Concanavalin A-induced receptor aggregation stimulates the tyrosine kinase activity of the insulin receptor in intact cells

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Concanavalin A (ConA) stimulated the phosphorylation of the β -subunit of the insulin receptor and an M_r -185000 protein on serine and tyrosine residues in intact H-35 rat hepatoma cells. This M_r -185000 protein whose phosphorylation was stimulated by ConA was identical to pp185, a protein reported previously to be a putative endogenous substrate for the insulin receptor tyrosine kinase in rat hepatoma cells. In Chinese hamster ovary (CHO) cells transfected with cDNA of the human insulin receptor, tyrosine-phosphorylation of pp185 was strongly enhanced by ConA compared with the controls, suggesting that the induction of tyrosine-phosphorylation of pp185 was due to stimulation of the insulin receptor kinase by ConA. Moreover, monovalent ConA only slightly induced the tyrosine-phosphorylation of pp185, which was enhanced by the addition of anti-ConA IgG, suggesting that ConA stimulated the insulin receptor kinase mainly by the receptor cross-linking or aggregation in intact cells. These data suggest that the insulin-mimetic action of ConA is related to the autophosphorylation and activation of the insulin receptor tyrosine kinase, as well as the subsequent phosphorylation of pp185 in intact cells.

INTRODUCTION

The insulin receptor is a membrane-spanning glycoprotein composed of α - and β -subunits (Kasuga *et al.*, 1982a). Insulin binding to the α -subunit stimulates the autophosphorylation and activation of the intrinsic tyrosine-specific protein kinase activity of the β -subunit (Kasuga *et al.*, 1982b, 1983; Roth & Cassell, 1983). Activation of the tyrosine kinase appears to be a prerequisite for many responses to insulin (Ellis *et al.*, 1986; Ebina *et al.*, 1987; Chou *et al.*, 1987; McClain *et al.*, 1987; Morgan & Roth, 1987; Izumi *et al.*, 1988). Several proteins have been reported to be putative substrates for the insulin receptor kinase (White *et al.*, 1985; Häring *et al.*, 1987; Perrotti *et al.*, 1987; Bernier *et al.*, 1987; Yu *et al.*, 1987). In particular, an M_r -185000 protein (pp185), or a related protein of similar M_r , is found in many cell types (Gibbs *et al.*, 1986; Kadowaki *et al.*, 1987; White *et al.*, 1987; Shemer *et al.*, 1987) and is a common substrate for both insulin and insulin-like growth factor I (IGF-I) receptor kinases (Izumi *et al.*, 1987). This protein is regarded as one of the important putative substrates in signal transduction by insulin (White *et al.*, 1988).

Several substances have been shown to mimic the actions of insulin. Concanavalin A (ConA), a plant lectin derived from jackbean, binds to cell surface glycoproteins with a high specificity for gluco- and manno-pyranosides. It has biological and morphological actions (Bittigen & Schnebli, 1976; Wakshull & Wharton, 1985; Faltynek *et al.*, 1988) and also mimics many actions of insulin (Kahn *et al.*, 1981). ConA stimulates the phosphorylation of the insulin receptor β -subunit in a cell-free system (Roth *et al.*, 1983) without inhibition of insulin binding (Kahn *et al.*, 1981; Katzen *et al.*, 1981), suggesting that it may exert its insulin-mimetic effects in a manner different from insulin. In this study, we show that ConA stimulates insulin

receptor autophosphorylation and the tyrosine-phosphorylation of pp185 by cross-linking or aggregation of receptors in intact cells. Thus the use of ConA further supports the role of tyrosine phosphorylation in the mechanism of insulin action.

EXPERIMENTAL

Materials

The following materials were obtained from the indicated sources. [32 P]P_i and Triton X-100 were from New England Nuclear, 125 I-labelled Protein A was from Amersham, pig insulin was kindly provided by Eli Lilly, tissue culture medium and fetal bovine serum were from Gibco or Flow, and all tissue culture supplies were from Nunc or Costar. Other reagents were obtained from the same sources as described previously (Izumi *et al.*, 1987, 1988; Kadowaki *et al.*, 1987).

Preparation of monovalent ConA

Monovalent ConA was prepared by the subunit-exchange method as described previously (Osawa & Beppu, 1987). The yield of monovalent hybrids was approx. 10% of the cross-linked dimers. The valency of monovalent ConA was confirmed by equilibrium dialysis against α -methyl α -[14 C]mannoside at pH 7.4. The binding activity and the carbohydrate specificity of this monovalent ConA were verified by the examination of 125 I-labelled monovalent ConA binding to H-35 cells and its displacement by native (tetraivalent) ConA and methyl α -mannopyranoside (Sigma). The minimum concentrations for haemagglutinating activity of the monovalent and the tetraivalent ConA against mouse erythrocytes were about 200 μ g/ml and 3.5 μ g/ml respectively.

Abbreviations used: IGF, insulin-like growth factor; ConA, concanavalin A; WGA, wheat-germ agglutinin; CHO, Chinese hamster ovary; anti-P-Tyr, anti-phosphotyrosine; PNPP, *p*-nitrophenyl phosphate.

