IMMUNOCHEMICAL APPROACH TO INSULIN RECEPTOR WITH USE OF SYNTHETIC PEPTIDES

CHIZUKO YANAIHARA¹, TOHRU MOCHIZUKI¹, TAKASHI INOUE¹,
KAZUAKI IGUCHI¹, MICHIIHIRO TOYOSHIGE², MINORU
HOSHINO², TOSHIHIKO IWANAGA³, TSUNEO FUJITA³,
SHINICHI IZUMI⁴, KAZUHO NAKANE⁴ AND
NOBORU YANAIHARA^{1,2}

*Shizuoka College of Pharmacy¹, Shizuoka 422, National Institute for Physiological
Sciences², Okazaki 444, Niigata University School of Medicine³, Niigata 951
and Tokai University School of Medicine⁴, Isehara 259-11*

Received for publication December 25, 1986

Based on the predicted amino acid sequence of human placental insulin receptor protein (HIRP), we prepared five synthetic peptides corresponding to purposely selected regions of HIRP; (9-25), (30-61), (48-71), (736-760) and (1139-1171). Antisera were generated against the respective synthetic peptides. Radioimmunoassays with anti-HIRP (30-61) and anti-HIRP (1139-1171) sera could recognize two forms, 270K and 850K daltons, of solubilized insulin receptor in gel filtration fractions, presumably HIRP $\alpha\beta$ -monomeric and its tetrameric forms. Immunostaining with the five antisera against the synthetic HIRP fragments revealed positive cells in human placental and hepatic tissues; particularly the antisera stained strongly the membrane regions of Hofbauer cells in the placenta. The results support the validity and usefulness of the region-specific anti-synthetic HIRP fragment sera in studies on the molecular basis of insulin receptor.

The amino acid sequence of human placental insulin receptor precursor protein has been predicted by Ullrich *et al.* (5) and Ebina *et al.* (1) independently. Such structural informations have opened a way to explore complex biologic systems, in which the receptor protein is involved, by chemical and immunochemical manipulations with use of synthetic peptides.

As a part of our immunochemical study on the molecular basis of insulin receptor, we have examined in this study human placental and hepatic tissues radioimmunologically and immunohistochemically with use of region-specific antisera which were elicited against synthetic peptides (Fig. 1) corresponding to purposely selected regions of human insulin receptor protein (HIRP) (1).

MATERIALS AND METHODS

Synthetic peptides

The synthetic peptides used as immunogens (Fig. 1) were prepared by a solid phase technique according to symmetrical anhydride method with an Applied

Correspondence: Chizuko Yanaihara, Shizuoka College of Pharmacy, 2-2-1 Oshika, Shizuoka 422.

HIRP (9-25)	: H-Pro-Gly-Met-Asp-Ile-Arg-Asn-Asn-Leu-Thr-Arg-Leu-His-Glu-Leu-Glu-Asn-OH
HIRP (30-61)	: H-Glu-Gly-His-Leu-Gln-Ile-Leu-Leu-Met-Phe-Lys-Thr-Arg-Pro-Glu-Asp-Phe-Arg-Asp-Leu-Ser-Phe-Pro-Lys-Leu-Ile-Met-Ile-Thr-Asp-Tyr-Leu-OH
HIRP (48-77)	: H-Asp-Leu-Ser-Phe-Pro-Lys-Leu-Ile-Met-Ile-Thr-Asp-Tyr-Leu-Leu-Leu-Phe-Arg-Val-Tyr-Gly-Leu-Glu-Ser-Leu-Lys-Asp-Leu-Phe-Pro-OH
HIRP (736-760)	: H-Ser-Leu-Gly-Asp-Val-Gly-Asn-Val-Thr-Val-Ala-Val-Pro-Thr-Val-Ala-Ala-Phe-Pro-Asn-Thr-Ser-Ser-Thr-Ser-OH
HIRP (1139-1171)	: H-Met-Val-Ala-His-Asp-Phe-Thr-Val-Lys-Ile-Gly-Asp-Phe-Gly-Met-Thr-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys-Gly-Gly-Lys-Gly-Leu-Leu-OH

FIG. 1. Amino acid sequences of synthetic peptides related to human insulin receptor protein (HIRP) which were prepared and used in this study.

Biosystems peptide synthesizer type 430B in the same manner as previously described (4). Crude peptide preparations were purified extensively by reverse-phase high performance liquid chromatography (HPLC) on a preparative TSK-GEL ODS-120T column (2.1×30 cm) using 0.01 N HCl/CH₃CN as eluent. High purity of each of the products was proved by amino acid analysis of acid hydrolysate and aminopeptidase-M digest of each product and analytical HPLC on a TSK-GEL ODS-120T column (0.46×25 cm) with 0.01 N HCl/CH₃CN and 0.1% TFA/CH₃CN as eluents. Details of the syntheses of the peptides and their physico-chemical characterization will be described elsewhere.

