Reprinted from Nature, Vol. 318, No. 6042, pp. 183-186, 14 November 1985

Insulin rapidly stimulates tyrosine phosphorylation of a M_r-185,000 protein in intact cells

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Phosphotyrosine-containing proteins are minor components of normal cells[1,2] which appear to be associated primarily with the regulation of cellular metabolism and growth[3,4]. The insulin receptor is a tyrosine-specific protein kinase[5,6], and one of the earliest detectable responses to insulin binding is activation of this kinase and autophosphorylation of its β-subunit[7–9]. Tyrosine autophosphorylation activates the phosphotransferase in the β-subunit and increases its reactivity toward tyrosine phosphorylation of other substrates[10,11]. When incubated *in vitro* with [γ-^{32}P]ATP and insulin, the purified insulin receptor phosphorylates various proteins on their tyrosine residues[12–16]. However, so far no proteins other than the insulin receptor have been identified as undergoing tyrosine phosphorylation in response to insulin in an intact cell. Here, using anti-phosphotyrosine antibodies, we have identified a novel phosphotyrosine-containing protein of relative molecular mass (M_r) 185,000 (pp185) which appears during the initial response of hepatoma cells to insulin binding. In contrast to the insulin receptor, pp185 does not adhere to wheat-germ agglutinin–agarose or bind to anti-insulin receptor antibodies. Phosphorylation of pp185 is maximal within seconds after exposure of the cells to insulin and exhibits a dose–response curve similar to that of receptor autophosphorylation, suggesting that this protein represents the endogenous substrate for the insulin receptor kinase.

To identify any phosphotyrosine-containing proteins which might appear in cells as a consequence of insulin binding, the well-differentiated and insulin-sensitive hepatoma cell line Fao[17,18] was labelled with ^{32}P-orthophosphate for 2 h[19]. Then the cells were either treated with 100 nM insulin or left untreated and the monolayers were solubilized with Triton X-100 as described in Fig. 1 legend. Purification of the resulting extract by wheat-germ agglutinin(WGA) chromatography and immunoprecipitation with anti-insulin receptor antibody showed a single insulin-stimulated phosphoprotein of M_r 95,000. This band was present in the basal state and increased about fourfold during insulin stimulation for 1 min (Fig. 1*a*, *b*). We have shown previously that this band corresponds to the β-subunit of the insulin receptor which undergoes tyrosine phosphorylation in response to insulin stimulation[7,9,19]. When the WGA-purified extract was immunoprecipitated with a polyclonal anti-phosphotyrosine antibody a similar result was observed, except that no phosphorylation of the β-subunit was seen under basal conditions because in the intact cells the β-subunit does not contain phosphotyrosine before insulin stimulation (Fig. 1*c*, *d*)[9,19]. During 1 h of incubation with insulin, the amount of ^{32}P-orthophosphate incorporated into the proteins eluted from the WGA–agarose was constant, suggesting that insulin stimulation of receptor phosphorylation does not result from a general increase in the specific activity of cellular ATP.

In an attempt to identify other phosphotyrosine-containing proteins, Fao cells were labelled and subjected to immunoprecipitation with anti-phosphotyrosine antibodies before WGA chromatography. In the absence of insulin, a single major phosphoprotein of M_r 120,000 was immunoprecipitated from the whole-cell extract (Fig. 1*e*). After incubation for 1 min with 100 nM insulin there was no change in pp120 (Fig. 1*f*), but we observed two new phosphoproteins of M_r 95,000 and 185,000 (Fig. 1*f*). pp95 is the β-subunit of the insulin receptor and was quantitatively immunoprecipitated by anti-insulin receptor serum (B2) (Fig. 1*g*, *h*). pp185, on the other hand was not recognized by this antibody, suggesting that it is a novel insulin-stimulated phosphotyrosine-containing protein that is antigenically distinct from the insulin receptor (Fig. 1*f*, *h*). The

