Vol. 116, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 31, 1983 Pages 417-422

INSULIN-STIMULATED PHOSPHORYLATION OF THE INSULIN RECEPTOR PRECURSOR

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Received September 6, 1983

SUMMARY. The α and β subunits of the insulin receptor, M_r=135K and 95K, appear to be synthesized via a single polypeptide precursor of M_r=190K. We have investigated whether insulin stimulates the phosphorylation of this proreceptor, as is the case with mature receptor. Rat liver endoplasmic reticulum membranes were solubilized in Triton X-100 and chromatographed sequentially on wheat-germ agglutinin-agarose and lentil lectin-agarose columns. Phosphorylation of the lentil eluate with [γ^{32}P]ATP revealed an insulin-stimulated phosphoprotein of M_r=192K, which was recognized by anti-receptor antibody, compatible with the receptor precursor. This suggests that further processing of the M_r=190K insulin receptor precursor is not necessary for insulin binding, kinase activation, and receptor phosphorylation.

The plasma membrane insulin receptor consists of M_r=135K (α) and 95K (β) subunits linked by disulfide bonds (1-4). The 135K subunit is generally believed to contain the insulin binding site. Recent evidence suggests that the 95K subunit contains a tyrosine kinase which is insulin-stimulated and phosphorylates the receptor as well as exogenous substrates (5-10).

Other recent studies have elucidated the insulin receptor's biosynthetic pathway (11-13). After translation and core glycosylation in the rough endoplasmic reticulum, the receptor precursor is a M_r~190K single polypeptide chain, which contains exclusively high mannose-type carbohydrate chains (12). This precursor is converted to the mature insulin receptor by proteolytic cleavage and carbohydrate maturation to complex type.

In terms of receptor kinase activity, the 190K precursor is analogous to a proenzyme, many of which require proteolytic cleavage for activation (14). In addition, the role of carbohydrate maturation in receptor kinase activity is unknown. To address these issues, we asked, does the insulin receptor precursor contain a functional kinase, capable of insulin stimulation and receptor phosphorylation?

0006-291X/83 $1.50

MATERIALS AND METHODS

Membrane Preparations. Liver rough and smooth microsomes were prepared from 200gm Sprague-Dawley rats according to the method of Adelman, et al. (15). The two final microsomal fractions were removed from the sucrose density step gradient separately, diluted in two volumes of 50 mM HEPES (pH 7.6), and recollected by centrifugation at 43,000 x g for 60 min at 4°C. The pellets were resuspended in the same buffer at a protein concentration of approximately 20 mg/ml and frozen at -70°C until further use. Previous marker enzyme studies on these microsomes by one of us (16) revealed appropriate recovery and enhancement of the endoplasmic reticulum enzyme glucose-6-phosphatase, as well as minor contamination with plasma membrane, mostly in the smooth microsomes.

Solubilization and Lectin Purification. Microsomes were solubilized with Triton X-100 and purified sequentially over wheat-germ agglutinin-agarose (Miles Laboratories, Elkhart, IN) and lentil lectin-agarose (P.L. Biochemicals, Milwaukee, WI) columns as previously described (17).

Phosphorylation of Lectin Purified Fractions. Phosphorylation of the lectin column eluates was performed by minor modifications of previously described techniques for the plasma membrane insulin receptor (8). Peak eluate fractions (containing 10-40 µg protein) were incubated for one hour at 4°C with or without 1×10^{-7} M porcine insulin in 300 µl solutions composed of 60 mM HEPES (pH 7.6), 100 mM NaCl, 0.07% Triton X-100, 0.01% bovine serum albumin, and 0.2 M eluting sugar (all final concentrations). Phosphorylation was initiated by the addition of [γ^{32}P]ATP (New England Nuclear, Boston, MA), CTP, and Mn acetate to final concentrations of 5 µM, 1 mM and 3 mM respectively. Final ATP specific activity was $\sim$ 5 µCi/nmole. After 15 min at 4°C, the reaction was terminated by addition of one-third volume of "stopping solution" (8).

