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Characterization of sites of serine phosphorylation in human placental insulin receptor copurified with insulin-stimulated serine kinase activity by two-dimensional thin-layer peptide mapping

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Insulin receptor was copurified from human placenta together with insulin-stimulated kinase activity that phosphorylates the insulin receptor on serine residues. Analysis of phosphorylated insulin receptor by two-dimensional tryptic peptide mapping showed that sites of insulin stimulated serine phosphorylation in the insulin receptor were recovered in the same peptides as those known to be phosphorylated on serine in vivo in response to insulin. This indicates that the serine kinase copurified with the insulin receptor represents a physiologically important enzyme involved in the insulin triggered serine phosphorylation of the insulin receptor in vivo.

Insulin; Receptor; Protein phosphorylation; Serine kinase; Peptide mapping

1. INTRODUCTION

The insulin receptor is an insulin-activated, tyrosine-specific protein kinase which catalyzes the autophosphorylation of multiple tyrosine residues in its own β-subunit [1–7], and subsequently the phosphorylation of other proteins [8,9] (for a review see [10]). In intact cells the insulin receptor is also well known to be phosphorylated on serine residues in response to insulin [11–14]. Most purified or partially purified preparations of the insulin receptor lack the serine kinase activity. Recently Smith et al. [15] have devised a protocol for isolating insulin receptor from human placental membranes with associated insulin-sensitive serine kinase activity. This serine kinase activity appears to be a novel species and distinct from Ca^{2+}/calmodulin, cyclic AMP and cyclic GMP-dependent protein kinases, casein kinases I and II and insulin-activated ribosomal S6 kinase [16]. Using this system Smith and Sale [16] have shown that inhibition of the tyrosine kinase activity of the insulin receptor by nine different methods, including use of peptide 1142–1153 of the insulin receptor as a competing substrate and antiphosphotyrosine antibody which locks the tyrosine kinase in a low activity form, all inhibit the ability of insulin to stimulate phosphorylation of the insulin receptor on serine. Additionally, direct stimulation of the receptor tyrosine kinase by vanadate increases phosphorylation of the insulin receptor on serine. Moreover, insulin-stimulated tyrosine phosphorylation precedes insulin-stimulated serine phosphorylation of the placental insulin receptor in vitro. Taken together this indicates that activation by insulin of the receptor serine kinase activity depends on the tyrosine kinase activity of the insulin receptor [16].

It is important to know whether the serine kinase that copurifies with the placental insulin receptor isolated by the protocol of Smith et al. [15] is one that acts on the insulin receptor physiologically. In the present work this has been investigated by characterizing the serine sites phosphorylated on the placental receptor in vitro by two-dimensional

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thin-layer peptide mapping of tryptic phosphopeptides and comparing them with those known to be phosphorylated in vivo.

2. MATERIALS AND METHODS

Insulin receptor was isolated with associated insulin-sensitive serine kinase activity by solubilization of human placental membranes in 2% Triton X-100 followed by chromatography on wheat-germ-agglutinin-agarose using the protocol of Smith et al. [15]. Cellulose thin-layer plates (20 × 20 cm) were from Kodak, Kirkby, Liverpool, England. Sources of other materials were as described in [15,16].

2.1. *Phosphorylation of insulin receptor and preparation of tryptic phosphopeptides*

Insulin receptor (1 mg protein/ml) was incubated for 15 min at 22°C with or without 150 nM insulin in 30 μl of 50 mM Hepes (pH 7.4)/0.1% Triton X-100 in the presence of 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol and 30 μM sodium vanadate, [γ-^{32}P]ATP (100 μM; 15 cpm/fmol) was then added, and after incubation for a further 15 min at 22°C phosphorylations were terminated by adding Laemmli [17] sample buffer (62.5 mM Tris-HCl, pH 7.4, 1% (w/v) SDS, 19 mg/ml dithiothreitol, 0.002% (w/v) bromophenol blue, 20% (w/v) sucrose) and boiling for 2 min [15]. Insulin receptor β-subunits were separated on 4% acrylamide stacking/7.5% acrylamide resolving gels and located by autoradiography [15]. The region of the gel containing the β-subunit was excised, counted in 5 ml of scintillation fluid [5-(4-biphenylyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole (6 g/l) in toluene], incubated with 10 ml of 20% (v/v) methanol for 18 h at 37°C and dried at 70°C for 2 h in an oven. Then 2 ml of 50 mM NH_4HCO_3 containing 100 μg of trypsin (treated with tosylphenylalanylchloromethane) was added. The mixture was incubated at 37°C for 6 h, a further 100 μg of trypsin was added and incubation continued for a further 18 h. The samples were freeze dried and either subjected to phosphoamino acid analysis as described in [15] or two-dimensional thin-layer peptide mapping.

