Vol. 195, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1993 Pages 237-241

NOVEL AFFINITY SUPPORT PREPARED BY CROSSLINKING PURIFIED INSULIN RECEPTORS TO AGAROSE [1]

Erik Helmerhorst [2] and Anne-Marie Nakhoul

School of Biomedical Sciences
Curtin University of Technology
GPO Box U1987,Perth WA 6001,AUSTRALIA

Received July 9, 1993

The insulin receptor interacts with other membrane proteins and possibly also a cytoplasmic second messenger(s) of insulin action. As an approach to identify and purify these ligands of the insulin receptor, we have developed an insulin receptor affinity support which retains the properties of the membrane associated insulin receptors. Insulin receptors first were purified from rat liver by affinity chromatography on wheat germ lectin agarose and insulin agarose. The purified insulin receptors then were crosslinked to agarose. The agarose immobilised insulin receptors had an affinity for insulin indistinguishable from the native receptors. In addition, insulin enhanced the autophosphorylation of the β-subunit of these agarose immobilised insulin receptors. The insulin binding and autophosphorylation activities were stable for at least one week when this novel affinity support was stored at 4°C.

The insulin receptor is a specific transmembrane glycoprotein of about 450 kDa that is minimally composed of two extracellularly located α-subunits of 130 kDa disulfide-linked to two transmembrane β-subunits of 90 kDa (1). Insulin binds to the α-subunits of the insulin receptor (2) promoting the autophosphorylation of the β-subunits (3). This leads to enhanced tyrosine kinase activity of β-subunit that is believed to be critical in promoting the intracellular effects of insulin (4). The physiological substrate(s) of the insulin receptor tyrosine kinase is unknown but various cytoplasmic proteins have been implicated (5, 6). There also is abundant evidence that the insulin receptor interacts with various membrane-associated proteins including G-proteins (7) and proteins that modulate the receptors affinity for insulin (8-12). However, the isolation and purification of these proteins that interact with the insulin receptor has proved elusive. We have prepared an insulin receptor affinity matrix that should prove useful for this purpose.

METHODS

Purification of Insulin Receptor Rat livers (20g) trimmed of excess fat were finely minced and then homogenised on ice in ten times their wet weight in 10mM Tris-HCl, pH 7.4

[1] This research has been supported by grants from the Australian Research Council (A08931905) and the Alphapharm / Diabetes Australia Research Trust.

[2] To whom correspondence should be addressed.

0006-291X/93 $4.00

containing 0.25M sucrose, 5mM ethylenediaminetetraacetate, 10mM benzamidine-HCl, 0.1mM phenylmethylsulfonylfluoride, 1mM bacitracin, 10mM aminohexanoate, 1mg/ml leupeptin and 1mg/ml pepstatin A. Triton X-100 (to a final concentration of 1%) was added to the homogenate which was then stirred for 2 hours at 4°C. This solution was centrifuged at 300, 000 x g for 50 min at 4°C. The supernatant was supplemented with 0.09% magnesium chloride and mixed gently overnight at 4°C with 5 ml wheat germ lectin pre equilibrated in 0.05mM Tris-HCl, pH 7.8 containing 0.15M sodium chloride, 0.1mM phenylmethylsulphonylflouride and 0.1% Triton X-100. The gel slurry was poured into a glass column and washed with 100 volumes of the buffer used to pre equilibrate the wheat germ lectin. Glycoproteins were eluted with this buffer containing 0.3M N-acetyl-D-glucosamine. The eluted glycoprotein fraction then was gently mixed overnight at 4°C with 2.5 ml of an insulin-agarose affinity support (prepared as described below) pre equilibrated in 0.05M Tris-HCl, pH 7.8 containing 0.1% Triton X-100 and 0.1mM phenylmethylsulphonylfluoride. The gel slurry then was poured into a glass column and washed with 100 volumes of this buffer containing 1M sodium chloride. This gel preparation was eluted (1 ml fractions) at room temperature with 0.05M sodium acetate, pH 5.0 containing 0.1% Triton X-100, 1M sodium chloride and 0.1mM phenylmethylsulphonylfluoride into test tubes containing 0.1 ml 1M Hepes, pH 7.8 containing 0.1% Triton X-100, 0.1mM phenylmethylsulphonylfluoride and 20mM ethyleneglycoltetra-acetate.

