INSULIN RECEPTOR IN HUMAN HEPATOMA PLC/PRF/5 CELLS AND ITS IMMUNOCHEMICAL CHARACTERIZATION WITH ANTI-SYNTHETIC PEPTIDE ANTIBODIES

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ABSTRACT

Human hepatoma PLC/PRF/5 cells were demonstrated to possess specific binding sites for insulin. Scatchard analysis of the binding of 125I-TyrA14-human insulin (125I-insulin) to the hepatoma cells afforded a linear plot with Kd of 1.69 x 10^{-9} M and Bmax of 1.28 x 10^{-10} M/1 x 10^5 cells. The binding of 125I-insulin to the cells was inhibited by unlabeled human insulin dose-dependently with ED_{50} of 5.6 x 10^{-10} M. The insulin receptors in the hepatoma cells were characterized immunochemically with use of anti-synthetic peptide antibodies. First, antibodies were raised against synthetic peptides corresponding to the (30-61), (48-77) and (734-760) portions of the predicted amino acid sequence of human placental insulin receptor protein (HIRP). The antibodies proved to cross-react specifically with components in solubilized human placental membrane preparation by radioimmunoassay and immunoblotting. The three anti-synthetic peptide antibodies cross-reacted with the cells and the binding of anti-HIRP (30-61) and anti-HIRP (48-77) antibodies to the cells was inhibited in the presence of insulin. Immunoprecipitates with the three antibodies were shown under non-reducing condition to contain three insulin-binding components with molecular weights of 320,000, 290,000 and 270,000, presumably corresponding to α_2β_2, α_2β(β_1) and α_2(β_1)2, respectively, and under reducing condition, they contained only one component with molecular weight of 125,000, probably corresponding to α. Anti-HIRP (30-61) and anti-HIRP (48-77) antibodies inhibited, though partially, 125I-insulin binding to the intact hepatoma cells. However, 125I-insulin binding to the cell membrane fraction was inhibited only by anti-HIRP (48-77) antibody, and anti-HIRP (30-61) antibody did not show any effect in the assay system. The results support implication in insulin-receptor interaction of the amino-terminal portion of the α-subunit and also indicate that the molecular arrangement of insulin binding sites in the isolated plasma membrane preparation is not necessarily identical with that in the intact cell membrane.

The prediction of the amino acid sequences of human placental insulin receptor precursor protein (HIRP) by cDNA analysis (3,15) has enabled us to use immunohistochemical manipulation with synthetic peptides and anti-synthetic peptide antibodies to explore molecular
basis of the functions of insulin receptor. In order to obtain region-specific antibodies against HIRP, we have already synthesized as immunogens forty-two peptides of HIRP fragments ranging various portions of the entire peptide chain (1-1,355) (3) of HIRP, and succeeded in raising antibodies against twelve of the synthetic peptides (11, 17, 18). The synthetic peptides were purposely designed to consist of 20-30 amino acid residues so that the peptides by themselves possess antigenicity high enough to elicit satisfactory antibodies without conjugating with macromolecules (16, 19, 20). Studies on insulin receptor with anti-synthetic peptide antibodies have been reported by others (7-9).

Several hepatoma cell lines in culture have been used to study insulin receptor and insulin action (2). Indeed, Crettaz has claimed hepatoma cells as a useful and valid alternative for study on the action of insulin in the liver (2).

The present communication describes some characterization of insulin receptor in a human hepatoma cell line, the PLC/PRF/5 cell, with use of three of our region-specific anti-HIRP antibodies raised against synthetic peptides, HIRP (30-61), (48-77) and (734-760).

MATERIALS AND METHODS

Peptides

Peptides HIRP (30-61), (48-77), (734-760) and 4-(3-hydroxyphenyl)propionylated HIRP (734-760) (Fig. 1) were prepared by a solid-phase technique using an Applied Biosystems automated peptide synthesizer type 430B as described previously (17). The peptides were extensively purified by gel filtration on a Bio-Gel P-6 column using 3M acetic acid as eluent followed by reverse-phase HPLC on a preparative TSK Gel ODS-120T column in a solvent system of 0.01N HCl/CH3CN. Purity of the peptides was carefully examined by TLC, HPLC, amino acid analysis of acid hydrolysate and enzymatic digest of each peptide and sequence analysis.

