Vol. 160, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1989 Pages 296-302

EVIDENCE FOR EXOGENOUS SUBSTRATE PHOSPHORYLATION AND DIMER FORMATION BY THE ACTIVATED 190 kDa INSULIN PRORECEPTOR *IN VITRO*

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Received February 10, 1989

SUMMARY The insulin receptor is synthesized as a single chain, 190 kDa glycoprotein precursor, which undergoes proteolytic cleavage, carbohydrate processing, and fatty acylation to generate the mature receptor on the plasma membrane. The relationship of these post-translational modifications to the acquisition of receptor function, i.e. ligand binding and phosphokinase activity, is not fully understood. Therefore, the 190 kDa proreceptor and mature receptor kinase activities were separately examined *in vitro*, and their phosphorylation properties compared. The solubilized receptor precursor from IM-9 lymphocytes was purified by sequential lectin chromatography and, following site specific anti-receptor antibody immunoprecipitation, phosphokinase studies performed. The isolated proreceptor was activated by insulin and phosphorylated exogenous substrate α-casein, as similarly observed for the mature receptor. Structurally, the phosphorylated proreceptor was identified as a 360 kDa homodimer under non-reducing condition.

The plasma membrane insulin receptor consist of α (135 kDa) and β (95 kDa) subunits in an $\alpha_2\beta_2$ heterotetrameric configuration [1-4]. Insulin binds to the extracellular α subunit and initiates an intramolecular event resulting in the autophosphorylation of the β subunit and tyrosine phosphokinase activation [5-7]. The receptor is synthesized as a single chain, 190 kDa precursor which undergo post-translational modification that include proteolytic cleavage, processing of high-mannose oligosaccharide chains to complex-type chains, and fatty acylation [8-12]. The relationship of each post-translational modification to receptor function is not fully understood. Processing of the N-linked oligosaccharides plays an important role in

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0006-291X/89 $1.50

the eventual translocation of the receptor to the plasma membrane [13,14]. In addition, N-linked glycosylation and proteolytic cleavage appears necessary to produce conformational changes for ligand binding [10,15,16,17]. To further define the role of proreceptor processing toward the acquisition of receptor function, we characterize phosphokinase activity of the 190 kDa proreceptor. We find the insulin receptor precursor to possess functional features similar to the fully processed mature receptor, i.e. insulin-stimulated endogenous and exogenous phosphorylation and early dimer formation.

MATERIALS AND METHODS

Cell culture and lectin Chromatography: IM-9 lymphocytes were grown in RPMI 1640 with 25 mM Hepes and 10% fetal bovine serum. Approximately 10^9 cells in suspension were harvested at stationary phase and solubilized with 1% (v/v) Triton-X 100 in 0.15 M NaCl, 50 mM Hepes buffer, pH 7.6 plus phenylmethylsulfonyl fluoride (2 mM) and aprotinin (1.5 trypsin inhibitor units/ml). The solubilized extract was passed several times over a lentil agarose column and extract flow through then placed over a Wheat Germ Agglutinin (WGA) agarose column [18]. The proreceptor fraction was obtained from the lentil agarose column by first eluting with eluant buffer [0.1 % (v/v) Triton-X 100 in 0.15 M NaCl, 50 mM Hepes, pH 7.6] containing 0.3 M methyl-α-D-mannopyranoside, followed by passing the eluate over a second WGA column. The flow through from this step contained the purified insulin proreceptor (lentil-WGA fraction). The mature receptor fraction was obtained from the first WGA column, which received the lentil agarose flow through, by eluting with 0.3 M GlcNac in eluant buffer (WGA fraction). The protein concentration for the WGA fraction containing the mature receptor was 1-2 mg/ml. The proreceptor or lentil agarose eluate-WGA flow through fraction concentration was 0.025-0.030 mg protein per ml.