Crosslinking Insulin to Agarose Affigel 10 (25ml) was washed with ice cold high-pure water and then with 75ml of 0.1M sodium phosphate, pH7.4 containing 6M urea at 4°C. The gel was mixed gently overnight with 25ml of a 1mg/ml solution of insulin in the same buffer. Unreacted active esters were blocked by the addition of 0.1ml 1M ethanolamine-HCl (pH 8.0) per ml of gel. Following 1 hour, the gel was transferred to a glass column and washed with 200 volumes of 0.05M Tris-HCl, pH 7.8 containing 0.1% Triton X-100, 1M sodium chloride and 0.1mM phenylmethylsulphonylfluoride.

Crosslinking Purified Insulin Receptors to Agarose A purified solution of insulin receptors (50µg protein in 1ml) was gently mixed for 4 hours at 4°C with 1 ml of affigel-10 pre equilibrated in 0.1M Hepes, pH 8.0. Unreacted ester groups then were blocked by the addition of 0.1ml of 1M ethanolamine, pH 8.0. After a 1 hour incubation at 4°C, the agarose support was washed with 100 volumes of 0.05M Tris-HCl, pH 7.8 containing 0.1% Triton X-100, 1M sodium chloride and 0.1mM phenylmethylsulphonylfluoride.

Insulin Binding Studies Samples containing soluble insulin receptors or insulin receptors immobilised on agarose (20 µl beads) were incubated with gentle mixing overnight at 4°C in 0.2 ml of 50 mM Tris-HCl, pH 7.5 containing 0.1% bovine serum albumin, 100 U/ml bacitracin, 0.1% Triton X-100, 8 fmoles [[^{125}I]Tyr^{A14}]insulin and a mM-pM range of unlabelled insulin. Following this incubation, soluble receptors were separated from free insulin by the addition of 50 µl of 0.4% bovine gamma globulin and 250 µl of 20% polyethylene glycol in ice cold 50 mM Tris-HCl, pH 7.5. This solution was vigorously vortex-mixed and incubated on ice for 15 min before centrifuging at 8500 x g, 4°C for 15 min. The supernatant was aspirated and the radioactivity of the pellet was monitored. Insulin receptors crosslinked to agarose were separated from free insulin following the overnight incubation by underlaying the incubation mixture with 1ml of ice cold 40% sucrose and centrifuging at 12, 000 x g for 1 min. The supernatant was aspirated and the radioactivity of the washed beads were monitored.

Analysis of Binding Data Binding data were analysed by the method of Scatchard (1949) using the `Ligand` program (Munson & Rodbard, 1980). The data were fitted with either a one site model or a two site model. The goodness of fit of a model was analysed with the `Ligand` program using the `Runs test` (Bennett & Franklin, 1954). This test predicts whether the scatter of points about a fit is likely due to chance and, therefore, whether a given model provides a significant fit to the data. Whether or not a two site model provided a statistically better fit over a one site model was tested with the `Ligand` program using an F-test criterion on the residual variances of the two models. Statistical comparisons between curves within a given model were also tested using the `Ligand` program. Non-specific binding was handled as a computer fitted parameter and all points within an analysis were weighted equally.

Autophosphorylation of Insulin Receptors Insulin receptors (40µl soluble preparation or immobilised receptors) were incubated for 15 min at 23°C in the presence or absence of 5µl of

1μM insulin in 50mM Hepes, pH 7.4 containing 0.15M sodium chloride, 10mM sodium orthovanadate, 1mM phenylmethylsulphonylfluoride, 100mM magnesium chloride, 20mM manganese chloride and 1% Triton X-100. Then 0.2MBq [γ-^{32}P]ATP (4 kBq/pmole) was added and the incubation continued for an additional 25 min at 23°C. Following the completion of this incubation an equal volume of 125mM Tris-HCl, pH 6.8 containing 6% sodium dodecyl sulphate, 20% glycerol and 200mM dithiothreitol was added and this solution was boiled for 3 min. The samples were centrifuged at 10, 000 x g to remove any precipitate or to sediment the agarose beads before resolving the proteins on a 7.5% polyacrylamide gel.

RESULTS

Insulin receptors were purified about 1500 fold from rat liver by chromatography firstly on wheat germ lectin agarose and then on an insulin agarose column. The purified insulin receptors then were crosslinked to an agarose support.