Production of Antibodies

Each of the synthetic peptides (500 μg/rabbit) was treated with 50% polyvinylpyrrolidone (0.5 ml) and the solution was emulsified with complete Freund's adjuvant (0.5 ml). The emulsion was injected intradermally into three female rabbits. Two weeks after the injection, the rabbits were bled and antibody titers in the sera were examined. Injection (peptide 300 μg/rabbit) and bleeding were repeated every two weeks, until antibody titers were elevated to satisfactorily high levels. Antibody titer was represented by binding capacity of diluted antiserum to 125I-labeled antigen peptide (≦5,000 cpm/0.1 ml diluted antiserum). Diluted (1:7,000-1:14,000) antiserum which could bind about 30-50% of labeled antigen added was used in this study.
Antisera thus obtained were partially purified by precipitation with 40% ammonium sulfate.

**Cell Culture**

Human hepatoma PLC/PRF/5 cells, a cell line established from human primary liver carcinoma (1), were cultured in monolayer at 37°C in RPMI-1640 medium (GIBCO Laboratories) supplemented with 10% fetal bovine serum (M. A. Bioproducts).

**Preparation of Hepatoma Cell Membranes**

Plasma membrane fraction of the PLC/PRF/5 cells was prepared in a manner similar to that described by Fujita-Yamaguchi and coworkers (6).

**Insulin-binding Assay**

The PLC/PRF/5 cells \((1 \times 10^5/2 \text{ cm}^2 \text{ well})\) were washed at 37°C 3 times with buffer, pH 7.4, (1 ml each) comprising 50 mM HEPES, 120 mM NaCl, 1.2 mM MgSO_4, 25 mM KCl, 15 mM sodium acetate, 10 mM glucose, 1 mM EDTA and 0.2% bovine serum albumin (buffer A). The cells were incubated in buffer A (0.3 ml) at 15°C for 180 min with 

\[ {^{125}}I-\text{Tyr}^{\alpha}_{13} \text{human insulin (}{^{125}}I\text{-insulin} \]

\((1,850 \text{ Ci/mmol, Amersham}) (4.8 \times 10^{-11} \text{ M})\) and increasing amounts of unlabeled human insulin (Eli Lilly). After washing twice with ice-cold buffer A (1 ml each), the cells were dissolved in 1 N NaOH and the cell-associated radioactivity was counted with a γ-counter. The radioactivity bound to the cells in the presence of \(1 \times 10^{-6} \text{ M}\) unlabeled human insulin was considered to be due to non-specific binding. Degradation of \( {^{125}}I\text{-insulin} \) during incubation was determined by the method of Freychet and coworkers (4).

**Binding of Anti-synthetic Peptide Antibody to Human Hepatoma Cells**

The PLC/PRF/5 cells were incubated with increasing amounts of anti-synthetic peptide antibody at 22°C for 120 min in buffer A (0.3 ml) as described above. After washing twice with buffer A (1 ml each) at 22°C, the cells were incubated with \( {^{125}}I\text{-protein-A (34 Ci/g, Amersham)} (1.2 \times 10^{-9} \text{ M}) \) (0.3 ml) at 22°C for 30 min. The cells were washed with ice-cold buffer A and dissolved in 1 N NaOH, and the radioactivity was counted with a γ-counter. Incubation of the cells with anti-synthetic peptide antibody was also carried out in the presence of unlabeled human insulin \((1 \times 10^{-7} \text{ M})\).

**Immunoprecipitation with Anti-synthetic Peptide Antibody**

Plasma membrane fraction prepared from the PLC/PRF/5 cells \((2 \times 10^9)\) was solubilized with Triton X-100 (14). The soluble membrane preparation was incubated with anti-synthetic peptide antibody or γ-globulin fraction of rabbit non-immune serum \((50 \mu\text{g} \text{ protein})\) in 50 mM HEPES buffer, pH 7.4, (1 ml) containing 154 mM NaCl, 0.1 mM phenylmethyl sulfonfyl fluoride (PMSF) and 0.1% Triton X-100 (buffer B). To the mixture was then added protein-A suspension \((40 \text{ mg/ml}, 53 \text{ mg IgG binding capacity/mg, Sigma}) (0.2 \text{ ml})\) and incubation was continued with shaking at 4°C for an additional 60 min. The immunoprecipitates were collected by centrifugation at 10,000 \(g\) for 5 min at 4°C.