2.2. *Two-dimensional thin-layer peptide mapping*

The ^{32}P-labelled tryptic phosphopeptides were dissolved in 20 μl of water and separated on a cellulose thin-layer plate by electrophoresis at 400 V for 4 h at pH 3.5 (pyridine/acetic acid/water, 1:10:189 by vol.) in the first dimension and ascending chromatography (pyridine/acetic acid/butanol/water, 10:3:15:12, by vol.) in the second dimension [6]. Autoradiograms were obtained at −70°C for 2–7 days using Amersham Hyperfilm-MP (Amersham International, Bucks, England) within cassettes containing Dupont Cronex intensifier screens. ^{32}P associated with phosphopeptides was determined by densitometric scanning with a Joyce-Loebl Chromoscan 3. For phosphoamino acid analysis of tryptic phosphopeptides purified by two-dimensional thin-layer analysis, the peptides were removed by scraping the cellulose and hydrolyzed in 6 M HCl at 110°C for 2 h. Phosphoamino acids were separated by electrophoresis on cellulose thin-layer plates at pH 3.5 for 1.5 h at 1 kV [15].

3. RESULTS AND DISCUSSION

Insulin receptor was copurified with associated insulin-stimulated insulin receptor serine kinase activity by the protocol of Smith et al. [15]. This involved solubilization of human placental membranes in Triton X-100 and affinity chromatography on wheat-germ-agglutinin-agarose. The insulin receptor was phosphorylated with [γ-^{32}P]ATP in the presence or absence of insulin and subjected to SDS gel electrophoresis. Phosphorylation of the β-subunit of the insulin receptor on tyrosine and serine was stimulated by insulin 4–5-fold and 6–7-fold, respectively, for preparations used in this study. Isolation of the β-subunits and direct phosphoamino acid analysis gave phosphoserine/phosphotyrosine ratios of approx. 0.2 for insulin receptor phosphorylated in the presence of insulin. These values are similar to those previously obtained using the placental insulin receptor preparation [15,16].

β-Subunits isolated from insulin receptor phosphorylated in the presence of insulin were digested with trypsin and subjected to two-dimensional thin-layer peptide mapping (fig.1a). ^{32}P-labelled tryptic phosphopeptides resolved by two-dimensional peptide mapping were scraped off and their phosphoamino acid content determined (table 1). Upon thin layer analysis a complex pattern of phosphopeptides was obtained. This arises because insulin-stimulated phosphorylation of the β-subunit occurs on at least seven tyrosines as well as on serine or serines [3–7]. The tyrosines are clustered in three domains: tyrosines 1316, 1322 in the C-terminal tail; 1146, 1150 and 1151 in the tyrosine kinase domain and possibly 953 and 960 or 972 near the transmembrane domain. Sites of insulin-stimulated serine phosphorylation have not been localized. Additionally, certain peptides are incompletely cleaved by trypsin, despite exhaustive digestion, adding further to the complexity. The identities of ^{32}P-labelled tryptic phosphotyrosyl peptides resolved by two-dimensional thin-layer mapping have been determined by Tavaré and Denton [6] and these assignments are used in the key to fig.1. The positions of the major phosphotyrosyl peptides resolved in the present work, using the autophosphorylated placental insulin receptor, correspond exactly with those obtained by Tavaré