Immunoprecipitation and Phosphorylation: Lectin purified fractions containing the proreceptor (1 ml/sample) and mature receptor (0.04 ml/sample) were incubated with site specific anti-receptor antibody, rAb-50 (1:100 dilution). This rabbit polyclonal antibody is directed to the carboxy terminal 21 amino acids of the β subunit and, similar to other antibodies, does not interfere with receptor phosphokinase activity [19,20]. The immune complexes were then adsorbed to protein A-Sepharose and resuspended in 50 mM Hepes, 0.1 % Triton, and 0.1 mg/ml bovine serum albumin (BSA) buffer, pH 7.6. For endogenous phosphorylation, the samples were pre-incubated with and without insulin for 1 h at 4°C and reaction solution [final concentration, [^{32}P]ATP (approximately 30 μCi/nmole), CTP (1 mM), unlabeled ATP (50 mM), $Mn(CH_3COO)_2$ (10 mM)] was added to initiate phosphorylation [21]. For exogenous phosphorylation, the samples were pre-incubated with insulin and α-casein (final concentration, 1 mg/ml) for 30 minutes at 22°C followed by the addition of reaction solution plus $MgCl_2$ (2 mM) [22]. Phosphorylation was terminated after 20 minutes at 22°C with stopping solution [0.4% (v/v) Triton-X 100, 200 mM Na Fluoride, 40 mM NaPyrophosphate, 20 mM EDTA, 40 mM NaPhosphate, 40 mM ATP, and 40 mM Hepes, pH 7.6] plus 5X sample buffer. The phosphorylated receptor components and α-casein were identified on SDS-polyacrylamide gel electrophoresis and autoradiography as described below.

Endoglycosidase H Treatment: Enzymatic digestion with endoglycosidase H (endo-β-N-acetylglucosaminidase, EC 3.2.1.96) was performed as previously described [23]. After immunoprecipitation and insulin-stimulated autophosphorylation, the

immunoadsorbed receptor components were released by boiling in 1% SDS, 10 mM dithiothreitol (DTT), and 10 mM phosphate buffer, pH 7.0. Samples were then diluted 1:4 in 0. 3 M citrate buffer, pH 5.0, and incubated with endoglycosidase H (final concentration of 0.2 units/ml) for 6 h at 37°C. The reaction was terminated with the addition of 5X sample buffer.

SDS-Polyacrylamide Gel Electrophoresis: The labeled receptors were released from immunoadsorbent by boiling in sample buffer (2 % SDS, 100 mM DTT, 0.002 % bromphenol blue, 10 % glycerol, and 10 mM phosphate buffer). The receptor components were separated by one dimensional SDS-polyacrylamide gel electrophoresis, according to the method of Laemmli [24]. For investigation of the proreceptor under non-reducing condidtion, DTT was eliminated from the sample buffer and 6 % SDS-polyacrylamide gel was used. α-Casein substrate phosphorylation was examined on a 10 % SDS-polyacrylamide gel and all other studies were analyzed using a 7.5 % gel. Autoradiography was performed at -70°C using Kodak XAR-5 film with Cronex Lightning Plus intensifying screen.

RESULTS

The insulin proreceptor from rat liver microsomes and monensin-treated 3T3-L1 adipocytes was demonstrated to exhibit ligand-stimulated autophosphorylation *in vitro* [15,25,26]. In this study, sequential lectin agarose columns and site specific anti-receptor antibody immunoprecipitation were used to isolate and enrich the low abundant proreceptor from IM-9 lymphocytes. In a purified lentil-WGA fraction incubated with [^{32}P]ATP, a single 190 kDa band was demonstrated (Figure 1, left). The intensity of this band increased in the presence of 10^{-7} M insulin. Under these conditions, phosphorylation of the 95 kDa β subunit band was not observed. In contrast, when the immunoprecipitated WGA eluate was incubated with [^{32}P]ATP, the 95 kDa β subunit was phosphorylated and the intensity of labeling increased 2-3 fold in the presence of insulin (10^{-7} M). Under similar conditions, a 210 kDa band was also phosphorylated in the WGA fraction. To further substantiate the 190 kDa band as the phosphorylated proreceptor with exclusively high-mannose oligosaccharide moieties, the [^{32}P] labeled lentil-WGA fraction was processed for treatment with endoglycosidase H. Following enzymatic digestion, the 190 kDa band shifted completely to 170 kDa (Figure 1, right). This characteristic change in electrophoretic mobility after removal of susceptable N-linked oligosaccharides identifies the insulin proreceptor [8,13].

Structurally, the mature insulin receptor resides in the plasma membrane as an $\alpha_2\beta_2$ heterotetramer [3,4]. Recently, analysis by binding to insulin affinity column identified the proreceptor to dimerize early in biogenesis [17]. Thus, we next wished to determine the state of intermolecular disulphide linkage of the phosphorylated proreceptor under non-reducing condition (Figure 2). Two distinct bands, approximately 380 and 340 kDa, were observed in the WGA eluate under non-reducing condition. The 95 kDa band was also noted, but the intensity of the label was reduced when compared to gels containing DTT. Only a 360 kDa band was observed

