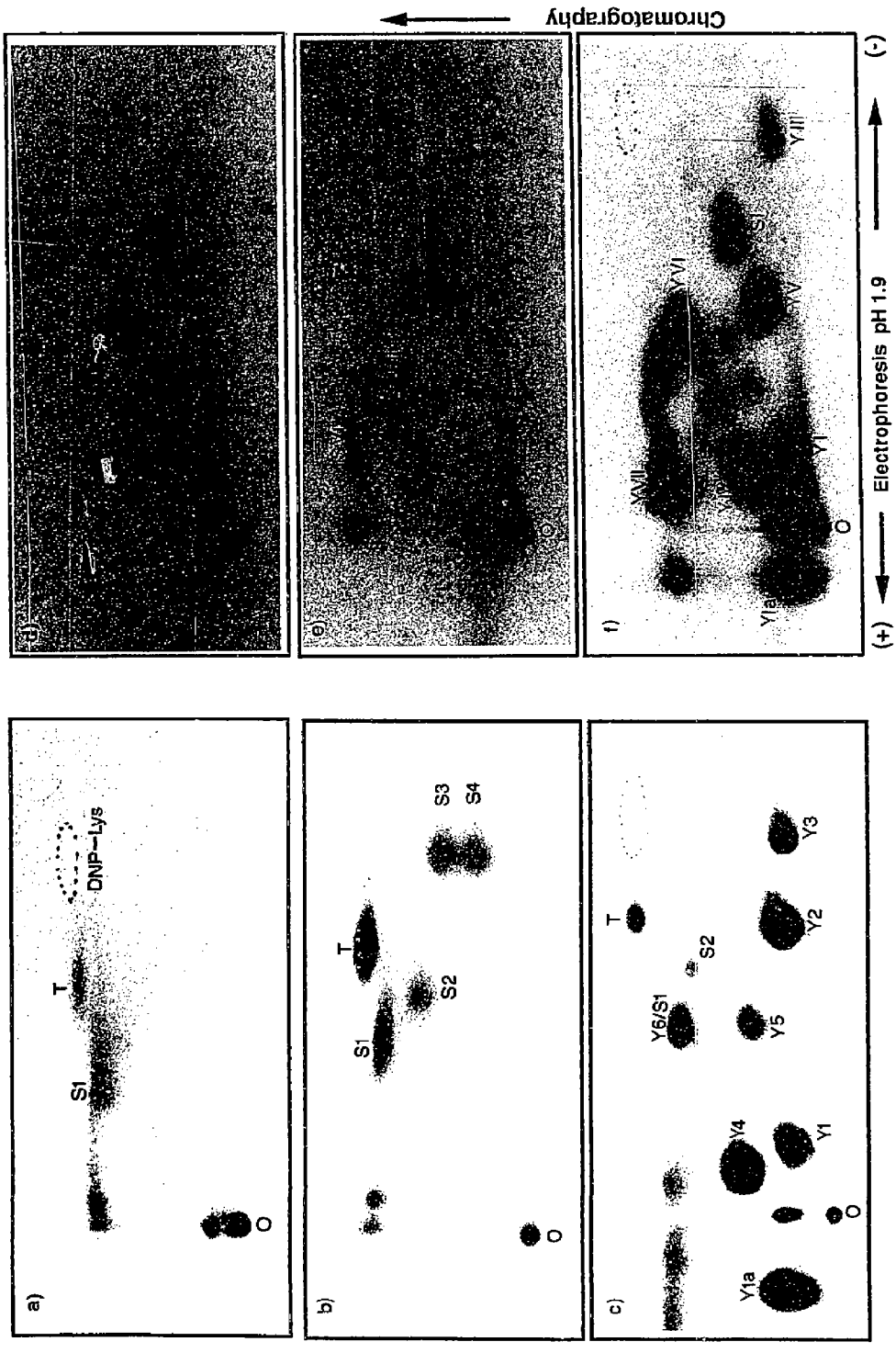

Fig. 2. Representative examples of 2D tryptic phosphopeptide maps of insulin receptor or IGF-I receptor β-subunit phosphorylated in response to PMA or insulin and IGF-I. ^{32}P-labeled NIH-3T3-HIR 3.5 or IGF-I-R/3T3 cells were exposed to PMA (1 μM) (panels b and e) or DMSO without PMA (denoted as basal, panels a and d) or insulin (1 μM) (panel c) and IGF-I (panel f), respectively. ^{32}P-labeled β-subunits were isolated and digested exhaustively with TPCK-trypsin. The resulting tryptic peptides were separated by high voltage electrophoresis in 30% formic acid pH 1.9 followed by ascending chromatography. ^{32}P-labeled peptides were detected by autoradiography; exposures shown were for 16 h at – 70°C (insulin receptor from NIH-3T3-HIR 3.5 cells) and 48 h at – 70°C (IGF-I receptor from IGF-I-R/3T3 cells). DNP-Lysine (DNP-Lys) was used as a marker to monitor the progress of separation in both dimensions. Peptide nomenclature (Y1–Y7) corresponds to the phosphopeptide sequences determined by White et al. [29] as related to those determined by Tavare and Denton [16] (i.e. Y1 = A1; Y1a = A2; Y4 = B3; Y5 = B2; Y2 = B1 and Y6 = C1). Peptides are named according to the predominant phosphoamino acid present. Insulin receptor peptides Y6 and S1 comigrate and are represented as a single peptide Y6/S1 in Fig. 2c. 'O' denotes the origin.

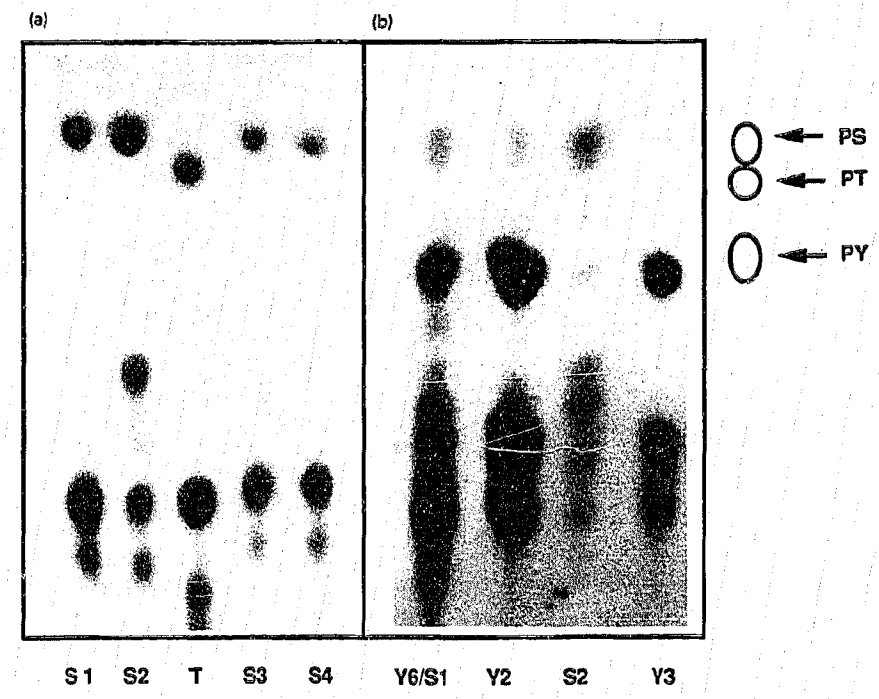

Fig. 3. Phosphoamino acid analysis of selected insulin receptor phosphopeptides after PMA (panel a) and insulin-stimulation (panel b). The tryptic phosphopeptides obtained after phosphorylation of the insulin receptor (Fig. 2b,c) were excised from the thin layer plate, eluted into 30% formic acid and lyophilised. The pepetides were then hydrolysed in 6 M HCl and the hydrolysates analysed by high voltage electrophoresis at pH 3.5. ^{32}P-phosphoamino acids were located by autoradiography and the unlabeled phosphoamino acids detected by ninhydrin staining. The origin lies at the cathodal end (bottom). PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