Immunoprecipitation and Analysis. Phosphorylated lectin eluates were then subjected to immunoprecipitation with anti-receptor antisera from patient B-d or control sera (8). The immunoprecipitates were analyzed by SDS-polyacrylamide electrophoresis on 7.5% resolving gels (18). Dried gels were autoradiographed at -70°C using Kodak X-Omat AR film and lightning-plus intensifying screens (Dupont, Wilmington, DE). M_r determinations were by comparison to Bio-Rad high molecular weight protein standards (Richmond, CA).

RESULTS

To test for insulin-stimulated phosphorylation of the M_r=190K insulin receptor precursor, a preparation relatively enriched in this component and depleted of mature plasma membrane receptor was required. Since the precursor is an intracellular protein (11,12) associated with endoplasmic reticulum (19), our starting material was rough and smooth microsomes from rat liver. To separate the precursor form from the mature insulin receptors we exploited the differences in carbohydrate composition of these components (12) using sequential chromatography on immobilized lectins. The microsomal membranes were solubilized in Triton X-100 and applied first to wheat-germ agglutinin-agarose (high affinity for sialic acid), to bind receptors with mature carbohydrates. The flow-through from this column, which should be relatively depleted in mature insulin receptor but still contain high mannose forms, was then adsorbed to

and eluted from lentil lectin-agarose (high affinity for mannose), to further purify the immature receptor species.

Wheat-germ and lentil column eluates were then tested for insulin-stimulated receptor phosphorylation by incubation with [γ^{32}P]ATP and subsequent analysis by precipitation with anti-receptor antibodies, electrophoresis, and autoradiography. Figure 1 shows a representative experiment with eluates derived from rough microsomes. In wheat-germ eluates (left), insulin stimulated the phosphorylation of one major protein recognized by the anti-receptor antibody. This M_r=95K phosphoprotein most likely represents the mature β subunit of the insulin receptor, present in the rough microsomal fraction as a result of contamination with plasma membrane. Analysis by densitometry revealed a 5.4-fold stimulation of ^{32}P incorporation into the 95K band by insulin. No higher molecular weight phosphoproteins compatible with insulin receptor precursors were observed in the wheat-germ eluates.

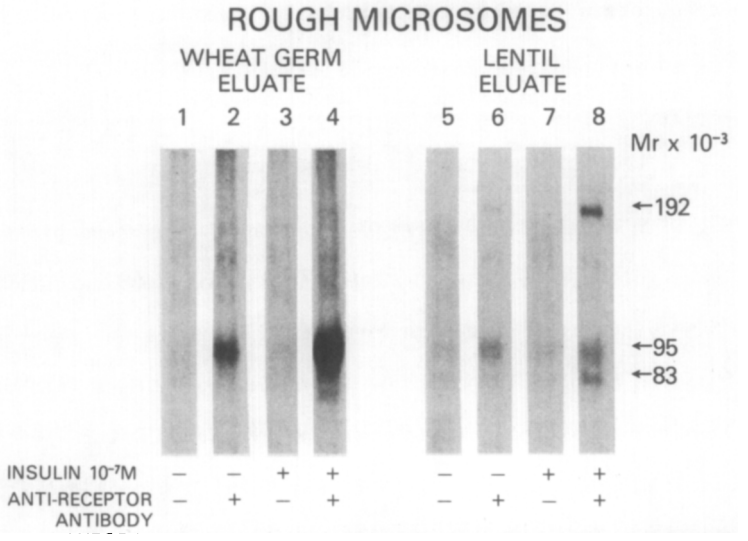

Figure 1. Insulin-stimulated phosphorylation of insulin receptors from rat liver endoplasmic reticulum. Rough microsomal membranes were prepared from rat liver by density gradient centrifugation, solubilized in Triton X-100, and chromatographed sequentially on immobilized wheat-germ and lentil lectins. Wheat-germ (lanes 1-4) and lentil (lanes 5-8) eluates were incubated in the presence or absence of 10^{-7} M pork insulin as indicated. Following phosphorylation with [γ^{32}P]ATP, the insulin receptors were immunoprecipitated with control sera (odd lanes) or anti-receptor sera (even lanes), and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Similar experiments were performed with lectin purified fractions from smooth microsomes, revealing a similar pattern of M_r=95K and lower phosphoproteins, but none of higher molecular weight compatible with the receptor precursor (data not shown).