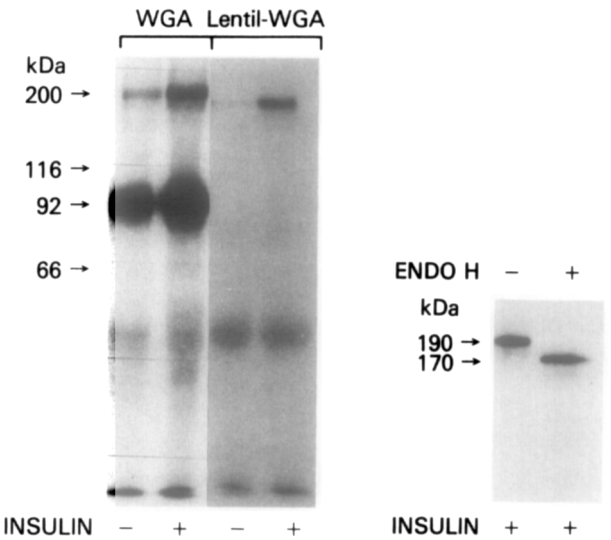

Figure 1. (Left) Autophosphorylation of immunoprecipitated insulin receptor from WGA and lentil-WGA fractions. Purified receptors (approximately 25-30 μg) contained in WGA and lentil-WGA fractions were immunoprecipitated with anti-receptor antibody, rAb-50. The immune complexes were then adsorbed to protein A-Sepharose, resuspended in 0.1 % Triton, 50 mM Hepes buffer with 0.1 mg/ml BSA, and incubated in the presence or absence of 10^{-7} M insulin. Phosphorylation reaction was then performed as described under "Method". The immune complexes were released by boiling in sample buffer and receptor components identified by SDS-polyacrylamide gel electrophoresis and autoradiography. (Right) Endoglycosidase H digestion of immunoprecipitated and autophosphorylated insulin proreceptor from lentil-WGA fraction. The phosphorylated proreceptor in lentil-WGA fraction was recovered by boiling in 1 % SDS, 100 mM DTT, 10 mM phosphate buffer, pH 7.0, and endoglycosidase H digestion was performed in 0.3 M citrate buffer, pH 5.5.

in the immunoprecipitated lentil-WGA fraction under non-reducing condition. This 360 kDa component probably represents the homodimer of the proreceptor. Thus, proreceptor phosphorylation occurs in the setting of established intermolecular disulphide linkage, which appears to be an early step in receptor biogenesis.

Given the association of the proreceptor to form a dimeric configuration similar to the $\alpha_2\beta_2$ tetramer of the mature receptor, we next investigated the ability of the insulin receptor precursor to act as a tyrosine kinase toward an exogenous substrate. In the WGA eluate incubated with α-casein, the addition of [^{32}P]ATP resulted in a marked increase in the labeling of both the 95 kDa (β subunit) and the 34 kDa (α-casein) bands with 10^{-7} M insulin stimulation (Figure 3). Phosphotransferase reaction in the buffer control produced no appreciable difference in the labeling of the 34 kDa bands, with or without insulin. When the lentil-WGA fraction with substrate was incubated with [^{32}P]ATP, the 190 kDa and 34 kDa bands were observed to increase in intensity with insulin treatment. Thus, the incorporation of labeled phosphate into α-casein occured in the presence of proreceptor phosphokinase activity. However, only at high ligand concentration (10^{-7} and 10^{-6} M insulin) was proreceptor and substrate