Competitive displacement and Scatchard plots of the equilibrium binding of insulin at 4°C to purified insulin receptors before and after crosslinking them to agarose are illustrated in Figure 1. A model assuming a single class of homogeneous binding sites provided a significant fit (P>0.05; Runs test of Bennett and Franklin,) to each plot. More complex models did not improve the fit. The affinities of the native (Kd = 1.3 ± 0.5 nM) and immobilised (Kd = 1.5 ± 0.2 nM) insulin receptors were indistinguishable since a Scatchard plot of the binding of insulin to the native receptor could be significantly fitted using the affinity constant derived from a Scatchard plot of the binding of insulin to the immobilised receptors and visa versa. The receptor numbers determined from the Scatchard plots were used to estimate that about 25 % of the purified insulin receptors were successfully crosslinked to the agarose support. The insulin binding activity of the immobilised receptors was stable for several weeks when this novel affinity support was stored at 4°C.

Insulin receptor preparations were incubated for 20 min in buffer containing [γ-^{32}P]ATP in the presence or absence 1 mM insulin either before or after crosslinking them to agarose. Each preparation was solubilised under reducing conditions and the proteins were resolved by

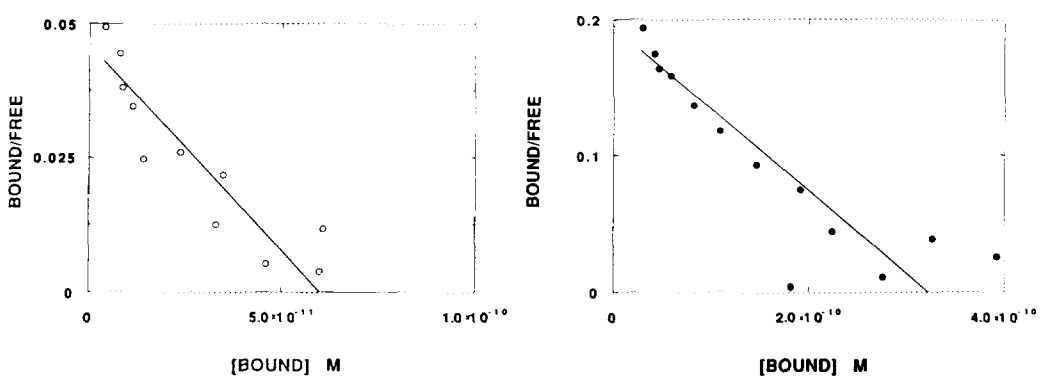

FIGURE 1. Scatchard plots of insulin binding to insulin receptors: Insulin receptors were purified from rat livers by wheat germ lectin and insulin agarose chromatography. Insulin binding to the purified insulin receptors was evaluated using Scatchard analysis before (•) or after (o) crosslinking them to an agarose support.

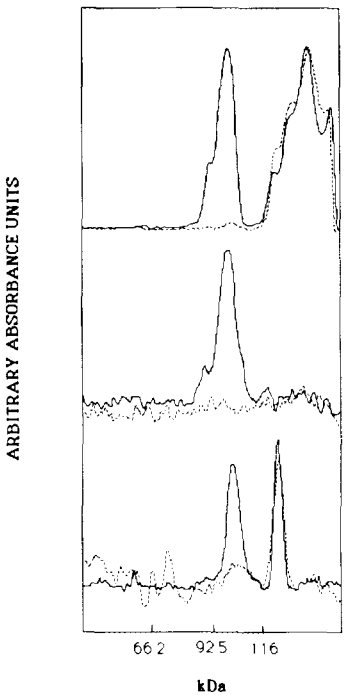

FIGURE 2. Autophosphorylation of insulin receptors: Proteins purified first on wheat germ lectin (upper caption) and then on insulin agarose (middle caption) were crosslinked to an agarose support (lower caption) and incubated with [γ-^{32}P]ATP in the presence (solid lines) or absence (dotted lines) of 125 nM insulin. The proteins were solubilised under reducing conditions and then resolved on a 7.5% polyacrylamide gel. A densitometric scan of the autoradiograph of this gel is illustrated.