In contrast, phosphorylation of the lentil column eluate derived from rough microsomes (Figure 1, right) showed a predominant insulin-stimulated phosphoprotein at M_r=192K which was immunoprecipitated by anti-receptor antibody, compatible with the high mannose precursor of the insulin receptor. Of note is the narrowness of this band, which suggests relative carbohydrate homogeneity. This band was also visible in eluate incubated without insulin (lane 6), indicating that basal phosphorylation of the M_r=192K protein occurs. By densitometry, insulin stimulated 192K phosphorylation by 7.3-fold, comparable to that seen with the 95K protein in wheat-germ eluates.

Two other specifically immunoprecipitated phosphoproteins, at M_r=83K and 95K, are noted in the lentil eluates. Insulin stimulated phosphorylation of the 83K band 2.7-fold. This phosphoprotein may represent β subunit which has not yet undergone full processing from immature to complex carbohydrate, as observed in studies of insulin receptor biosynthesis (12,13). The identity of the 95K phosphoprotein is unclear. This band showed minimal insulin stimulation (1.2-fold), making the presence of mature β subunit in the lentil eluate unlikely.

DISCUSSION

Starting with a relatively pure preparation of rough endoplasmic reticulum from rat liver, followed by sequential lectin affinity chromatography purification, we have visualized a protein of M_r=192K which is phosphorylated in an insulin-stimulated manner. By multiple criteria, we believe this protein is the high mannose form of the insulin receptor precursor. Its electrophoretic mobility coincides with that of the precursor labeled with radioactive sugars or amino acids (12). The 192K phosphoprotein is visualized only in the preparation derived from rough endoplasmic reticulum. It is not retained by immobilized wheat-germ agglutinin, as receptor with mature carbohydrate would be, but it is adsorbed to and eluted from immobilized lentil, which together suggests the presence of only high mannose type oligosaccharides. Finally, it is recognized by anti-receptor antibody. Thus, we conclude that the insulin receptor, after translation and core glycosylation in the rough endoplasmic

reticulum, and before further processing, is capable of undergoing insulin stimulated phosphorylation.

Such phosphorylation may have resulted from two different mechanisms. First, the M_r=190K precursor may be capable of binding insulin, as suggested by studies using ^{125}I-insulin crosslinking in IM-9 cells (12,13) and adipose cells.[1] After insulin binding, the precursor may activate the receptor kinase and serve as substrate. This would suggest that further processing of the receptor precursor by cleavage and carbohydrate maturation is not necessary qualitatively for these functions, i.e. to couple binding to kinase activation or to uncover substrate domains.

Alternatively, it is possible that the insulin receptor precursor only served as a substrate for the receptor kinase of more mature receptors also present in the lentil column eluates. However, we feel this possibility is unlikely, judging from the fold stimulation of the 192K band compared to the 95K and 83K bands in the lentil eluate. Other investigations have also suggested that receptor phosphorylation is an intramolecular event, i.e. the kinase of one receptor phosphorylates itself but not other receptors (20,21).

If the insulin receptor is functional both as a kinase and substrate in its initial form in the endoplasmic reticulum, the possibility arises for some function of the kinase at this early stage in the receptor life cycle. For example, the kinase activity or its product (phosphorylated receptor) might be a signal utilized during cell processing after initial translation. Mannose-6-phosphate appears to be a sorting signal for delivery of enzymes to lysosomes (22); perhaps phosphotyrosine subserves a similar sorting function. Only further experimentation can resolve this speculation.

Finally, our experimental protocol yields a preparation enriched in insulin receptor precursor and depleted of mature receptors. This preparation may prove useful in further characterization of the structure and function of the insulin receptor precursor.

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1. Hedo, J.A. and Simpson, I.A., unpublished observations.

ACKNOWLEDGEMENTS

We thank S.I. Taylor for helpful discussion and review of the manuscript, P. Pilch and C.R. Kahn for providing data prior to publication, and C. Culwell for secretarial assistance.

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