Biogenesis, Transit, and Functional Properties of the Insulin Proreceptor and Modified Insulin Receptors in 3T3-L1 Adipocytes. Use of Monensin To Probe Proreceptor Cleavage and Generate Altered Receptor Subunits†

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ABSTRACT: The biogenesis, intracellular transport, and functional properties of the insulin proreceptor and modified insulin receptors were studied in hormone-responsive 3T3-L1 adipocytes. After control cells were labeled with [^{35}S]Met for 7 min, the principal polypeptide that was precipitated by anti-insulin receptor antibodies had a molecular weight (M_r) of 180 000. This initial precursor was rapidly converted ($t_{1/2}$ = 35 min) to a 200-kilodalton (kDa) polypeptide, designated the insulin proreceptor, by the apparent posttranslational addition of N-linked, high mannose core oligosaccharide units. Mature α (M_r 130 000) and β (M_r 90 000) subunits were derived from sequences within the proreceptor by proteolytic cleavage and late processing steps, and these subunits appeared on the cell surface 2–3 h after synthesis of the 180-kDa precursor. The cation ionophore monensin was used in combination with metabolic labeling, affinity cross-linking, and external proteolysis to probe aspects of proreceptor function, transit, and the development of insulin sensitivity at the target cell surface. At 5 μg/mL, monensin potently inhibited the proteolytic cleavage step, and the 200-kDa polypeptide accumulated. Lower concentrations of the ionophore selectively blocked late processing steps in 3T3-L1 adipocytes so that apparently smaller α' (M_r 120 000) and β' (M_r 85 000) subunits were produced. Proreceptor and α' and β' subunits were translocated to the cell surface, indicating that the signal for intracellular transit occurs in the 200-kDa polypeptide and is independent of the posttranslational proteolysis and late processing steps. The α' subunit bound insulin both at the surface of intact cells and after solubilization with Triton X-100; the β' subunit was phosphorylated in an insulin-stimulated manner. The detergent-solubilized 200-kDa proreceptor also exhibited both functional properties. However, the proreceptor that was transported to and exposed on the cell surface was incapable of binding insulin in intact adipocytes. Thus, late processing is not essential for the expression of functions associated with mature α and β subunits. In contrast, it appears that the proteolytic generation of subunits is required for the correct orientation of the hormone binding site in the plasma membrane bilayer and the development of insulin responsiveness in 3T3-L1 adipocytes.

The cell surface insulin receptor is thought to be a tetramer composed of two α (M_r 130 000) and two β (M_r 90 000) subunits that are covalently joined by disulfide bonds and further stabilized by noncovalent interactions (Jacobs et al., 1977, 1980; Massague et al., 1980; Harrison & Itin, 1980; Siegel et al., 1981; Van Obberghen et al., 1981). Specific functional properties are associated with the individual subunits: the α subunit contains the hormone binding site (Massague et al., 1980; Siegel et al., 1981; Jacobs et al., 1979; Pilch & Czech, 1980; Wisher et al., 1980; Yeung et al., 1980; Kasuga et al., 1981b) while serine and tyrosine residues in the β subunit serve as phosphate acceptors in insulin-stimulated phosphorylation reactions (Kasuga et al., 1982a,b; Petruzzelli et al., 1982; Avruch et al., 1982). Recent reports suggest that the β subunit exhibits an intrinsic tyrosine protein kinase activity and catalyzes the autophosphorylation of the receptor (Roth & Cassell, 1983; Van Obberghen et al., 1983; Shia et al. 1983; Roth et al. 1983).

Since the α and β subunits of the receptor (a) account for multiple functional activities, (b) are both glycosylated (Hedo et al., 1981), and (c) are degraded at the same rates (Kasuga et al., 1981a) and since (d) there is no evidence suggesting the occurrence of free α or β polypeptides, it is of considerable interest to determine how the biosynthesis, processing, and kinetics of intracellular transit are integrated to produce equimolar amounts of the mature subunits at the plasma membrane. Murine 3T3-L1 cells provide an excellent system for studying the biosynthesis and processing of the insulin receptor in hormone-responsive and/or differentiating cells. After confluent 3T3-L1 preadipocytes are primed with dexamethasone and methylisobutylxanthine, they differentiate into adipocytes that have approximately 200 000 high-affinity binding sites at the cell surface (Rubin et al., 1978; Reed et al., 1977). The adipocytes also become physiologically responsive to low concentrations of insulin (Rubin et al., 1978). Previous preliminary studies (Deutsch et al., 1983) indicated that the biogenesis of the insulin receptor in 3T3-L1 adipocytes is atypical when compared with observations made on other integral plasma membrane proteins and secreted proteins (Lodish et al., 1981; Hubbard & Ivatt, 1981; Rothman, 1981; Strous & Lodish, 1980; Krangel et al., 1979). By employing either affinity cross-linking or metabolic labeling in combination with immunoprecipitation by anti-insulin receptor IgG, we identified two putative, large precursors (M_r 180 000 and 200 000) of the mature insulin receptor subunits and documented that the 200-kilodalton (kDa)[1] protein had an insulin binding site (Deutsch et al., 1983). In earlier studies, Hedo, Kasuga, and colleagues (Hedo et al., 1981; Kasuga et al., 1981a, 1982a) described a glycosylated 210-kDa protein in transformed human IM-9 lymphocytes that was complexed by human anti-insulin receptor antibodies, but the ability of

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[1] Abbreviations: SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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this polypeptide to bind insulin could not be demonstrated. More recent studies by Hedo et al. (1983) and Jacobs et al. (1983) have described an earlier 180–190-kDa precursor of insulin receptor subunits in IM-9 cells that appears to differ significantly (see Results) from the 180-kDa polypeptide that we previously described in 3T3-L1 adipocytes (Deutsch et al., 1983).