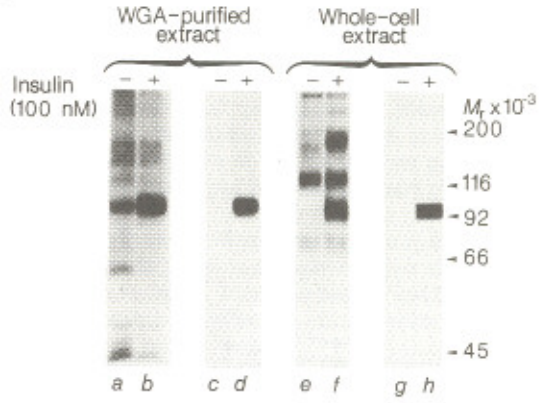

Fig. 1 Purification of phosphotyrosine-containing proteins from ^{32}P-orthophosphate-labelled Fao cells by immunoprecipitation with anti-insulin receptor and anti-phosphotyrosine antibodies. *a–d*, WGA-purified extracts immunoprecipitated with anti-receptor antibody (*a*, *b*) or anti-phosphotyrosine antibody (*c*, *d*). *e–h*, Whole-cell extracts immunoprecipitated with anti-phosphotyrosine antibody (*e*, *f*) or with anti-phosphotyrosine antibody, followed by anti-receptor antibody (*g*, *h*).

Methods. Fao cells were grown in plastic tissue culture dishes (15-cm diameter) containing 30 ml of RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco); 12 h before each experiment, the culture medium was changed to serum-free RPMI 1640. Confluent Fao cells were labelled for 2 h in 10 ml of phosphate-free and serum-free RPMI 1640 medium containing carrier-free ^{32}P-orthophosphate (0.5 mCi ml^{-1}; NEN). Cells were incubated without (−) or with (+) insulin (100 nM) at 37 °C for 1 min, then the experiments were stopped quickly by removing the incubation medium and freezing the cell monolayers with liquid nitrogen. The monolayers were thawed and solubilized immediately at 4 °C with 2 ml of a solution containing 50 mM HEPES *p*H 7.4, 1% Triton X-100, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium vanadate, 1 mg ml^{-1} aprotinin, and 2 mM phenylmethylsulphonyl fluoride. A supernatant of the whole-cell extract was prepared by scraping the cells from the dishes and sedimenting the insoluble material by centrifugation at 50,000 r.p.m. in a Beckman 70.1 Ti rotor for 60 min. WGA-purified extracts were obtained by applying the supernatant to a 0.5-cm diameter disposable column (BioRad) containing 0.2 ml of WGA–agarose (Vector). The agarose was washed with 100 ml of 50 mM HEPES *p*H 7.4 containing 0.1% Triton X-100, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 4 mM EDTA and 2 mM sodium vanadate, and the bound glycoproteins were eluted with two 0.5-ml portions of this wash solution containing 300 mM *N*-acetylglucosamine (Sigma). Specific immunoprecipitates were obtained from WGA-purified extracts (*a–d*) or from whole-cell extracts (*e–h*) using anti-phosphotyrosine antibodies prepared according to the method of Pang *et al.*[34], or anti-insulin receptor antibodies (B2) obtained from patients[31]. After incubation with the extract at 4 °C for 2 h, the antibodies were immobilized on Pansorbin (Calbiochem) and the precipitates were washed three times with a solution containing 50 mM HEPES, 1% Triton X-100 and 0.1% SDS. Proteins were eluted from the phosphotyrosine–antibody complex by addition of 10 mM *p*-nitrophenylphosphate to the wash solution [9] and from the anti-receptor antibody with SDS-polyacrylamide gel electrophoresis(PAGE) sample buffer[31]. The eluted proteins were reduced with 100 mM dithiothreitol (DTT; BioRad) and the phosphoproteins were separated by SDS-PAGE on 7.5% resolving polyacrylamide gels as described previously[32]. The phosphoproteins were identified by autoradiography (6 h exposure) of the stained and dried gels using Kodak X-Omat film and an intensifying screen. The WGA-purified extract was immunoprecipitated with anti-receptor antibody (*a*, *b*) or anti-phosphotyrosine antibody (*c*, *d*). The whole-cell extract was immunoprecipitated with anti-phosphotyrosine antibody and the precipitated phosphoproteins were either separated by PAGE (*e*, *f*) or immunoprecipitated a second time with anti-receptor antibodies before SDS-PAGE (*g*, *h*).

