The in vitro synthesized and processed human insulin receptor precursor binds insulin

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Received 12 May 1999

Abstract The cell-free examination of the human insulin receptor during biogenesis may provide a greater understanding of the elements that contribute to the acquisition of receptor function. The insulin receptor precursor components were produced in a cell-free system and the insulin binding ability of the [^{35}S]methionine-labeled translation products was determined. The processed proreceptor represented by a 190 kDa band was retained on insulin-linked biotin-streptavidin agarose or an insulin column. The insulin binding 190 kDa band migrated slower than the non-binding 190 kDa band on SDS-PAGE which suggests that covalent modifications account for these differences. The trypsin-digested product of the 190 kDa proreceptor was also retained on insulin-linked biotin-streptavidin agarose, however the α-subunit precursor was retained on insulin agarose to a much lesser degree. We conclude that a significant fraction of the processed, in vitro translated insulin proreceptor acquires insulin binding ability.

Key words: Insulin proreceptor; Ligand binding; Biosynthetic processing; Reticulocyte lysate

1. Introduction

The human insulin receptor (hINSR) is present on the cell surface in an $\alpha_2\beta_2$ heterotetrameric configuration linked by disulfide bonds. This quaternary structure of the fully functional hINSR results from a series of post-translational processing steps which include *N*-linked and *O*-linked glycosylation, dimerization, proteolytic cleavage, and fatty acylation [1,2]. Studies by others have demonstrated that insulin binding induces intramolecular changes that activate the β-subunit of an α-β heterodimer to phosphorylate tyrosine residues in the cytoplasmic domain of the other α-β heterodimer which then results in receptor activation [3]. This intramolecular interaction underscores the importance of post-translational modifications that yield the heterotetrameric configuration of the hINSR on the cell surface. Therefore, studies that examine the receptor in various stages of biogenesis may provide a greater understanding of the elements that contribute to the acquisition of receptor function. However, biosynthetic processing events occur rapidly in a cell, which makes in vivo examination of receptor precursors at different stages of biogenesis difficult. The in vitro translation systems have been used to examine protein folding, subunit assembly and interaction with molecular chaperones [4–6]. In vitro translation of the luciferase protein in reticulocyte lysate results in proper protein folding and associated enzymatic activity [5,7]. The eukaryotic rabbit reticulocyte system has been utilized to examine conformational status of the hINSR precursor components but the functional examination of in vitro synthesized and processed membrane-bound proteins have not been previously reported [8,9]. In this study, we present insulin binding results of receptor precursors synthesized and processed in a cell-free system.

2. Materials and methods

2.1. Materials

The pSP64 plasmid which contains the 4.6 kb hINSR cDNA was a generous gift of Dr. Graeme Bell (Howard Hughes Medical Institute, University of Chicago, IL, USA). The cDNA sequence encoding the α-subunit of the hINSR (sequence 46–2246) was subcloned into a pSP64polyA plasmid using appropriate restriction endonuclease sites, *Avr*II and *Afl*II [10]. Crystalline biosynthetic human insulin was kindly provided by Dr. R. Chance of Eli Lilly Company (Indianapolis, IN, USA). The materials for the coupled transcription and translation reactions, and pancreatic microsomes were purchased from Promega (Madison, WI, USA). Anti-hINSR antibody, a rabbit polyclonal antibody directed at amino acids 441–620 of the β-subunit of the hINSR was obtained from Transduction Laboratories (Lexington, KY, USA) [10]. The materials for lectin chromatography, insulin-linked and monoclonal antibody-immobilized biotin-streptavidin agarose, phenylmethylsulfonyl fluoride (PMSF) and aprotinin were obtained from Sigma (St. Louis, MO, USA). Translation grade [^{35}S]methionine (1250 Ci/mmol), [^{3}H]leucine (60 Ci/mmol) and En[^{3}H]ance were purchased from Dupont NEN (Boston, MA, USA), and the Enhanced Chemiluminescence (ECL) detection system was obtained from Amersham (Arlington Heights, IL, USA). Affi-gel 15 resin and materials for polyacrylamide gel electrophoresis (PAGE) were obtained from Bio-Rad (Richmond, CA, USA). All other chemicals were reagent grade.