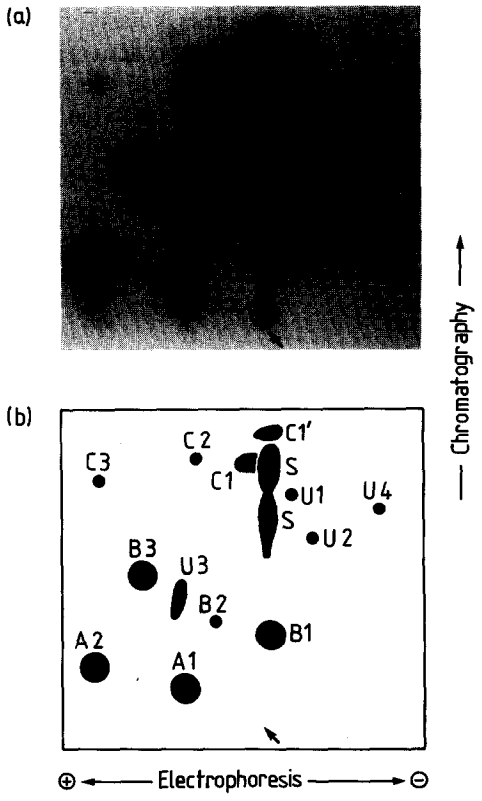

Fig.1. Two-dimensional thin-layer peptide mapping of phosphorylated insulin receptor. Insulin receptor with associated insulin stimulated serine kinase activity was partially purified from human placental membranes and phosphorylated with [γ-^{32}P]ATP in the presence of insulin. The β-subunits of the insulin receptor were isolated by SDS gel electrophoresis, digested with trypsin and ^{32}P-labelled tryptic phosphopeptides separated on a cellulose thin-layer plate by electrophoresis at pH 3.5 and ascending chromatography. (a) An autoradiograph of the result which is representative of several experiments on different preparations of the insulin receptor. The origin of sample application is marked by an arrow. (b) A key to the identification of the ^{32}P-labelled phosphopeptides. Phosphotyrosyl peptides are: C1, 1143–1153, mixture of monophosphorylated forms; B2, 1143–1156 and B3, 1143–1153, mixture of diphosphorylated forms; A1, 1143–1156 and A2, 1143–1153, triphosphorylated; B1, 1315–1329, diphosphorylated; C1′, C2 and C3 are believed to be phosphorylated at tyrosine residues 953 and 960 or 972 [6,7]. C2 and C3 are barely evident. Phosphoserine is predominantly recovered in peptides labelled S and also the C1/C1′ region (table 1). These phosphoseryl peptides correspond to those derived from insulin receptor phosphorylated in vivo. A small amount of phosphoserine is also recovered in peptides U1 and U2. The identities of U1, U2, U3, U4 and phosphopeptides not illustrated in the key are unknown.

Table 1

Phosphoamino acid analysis of ^{32}P-labelled tryptic phosphopeptides separated by two-dimensional thin-layer peptide mapping

Peptide	^{32}P (arbitrary units)		
	Total	Phosphotyrosine	Phosphoserine
A1	27.7 ± 6.7	27.7 ± 6.7	0.0 ± 0.0
A2	28.7 ± 5.2	28.7 ± 5.2	0.0 ± 0.0
B1	35.3 ± 6.5	35.3 ± 6.5	0.0 ± 0.0
B2	2.3 ± 0.3	ND	ND
B3	29.7 ± 5.2	29.7 ± 5.2	0.0 ± 0.0
C1′	13.7 ± 0.7	8.0 ± 0.6	5.7 ± 0.9
C1	14.3 ± 2.0	8.3 ± 1.8	6.0 ± 0.6
C2	3.7 ± 1.4	ND	ND
C3	1.3 ± 0.7	ND	ND
S (upper)	16.7 ± 1.5	7.7 ± 0.9	9.0 ± 0.6
S (lower)	15.7 ± 1.2	6.3 ± 0.3	9.3 ± 0.9
U1	6.3 ± 2.4	5.7 ± 2.2	0.6 ± 0.2
U2	6.8 ± 1.6	5.7 ± 1.2	1.1 ± 0.5
U3	15.7 ± 2.6	15.7 ± 2.6	0.0 ± 0.0
U4	9.3 ± 5.3	9.3 ± 5.3	0.0 ± 0.0

Human placental insulin receptor copurified with insulin-stimulated serine kinase activity was phosphorylated with [γ-^{32}P]ATP in the presence of insulin. The β-subunits of the insulin receptor were isolated, digested with trypsin and subjected to two-dimensional thin-layer analysis. ^{32}P-labelled tryptic phosphopeptides were located by autoradiography and subjected to phosphoamino acid analysis. Total ^{32}P present in the various phosphopeptides and ^{32}P recovered in phosphoamino acids was quantitated by densitometric scanning of autoradiographs. Values are means ± SE for 3 thin layer analyses. ND, not determined

and Denton [6] and Tavaré et al. [7] who used autophosphorylated insulin receptors from human placenta, CHO.T cells and NIH 3T3 HIR3.5 cells.

Using both intact CHO.T cells and NIH 3T3 HIR 3.5 cells the in vivo site(s) of insulin-stimulated serine phosphorylation were shown by Tavaré et al. [7] to be recovered in peptide(s) that resolved poorly from monophosphotyrosyl peptide 1143–1153 (C1). This resulted in phosphoserine being detected in the C1 spot as well as phosphopeptide(s) designated S which migrated to a position that overlapped with the right hand side of the C1 spot [7]. This distinctive pattern was also obtained upon analysis of placental insulin receptor copurified with serine kinase activity and phosphorylated in vitro in the presence of insulin (fig.1a, table 1). This indicates that the serine kinase that associates with the insulin receptor during purification by the protocol of Smith et al.