major phosphopeptides YI, YIa, YIV, YV, YVI, YIII and minor peptide YVII. Peptides YI, YIa, YIV, YV and YVI appeared to be similar to their counterparts derived from the insulin receptor. The labeling of SI was only modestly increased by IGF-I while the addition of PMA resulted in a 2-fold increase in the labeling of SI and the appearance of a further minor peptide SVI. This was associated with the concomitant disappearance of peptides SIII, SIV and SV.

4. DISCUSSION

Serine/threonine phosphorylation of insulin and IGF-I receptors has been studied previously in several cell types [7,10–13,21,22]. However, the sites of phosphorylation in response to different stimuli have seldom been compared directly within a single cell type [21]. The use of transfected fibroblasts allows such direct comparison, and the high level of receptor expression in these cells facilitates analysis of phosphopeptides. We modified the conditions for separating tryptic phosphopeptides by 2D thin layer methods, compared to previous studies on transfected cells [10–12]. Firstly, by not using a preliminary HPLC fractionation step, we eliminated the possibility of losing markedly hydrophobic phosphopeptides by failure to elute from reverse-phase columns, and we were able to compare the mobility and relative labeling of all peptides from a given receptor preparation on a single map. Secondly, it was found that resolution of the major serine/threonine phosphopeptides during high voltage electrophoresis was significantly improved at pH 1.9 over that at pH 3.5 [10,16] and consequently additional serine phosphopeptides were identified compared with those observed previously [10]. In particular, it was found that a major phosphoserine peptide S1 (Fig. 2) comigrated with phosphopeptide Y6 [17]. Furthermore, to extend upon studies of insulin receptor phosphorylation we report, for the first time, a partial characterization of IGF-I and PMA stimulated serine/threonine phosphorylation sites of the IGF-I receptor in a transfected cell line, IGF-I-R/3T3 [23].