**Cross-linking of \(^{125}I\text{-Insulin Bound****

The immunoprecipitates were incubated at 22°C for 60 min in 50 mM HEPES buffer, pH 7.4, (60 μl) containing 154 mM NaCl, 0.1 mM PMSF and 0.5% Triton X-100 and the mixture was centrifuged at 10,000 \(g\) for 5 min at 4°C. The supernatant was incubated at 4°C for 16 h with \( {^{125}}I\text{-insulin (final concentration, 3.6} \times 10^{-11} \text{ M}) \) in buffer B (0.4 ml) in the presence or absence of unlabeled insulin \((1 \times 10^{-6} \text{ M})\). The bound insulin was cross-linked to the binding receptor protein according to the method of Pilch and Czech (14) using 10 mM disuccinimidyl suberate in dimethyl sulfoxide. The same insulin cross-linking experiment was also carried out with solubilized membrane preparation of the PLC/PRF/5 cells without immunoprecipitation.

**Electrophoresis and Autoradiography**

The insulin cross-linked preparation was boiled for 3 min in 62.5 mM Tris-HCl, pH 6.8,
Effect of Anti-synthetic Peptide Antibody on Insulin-binding

Human hepatoma cell  The PLC/PRF/5 cells were incubated with various amounts of anti-synthetic peptide antibody in buffer A (0.3 ml) at 22°C for 120 min and 125I-Tyr\textsubscript{A14} human insulin (final concentration 3.6 \times 10^{-11} \text{ M} in 0.4 ml) was added. Incubation was continued at 15°C for an additional 180 min. After washing, the cells were dissolved in 1 N NaOH and counted with a \(\gamma\)-counter as described above.

Human hepatoma cell membrane  The plasma membrane fraction (31 \mu g protein) from the PLC/PRF/5 cells was incubated at 4°C for 4 h with various amounts of anti-synthetic peptide antibody in buffer, pH 7.4, (0.3 ml) comprising 50 mM Tris-HCl, 10 mM MgCl\(_2\), 0.1 mM PMSF, 0.5 mg/ml bacitracin and 0.1% bovine serum albumin. 125I-Tyr\textsubscript{A14} human insulin (final concentration, 3.6 \times 10^{-11} \text{ M} in 0.4 ml) was then added. The mixture was incubated at 4°C for 20 h and the membrane was separated by precipitating with 0.4% bovine \(\gamma\)-globulin (0.1 ml) and 20% polyethylene glycol 6000 (0.5 ml) (6), followed by centrifugation at 1,500 g for 20 min. The membrane-associated radioactivity was counted with a \(\gamma\)-counter. Non-specific binding was determined in the presence of unlabeled human insulin (1 \times 10^{-6} \text{ M}).

RESULTS

Anti-synthetic Peptide Antibodies

Specificities of anti-HIRP (30–61), anti-HIRP (48–77) and anti-HIRP (734–760) antibodies were assessed by radioimmunoassay and immunoblot analysis using solubilized human placental membranes prepared according to the method by Fujita-Yamaguchi and coworkers (6). The radioimmunoassay was developed as described previously (13) with use of the respective antibodies, peptide antigens as standard and 125I-labeled peptide antigens as tracer. In the case of HIRP (734–760), 125I-4-(3-hydroxyphenyl)propionyl-HIRP (734–760) was used as tracer. Solubilized human placental membrane preparation was demonstrated to inhibit binding of the labeled antigens to the respective anti-synthetic peptide antibodies in all three radioimmunoassays examined. The dilution curves of the membrane preparations were parallel to the standard curves in all
Fig. 4 Inhibition of $^{125}$I-insulin binding to the PLC/PRF/5 cells with insulin, IGF-1, EGF or VIP. Human IGF-1 and human EGF had been prepared by gene technology by Fujisawa Pharmaceutical Co., Osaka, and Earth Chemical Co., Ako, Japan, respectively. VIP had been chemically synthesized by solid-phase method in our laboratory.

the assay systems. In addition, immunoblot analysis confirmed the specific binding of the three anti-synthetic peptide antibodies to the expected placental receptor components. The details will be described elsewhere.