[15] is active on the insulin receptor in vivo. Additionally, in the present work resolution in the chromatography direction of the thin-layer analysis was better than that obtained by Tavaré et al. [7] and phosphoseryl peptide S more clearly separated into two peptides. Moreover both S spots and the C1 spot contained phosphotyrosine and phosphoserine (phosphoserine/phosphotyrosine ratios: S (upper) = 1.2; S (lower) = 1.5; C1 = 0.72) as found for S and C1 derived from insulin receptor phosphorylated in vivo [7]. The C1′ spot obtained from placental insulin receptor phosphorylated in vitro also contained both phosphoserine and phosphotyrosine (phosphoserine/phosphotyrosine ratio = 0.71); C1′ obtained from insulin receptor phosphorylated in vivo was not analyzed [7]. There appear to be two possible explanations for the presence of phosphotyrosine and phosphoserine in the same spots. Firstly, phosphoserine and phosphotyrosine could reside in distinct phosphopeptides that are incompletely resolved. Secondly, phosphoserine and phosphotyrosine could be present within the same peptide. It is noteworthy that phosphotyrosyl peptides derived from both the C-terminal domain (contains tyrosine residues 1316, 1322) and the juxtamembrane domain (contains tyrosine residues 950 and 960 or 972) contain serine residues. The juxtamembrane domain has been postulated to contain a serine site phosphorylated by Ca^{2+}/phospholipid-dependent protein kinase [18].

In the present study spots S, C1 and C1′ accounted for >90% of the phosphoserine recovered after two-dimensional mapping of tryptic phosphopeptides derived from placental insulin receptor phosphorylated in vitro in the presence of insulin. Further study is required to determine whether this actually represents phosphorylation of more than one serine site. The remainder of the phosphoserine was located in two minor spots, U1 and U2. Phosphoserine was barely detected when insulin receptor was phosphorylated in the absence of insulin. Additionally two phosphotyrosyl peptides labelled U3 and U4 were resolved in the present work that was not discernible in the studies of Tavaré and co-workers [6,7]. The detection of U3 could again reflect the greater resolution achieved in this study in the chromatographic direction. U4 was relatively minor.

In conclusion the specific copurification of the insulin receptor with a serine kinase activity, the insulin-dependent nature of the activation of the serine kinase and the ability of the copurified serine kinase to phosphorylate in vitro serines in the same peptides that are phosphorylated in vivo all indicate that the serine kinase copurified with the placental insulin receptor is a physiologically relevant kinase involved in the insulin triggered serine phosphorylation of the insulin receptor in vivo.

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REFERENCES

[1] Kasuga, M., Zick, Y., Blithe, D.L., Crettaz, M. and Kahn, C.R. (1982) Nature 298, 667–669.
[2] 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. and Ramachandran, J. (1985) Nature 313, 756–761.
[3] Tornqvist, H.E., Pierce, M.W., Frackleton, A.R., Nemenoff, R.A. and Avruch, J. (1987) J. Biol. Chem. 262, 10212–10219.
[4] Tornqvist, H.E., Gunsalus, J.R., Nemenoff, R.A., Frackleton, A.R., Pierce, M.W. and Avruch, J. (1988) J. Biol. Chem. 263, 350–359.
[5] White, M.F., Shoelson, S.E., Keutmann, H. and Kahn, C.R. (1988) J. Biol. Chem. 263, 2969–2980.
[6] Tavaré, J.M. and Denton, R.M. (1988) Biochem. J. 252, 607–615.
[7] Tavaré, J.M., O'Brien, R.M., Siddle, K. and Denton, R.M. (1988) Biochem. J. 253, 783–788.
[8] Rosen, O.M., Herrera, R., Olowe, Y., Petruzzelli, L.M. and Cobb, M.H. (1983) Proc. Natl. Acad. Sci. USA 80, 3237–3240.
[9] Yu, K.-T. and Czech, M.P. (1984) J. Biol. Chem. 259, 5277–5286.
[10] Sale, G.J. (1988) Int. J. Biochem. 20, 897–908.
[11] Gazzano, H., Kowalski, A., Fehlman, M. and Van Obberghen, E. (1983) Biochem. J. 216, 575–582.
[12] Takayama, S., Morris, M.F., Lauris, V. and Kahn, C.R. (1984) Proc. Natl. Acad. Sci. USA 81, 7797–7801.
[13] White, M.F., Takayama, S. and Kahn, C.R. (1985) J. Biol. Chem. 260, 9470–9478.
[14] Stadtmauer, L. and Rosen, O.M. (1986) J. Biol. Chem. 261, 3402–3407.
[15] Smith, D.M., King, M.J. and Sale, G.J. (1988) Biochem. J. 250, 509–519.
[16] Smith, D.M. and Sale, G.J. (1988) Biochem. J., 256, 903–909.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Takayama, S., White, M.F. and Kahn, C.R. (1988) J. Biol. Chem. 263, 3440–3447.