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Cells

The experiments were performed with well-differentiated and insulin-sensitive H-35 rat hepatoma cells, provided by Dr. Y. Kitagawa (University of Nagoya, Nagoya, Japan), and with Chinese hamster ovary (CHO) cells (American Tissue Culture Collection) transfected with the human insulin receptor cDNA as described below. All cultured cells were maintained at 37 °C in a humidified atmosphere composed of air/CO₂ (19:1). The H-35 cells were grown in plastic tissue culture dishes containing 10 ml of Eagle's modified essential medium supplemented with 6% fetal bovine serum. The CHO cells were grown in plastic tissue culture dishes (10 cm diam.) containing 10 ml of F12 medium supplemented with 10% fetal bovine serum.

Transfection of the CHO cells with wild-type human insulin receptor cDNA

Two insulin receptor cDNA clones, pHIR18-2 and pHIR13-1 (Whittaker *et al.*, 1987), were kindly provided by Dr. G. I. Bell (University of Chicago, Chicago, IL, U.S.A.). The *EcoRI* fragment of pHIR18-2 was cloned into the *EcoRI* site of pSP65 (pSP65-HIR18-2). To add the *HindIII* site, the consensus initiation sequence of ATG and the *Eco52I* site to the 5' end of pHIR18-2, oligodeoxynucleotides (AGCTTATGGGCACCGG-GGGCCGGCGGGGGGCGGC and GGCCGCCGCCCCC-GCCGGCCCCCGGTGCCATAAGCT) were phosphorylated, annealed and ligated into the *HindIII*- and *Eco52I*-digested pSP65-HIR18-2 clone.

pHIR13-1 was cloned into the *EcoRI* site of pBR327. To delete the *EcoRI* site of pHIR13-1, pHIR13-1 was digested with *NspV* and *ClaI*, and re-ligated. The modified 13-1 clone and the modified 18-2 clone were digested with *EcoRI* and *HindIII* and ligated into the *HindIII* site of pSV2neo(pSV2HIR) (Mulligaw & Berg, 1981).

CHO-K1 cells (10⁶ cells/100 mm plate) were cotransfected with 10 µg of pSV2HIR plus 1 µg of pSV2neo using a calcium phosphate precipitation technique and selected by culture in the presence of G418 (Gibco) at 600 µg/ml. After positive colonies were enriched, cells were analysed by a fluorescence-activated cell sorter (FACStar; Becton & Dickinson, Mountain View, CA, U.S.A.) according to the method of Ellis *et al.* (1986) using a monoclonal anti-(insulin receptor) antibody (α -IR-1) kindly provided by Dr. S. Jacobs (Burroughs-Wellcome Research Lab., Research Triangle Park, NC, U.S.A.). After enrichment by sorting five times, cells were cloned by limiting dilution and used for experiments.

Antibodies

The anti-phosphotyrosine (anti-*P*-Tyr) antibody was prepared in rabbits by injection of *N*-bromoacetyl-*O*-phosphotyramine conjugated to keyhole-limpet haemocyanin as described previously (White *et al.*, 1985; Kadowaki *et al.*, 1987). The antibody was purified from serum by affinity chromatography on a phosphotyramine-Sepharose column. The final protein concentration of affinity-purified antibody was approx. 250 µg/ml. The site-specific antibody for the insulin receptor named Ab-IRC (Izumi *et al.*, 1988) was provided by Dr. T. Izumi in our laboratory. The final concentration of affinity-purified Ab-IRC was approx. 100 µg/ml. The rabbit anti-ConA IgG was provided by Dr. I. Yahara (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan).

Labelling of cells with [³²P]P_i and immunoprecipitation with anti-*P*-Tyr antibodies and anti-(insulin receptor) antibodies

Cells were labelled with [³²P]P_i, stimulated with ConA or insulin for the indicated time period and solubilized with buffer

A (50 mM-Hepes, pH 7.4, 10 mM-sodium pyrophosphate, 100 mM-NaF, 4 mM-EDTA, 2 mM-sodium orthovanadate, 1 mM-phenylmethanesulphonyl fluoride and 200 units of aprotinin/ml) containing 1% (v/v) Triton X-100 as described previously (Izumi *et al.*, 1987). A whole-cell extract was prepared by scraping the cells from the dishes and sedimenting insoluble materials by centrifugation at 15000 *g* for 15 min. ConA-stimulated phosphorylation of pp185 was not demonstrable by direct immunoprecipitation with anti-*P*-Tyr antibodies because of the high background level of ³²P on SDS/PAGE. Since pp185 bound to hydroxyapatite and was almost fully recovered by boiling in 2% SDS (K. Tobe & M. Kasuga, unpublished work), we partially purified pp185 by the following method. The whole-cell extract was incubated with 150 µl of hydroxyapatite [60% (v/v) slurry] at 4 °C for 30 min, and the hydroxyapatite was washed twice with the buffer A containing 1% Triton X-100. The hydroxyapatite was then incubated with 100 µl of buffer A containing 2% SDS and boiled immediately for 3 min. The eluted proteins were diluted to 1 ml and immunoprecipitated with anti-*P*-Tyr antibodies (1:100) as described previously (Izumi *et al.*, 1987). The phosphoproteins were eluted, reduced with 10 mM-dithiothreitol and separated by SDS/PAGE as described previously (Izumi *et al.*, 1987). The phosphoproteins were identified by autoradiography of the stained and dried gels using Kodak X-Omat film and an intensifying screen.