In terms of the major sites phosphorylated, insulin- and PMA-stimulated phosphorylation of the same peptides. However, differences were clearly evident in the extent of phosphorylation and the effects of insulin and PMA on overall phosphorylation were almost additive (Fig. 1). The insulin-stimulated increases in receptor serine/threonine phosphorylation may partly be explained by a weak activation of PKC [19,20]. The fact that peptides S3 and S4 are not seen in insulin-stimulated cells may be a reflection of the different time courses and extent of enzyme activation in response to the 2 agonists. However, it is also possible that distinct enzymes (PKC isoenzymes or other kinases) are activated in response to PMA and insulin. The increase in

labeling of peptide T was greater with PMA than with insulin suggesting that this site was relatively more specific for protein kinase C and possibly less specific for the insulin-stimulated insulin receptor serine kinase. In an immunoaffinity-purified insulin receptor preparation that retains serine kinase activity towards the insulin receptor phosphopeptide T is absent [17]. However, studies involving inhibition or downregulation of PKC in HepG2 cells suggest that PKC is unlikely to be significantly involved in insulin-stimulated insulin receptor phosphorylation [25].

Differences between the pattern of insulin and PMA-induced insulin receptor phosphorylations are also seen in other cell types. In IM9 and HepG2 cells [21,25] a hydrophobic phosphothreonine peptide was observed in the basal state, whose phosphorylation was stimulated by PMA and to a lesser extent by insulin. In these cells PMA stimulated the appearance of 4 other phosphoserine peptides. Based on synthetic peptide comigration [11,13] the appearance of phosphopeptide T reflects the phosphorylation of Thr-1348 (1336). In Fao rat hepatoma cells [7], PMA and insulin were both shown to stimulate the phosphorylation of 9 phosphopeptides of which 3 were specific to each stimulus. In rat H-35 cells [13] phorbol ester-induced phosphorylation of the insulin receptor occurred on a single phosphothreonine peptide and 2 phosphoserine peptides. Although direct comparison with the present work is difficult, the phosphoserine peptides appeared to correspond to phosphopeptides S3 and S4 although they may represent phosphopeptides S1 and S2. It is unlikely that any of these phosphopeptides reflect phosphorylation at serines-1305/1306 (1293/1294), the only serine phosphorylation site defined to date [12] because this phosphopeptide is fairly hydrophilic and highly charged at pH 1.9, and consequently would be expected to migrate near phosphopeptide Y3. The identities of the other phosphorylation sites remain unknown. It is not clear whether these additional serine phosphopeptides represent distinct sites of phosphorylation or are the result of differential tryptic cleavage because of contiguous basic residues.

The major phorbol-ester induced phosphorylation site in the IGF-I receptor is located in a phosphoserine-containing peptide SI (Fig. 2e) that has no clear counterpart in the insulin receptor suggesting that this site is located in a region that is not or poorly conserved in the IGF-I receptor. PMA was more effective at increasing phosphorylation of this site than IGF-I. There was no counterpart to the insulin receptor T peptide in the IGF-I receptor. This would be expected as threonine-1348 of the insulin receptor is replaced by proline at the equivalent position in the IGF-I receptor. The pattern of serine phosphorylation of IGF-I receptor in transfected NIH 3T3 cells appeared less complex than previously reported in IM9 and HepG2 cells. None of these sites of serine phosphorylation has yet been identified.

It would appear that insulin or IGF-I-stimulated serine/threonine phosphorylation would be of fairly low stoichiometry compared to tyrosine autophosphorylation in these cells, assuming that ^{32}P equilibrates equally at all sites under the conditions used. If this were true, then it suggests that functional effects on tyrosine kinase activity are unlikely to be of significance if only a small proportion of receptors are involved. Alternatively, serine/threonine phosphorylation could significantly affect a distinct cellular subfraction of receptors, eg. influencing subcellular distribution [26]. Given the multiple sites involved and the existence of multiple PKC isoenzymes [27], it is possible that there is an element of tissue specificity in the response to phorbol esters affecting either the extent of phosphorylation or specific sites. This would explain the apparent inhibition of tyrosine kinase activity in some cell types [7,8] but not in others [21,22,28]. Future studies should be directed towards identifying these additional sites of serine phosphorylation and assessing their relative contributions towards insulin receptor regulation as well as determining the stoichiometries of increases in receptor serine/threonine phosphorylation in response to insulin, IGF-I and PMA.