Antisera

Antisera were raised against the five synthetic HIRP peptide fragments in rabbits. Three animals were used for immunization with each peptide. Synthetic peptide (0.5–1.0 mg/rabbit) was stirred in 50% polyvinylpyrrolidone (PVP) in saline (0.5 ml) at room temperature for 2 hr, which was then emulsified with complete Freund's adjuvant (0.5 ml). The emulsion was injected intracutaneously into the rabbit. Injection was repeated every two weeks using half of the amount of the immunogen used for the initial injection. Ten days after the 3rd–6th immunization when sufficiently high titer was detected, the blood was collected, from which serum was obtained in the usual manner. The antisera used in the present study include anti-HIRP(9–25) serum HIR1-1, anti-HIRP(30–61) serum HIR2-2, anti-HIRP(48–77) serum HIR3-2, anti-HIRP(736–760) serum HIR31-1 and anti-HIRP(1139–1171) serum HIR25-1.

Radioimmunoassay

Using the anti-HIRP(30–61) serum HIR 2-2 and anti-HIRP(1139–1171) serum HIR25-1, region-specific radioimmunoassays were developed, respectively. The tracers used for the assays were prepared by radioiodinating the synthetic peptides HIRP(30–61) and (1139–1171), respectively, according to chloramine T method followed by purification by gel filtration on Sephadex G10 column. The assays were performed by double antibody method as described for PHI radioimmunoassay (7). The minimum detectable doses of the two assay systems were both 5 fmol.

Gel filtration

Human placental membrane preparation was examined by gel filtration and

radioimmunoassays. Solubilized human placental membrane was prepared according to the method described by Fujita-Yamaguchi *et al.* (2). The solubilized preparation was fractionated by gel filtration on a Sepharose 6B column (1.2×100 cm) with 50 mM Tris-HCl buffer /150 mM NaCl 1/0.1% Triton X-100/0.1 mM phenylmethylsulfonyl fluoride (pH 7.4) as eluent. Each fraction (2 ml) was assayed by the two region-specific radioimmunoassays for HIRP described above. Insulin-binding activity was also assayed with each fraction according to the method of Fujita-Yamaguchi *et al.* (2).

Immunohistochemistry

Human placenta in the 6th month of gestation and needle-biopsied human liver tissue (diagnosed as normal) from a 32-year-old male patient were used for immunostaining with anti-HIRP fragment sera HIR1-1, HIR2-2, HIR3-2, HIR31-1 and HIR25-1. Formalin-fixed cryostat sections of the placental tissue and formalin-fixed paraffin embedded sections of the hepatic tissue were immunostained by the five anti-HIRP fragment sera according to peroxidase-antiperoxidase method and according to peroxidase-labeled antibody method, respectively. The anti-HIRP fragment sera were used at a dilution of 1 : 3000 for the placental tissue and 1 : 200 for the hepatic tissue.

RESULTS AND DISCUSSION

Synthetic peptides HIRP (9-25), (30-61) and (48-77) correspond to extremely N-terminal regions of the α -subunit of HIRP and HIRP(736-760) to the most N-terminal region of the β -subunit. These regions are supposed to be in the extracellular structure of the receptor. On the other hand, HIRP(1139-1171) corresponds to a middle portion of the β -subunit which is one of the predicted intracellular functional regions, i.e. a kinase activity domain. These synthetic peptides consist of 17-33 amino acid residues and were sufficiently immunogenic by themselves; antisera of titers high enough to be used for either radioimmunoassay or immunohistochemistry could be elicited against the peptides in rabbits. We purposely used these synthetic peptides as immunogens without conjugating with macromolecules to preserve their intact chemical structures. This procedure for antiserum production has been well established in our laboratory (6).

On gel filtration of solubilized human placental membrane preparation, radioimmunoassays with use of anti-HIRP(30-61) serum HIR2-2 and anti-HIRP (1139-1171) serum HIR25-1 both revealed a main peak of immunoreactivity, which eluted in a position corresponding to a molecular size of approximately 270 K daltons. This immunoreactive peak was overlapped with one of the two peak components of insulin-binding activity. The other peak component of insulin-binding activity was in a molecular size of approximately 850 k daltons and showed very low immunoreactivity when measured by the HIR2-2 and HIR25-1 radioimmunoassays. It may be presumed that the smaller-size component represents a monomeric form of combined α - and β -subunits of HIRP and the larger-size component a tetramerized form of the monomer (3). The (31-60) and (1139-1171) sequence regions in the tetrameric form of $\alpha\beta$ -monomer may hardly be recognized by either antiserum HIR2-2 or antiserum HIR25-1 conceivably because of steric hindrance in the aggregated form or marked alteration of the steric conformations of

FIGS. 2a, b. Immunostaining of human placenta (a) with anti-HIRP (48-77) serum HIR3-2 ($\times 3000$) and (b) with anti-HIRP (1139-1171) serum HIR25-1 ($\times 3000$).