2.2. Cell culture

IM-9 lymphocytes (ATCC, Rockville, MD, USA) were grown in suspension using RPMI 1640 medium containing 25 mM HEPES and 10% fetal bovine serum and supplemented with 10 mg/ml L-glutamine. Approximately 10^9 cells were washed several times in PBS, and solubilized with 1% Triton X-100 in 50 mM HEPES, 150 mM NaCl buffer, pH 7.6 containing 2 mM PMSF and 0.5 TIU/ml of aprotinin for 30 min on ice. The solubilized extract which contained mature insulin receptors was subsequently obtained by centrifugation at 16 000 *g* for 30 min at 4°C.

2.3. Preparation of Xenopus egg extract (XEE)

This protocol was approved by the Laboratory of Animal Services of the University of Hawaii-Manoa. Adult female *Xenopus laevis* were obtained from Nasco (Fort Atkinson, WI, USA). The *Xenopus* eggs were obtained from frogs by injection of 100 U of PMSG for oocyte maturation followed by induced ovulation with an injection of 750 U of hCG, 4–5 days following PMSG injection. Eggs were dejellied by incubation for 10–20 min at room temperature in 2% L-cysteine-HCl, pH 7.7 solution, washed with extraction buffer containing 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM sucrose, and 10 mM HEPES-KOH, pH 7.7. About 1 ml of eggs were transferred to a

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PII: S0014-5793(99)00995-3

1.5 ml microcentrifuge tube, and the eggs were covered with Nyosil M20 oil (Nye Lubricants, New Bedford, MA, USA). After a 2 min centrifugation at 2000 rpm, the supernatant buffer and the oil were removed, the eggs crushed and the cytoplasm isolated by centrifugation for 15 min at 10 000 rpm in 4°C. The middle layer extract was collected by side-puncturing the tube with a needle [11]. The extract was supplemented with cytochalasin B (50 mg/l) and aprotinin (76 TIU/l), and centrifuged again for 5 min at 10 000 rpm. The middle layer was again collected and treated with RNase A (0.1 mg/l) for 15 min in 10°C to deplete endogenous mRNA. RNase was then inactivated with RNase inhibitor (500 U/ml) and 1 mM DTT. The extract was then supplemented with calf tRNA (100 μg/l) and glycerol (5%) or sucrose (250 mM) and aliquoted and stored at −70°C for later use.

2.4. In vitro transcription/translation and processing

The hINSR precursor and the α-subunit precursor of the hINSR were synthesized in a cell-free coupled transcription and translation system by adding 0.5 μg of pSP64-hINSR or pSP64-polyAα-subunit plasmid in a reaction that contained rabbit reticulocyte lysate, RNAse inhibitor, methionine-free amino acid mixture, and SP6 RNA polymerase in a final volume of 25 μl according to the manufacturer's instructions [12]. The translation products were radiolabeled with [^{35}S]methionine (20 μCi/reaction) or [^{3}H]leucine (5 μCi/reaction), and post-translational processing was performed with the addition of canine pancreatic microsomal membranes (2 EQ/25 μl reaction) or XEE (1.0–1.5 μl/reaction). The coupled transcription and translation reactions were performed for 1.5 h at 30°C [13].

Trypsin digestion of the in vitro translated precursor was performed with the addition of 5 μg/ml trypsin for 5 min at 4°C. The digestion was terminated with the addition of 0.5 M EDTA and aprotinin (0.5 TIU/ml).

2.5. Lectin and insulin affinity chromatography

Approximately 2 ml of wheat germ agglutinin (WGA) lectin (8 mg) coupled to agarose was packed into a 15 ml column. These columns were prepared by washing with a non-denaturing detergent buffer, buffer II (50 mM HEPES, 150 mM NaCl, 0.1% Triton X-100 buffer, pH 7.6), followed by the addition of 0.01% sodium dodecyl sulfate (SDS) to buffer II (buffer I) [14]. Approximately 2 mg/ml of solubilized IM-9 cell extract containing the mature hINSR was diluted with 10-fold excess of buffer III (10 mM $MgSO_4$ to buffer II) supplemented with PMSF (2 mM) and aprotinin (0.5 TIU/ml) and passed over the WGA lectin column, and the bound material eluted with 2 ml of buffer III containing 0.3 M *N*-acetyl glucosamine.