For immunoprecipitation with anti-(insulin receptor) antibodies, we solubilized cells with a solution containing SDS, since we have encountered difficulties immunoprecipitating the ConA-stimulated insulin receptor solubilized only by Triton. For example, when the cells were labelled with [³⁵S]methionine and solubilized by a buffer containing 1.5% Triton X-100, we could not detect the ³⁵S-labelled insulin receptor after stimulation with ConA. To decrease volumes, the monolayer cells were ruptured at 4 °C with 400 µl of buffer A containing 0.1% Triton X-100 and sedimented by centrifugation at 15000 *g* for 15 min. The membrane pellet was solubilized with 100 µl of buffer A containing 1.5% Triton X-100 and 2% SDS at 4 °C for 15 min, boiled for 3 min, sonicated 10 times for 6 s each, and diluted to 1 ml with buffer A. Insoluble materials were sedimented by centrifugation at 15000 *g* for 15 min and the supernatant was incubated with anti-(insulin receptor) antibodies (Ab-IRC) (1:50) at 4 °C for 2 h, and the antibodies were immobilized on Pansorbin. The precipitates were analysed by SDS/PAGE and autoradiography (Izumi *et al.*, 1987).

Immunoblotting of pp185 with anti-*P*-Tyr antibodies

The cell monolayer was treated with ConA or insulin and solubilized with buffer A containing 1% Triton X-100. Total cell lysates were incubated with 150 µl of hydroxyapatite, and then bound proteins were eluted with Laemmli's sample buffer (Laemmli, 1970) containing 10 mM-dithiothreitol, and boiled immediately for 3 min. They were then separated by SDS/PAGE and transferred on to nitrocellulose sheets (Schleicher & Schuell) as described previously (Momomura *et al.*, 1988). The sheets were incubated with anti-*P*-Tyr (3 µg/ml) and ¹²⁵I-labelled Protein A (specific radioactivity 30 mCi/mg) as described previously (Momomura *et al.*, 1988).

Phosphoamino acid analysis and peptide mapping

Analysis of the phosphoamino acid composition of the proteins separated by SDS/PAGE was carried out as described previously (Cooper *et al.*, 1983). Peptide mapping was performed according to the method described previously (Bordier & Crettol-Järviner, 1979; Izumi *et al.*, 1987).

Fig. 1. Lectin- and insulin-stimulated tyrosine-phosphorylation in intact H-35 cells

Serum-starved H-35 cells were labelled with [32 P]P_i and treated with buffer (lane a), ConA (1 mg/ml) for 5 min (lane b), WGA (1 mg/ml) for 5 min (lane c) or insulin (100 nM) for 1 min (lane d) at 37 °C. The cells were solubilized with buffer containing 1% Triton X-100, incubated with hydroxyapatite and eluted with 2% SDS with immediate boiling. The supernatants were diluted and immunoprecipitated with anti-*P*-Tyr antibodies and Pansorbin. 32 P-labelled immunoprecipitated proteins were eluted with 10 mM-PNPP, reduced with 10 mM-dithiothreitol and separated by SDS/PAGE. The autoradiogram was exposed at -70 °C for 36 h with an intensifying screen.

Fig. 3. Peptide maps of pp185 and pp120

H-35 cells were labelled with [32 P]P_i and treated with ConA (1 mg/ml) for 5 min or insulin (100 nM) for 1 min at 37 °C. Total cell lysates were incubated with hydroxyapatite. The eluted proteins from hydroxyapatite were immunoprecipitated with anti-*P*-Tyr antibodies and Pansorbin and the precipitated proteins were separated by SDS/PAGE. Proteolysis on an SDS/polyacrylamide gel was performed as described previously (Bordier & Crettol-Järvinen, 1979; Izumi *et al.*, 1987). Peptide maps were performed with 10 mg of *S. aureus* V8 protease/ml. The autoradiogram of the ConA-stimulated pp185 digest was exposed at -70 °C for 6 days, while the autoradiogram of the insulin-stimulated pp185 was exposed for 2 days.

Fig. 2. Identification of phosphoamino acids in the M_r -185,000 protein

H-35 cells were labelled with [32 P]P_i and treated with insulin (100 nM) for 1 min (a) or ConA (1 mg/ml) for 5 min at 37 °C (b). Phosphoamino acid analysis was performed as described in the Experimental section.