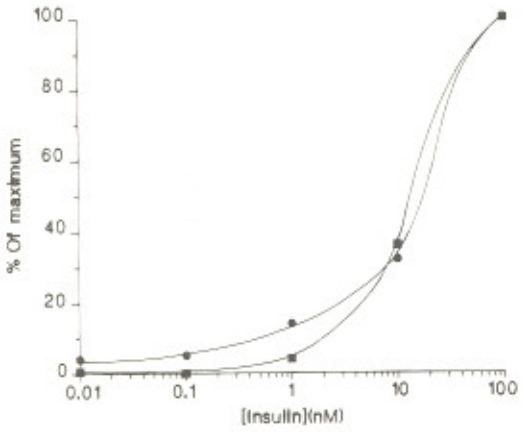

Fig. 2 Dose-response curve for effect of insulin on tyrosine phosphorylation. Labelled Fao cells were incubated in the presence of insulin for 10 min. Phosphoproteins were immunoprecipitated from the whole-cell extracts with the anti-phosphotyrosine antibody, eluted, separated by SDS-PAGE and identified by autoradiography as described in Fig. 1 legend. The intensities of pp185 (●) and the β-subunit (■) were measured by scanning densitometry and are presented as a percentage of the maximum stimulation. The maximum values, measured at 100 nM insulin, were 603 and 1,680 for pp185 and the β-subunit, respectively. The background measured in the absence of insulin has been subtracted.

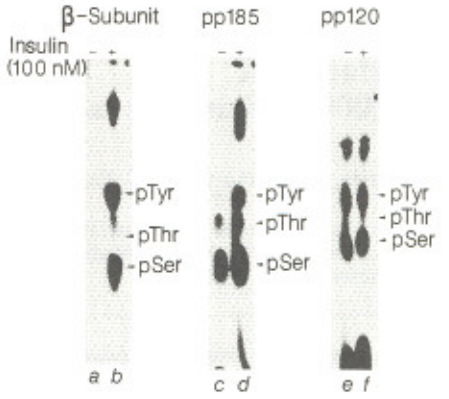

Fig. 3 Phosphoamino-acid analysis of pp185, pp120 and the β-subunit of the insulin receptor immunoprecipitated from whole-cell extracts with the anti-phosphotyrosine antibody. Fao cells were incubated in the absence (−) or presence (+) of insulin (100 nM) for 1 min. Phosphoproteins were immunoprecipitated from the whole-cell extracts with the anti-phosphotyrosine antibody and specifically eluted with *p*-nitrophenylphosphate, reduced with DTT and separated by SDS-PAGE. Dried gel fragments containing the β-subunit (*a*, *b*), pp185 (*c*, *d*) and pp120 (*e*, *f*) were incubated with trypsin (50 μg ml^{-1}) as described previously[9,31]. The phosphoamino-acid composition of the eluted tryptic peptides was determined as described previously[31]. The phosphoamino-acid standards were visualized by reaction with ninhydrin and the radioactive amino acids were detected by autoradiography for 24 h.

insulin dose-response curve for phosphorylation of pp185 was nearly identical to that for the β-subunit, supporting the notion that pp185 is a substrate for tyrosine phosphorylation by the insulin receptor kinase (Fig. 2).