Human insulin was dissolved in 0.1 M MOPS buffer, pH 7.5 or 6 M urea in 0.1 M Na-phosphate buffer, pH 7.4 in a concentration of 40 mg/ml, and incubated with 2 ml of activated Affi-gel 15 resin for 4 h at 4°C. The efficiency of insulin crosslinking to Affi-gel 15 was approximately 35%. BSA was similarly linked to the Affi-gel 15 resin and served as the non-specific binding control column. Equally divided aliquots of the WGA eluate and the in vitro translation reaction mixture were applied to insulin-linked and BSA-linked columns in separate experiments. Bound products were then eluted with 0.05 M acetic acid, 1.0 M NaCl and 0.1% Triton X-100, pH 5 buffer, and the columns washed with 50 volumes of 0.05 M Tris-HCl, 0.1% Triton X-100, pH 7.4 buffer. A Tris-HCl buffer with 0.1% SDS was then used to remove the remaining bound materials off the columns.

Additionally, the in vitro synthesized, processed, and [^{3}H]leucine-labeled hINSR precursor components were incubated with insulin-linked biotin or non-specific goat, anti-mouse immunoglobulin-linked biotin for 14 h at 4°C. The bound products were then isolated with the addition of streptavidin-linked agarose and the products released with 4× sample buffer (see below).

2.6. SDS-polyacrylamide gel electrophoresis

The final products of in vitro translation and lectin and insulin chromatography were mixed with sample buffer (2% SDS, 10% glycerol, 0.002% bromophenol blue, 0.1 M DTT, and 0.01 M phosphate buffer), boiled for 5 min and the receptor components were separated by SDS-PAGE (7.5%) under reduced condition by the discontinuous method of Laemmli [15]. Following electrophoresis, the radiolabeled bands on gels were identified by autoradiography or fluorography with Fuji Medical X-ray Film. The SDS-PAGE gels which contained non-radiolabeled proteins were processed for immunoblot analysis as described below.

2.7. Protein immunoblot analysis

Following SDS-PAGE, the non-radiolabeled proteins were transferred onto PVDF membranes using a Bio-Rad electroblotter for 45 min at 100 V in transfer buffer [50 mM Tris-HCl, 380 mM glycine, 0.1% SDS, and 20% methanol (v/v)]. The membranes were then incubated with 5% non-fat dry milk in 0.1% Tween 20 and PBS (PBS1T) buffer, pH 7.4 for 1 h at 23°C, followed by incubation with anti-insulin receptor antibody [1:250 dilution (v/v)] in PBS1T buffer with 1% non-fat dry milk for 14 h at 4°C. The membranes were subsequently washed with 0.3% Tween 20, PBS buffer and incubated with horseradish peroxidase-conjugated anti-rabbit goat antibody (1:1000 in PBS1T) for 1 h at 23°C, and the immunocomplexes identified by the ECL detection method.

3. Results and discussion

The acquisition of ligand binding ability of the hINSR during biogenesis occurs by a conformational change within the monomeric proreceptor prior to disulfide-linked dimerization [16]. To assess the post-translational processing events that confer insulin recognition to the single-chain proreceptor, a cell-free system was used to readily produce and identify the precursor components. The insulin-linked columns used to determine insulin binding function of the hINSR precursors were first assessed for retention of mature insulin receptors. IM-9 cells expressing high concentrations of cell surface hINSR were processed by non-denaturing detergent extraction, and the receptors purified with WGA chromatography [17]. The non-radiolabeled, WGA-purified material was applied to the insulin-linked or BSA-linked columns, and the bound material eluted with 0.05 M Na-acetate buffer, pH 5 and 0.1% SDS buffer. The products off the insulin and BSA columns were then processed by SDS-PAGE and the mature receptor identified by immunoblot analysis using an antibody specific for the 95 kDa β-subunit. Elution of WGA-purified receptors off the insulin column using Na-acetate buffer, pH 5.0 readily demonstrated the β-subunit, but the elution was not complete since the 95 kDa band was additionally observed in the detergent buffer eluate (Fig. 1). However, a large fraction of mature receptors was observed in the flow-through fraction of the insulin column. The β-subunit 95 kDa band was only detected in the flow-through fraction of the BSA-linked column, and not in the low pH or SDS eluate fractions. These results validated the insulin column's capacity to specifically retain the hINSR.