RESULTS

Phosphorylation of pp185 by ConA

To examine the possibility that ConA produces its insulin-like effects by tyrosine phosphorylation of pp185, confluent H-35 cells were labelled with [32 P]P_i, stimulated with insulin for 1 min or with ConA for 5 min at 37 °C and extracted with Hepes-buffered 1% Triton X-100. These extracts were purified on hydroxyapatite and immunoprecipitated with anti-*P*-Tyr antibodies. In the absence of stimulation, one major phosphoprotein

of M_r 120,000 (pp120) was observed (Fig. 1, lane a). After incubation with 100 nM-insulin, phosphorylation of pp185 was also observed (Fig. 1, lane d). After incubation of H-35 cells with 1 mg of ConA/ml, phosphorylation of the M_r -185,000 protein was clearly observed (Fig. 1, lane b). The ConA-stimulated pp185 could be extracted with Triton X-100-free solution (results not shown). Without partial purification by hydroxyapatite, however, the phosphorylation of pp185 after stimulation by ConA was not demonstrable. This result was assumed to be due to the high background level of [32 P]P_i. In the supernatant solution after hydroxyapatite treatment, we did not detect pp185 or pp120 after stimulation by insulin, suggesting that the hydroxyapatite was present in excess for adsorption of these phosphotyrosine-containing proteins. In some experiments, we observed phosphorylation of an M_r -95,000 protein (pp95) after stimulation by insulin or ConA in preparations adsorbed on to hydroxyapatite and eluted by SDS as shown in Fig. 8. This pp95 was immunoprecipitated specifically by anti-(insulin receptor) antibody, indicating that pp95 stimulated by insulin or ConA was the β -subunit of the insulin receptor (results not shown). Since the insulin receptor was also found in the flow-through fraction of the hydroxyapatite, only a fraction of the insulin receptor was adsorbed on the hydroxyapatite or associated with pp185 in some cases.

Wheat-germ agglutinin (WGA), another lectin which has insulin-like effects on intact cells and binds to insulin receptors (Livingston & Purvis, 1980; Hedo *et al.*, 1981), was also studied. WGA (1 mg/ml) stimulated the phosphorylation of pp185 in H-35 rat hepatoma cells to the same degree as did ConA (Fig. 1, lane c).

The phosphoamino acid composition of pp185 was determined by partial acid hydrolysis and separation by two-dimensional

Fig. 4. Dose-dependent phosphorylation of pp185

H-35 cells were labelled with [32 P]P_i and treated with the indicated doses of ConA for 10 min at 37 °C. Proteins immunoprecipitated with anti-*P*-Tyr antibodies were analysed as described in the Experimental section. The relative intensity of pp185 was evaluated by scanning densitometry.

high-voltage electrophoresis. After stimulation by insulin, pp185 showed phosphorylated spots at phosphoserine, phosphotyrosine and phosphothreonine, as described previously (White *et al.*, 1985) (Fig. 2a). After stimulation by ConA, we could also recognize phosphorylated residues at phosphoserine, phosphothreonine and phosphotyrosine (Fig. 2b). On immunoblotting with anti-*P*-Tyr antibodies, tyrosine-phosphorylation of the M_r -185000 protein after ConA stimulation (1 mg/ml) was found (results not shown). These findings suggested that ConA stimulates tyrosine-phosphorylation of pp185 in intact H-35 cells.

Phosphopeptide mapping of pp185

To examine whether pp185 phosphorylated by ConA was structurally identical to the pp185 identified previously in insulin-stimulated H-35 rat hepatoma cells, we carried out Cleveland mapping of the pp185 stimulated by ConA or insulin. At the same time we carried out mapping of pp120, a constitutive tyrosine-phosphorylated protein, for comparison. After digestion in an SDS/polyacrylamide gel by *Staphylococcus aureus* V8 protease, we found that the fragments from pp185 phosphorylated under both conditions were indistinguishable in pattern from each other; similarly, the maps of pp120 were also indistinguishable (Fig. 3). This result suggested that the M_r -185000 protein from ConA-stimulated cells was identical to the pp185 from insulin-stimulated cells.

Dose-dependence and kinetics of the phosphorylation of pp185 by ConA

The dose-dependency of the ConA effect on the phosphorylation of pp185 was studied in the experiment shown in Fig. 4. H-35 rat hepatoma cells were stimulated for 10 min with increasing concentrations of ConA. The autoradiogram was analysed by scanning densitometry which showed that a half-maximal effect of ConA occurred at about 40 μg/ml, and a maximal effect occurred at about 200 μg/ml (Fig. 4). However, the maximum effect of ConA was the same or slightly less potent than the effect of 1 nM-insulin in comparisons with the dose-dependence of the insulin effect on pp185 phosphorylation (results not shown).

The time course of the phosphorylation of pp185 by ConA (Fig. 5) was distinct from that by insulin, which phosphorylated pp185 immediately, reaching a maximum level after 30 s and decreasing rapidly (Kadowaki *et al.*, 1987). The phosphorylation

Fig. 5. Time course of the stimulation by ConA of the phosphorylation of pp185

32 P-labelled cells were treated with 1 mg of ConA/ml at 37 °C for the indicated time periods and immunoprecipitated as described in the Experimental section.