Kinetic Properties of Insulin Binding to PLC/PRF/5 Cells

The binding of $^{125}$I-insulin to the hepatoma cells was a saturable process (Fig. 2). Non-specific binding was less than 1% of the total radioactivity added and degradation of $^{125}$I-insulin in assay buffer was less than 2%. Scatchard analysis of insulin binding to the cells afforded a linear plot over the insulin concentration range examined with Kd of $1.69 \times 10^{-9}$ M and $B_{max}$ of $1.28 \times 10^{-10}$ M/1 x 10$^5$ cells (Fig. 3). The binding of $^{125}$I-insulin to the cells was inhibited by unlabeled insulin dose-dependently with ED$_{50}$ of $5.6 \times 10^{-10}$ M (Fig. 4). Structurally-related human IGF-I also showed inhibitory activity against $^{125}$I-insulin binding, but the potency was 200-fold lower than that of insulin. Human EGF and VIP had no effect on $^{125}$I-insulin binding.

Fig. 5 Binding of anti-synthetic peptide antibodies to the PLC/PRF/5 cells in the absence (---) or presence (---) of insulin. Anti-HIRP (30-61) antibody (a), anti-HIRP (48-77) antibody (b) and anti-HIRP (734-760) antibody (c)

Binding of Anti-synthetic Peptide Antibodies to PLC/PRF/5 Cells

Anti-HIRP (30-61), anti-HIRP (48-77) and anti-HIRP (734-760) antibodies bound to the hepatoma cells in dose-dependent manners which were demonstrated by $^{125}$I-protein A binding to the antibodies bound to the cells (Fig. 5, a, b and c). The binding of $^{125}$I-protein A to the antibody-cell complexes increased up to $77.6 \times 10^{-12}$, $68.2 \times 10^{-12}$ and $23.0 \times 10^{-12}$ M, respectively. In the presence of unlabeled insulin, the antibody binding to the cells was apparently decreased in the cases of anti-HIRP (30-61) and anti-HIRP (48-77) antibodies, the decrease in antibody binding being 31.0 and 41.3%, respectively (Fig. 5, a and b). The presence of unlabeled insulin had no effect on anti-HIRP (734-760) antibody binding to the cells (Fig. 5c).

Immunoprecipitation with Anti-synthetic Peptide Antibodies and Cross-linking of $^{125}$I-Insulin

Immunoprecipitates from solubilized membrane preparation of the PLC/PRF/5 cells with anti-HIRP (30-61), anti-HIRP (48-77) or anti-HIRP (734-760) antibody were shown,
after being dissociated from the antibodies, to be able to bind $^{125}$I-insulin specifically. SDS-polyacrylamide gel electrophoresis followed by autoradiography of $^{125}$I-insulin-cross-linked immunoprecipitates showed three radioactive components under non-reducing condition (Fig. 6). The molecular weight of a major component was estimated to be 320,000 and that of two minor components to be 290,000 and 270,000. Under reducing condition, only one $^{125}$I-insulin-cross-linked component of 125,000 was detected in any immunoprecipitates produced by the three anti-synthetic peptide antibodies (Fig. 7). In the presence of $1 \times 10^{-6}$ M unlabeled insulin, no radioactive insulin-bound components were observed. Parallel control experiment with γ-globulin fraction from non-immune serum revealed no immunoprecipitated proteins with insulin-binding activity (Figs. 6 and 7). Electrophoretic patterns of $^{125}$I-insulin-cross-linked components in solubilized membrane preparation of the hepatoma cells without immunoprecipitation were comparable with those of the immunoprecipitates described above.

**Inhibition of $^{125}$I-Insulin Binding with Anti-synthetic Peptide Antibodies**

Anti-HIRP (30–61) and anti-HIRP (48–77) antibodies inhibited $^{125}$I-insulin binding to the PLC/PRF/5 cells dose-dependently up to 25.5 and 23.4%, respectively (Fig. 8, a and b), while anti-HIRP (734–760) antibody showed no effect on the binding (Fig. 8c). In insulin binding assay with use of plasma membrane fraction of the hepatoma cells, on the other hand, only anti-HIRP (48–77) antibody inhibited $^{125}$I-insulin binding in a dose-dependent manner up to 28.1% (Fig. 8e) and anti-HIRP (31–60) and anti-HIRP (734–760) antibodies had no inhibitory effect on the binding (Fig. 8, d and f).