To determine the insulin binding ability of the cell-free translated and processed proreceptor, [^{35}S]methionine-labeled translation products were examined for retention on the in-

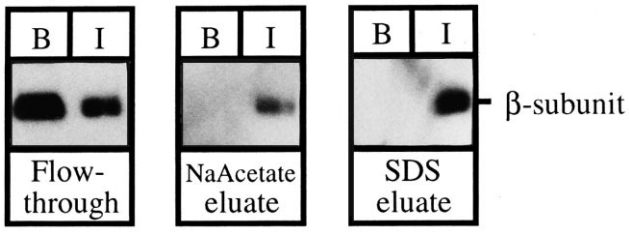

Fig. 1. Insulin column retention of mature hINSR isolated from IM-9 lymphocytes. The WGA-purified hINSR from IM-9 cells was examined for retention on insulin (I) or BSA (B) columns. The flow-through fraction, and both 0.05 M Na-acetate buffer, pH 5 and 0.1% SDS eluate fractions were examined by 6% SDS-PAGE and immunoblot analysis using the anti-hINSR antibody and ECL detection method.

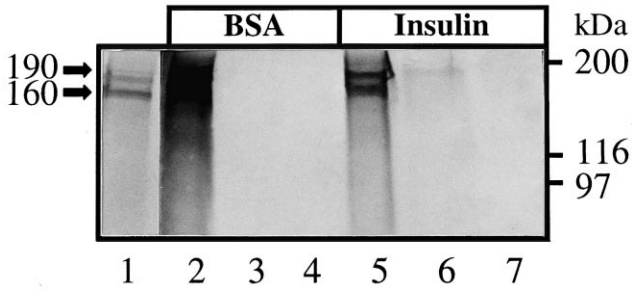

Fig. 2. Insulin affinity column processing of the in vitro synthesized hINSR precursor. The pSP64-hINSR plasmid was transcribed with SP6 RNA polymerase and the mRNA translated in a coupled system with rabbit reticulocyte lysate. The translation products were labeled with [^{35}S]methionine and co-translational processing performed with the addition of canine pancreatic microsomes. Ligand binding ability of proreceptor components was examined by application to insulin- or BSA-linked Affi-gel 15 columns as described in Section 2. The translated products retained on the column were sequentially eluted with 0.05 M Na-acetate buffer, pH 5 and 0.1% SDS buffer. The products in the original translation reaction and the insulin column flow-through, and Na-acetate and SDS eluates were identified by 7.5% SDS-PAGE under reduced condition and autoradiography. Lane 1: original translation reaction; lanes 2 and 5: column flow-through; lanes 3 and 6: Na-acetate eluate; lanes 4 and 7: 0.1% SDS eluate.

sulin column. Equal aliquots of the translation reaction mixture were applied to the insulin-linked or BSA-linked Affi-gel 15 columns, and the retained products were sequentially eluted with Na-acetate buffer, pH 5 and 0.1% SDS buffer. The radiolabeled 190 kDa and 160 kDa bands, which represent the processed proreceptor and the nascent precursor components respectively, were primarily found in the flow-through fraction of both the insulin and BSA columns (Fig. 2, lanes 2 and 5). A 190 kDa band of reduced intensity was the only radiolabeled band observed in the low pH eluate fraction of the insulin column which suggested that a small fraction of the processed 190 kDa component-bound insulin (lanes 3 and 6).

The low yield of the 190 kDa component with in vitro translation and the insulin column retention of the 190 kDa fraction required improved methods to isolate the *N*-linked glycosylated 190 kDa processed proreceptor. We have recently found that translocation and processing of the hINSR precursor was enhanced by the addition of XEE as compared to MM in the RRL translation reactions [12]. The processing efficiency of the combined RRL and XEE reactions was near-complete, allowing for the exclusive production of the 190 kDa precursor component. Furthermore, the limitations of the insulin-linked column method to examine ligand binding capacity of the in vitro translated and processed hINSR precursor was overcome by the use of insulin-linked biotin-streptavidin agarose matrices which allowed longer incubation times. Incubation of combined RRL and XEE synthesized product with insulin-linked biotin resulted in the isolation with streptavidin agarose matrix of approximately 30–40% of the radiolabeled 190 kDa bands of the total reaction mixture (Fig. 3, top panel). Incubation of the reaction mixture with immunoglobulin-linked biotin failed to produce any 190 kDa bands with streptavidin agarose adsorption (Fig. 3, bottom panel). These results confirm the initial observation that the in vitro synthesized and processed hINSR proreceptor bind insulin. The hINSR undergoes proteolytic cleavage during biogenesis to yield the α-β heterodimer. To determine if proteolytic cleavage of the 190 kDa precursor component increased ligand binding ability, the in vitro synthesized product was digested with trypsin and incubated with insulin-linked biotin. Trypsin treatment of the reaction mixture resulted in incomplete digestion of the 190 kDa component and yielded a small fraction of the 120 kDa band which represents the pre-α-subunit (Fig. 3, top panel). Isolation of the insulin-linked biotin-bound fraction identified a weak 120 kDa radiolabeled band which also appeared to migrate slower than the non-insulin-bound 120 kDa band. Due to the inefficiency of tryptic digestion, the percentage of the insulin binding component of the 120 kDa band was difficult to determine. However, the results appeared similar to the 30–40% binding fraction seen with the 190 kDa precursor.