The phosphoamino-acid composition of pp185, pp120 and the β-subunit of the insulin receptor was determined by partial acid hydrolysis and separation of the amino acids by high-voltage electrophoresis[19]. After insulin stimulation, each protein contained about equal amounts of phosphotyrosine and phosphoserine (Fig. 3); pp185 also contained substantial amounts of phosphothreonine. Although there was no distinct evidence of pp185 before insulin stimulation, there was a faint band that migrated slightly faster than pp185 which contained a small amount of phosphotyrosine (Figs 1*e*, 3*c*). Assuming that this is the same protein, insulin stimulated its phosphotyrosine content at least 10-fold (Fig. 3*d*). A significant increase in the amount of phosphoserine and phosphothreonine was also observed in pp185 after insulin stimulation, but whether this arises from *de novo* phosphorylation or tyrosine phosphorylation of previously phosphorylated pp185 is unknown. Before and after insulin stimulation, pp120 contained nearly equal amounts of phosphotyrosine, suggesting that it may be a substrate for an unidentified and constitutively activated tyrosine kinase in Fao cells.

Phosphorylation of the β-subunit is one of the earliest molecular responses that occurs in cells following insulin binding[9,19]. As we have reported previously[19], within 20 s after insulin stimulation (100 nM), phosphorylation of the β-subunit is maximal and remains at this elevated level for at least 1 h during uninterrupted exposure to insulin (Fig. 4). Phosphorylation of pp185 was also maximal by 30 s after exposure of the Fao cells to insulin, but it decreased during 60 min of continued insulin stimulation (Fig. 4); this decrease was not the result of a change in the specific activity of cellular [γ-^{32}P]ATP as there was no change in the level of phosphorylation of pp120 or of the β-subunit, which is rapidly dephosphorylated when the labelled ATP pool is diluted with a chase of unlabelled phosphate[20]. Phosphoamino-acid analysis of pp185 indicated that concentrations of phosphoserine, phosphothreonine and phosphotyrosine decreased in parallel during this time interval (data not shown). These results suggest that following phosphorylation of pp185 there may be activation of a phosphoprotein phosphatase that recognizes pp185, but not the insulin receptor or pp120, as a substrate for dephosphorylation. These findings are consistent with the notion that the action of insulin may be regulated in the intact cell by both phosphorylation and dephosphorylation reactions[21].

In the experiment shown in Fig. 4, phosphoproteins of M_r 75,000 and >200,000 were also detected by immunoprecipitation with the anti-phosphotyrosine antibody. The band of higher relative molecular mass showed similar kinetics to pp185, whereas pp75 was present in the basal state and tended to increase after 30–60 min of insulin stimulation. These phosphoproteins were less abundant than the others described above and were observed only after prolonged exposure of the autoradiograms. Phosphoproteins with M_r s of 300,000–200,000 were detected by anti-phosphotyrosine antibodies in human fibroblasts stimulated with platelet-derived growth factor (PDGF)[22].

Like the epidermal growth factor (EGF) receptor, PDGF receptor and several oncogene products, the insulin receptor is a tyrosine-specific protein kinase. Previous studies have successfully identified several cellular substrates (pp34, pp41, pp81, enolase, lactate dehydrogenase, phosphoglycerate mutase, vinculin) for the EGF receptor[23–26], PDGF receptor[22,27] and pp60^{src} (refs 23, 24, 26, 28), but so far no endogenous substrates for the insulin receptor have been identified. The present study indicates that in addition to stimulating autophosphorylation of the insulin receptor, insulin stimulates tyrosine phosphorylation of a protein in intact hepatoma cells that has a M_r of 185,000. This protein (pp185) may have gone undetected by previous studies for several reasons. First, its rapid phosphorylation and dephosphorylation in insulin-stimulated cells mean that it can be detected only at early time points. Second, WGA chromatography is often used to purify cell extracts before studying insulin receptor phosphorylation[29], but as pp185 does not bind to this lectin it would not be detected in such experiments. Third, sodium vanadate, an inhibitor of dephosphorylation[19,30], was used in our studies during cell lysis and protein purification, but it has not been used routinely in the past. Finally, two-dimensional gel electrophoresis, which has been successful in identifying many tyrosine kinase substrates[23,28], may miss phosphoproteins with *p*I values <5.5 (ref. 27) or high-M_r phosphoproteins due to the background phosphoryla-