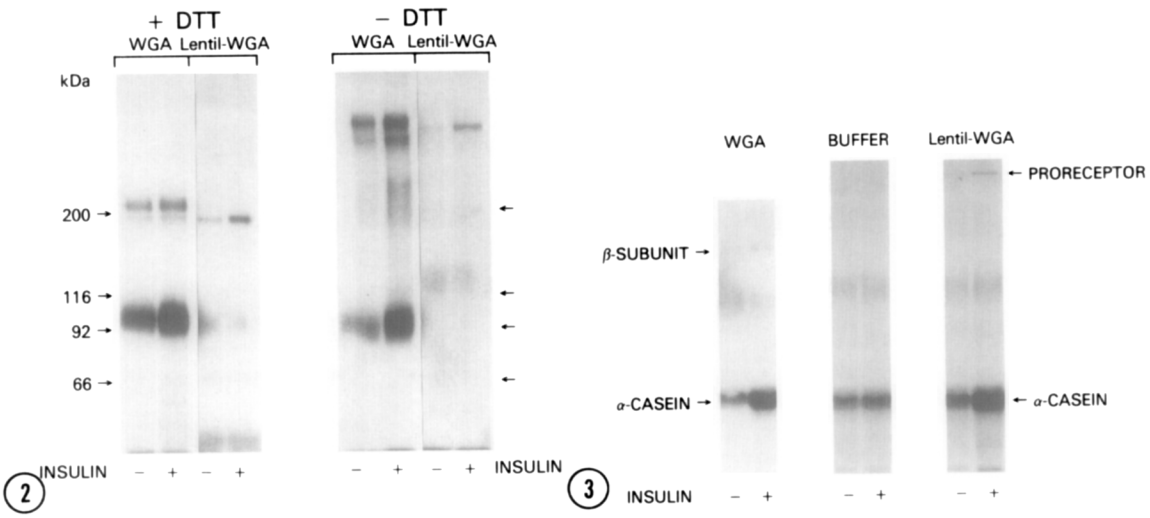

Figure 2. Phosphorylated insulin receptor from WGA and lentil-WGA fractions examined under reducing and non-reducing conditions. Purified receptor components from WGA and lentil-WGA fractions were processed as described for Figure 1. Phosphorylated proreceptor and β subunit of the mature receptor were released by boiling in sample buffer with and without 100 mM DTT and analyzed by SDS-polyacrylamide gel (6 %) electrophoresis and autoradiography.

Figure 3. α-Casein phosphorylation by immunoprecipitated insulin receptor from WGA and lentil-WGA fractions. Partially purified receptor in lectin eluates were immunoprecipitated with site specific anti-receptor antibody, rAb-50. The immune complexes were adsorbed to protein A-Sepharose and then resuspended in 0.1 % Triton, 50 mM Hepes, 0.1 mg/ml BSA buffer containing α-casein (1 mg/ml), in the presence or absence of insulin (WGA, 10^{-7} M; lentil-WGA, 10^{-6} M) for 1 h. The phosphorylation reaction was initiated with [^{32}P]ATP (30 μCi/nmole) and terminated with the addition of stopping solution and 5X sample buffer. The receptor components and α-casein were identified by SDS-polyacrylamide gel (10%) electrophoresis and autoradiography (slightly longer exposure for buffer control and lentil-WGA fractions).

phosphorylation demonstrable. PolyGT ($GluNa^{80}Tyr^{20}$) substrate phosphorylation by the immunoprecipitated lentil-WGA fraction showed little measurable phosphate incorporation and could not be used to produce meaningful kinase profiles.

DISCUSSION

The recent report of a patient with insulin resistance and abnormal proreceptor processing underscores the significance of receptor post-translational modification [27-30]. To further understand the association of the structural changes during biogenesis and the acquisition of receptor activity, we investigated *in vitro*, phosphorylation of the 190 kDa proreceptor and compared findings to the fully processed mature receptor.

The receptor precursor was purified from solubilized IM-9 cell extracts by sequential lectin affinity chromatography. The reduced concentration of the proreceptor after elution necessitated an immunoprecipitation step prior to

examination for phosphokinase activity. The lentil-WGA fraction was found to be free of the mature receptor and the identified insulin-stimulated 190 kDa band was demonstrated to possess the characteristic shift of the insulin proreceptor after endoglycosidase H digestion. Furthermore, there was a ligand-stimulated increase in [^{32}P]-labeling of α-casein when incubated with lentil-WGA fraction, clearly demonstrating phosphotransferase activity of the proreceptor. Thus, the insulin proreceptor has acquired the ability to phosphorylate exogenous substrate once activated by insulin. We observed the receptor precursor in a homodimeric configuration (approximately 360 kDa) in a non-reduced, SDS-polyacrylamide gel. The intermolecular disulphide linkage between two single chain proreceptors occurs early in biosynthesis and may be required for kinase activity or possibly post-translational processing and routing to appropriate intracellular locations.

In the insulin resistant patient with abnormal proreceptor processing, endogenous phosphorylation of the uncleaved 210 kDa insulin receptor was demonstrated [30]. However, high concentration of insulin was required to obtain a maximal response, similar to that observed in this study for the 190 kDa proreceptor. The altered insulin-stimulated phosphorylation response of the abnormal uncleaved mature receptor and the proreceptor probably result from reduced insulin binding, a consequence of the absence of proteolytic cleavage. This is supported by studies of the insulin resistant patient where mild trypsinization of cells produced distinct receptor subunits and corrected the reduced ligand binding to cells [27,28].

In conclusion, we find the insulin proreceptor to exhibit endogenous and exogenous phosphokinase activity and to associate in a dimeric configuration, features similar to the mature receptor. Thus, the insulin receptor is functional early in biogenesis and the post-translational modifications appear to be important in conferring to the receptor ligand sensitivity.

Acknowledgment: We wish to thank Dr. Jose A. Hedo for his helpful comments and discussion during the course of this study.

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