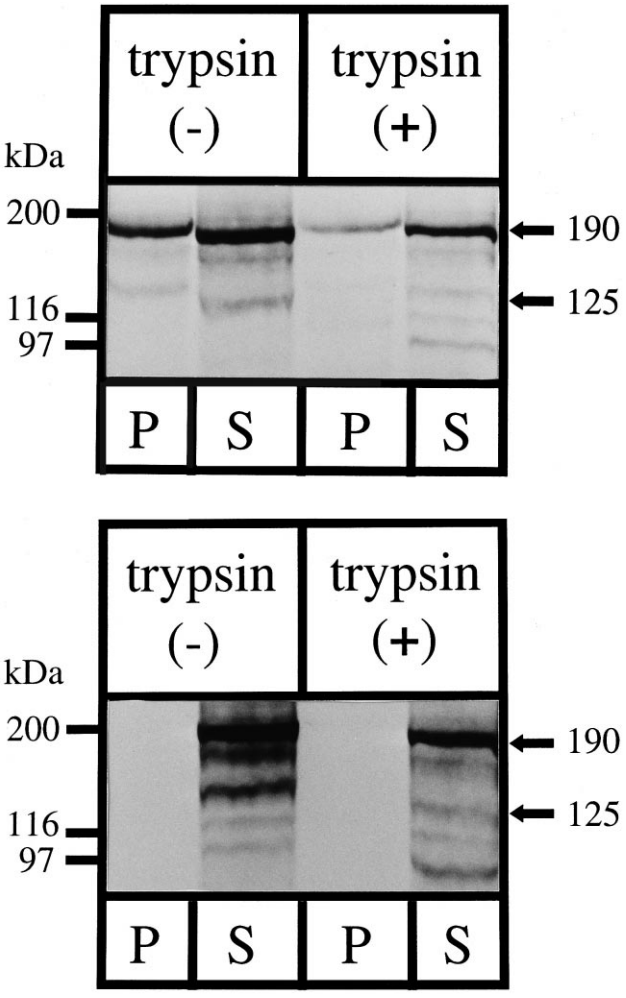

Fig. 3. Insulin binding of the in vitro synthesized and trypsin-digested hINSR precursor. The hINSR precursor components were produced and labeled with [^{3}H]leucine as described in Section 2. Co-translational processing of the hINSR nascent precursor was performed with the addition of XEE. Ligand binding ability of the proreceptor components was examined by application to insulin-linked biotin or immunoglobulin-linked biotin for 14 h at 4°C. The bound products were purified with the addition of streptavidin-linked agarose, and the products released with SDS buffer. The products were identified by 7% SDS-PAGE and fluorography. Trypsin digestion of the hINSR precursor was performed after in vitro translation with 5 μg/ml trypsin for 5 min at 4°C. Top panel: insulin-linked biotin/streptavidin agarose (P) or supernatant (S). Bottom panel: Ig-linked biotin/streptavidin supernatant.

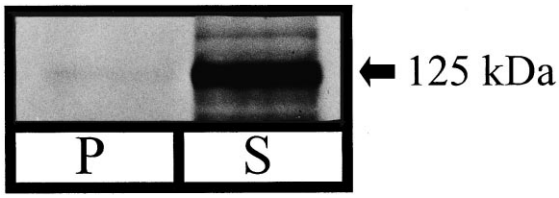

Fig. 4. Insulin binding of the in vitro synthesized α-subunit precursor. The hINSR α-subunit precursor was produced and labeled with [^{3}H]leucine and ligand binding ability of the α-subunit precursor was examined as described in Fig. 3. The products were identified by 7% SDS-PAGE and fluorography [insulin-linked biotin/streptavidin agarose (P) or supernatant (S)].