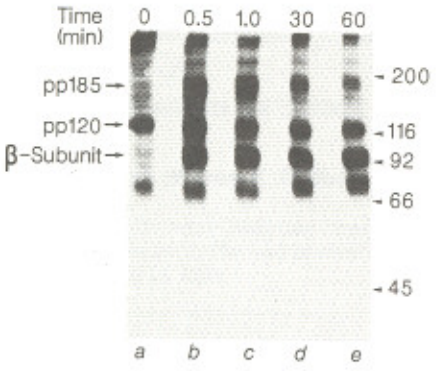

Fig. 4 The time course of insulin-stimulated tyrosine phosphorylation. Fao cells were incubated in the absence (0 min) or presence of insulin for the indicated time intervals. Phosphoproteins were immunoprecipitated from the whole-cell extracts with the anti-phosphotyrosine antibody, eluted with *p*-nitrophenylphosphate and identified by SDS-PAGE and autoradiography as described in Fig. 1 legend.

tion that occurs in this region[22]. These limitations can be avoided by using anti-phosphotyrosine antibodies, which provides specific probes for studying tyrosine phosphorylation events that occur during the initial response of cells to insulin.

Although the exact nature of pp185 is unknown, we can eliminate certain possibilities. pp185 does not bind to WGA and was not immunoprecipitated by the anti-insulin receptor serum B2, which recognized the mature receptor and the glycosylated and non-glycosylated (H. A. Hedo, personal communication) forms (M_r 155,000–190,000) of the precursor[31,32]. Furthermore, the tryptic phosphopeptide map of pp185 does not correspond to the profile of β-subunit (data not shown). Thus, pp185 is not the insulin receptor. Fao cells show no specific EGF binding and no detectable tyrosine phosphorylation during EGF stimulation (data not shown), suggesting that pp185 is not related to the EGF receptor (M_r 175,000). pp185 is also probably not the PDGF receptor (M_r 185,000) as the latter binds to WGA[26,33].

Immunoprecipitation of ^{35}S-methionine-labelled Fao cells by the anti-phosphotyrosine antibody did not reveal any band at M_r 185,000, although both the α- and β-subunits were easily detected (data not shown). Thus, pp185 is possibly a minor protein that is recognized with high affinity by the insulin receptor. Whether pp185 is directly phosphorylated by the insulin receptor kinase or is the result of activation of another tyrosine kinase in response to insulin is not yet known.

The only other prominent phosphoprotein in Fao cells that is recognized by the anti-phosphotyrosine antibody has a M_r of 120,000 and is constitutively phosphorylated on tyrosine residues. The nature of this phosphoprotein is unknown. Others have reported that ATP citrate lyase (M_r 116,000) is equally immunoprecipitated with a monoclonal antibody against phenylphosphonate before and after EGF stimulation of A431 cells because it contains phosphohistidine, which cross-reacts with this antibody[25]; however, phosphoamino-acid analysis confirmed the presence of phosphotyrosine in pp120. Insulin stimulates tyrosine phosphorylation of a M_r-120,000 protein in a Triton X-100 extract of rat hepatocyte plasma membranes purified by WGA chromatography[29]. It is unknown whether pp120 bears any similarity to the hepatocyte protein but they are probably different as pp120 identified here does not bind to WGA.

Our results provide the first demonstration, in an intact cell, of insulin-stimulated tyrosine phosphorylation of a protein other than the insulin receptor. Identification of pp185 should elucidate the relationship between the insulin receptor kinase and the mechanism of insulin action.

This work was supported in part by grants AM31036 and AM29770 to C.R.K. and a fellowship (AM0716301) to M.F.W. from the Institute of Health and Human Development, NIH, USPHS. We thank Drs D. T. Pang, B. R. Sharma and J. A. Shafer for the gift of phosphotyrosine binding antibody and for making available prior to publication the procedures for preparation of the antibody.

Received 22 July; accepted 18 September 1985.