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REFERENCES

[1] Houslay, M.D. and Siddle, K. (1989) Brit. Med. Bull. 45, 264-284.
[2] Kasuga, M., Zick, Y., Blith, D.L., Karlsson, F.A., Haring, H.U. and Kahn, C.R. (1982) J. Biol. Chem. 257, 9891-9894.
[3] Zick, Y., Grunberger, G., Podskalny, J.M., Moncada, V., Taylor, S.I., Gorden, P. and Roth, J. (1983) Biochem. Biophys. Res. Commun. 116, 1129-1135.
[4] Gazzano, H., Kowalski, A., Fehlmann, M. and Van Obberghen, E. (1983) Biochem. J. 216, 575-582.
[5] Smith, D.M., King, M.J. and Sale, G.J. (1988) Biochem. J. 250, 509-519.
[6] Stadtmauer, L. and Rosen, O.M. (1986) J. Biol. Chem. 261, 3402-3407.
[7] Takayama, S., White, M.F., Lauris, V. and Kahn, C.R. (1984) Proc. Natl. Acad. Sci. USA 81, 7797-7801.
[8] Takayama, S., White, M.F. and Kahn, C.R. (1988) J. Biol. Chem. 263, 3440-3447.
[9] Whittaker, J., Okamoto, A.K., Thys, R., Bell, G.I., Steiner, D.F. and Hofmann, C.A. (1987) Proc. Natl. Acad. Sci. USA 84, 5237-5241.
[10] Tavare, J.M., O'Brien, R.M., Siddle, K. and Denton, R.M. (1988) Biochem. J. 253, 783-788.
[11] Lewis, R.E., Cao, L., Perregaux, D. and Czech, M.P. (1990) Biochemistry 29, 1807-1813.
[12] Lewis, R.E., Wu, G.P., MacDonald, R.G. and Czech, M.P. (1990) J. Biol. Chem. 265, 947-954.
[13] Koshio, O., Akanuma, Y. and Kasuga, M. (1989) FEBS Lett. 254, 22-24.
[14] Soos, M.A., O'Brien, R.M., Brindle, N.P.J., Stigter, J.M., Okamoto, A.K., Whittaker, J. and Siddle, K. (1989) Proc. Natl. Acad. Sci. USA 86, 5217-5221.

[15] Schägger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368-379.
[16] Tavare, J.M. and Denton, R.M. (1988) Biochem. J. 252, 607-615.
[17] Pillay, T.S. (1990) PhD thesis, University of Cambridge.
[18] Pillay, T.S., Whittaker, J. and Siddle, K. (1990) Biochem. Soc. Trans. 18, 494-495.
[19] Walaas, S.I., Horn, R.S., Adler, A., Albert, K.A. and Walaas, O. (1987) FEBS Lett. 220, 311-318.
[20] Cooper, D.R., Konda, T.S., Standaert, M.L., Davis, J.S., Pollet, R.J. and Farese, R.V. (1987) J. Biol. Chem. 262, 3633-3639.
[21] Jacobs, S. and Cuatrecasas, P. (1986) J. Biol. Chem. 261, 934-939.
[22] Jacobs, S., Sahyoun, N.E., Saltiel, A.R. and Cuatrecasas, P. (1983) Proc. Natl. Acad. Sci. USA 80, 6211-6213.
[23] Lammers, R., Gray, A., Schlessinger, J., Ullrich, A. (1989) EMBO J. 8, 1369-1375.
[24] Kull Jr, F.C., Jacobs, S., Su, Y.F., Svoboda, M.E., Van Wyk, J.J., Cuatrecasas, P. (1983) J. Biol. Chem. 258, 6561-6566.
[25] Duronio, V. and Jacobs, S. (1990) Endocrinology 127, 481-487.
[26] Bottaro, D.P., Bonner-Weir, S. and King, G.L. (1989) J. Biol. Chem. 264, 5916-5923.
[27] Nishizuka, Y. (1988) Nature 334, 661-665.
[28] Hachiya, H.L., Takayama, S., White, M.F. and King, G.L. (1987) J. Biol. Chem. 262, 6417-6424.
[29] White, M.F., Shoelson, S.E., Keutman, H. and Kahn, C.R. (1988) Biochem. J. 252, 607-615.