Fig. 6. Phosphorylation of the insulin receptor

H-35 cells were labelled with [32 P]P_i and treated with buffer (lane a), ConA (1 mg/ml) for 5 min (lane b) or insulin (100 nM) for 1 min (lane c) at 37 °C. Solubilized extracts prepared as described in the Experimental section were immunoprecipitated with site-specific antibodies to the insulin receptor [Ab-IRC (1:50)] (Izumi *et al.*, 1988) and Pansorbin and separated by SDS/PAGE.

of pp185 by ConA (1 mg/ml) was detectable within 1 min, reached a maximum after 10 min and decreased to 60% of the maximum level after 1 h. The pp185 band migrated at a higher M_r (approx. 195000) after 30 min of treatment of cells with ConA as described previously for insulin treatment (White *et al.*, 1987). As shown in Fig. 5, in some preparations the phosphorylation of proteins of M_r 60000, 140000 and 210000 was slightly stimulated by ConA.

Phosphorylation of the insulin receptor by ConA

ConA and WGA are known to bind to insulin receptors (Hedo *et al.*, 1981), but such binding does not appear to occur at the insulin-binding site because of the specificity of these lectins for specific carbohydrate determinants and their inability to inhibit 125 I-insulin binding (Kahn *et al.*, 1981; Katzen *et al.*, 1981). Since

Fig. 7. Identification of phosphoamino acids in the insulin receptor

^{32}P -labelled M_r -95000 bands of the insulin receptor in Fig. 6 were excised from the gel, washed, trypsin-treated and hydrolysed as described in the Experimental section. To separate phosphoamino acids, vertical electrophoresis was performed at pH 1.9 by 2000 V for 20 min, followed by horizontal electrophoresis at pH 3.5 by 2000 V for 15 min. H-35 cells were treated with buffer (a), ConA (1 mg/ml) for 5 min (b), or insulin (100 nM) for 1 min (c) at 37 °C.

H-35 cells lack IGF-I receptors (Massague & Czech, 1982), we speculated that the phosphorylation of pp185 by ConA and WGA was catalysed through insulin receptor tyrosine kinase. To test this hypothesis, we have examined the phosphorylation of the insulin receptor after stimulation of H-35 cells by ConA. H-35 cells were labelled with ^{32}P , stimulated by ConA (1 mg/ml) or insulin (100 nM), solubilized with 1.5% Triton X-100 and 2% SDS, and boiled for 3 min to ensure solubilization of the ConA-stimulated insulin receptor. Samples were sonicated, diluted and immunoprecipitated by site-specific anti-(insulin receptor) antibodies (Ab-IRC) (Izumi *et al.*, 1988) (Fig. 6). The M_r -95000 band specifically immunoprecipitated by the antibodies was labelled with ^{32}P in the absence of insulin or ConA (Fig. 6, lane a). ^{32}P -labelling of the M_r -95000 band was increased by incubation with ConA for 5 min (Fig. 6, lane b) or insulin for 1 min (Fig. 6, lane c) at 37 °C. These bands were excised from the gel, hydrolysed and then analysed by two-dimensional high-voltage electrophoresis (Fig. 7). This analysis revealed that ConA stimulated the formation of both phosphoserine and phosphotyrosine (Fig. 7b); however, insulin mainly stimulated formation of phosphotyrosine (Fig. 7c). Thus the unexpectedly high degree of phosphorylation of the ConA-stimulated insulin receptor could be explained by unusually strong phosphorylation of serine residues.

Phosphorylation of pp185 in CHO cells transfected with human insulin receptor

As described previously, CHO cells contained an M_r -175000 protein (pp175) that was apparently identical to pp185 in H-35 rat hepatoma cells (White *et al.*, 1987). We have transfected the cDNA of the human insulin receptor into CHO cells (CHO-HIR), and examined the resultant insulin-binding capacity. Scatchard plot analysis indicated that the total insulin-binding capacity of the transfected cells was 40-fold higher than in the control cells (CHO-WT) (results not shown). These cells were

Fig. 8. Phosphorylation of pp185 in CHO cells transfected with human insulin receptor

CHO cell lines, which express about 5×10^5 human insulin receptors/cell (CHO-HIR) and wild-type CHO cells (CHO-WT) were labelled with ^{32}P and treated with buffer (lanes a and d), ConA (1 mg/ml) for 5 min (lanes b and e), or insulin (100 nM) for 1 min (lanes c and f). Total cell lysates were incubated with hydroxyapatite, eluted with 2% SDS and immunoprecipitated with anti-*P*-Tyr antibodies and Pansorbin. Immunoprecipitates were eluted, reduced with 10 mM-dithiothreitol, separated by SDS/PAGE and analysed by autoradiography.

labelled with ^{32}P , incubated with ConA (1 mg/ml) for 5 min or insulin (100 nM) for 1 min, solubilized and immunoprecipitated with anti-*P*-Tyr antibodies (Fig. 8). In control cells (CHO-WT), ConA did not induce any major ^{32}P -labelled band, but insulin induced one major ^{32}P -labelled band of M_r 175000. However, in transfected cells (CHO-HIR), ConA and insulin clearly induced ^{32}P -labelling of bands of M_r 175000 and 95000. As mentioned previously, this M_r -95000 protein is the β -subunit of the insulin receptor. Thus this experiment also suggested that ConA stimulated the tyrosine-phosphorylation of the insulin receptor. Interestingly, insulin induced the tyrosine-phosphorylation of several other cellular proteins of M_r values of 30000–60000 in the transfected cells (CHO-HIR). The extent of insulin-stimulated phosphorylation of pp175 in transfected cells was 6-fold higher than in the controls when the relative intensity of phosphorylation was measured by scanning densitometry. In parallel, the ConA-stimulated phosphorylation of pp175 was enhanced by 8-fold. According to immunoblotting utilizing anti-*P*-Tyr antibodies, the tyrosine phosphorylation of pp185 by ConA was also enhanced in CHO-HIR cells (results not shown). Since the difference between these two sets of cells was only the expressed human insulin receptor, we conclude that ConA stimulated the tyrosine phosphorylation of pp175 through the insulin receptor tyrosine kinase.