Fig. 2a. $\times 700$, Fig. 2b. $\times 450$

the peptide regions in the large molecule from those of synthetic peptides used as antigens.

The five anti-synthetic peptide sera successfully stained both human placental and hepatic tissues. In the placenta, Hofbauer cells in the chorionic villi were stained with any of the five antisera used; particularly strong immunostaining was observed around the cell membranes (Figs. 2a, b). In the liver, on the other hand, strongly and slightly stained hepatocytes were intermingled; especially sinusoidal sites of the hepatocytes were strongly stained (Figs. 3a, b). It showed small granular

FIG. 3. Immunostaining of human liver (a) with anti-HIRP (9-25) serum HIR1-1 ($\times 200$) and (b) with anti-HIRP (736-760) serum HIR31-1 ($\times 200$). $\times 750$

staining pattern and sinusoidal endothelia, Kupffer's stellate cells and fat storing cells were negative. The positive staining of the hepatocytes was completely abolished when the HIRP antisera were preincubated with the respective antigen peptides. The immunostaining of Hofbauer cells in the placenta, on the other hand, could hardly be abolished by preincubation of the antisera with the synthetic antigens. However, the present anti-synthetic HIRP fragment sera which independently recognize distantly-located regions in the HIRP could stained the same Hofbauer cells and they reacted most strongly with the plasma membrane components of the cells. In addition, any of our antisera which had been generated against various peptide hormones and neuropeptides failed to stain Hofbauer cells in the placenta. It seems therefore unlikely that the immunostaining of the placenta-specific cells with the present anti-synthetic HIRP fragment sera resulted from non-specific binding of the antisera. An attempt is now being made to solve the problem.

In the present study, we succeeded for the first time in detecting solubilized HIRP by radioimmunoassays and also in demonstrating by immunohistochemistry HIRP or the related protein in Hofbauer cells in human placenta as well as in human hepatocytes with use of region-specific antisera raised against synthetic HIRP-related peptides. The present results of radioimmunoassay and immunohistochemistry strongly support the validity and usefulness of the region-specific anti-synthetic HIRP fragment sera in studies on the molecular basis of insulin receptor.

Advances in gene technology have made it possible to predict amino acid sequences of complex biomolecules including biosynthetic precursors of biologically active peptides and even receptor proteins. These informations by themselves,

however, do not provide us sufficient definite clues for our understanding of the functional features of such proteins in physiological as well as pathophysiological states. Our immunochemical manipulations with use of anti-synthetic peptide sera will be a method of choice for studies on the functions and molecular mechanisms of not only insulin receptor but also other functional proteins which amino acid sequences have been predicted.

ACKNOWLEDGEMENTS

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.-H., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A. and Rutter, W. J.: The human insulin receptor cDNA: the structural basis for hormone-activated transmembrane signalling. *Cell* 40; 747-758, 1985.
2. Fujita-Yamaguchi, Y., Choi, S., Sakamoto, Y. and Itakura, K.: Purification of insulin receptor with full binding activity. *J. Biol. Chem.* 258; 5045-5049, 1983.
3. Ginsberg, B. H., Kahn, C. R., Roth, J. and De Meyts, P.: Insulin-induced dissociation of its receptor into subunits. *Biochem. Biophys. Res. Commun.* 73; 1068-1074, 1976.
4. Iguchi, K., Mochizuki, T., Inoue, T., Yanaihara, C., Naruse, S., Nokihara, K., Forssmann, W. G., Mutt, V., Kanno, T. and Yanaihara, N.: Synthesis of human secretin and comparison of biological activities of synthetic human, porcine and chicken secretin in the rat and dog. In *Peptide Chemistry 1985*, ed. by Y. Kiso, Protein Research Foundation, Osaka, 1986, p. 191-196.
5. 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.: Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature* 313; 756-761, 1985.
6. Yanaihara, N., Sakagami, M., Sato, H., Yamamoto, K., Hashimoto, T., Yanaihara, C., Ito, Z., Yamaguchi, K. and Abe, K.: Immunological aspects of secretin, substance P, and VIP. *Gastroenterology* 72; 803-810, 1977.
7. Yanaihara, N., Yanaihara, C., Nokihara, K., Iguchi, K., Fukata, S., Tanaka, M., Yamamoto, Y. and Mochizuki, T.: Immunochemical study on PHI/PHM with use of synthetic peptides. *Peptides* 5; 247-254, 1984.