1. Hunter, T. & Sefton, B. M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1311–1315 (1980).
2. Sefton, B. M., Hunter, T., Beemon, K. & Eckhart, W. *Cell* **20**, 807–816 (1980).
3. Bishop, M. *A. Rev. Biochem.* **52**, 301–354 (1983).
4. Heldin, C.-H. & Westermark, B. *Cell* **37**, 9–20 (1984).
5. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L. & Kahn, C. R. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2137–2141 (1980).
6. Petruzzelli, L., Herrera, R. & Rosen, O. M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3327–3331 (1984).
7. Kasuga, M., Karlsson, F. A. & Kahn, C. R. *Science* **215**, 185–187 (1982).
8. Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M. & Kahn, C. R. *Nature* **298**, 667–669 (1982).
9. Pang, D. T., Sharma, B. R., Shafer, J. A., White, M. F. & Kahn, C. R. *J. biol. Chem.* **260**, 7131–7136 (1985).
10. Rosen, O. M., Herrera, R., Olowe, Y., Petruzzelli, L. M. & Cobb, M. H. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3237–3240 (1983).
11. Yu, K.-T. & Czech, M. P. *J. biol. Chem.* **259**, 5277–5286 (1984).
12. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L., White, M. F. & Kahn, C. R. *J. biol. Chem.* **258**, 10973–10979 (1983).
13. Stadtmauer, L. A. & Rosen, O. M. *J. biol. Chem.* **258**, 6682–6685 (1983).
14. Zick, Y. *et al. Eur. J. Biochem.* **137**, 631–637 (1983).
15. Haring, H. U. *et al. J. Cell Biochem.* **27**, 171–182 (1985).
16. White, M. F. & Sale, E. M. *Diabetes* **33**(Suppl. 1), 31A (1984).
17. Deschatrette, J., Moore, E. E., Dubois, M., Cassio, D. & Weiss, M. C. *Somat. Cell Genet.* **5**, 697–718 (1979).
18. Deschatrette, J. & Weiss, M. C. *Biochimie* **11**, 1603–1611 (1974).
19. White, M. F., Takayama, S. & Kahn, C. R. *J. biol. Chem.* **260**, 9470–9478 (1985).
20. Haring, H. U., Kasuga, M., White, M. F., Crettaz, M. & Kahn, C. R. *Biochemistry* **23**, 3298–3306 (1984).
21. Denton, R. M., Brownsey, R. W. & Belshan, G. J. *Diabetologia* **21**, 347–362 (1981).
22. Ek, B. & Heldin, C.-H. *J. biol. Chem.* **259**, 11145–11152 (1984).
23. Cooper, J. A. & Hunter, T. *J. Cell Biol.* **91**, 878–893 (1983).
24. Erikson, E., Shealy, D. J. & Erikson, R. L. *J. biol. Chem.* **256**, 11381–11384 (1981).
25. Frackleton, A. R., Ross, A. H. & Eisen, H. N. *Molec. cell. Biol.* **3**, 1343–1352 (1983).
26. Sefton, B. M. & Hunter, T. *Cell* **24**, 165–174 (1981).
27. Frackelton, A. R., Tremble, P. M. & Williams, L. T. *J. biol. Chem.* **259**, 7909–7915 (1984).
28. Cooper, J. A., Reiss, N. A., Schwartz, R. J. & Hunter, T. *Nature* **302**, 218–223 (1983).
29. Rees-Jones, R. & Taylor, S. *J. biol. Chem.* **260**, 4461–4467 (1985).
30. Swarup, G., Cohen, S. & Garbers, D. L. *Biochem. biophys. Res. Commun.* **113**, 80–86 (1982).
31. Kasuga, M., White, M. F. & Kahn, C. R. *Meth. Enzym.* **109**, 609–621 (1985).
32. Ronnett, G. V., Knutson, V. P., Kohanski, R. A., Simpson, T. L. & Lane, M. D. *J. biol. Chem.* **259**, 4566–4575 (1984).
33. Daniel, T. O., Tremble, P. M., Frackelton, A. R. & Williams, L. T. *Proc. natn. Acad. Sci. U.S.A.* **82**, 2684–2687 (1985).
34. Pang, D. T., Sharma, B. R. & Schafer, J. A. *Archs. Biochem. Biophys.* **242**, 176–186 (1985).