Effect of valency of ConA on the induction of pp185

On the basis of the finding that cross-linking or aggregation of insulin receptors is important for the insulin-like action of ConA (Kahn *et al.*, 1978, 1981), we hypothesized that ConA would activate the insulin receptor tyrosine kinase through receptor cross-linking or aggregation. To test this hypothesis, we have examined the phosphorylation of pp175 in the CHO-HIR cells after treatment with monovalent ConA using an immunoblotting method. As shown in Fig. 9(a), tetravalent ConA stimulated the tyrosine-phosphorylation of pp175, while increasing the dose of

Fig. 9. Effect of valency of ConA on the induction of pp175

(a) CHO cells transfected with the cDNA of the human insulin receptor were treated with the indicated dose of monovalent (lanes a-c) or tetravalent (native) (lanes d-g) ConA for 5 min at 37 °C. Total cell lysates were incubated with hydroxyapatite and eluted with Laemmli (1970) sample buffer with 10 mM-dithiothreitol. The samples were separated by SDS/PAGE and analysed by immunoblotting utilizing anti-*P*-Tyr antibodies as described in the Experimental section. (b) CHO-HIR cells were exposed to monovalent ConA (25 µg/ml) for 30 min at 37 °C, and then rabbit anti-ConA IgG (200 µg/ml; lane a) or control rabbit IgG (200 µg/ml; lane b) was added and incubated for 20 min at 37 °C. The samples were analysed by immunoblotting utilizing anti-*P*-Tyr antibodies as described in the Experimental section.

monovalent ConA only weakly phosphorylated pp175. The extent of phosphorylation of pp175 by 200 µg of monovalent ConA/ml was clearly less than that by 50 µg of tetravalent ConA/ml, which should have the same number of binding sites as 200 µg of monovalent ConA/ml; in fact the phosphorylation was also less than that caused by 12.5 µg of tetravalent ConA/ml. The weak phosphorylation of pp175 by the monovalent ConA preparation might be attributable to its residual aggregating activity, since our monovalent ConA fraction had 1.7–2.0% of the haemagglutinating activity against mouse erythrocyte of tetravalent ConA (results not shown). The weak residual aggregating activity of the monovalent derivative could be explained by several possibilities as discussed previously (Beppu *et al.*, 1979).

To explore the role of valency in stimulating the insulin receptor kinase, reconstitution of valency was attempted by exposing the receptor to the monovalent ConA and then cross-linking them by addition of anti-ConA antibodies in intact cells. As shown in Fig. 9(b), addition of antibodies against ConA clearly enhanced the ability of monovalent ConA to stimulate the tyrosine phosphorylation of pp175 in CHO-HIR cells, although the lower- M_r band, which presumably represents IgG, is labelled less by ^{125}I -Protein A in the presence of anti-ConA IgG. These data suggest that ConA stimulates the insulin receptor kinase mainly by the receptor cross-linking or aggregation in intact cells.

DISCUSSION

The insulin receptor has a tyrosine-specific protein kinase activity associated with the cytoplasmic domain of its β -subunit. Recent studies utilizing site-directed mutagenesis of tyrosine residues or the ATP-binding site (Ebina *et al.*, 1987; Chou *et al.*, 1987; McClain *et al.*, 1987) and tyrosine kinase inhibitory antibodies (Morgan & Roth, 1987; Izumi *et al.*, 1988) indicate that the activation of the tyrosine kinase is a key step in insulin signal transduction. Furthermore, it has been hypothesized that

this signal transduction may occur in the intact cell by a cascade of substrate phosphorylation steps. The pp185 protein is known as one of the putative important endogenous substrates specific for the insulin and IGF-I receptor kinase (White *et al.*, 1985, 1987, 1988; Gibbs *et al.*, 1986; Kadowaki *et al.*, 1987; Izumi *et al.*, 1987; Shemer *et al.*, 1987). In this present study, we have demonstrated that ConA stimulated the tyrosine-phosphorylation of an M_r -185000 protein in intact H-35 rat hepatoma cells. This phosphoprotein of M_r 185000 shared the following common characteristics with the pp185 observed during the initial response of H-35 cells to insulin. First, the phosphopeptide map of the ConA-stimulated M_r -185000 protein was identical to that of the insulin-stimulated pp185. Secondly, prolonged exposure of cells to ConA also caused retarded migration of this protein in SDS/PAGE and decreased its phosphorylation. Thirdly, this protein could be extracted by detergent-free solution and adsorbed on hydroxyapatite.