polyacrylamide gel electrophoresis (Figure 2). Several protein bands were radiolabelled with phosphate in the wheat germ purified extract (Figure 2, upper caption), however only the phosphorylation of a 95 kDa band was specifically promoted by insulin. Insulin also specifically promoted the phosphorylation of a 95 kDa band in preparations that were further purified by chromatography on insulin agarose (Figure 2, middle caption) or following immobilisation of the insulin-agarose purified preparation on an agarose support (Figure 2, lower caption). The phosphorylation of a 120 kDa band in the immobilised insulin receptor preparation was independent of insulin and seemed to be related to the solubilisation of the agarose support. The kinase activity of the immobilised receptors declined with storage at 4°C but the support could be stored for up to one week at 4°C.

DISCUSSION

There is a substantial body of evidence that a number of membrane and cytoplasmic proteins interact with the insulin receptor (5-12). However, to date isolation and

characterisation of these proteins has proved elusive. Affinity chromatography is a powerful approach to protein purification. Thus we developed an insulin receptor affinity support as an approach to isolating and characterising ligands of the insulin receptor.

Insulin receptors purified from rat livers were crosslinked to Affigel 10. This support was chosen because of the simple and specific chemistry involved. An N-hydroxysuccinimide ester is attached to a 10 atom spacer arm to distance the attached ligand from the agarose matrix making it less susceptible to steric hindrance. Primary amino groups of the ligand react specifically with the ester to form a stable amide bond with the agarose support. The immobilised insulin receptors were covalently attached through linkage to either their α or β subunits since autophosphorylated β-subunits were liberated into solution following reduction and the remaining receptor-agarose support also was radioactive.

The insulin receptor affinity support developed in this study retained the functional properties of the native insulin receptor molecules. Insulin bound to both the soluble and immobilised insulin receptors in a reversible manner and with high affinity. The kinetics of binding at 4°C were consistent with the presence of a single class of homogeneous binding sites, as reported in several other studies (8, 13-15), both before and following immobilisation of the receptors on the agarose support. Secondly, the immobilised insulin receptors retained their ability to bind ATP and to autophosphorylate the receptors β subunits. We now plan to use a transfected cell line to express mg quantities of insulin receptors for the preparation of a high capacity, insulin receptor affinity support.

REFERENCES

1 Helmerhorst, E., Ng, D.S., Moule, M.L., and Yip, C.C. (1986) Biochemistry 25, 2060-2065.
2 Yip, C.C., Yeung, C.W.T., & Moule, M.L. (1978) *J. Biol. Chem.* 253, 1743-1745.
3 Avruch, J., Nemenoff, R.A., Blackshear, P.J., Pierce, M.W., and Osathanondh, R. (1982) J. Biol. Chem. 257, 15162-15166.
4 Chou, C., Dull,T., Russell, D.S., Gherzi, R., Lebwohl, D., Ullrich, A., and Rosen, O.M. (1987). J. Biol. Chem. 262, 1842-1847.
5 Roth, R. A., Zhang, B., Chin, J. E., and Kovacina, K. (1992) J. Cellular Biochem. 48, 12-18.
6 Rothenberg, P. L., Lane, W. S., Karasik, A., Backer, J., White, M., and Kahn, C. R. (1991). J. Biol. Chem. 266, 8302-8311.
7 Mortensen, E. R., Drachman, J., and Guidotti, G. (1992) Biochem.J. 281, 735-743.
8 Helmerhorst, E., and Yip, C. C. (1993). Biochemistry 32, 2356-2362.
9 Harmon, J., Hedo, J., and Kahn, C. R. (1983) J. Biol. Chem. 258, 6875-6881.
10 Baldini, G., Hohman, R., Charron, M.J., & Lodish, H.F. (1991) J. Biol. Chem. 266, 4037-4040.
11 Ciaraldi, T. P. & Maisel, A. (1989) Biochem. J. 264, 389-396.
12 Davis, H.W., & McDonald, J.M. (1990) Biochem. J. 270, 401-407.
13 Lipkin, E.W., Teller,D.C., and de Haën, C. (1986) J. Biol. Chem. 261, 1694-1701.
14 Kohanski, R.A., and Lane, M.D.(1985) J. Biol. Chem. 260, 5014-5025.
15 Helmerhorst, E. (1987) Biochem. Biophys. Res. Commun. 147, 399-407.