The fraction of the 190 kDa component that binds insulin appears to migrate slower in SDS-PAGE than the non-binding fraction. This electrophoretic pattern suggests an approximately 5 kDa increase in molecular mass of the ligand binding component which can only be attributed to covalent modifications. Since the SDS-PAGE is performed under reduced and denaturing conditions, disulfide bridging or conformational changes would not account for this slower migrating pattern. The co-translational *N*-linked glycosylation of the hINSR precursor may account for this difference. The addition of core oligosaccharides to the nascent precursor in the in vitro translation reactions is probably an incomplete process. Full glycosylation of the α-subunit may result in conformational changes that allow for the acquisition of ligand binding as well as the differences in molecular mass [18]. Another consideration for this apparent increase in molecular mass is the covalent modification of the hINSR precursor by phosphorylation. The mature hINSR on the cell surface is phosphorylated on serine and threonine residues in the basal state, and autophosphorylated on tyrosine residues with insulin stimulation [1]. Moreover, phosphorylation of proteins account for significant differences in molecular mass as demonstrated for IRS-1 [19]. Ester-linked phosphorylations may occur during biogenesis of the hINSR, accounting for the difference in molecular size. Tyrosine phosphorylation could also occur during biosynthesis and possibly be removed by cytoplasmic phosphatases prior to insertion into the plasma membrane. These possibilities would suggest that phosphorylation plays a role in biogenesis or regulates acquisition of receptor function. Other covalent modifications such as myristylation also need to be considered as well as modifications that have yet to be defined [20].

Combined RRL and XEE synthesis and insulin binding studies of the α-subunit precursor demonstrated results that were quite different than that observed of the 190 kDa proreceptor and the trypsin-digested 120 kDa product (Fig. 4). The 120 kDa α-subunit band showed a markedly reduced insulin binding fraction which was estimated to be less than 10% of the synthesized 120 kDa band. However, the insulin binding fraction showed a similarly slower migration pattern with protein electrophoresis. Thus, the insulin binding component of the hINSR precursor has an increased apparent molecular mass which suggest that the covalent modification accounting for this change allows for the acquisition of ligand binding.

The demonstration of the in vitro translated and processed proreceptor to bind insulin raised the possibility that the tertiary conformational status of the ligand binding precursor component is different from that of the predominant, non-ligand binding component. The hINSR precursor produced in the cell-free rabbit reticulocyte lysate system has been previously identified to be in monomeric conformation [8]. The possibility that the ligand binding component of the 190 kDa hINSR precursor is processed to a quaternary homodimeric form required the examination of the insulin column eluates under non-reducing condition. Radiolabeled bands larger than 200 kDa were not observed when analyzed on a 6% non-reduced gel which suggests the absence of multimeric proreceptor components (data not shown). Thus, the ligand binding 190 kDa component of the cell-free produced hINSR precursor is a monomer.

This study demonstrate that proteolytic cleavage to separate the α-subunit from the β-subunit of the in vitro produced hINSR precursor does not appear to enhance or reduce insulin binding capacity, and production of the insulin binding subunit alone results in an α-subunit fraction with reduced binding capacity. This ligand binding fraction of the hINSR precursor suggests that tertiary changes occur with cell-free translation which allows for proper conformational status for insulin binding. The in vitro methods used in this study do not entirely reproduce cellular processing, but many cytoplasmic and secreted proteins synthesized with this system are completely processed and functionally active. This study utilizing cell-free methods offers an initial attempt at identifying the early stages of functional acquisition of membrane-bound proteins such as the hINSR which undergo extensive post-translational modifications. The acquisition of function of the hINSR occurs with co-translational processing, and optimal or full functional status of the receptor is conferred by extensive post-translational modifications. The identification of mutations of the insulin receptor that alter biosynthetic processing and translocation to the plasma membrane resulting in decreased cell surface receptors further underscore the importance of modifications that yield the heterotetrameric configuration of the hINSR on the cell surface [18,21,22]. The demonstration of ligand binding ability of the monomeric proreceptor elicits consideration of other receptor functions such as ligand-induced dimerization, autophosphorylation, and tyrosine kinase activation which may be acquired with early biosynthetic processing. These functional characteristics can be readily examined with in vitro translation methods, and possibly yield a better understanding of receptor structure changes that result in acquisition of function.

Acknowledgements: Richard Arakaki was a recipient of a KO8 Award from the NIDDK (DK-02120). This project was supported in part by a University Research Council Award. Dean Britos was supported by a grant from the Minority Access to Research Careers Program of the NIGMS to the University of Hawaii-Manoa. The authors wish to thank Drs. Y. Ban and M. Taniyama for their support and encouragement.

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