**DISCUSSION**

Human hepatoma PLC/PRF/5 cell line had been established by Alexander and coworkers from a primary liver carcinoma tissue and the morphology of the cells indicates a resemblance to liver cells in most respects (1). We first demonstrated in this study that the cells possess specific binding sites for insulin (insulin receptors). The number of the receptors is large enough to use the cells for human insulin radioreceptor assay. In fact, we could develop a sensitive and specific insulin receptor assay system with the hepatoma cells.

We have been working on human placental insulin receptor proteins by immunochemical...
Fig. 8 Inhibition of $^{125}$I-insulin binding to the PLC/PRF/5 cells (a, b and c) or to the cell membranes (d, e and f) with anti-synthetic peptide antibodies. Anti-HIRP (30–61) antibody (a and d), anti-HIRP (48–77) antibody (b and e) and anti-HIRP (734–760) antibody (c and f).

In the present study, the insulin receptors of the human hepatoma PLC/PRF/5 cells were characterized by using three of anti-synthetic peptide antibodies, anti-HIRP (30–61), anti-HIRP (48–77) and anti-HIRP (734–760) antibodies. The three antibodies were first confirmed to recognize specifically receptor protein components in human placental membrane fraction. With solubilized membrane fraction of the PLC/PRF/5 cells, the anti-synthetic peptide antibodies were demonstrated to immunoprecipitate, under non-reducing condition, three insulin-binding components with molecular weights of 320,000, 290,000 and 270,000. Under reducing condition, insulin binding components that could be immunoprecipitated with the three antibodies all showed molecular weight of 125,000. The three components detected under non-reducing condition are likely to correspond to $\alpha_2\beta_2$, $\alpha_2\beta_1\beta_1$ and $\alpha_2(\beta_1)_2$, respectively, of human placental insulin receptor (5, 6, 12) and the component revealed under reducing condition to $\alpha$. These results indicate molecular weight profile and molecular features of insulin receptor proteins of human hepatoma PLC/PRF/5 cells identical with or at least very similar to those of human placental insulin receptor. Crettaz has identified both the $\alpha$ and $\beta$ subunits in Fao hepatoma cells (2). Similar molecular features of insulin receptor proteins in other hepatoma cells have also been described (10). In addition, the fact that the antibodies raised against the partial sequences of human placental insulin receptor cross-reacted with the insulin binding components in the PLC/PRF/5 cell membranes supports the identical amino acid sequences in insulin receptors in the membranes of the hepatoma cell and human placenta regarding the antigenic determinants of the antibodies.

The (30–61) and (48–77) sequences in HIRP correspond to amino-terminal portions of the $\alpha$-subunit of the placental receptor protein and the (734–760) sequence to an amino-terminal portion of the $\beta$-subunit. The three portions of the receptors are supposed to be located outside the cell membranes (3). Anti-HIRP (30–61) and anti-HIRP (48–77) antibodies inhibited partially but apparently insulin binding to the intact PLC/PRF/5 cells, while anti-HIRP (734–760) antibody had no effect on the binding. Assuming that the insulin receptor of the PLC/PRF/5 cells has the same molecular construction as that of the placental receptor, these data together with the above-mentioned results shown in Fig. 5, a and b support implication in insulin-receptor interaction of the amino-terminal portion of the $\alpha$-subunit of insulin receptor. In addition, such implication of the (30–61) portion of the $\alpha$-subunit in insulin binding was no longer observed when plasma membrane preparation of the cells was used for binding assay. This indicates distinct difference in insulin receptors in the intact hepatoma cells and in plasma membrane preparation of the cells in terms of their interaction with insulin. Specifically, the (30–61), more probably (30–47), region of the $\alpha$-subunit seems to be modified in its steric conformation during preparation of plasma membrane fraction from the PLC/PRF/5 cells. This is an important indication that the molecular arrangement of insulin binding sites in the isolated plasma membrane preparation is not necessarily identical with that in
the intact cell membrane. No inhibitory effect of anti-HIRP (734–760) antibody on insulin binding together with the result shown in Fig. 5c may indicate that the amino-terminal portion of the β-subunit is little involved in the insulin-receptor interaction. However, this is not conclusive at present, since the binding affinity of the antibody was not so high as those of the other two as shown in Fig. 5c.

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