In this study, we have also demonstrated that ConA stimulated tyrosine-phosphorylation of the β -subunit of the insulin receptor, and it also stimulated serine-phosphorylation of the insulin receptor. Interestingly, the degree of serine-phosphorylation was stimulated more by ConA than by insulin, suggesting that ConA stimulated several cellular serine kinases or decreased phosphatase activities specific for serine residues in intact cells. This may be explained by the fact that ConA interacts with many kinds of glycoproteins on the cell surface, and the insulin receptor is only one of the glycoproteins to which it binds (Bittigen & Schnebli, 1976). This fact also made it difficult to identify the tyrosine kinase(s) responsible for the tyrosine-phosphorylation of pp185. However, our finding that the tyrosine-phosphorylation of pp185 induced by ConA was demonstrated only in the CHO cells transfected with human insulin receptor cDNA suggested that the insulin receptor kinase was responsible for the tyrosine-phosphorylation of pp185 induced by ConA.

Next we investigated the role of the valency of ConA in the activation of the receptor kinase. For this purpose, we produced monovalent ConA, and confirmed its valency by equilibrium dialysis and its binding activity to glycoproteins using an ^{125}I -labelled fraction. The monovalent ConA fraction was less

potent in inducing the tyrosine-phosphorylation of pp185 than was tetravalent ConA. The slight phosphorylation of pp185 induced by monovalent ConA may be explained by the residual aggregating activity of monovalent ConA (Beppu *et al.*, 1979). Reconstitution of valency by the addition of the anti-ConA antibodies enhanced the ability of monovalent ConA to stimulate the tyrosine phosphorylation of pp175 in CHO-HIR cells. Thus ConA may activate the tyrosine-kinase activity of the insulin receptor by aggregation or cross-linking of the receptor. However, it cannot be fully discounted that the monovalent and tetravalent ConA act differently on the tyrosine-phosphorylation of pp185, since the direct effects of ConA on receptor auto-phosphorylation cannot be demonstrated for technical reasons.

In a Triton-solubilized system, Heffetz & Zick (1986) demonstrated that receptor cross-linking or aggregation constituted a sufficient trigger for activation of the insulin receptor kinase using monovalent and divalent anti-receptor Fab fragments. Thus receptor aggregation may activate the tyrosine kinase activity of the receptor both in Triton-solubilized fractions and in the intact membrane. From studies of truncated receptors (Ellis *et al.*, 1987) or trypsin-treated receptors (Shoelson *et al.*, 1988), it was proposed that the α -subunit of the insulin receptor inhibited the kinase activity of the β -subunit. That is, elimination of inhibitory constraints imposed by the α -subunit might permit a conformational relaxation that enables the β -subunit to adopt an unconstrained, uninhibited, and thus activated conformation (Shoelson *et al.*, 1988). According to this hypothesis, receptor aggregation may also release the kinase activity of the β -subunit from inhibitory control.

Half-maximal stimulation of pp185 phosphorylation by ConA occurred at about 40 μ g of ConA/ml. This value was slightly higher than that reported previously for glucose oxidation of rat adipocytes treated by ConA (Kahn *et al.*, 1981) and for induction of tyrosine aminotransferase activity by WGA (Smith & Liu, 1981), and was similar to the value for ConA-stimulated auto-phosphorylation of the insulin receptor in a cell-free system (Roth *et al.*, 1983). Furthermore, Kahn *et al.* (1981) have characterized the role of valency and receptor aggregation in the insulin-like effects of ConA in rat adipocytes and found that monovalent ConA totally lacked insulin-like effects. Considering these previous data, our present results, including the data with monovalent ConA, raised the possibility that ConA produces insulin-like effects through activation of the insulin receptor kinase and the tyrosine-phosphorylation of pp185 initiated by the aggregation or cross-linking of the receptor.

In conclusion, ConA stimulated the phosphorylation of the insulin receptor on tyrosine residues and stimulated tyrosine kinase activity, and it resulted in tyrosine phosphorylation of pp185, a putative cellular substrate for the insulin-stimulated insulin receptor kinase; these steps occurred in a dose- and time-dependent manner in intact cells. The mechanism of ConA activation of insulin receptor kinase is suggested to be mainly by cross-linking or aggregation of the receptors in intact cells.

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REFERENCES

- Beppu, M., Terao, T. & Osawa, T. (1979) *J. Biochem. (Tokyo)* **85**, 1275–1287.
- Bernier, M., Laird, D. M. & Lane, M. D. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1844–1848.
- Bittigen, H. & Schnebli, H. P. (eds.) (1976) *Concanavalin A as a Tool*, Wiley, New York.
- Bordier, C. & Crettol-Järvinen, A. (1979) *J. Biol. Chem.* **254**, 2565–2567.
- Chou, C. K., Dull, T. J., Russell, D. S., Gherzi, R., Lebowitz, D., Ullrich, A. & Rosen, O. M. (1987) *J. Biol. Chem.* **259**, 5277–5286.
- Cooper, J. A., Sefton, B. M. & Hunter, T. (1983) *Methods Enzymol.* **99**, 387–402.
- Ebina, Y., Araki, E., Taira, M., Shimada, F., Mori, M., Craik, C. S., Siddle, K., Pierce, S. B., Roth, R. A. & Rutter, W. J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 328–332.
- Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A. & Rutter, W. J. (1986) *Cell* **45**, 721–732.
- Ellis, L., Morgan, D. O., Clauser, E., Roth, R. A. & Rutter, W. J. (1987) *Mol. Endocrinol.* **1**, 15–24.
- Faltynek, C. R., Princier, G. L., Ruscetti, F. W. & Birchenall-Sparks, M. (1988) *J. Biol. Chem.* **263**, 7112–7117.
- Gibbs, E. M., Allard, W. J. & Lienhard, G. E. (1986) *J. Biol. Chem.* **261**, 16597–16603.
- Häring, H. U., White, M. F., Machicao, F., Erme, B., Schleicher, E. & Obermaier, B. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 113–117.
- Hedo, J. A., Harrison, L. C. & Roth, J. (1981) *Biochemistry* **20**, 3385–3393.
- Heffetz, D. & Zick, Y. (1986) *J. Biol. Chem.* **261**, 889–894.
- Izumi, T., White, M. F., Kadowaki, T., Takaku, F., Akanuma, Y. & Kasuga, M. (1987) *J. Biol. Chem.* **262**, 1282–1287.
- Izumi, T., Saeki, Y., Akanuma, Y., Takaku, F. & Kasuga, M. (1988) *J. Biol. Chem.* **263**, 10386–10393.
- Kadowaki, T., Koyasu, S., Nishida, E., Tobe, K., Izumi, T., Takaku, F., Saeki, H., Yahara, I. & Kasuga, M. (1987) *J. Biol. Chem.* **262**, 7342–7350.
- Kahn, C. R., Baird, K. L., Jarrett, D. B. & Flier, J. S. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4209–4213.
- Kahn, C. R., Baird, K. L. & Van Obberghen, E. (1981) *FEBS Lett.* **129**, 131–134.
- Kasuga, M., Hedo, J.-A., Yamada, K. M. & Kahn, C. R. (1982a) *J. Biol. Chem.* **257**, 10392–10399.
- Kasuga, M., Karlsson, F. A. & Kahn, C. R. (1982b) *Science* **215**, 185–187.
- Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. & Kahn, C. R. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2137–2141.
- Katzen, H. M., Selderman, D. D. & Green, B. G. (1981) *Biochem. Biophys. Res. Commun.* **115**, 410–416.
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
- Livingston, J. N. & Purvis, B. J. (1980) *Am. J. Physiol.* **238**, E267–E275.
- Massague, J. & Czech, M. P. (1982) *J. Biol. Chem.* **257**, 5038–5045.
- McClain, D. A., Maegawa, H., Lee, J., Dull, T. J., Ullrich, A. & Olefsky, J. M. (1987) *J. Biol. Chem.* **262**, 14663–14671.
- Momomura, K., Tobe, K., Seyama, Y., Takaku, F. & Kasuga, M. (1988) *Biochem. Biophys. Res. Commun.* **155**, 1181–1186.
- Morgan, D. O. & Roth, R. A. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 41–45.
- Mulligaw, R. C. & Berg, P. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2072–2076.
- Osawa, T. & Beppu, M. (1987) *Methods Enzymol.* **150**, 17–28.
- Perrotti, N., Accili, D., Marcus-Samuels, B., Rees-Jones, R. W. & Taylor, S. I. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3137–3140.
- Roth, R. A. & Cassell, D. J. (1983) *Science* **219**, 299–301.
- Roth, R. A., Cassell, D. J., Maddux, B. A. & Goldfine, I. D. (1983) *Biochem. Biophys. Res. Commun.* **115**, 245–252.
- Shemer, J., Adamo, M., Wilson, G. L., Heffetz, D., Zick, Y. & LeRoith, D. (1987) *J. Biol. Chem.* **262**, 15476–15482.
- Shoelson, S. E., White, M. F. & Kahn, C. R. (1988) *J. Biol. Chem.* **263**, 4852–4860.
- Smith, J. D. & Liu, A. Y.-C. (1981) *Science* **214**, 799–800.

- Wakshull, E. M. & Wharton, W. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8513–8517
- White, M. F., Maron, R. & Kahn, C. R. (1985) *Nature (London)* **318**, 183–186
- White, M. F., Stegmann, E. W., Dull, T. J., Ullrich, A. & Kahn, C. R. (1987) *J. Biol. Chem.* **262**, 9769–9777
- White, M. F., Livingston, J. N., Backer, J. M., Lauris, V., Dull, T. J., Ullrich, A. & Kahn, C. R. (1988) *Cell* **54**, 641–649
- Whittaker, J., Okamoto, A. K., Thys, R., Bell, G. I., Steiner, D. F. & Hoffman, C. A. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5237–5241
- Yu, K. T., Khalaf, N. & Czech, M. P. (1987) *J. Biol. Chem.* **262**, 7865–7873

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