In the present report, we have studied the biosynthesis, processing, proteolytic cleavage, and transit of the insulin proreceptor in 3T3-L1 adipocytes. A comparison of our current and previous (Deutsch et al., 1983) results with the work of Jacobs et al. (1983) and the comprehensive studies of Kasuga et al. (1982a) and Hedo et al. (1983) supports and strengthens the generality of the idea that insulin receptor subunits are derived from a common precursor. However, significant differences in the processing of the proreceptor in the hormone-sensitive 3T3-L1 adipocytes were also apparent. In addition, some structural and functional properties of the 200-kDa proreceptor have been established, and we have used various concentrations of the cation ionophore monensin to alter proreceptor cleavage and processing in order to generate cells with abnormally small receptor subunits for functional studies.

Experimental Procedures

Materials. Aprotinin, leupeptin, phenylmethanesulfonyl fluoride, and tunicamycin were purchased from Sigma; α-chymotrypsin was obtained from Worthington; monensin was from Calbiochem; and disuccinimidyl suberate was from Pierce Chemical. L-[^{35}S]Methionine (800 Ci/mmol) and [γ-^{32}P]ATP (300 Ci/mmol) were purchased from Amersham. Anti-insulin receptor IgG (Jacobs et al., 1978) was a generous gift from Dr. Steven Jacobs (Wellcome Research, Research Triangle Park, NC). Purified endoglycosidase H from *Streptomyces plicatus* that was free of contaminating glycosidases was generously provided by Dr. Paul Atkinson (Department of Developmental Biology and Cancer, Albert Einstein College of Medicine). ^{125}I-Insulin was prepared and purified as previously described (Rubin et al., 1978).

Cell Culture and Differentiation of 3T3-L1 Preadipocytes to Adipocytes. 3T3-L1 cells were grown and induced to differentiate as previously described (Rubin et al., 1978).

Metabolic Labeling and Identification of Insulin Receptor Subunits and Precursors. Monolayers of 3T3-L1 adipocytes were rinsed twice with 0.15 M NaCl in 10 mM sodium phosphate, pH 7.4 (buffer A), and then were incubated for various periods of time (see figure legends for details) in methionine-free Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal calf serum and L-[^{35}S]Met at 30 μCi/mL. After the cells were pulse labeled, they were washed twice with buffer A and then were subjected to chase periods of 0–5 h in complete Dulbecco's modified Eagle's medium containing 10% fetal calf serum. When the incubations were terminated, the adipocytes were washed 3 times in buffer A and then were scraped with a rubber policeman into ice-cold 0.1 M Hepes buffer, pH 7.9, containing 0.12 M NaCl, 1.2 mM $MgCl_2$, 2.5 mM KCl, 1 mM EDTA, and 15 mM sodium acetate (buffer B). All further procedures were performed at 4 °C. Cells were disrupted with a Polytron homogenizer, and the homogenate was subjected to centrifugation at 40000*g* for 20 min. The supernatant solution was discarded, and the particulate material from 1.3×10^7 cells was then extracted with 1 mL of 50 mM Hepes buffer, pH 7.6, containing 1% Triton X-100. After centrifugation at 40000*g* for 20 min, the supernatant fraction was partially purified by affinity chromatography on a column of wheat germ agglutinin–agarose as described by Van Obberghen et al. (1981) to lower the background of ^{35}S-labeled proteins. The glycoprotein fraction eluted with 0.3 M *N*-acetylglucosamine and 5 mM EDTA was immunoprecipitated with 1 μL (6 μg) of anti-insulin receptor IgG and 30 μL of protein A bearing *Staphylococcus aureus* suspension (IgG-sorb, The Enzyme Center, Inc.) as indicated by Deutsch et al. (1982). Immunoprecipitated proteins were denatured, reduced, and released from *S. aureus* by boiling for 3 min in 50 μL of 62 mM Tris-HCl buffer, pH 6.7, containing 2% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, and 10% glycerol. Samples were applied to the lanes of a 0.1% SDS–7.5% polyacrylamide gel and were subjected to electrophoresis, staining, destaining, and fluorography as previously described (Deutsch et al., 1982, 1983). The determination of molecular weights of the labeled polypeptides was carried out as previously described (Deutsch et al., 1982). Immunoprecipitated polypeptides from 1.5×10^7 cells were analyzed in each lane unless otherwise noted.

In some experiments, 3T3-L1 adipocytes were treated with chymotrypsin (0.5 mg/mL) according to the procedure of Deutsch et al. (1982) to inactivate cell surface insulin binding sites and/or probe the accessibility of the α and β subunits and insulin receptor precursors at the external surface of the plasma membrane.

In studies on the effects of monensin on insulin receptor biosynthesis, 3T3-L1 adipocytes were preincubated with the indicated concentrations of the ionophore for 1 h and the media used for the subsequent pulse and chase incubations also contained the same concentrations of monensin.

Substitution of affinity chromatography on lentil lectin–agarose for the wheat germ agglutinin–agarose step did not qualitatively or quantitatively alter the results of the metabolic labeling experiments.

Affinity Cross-Linking of Insulin Receptors to Insulin. High-affinity insulin binding sites on the plasma membrane of intact 3T3-L1 adipocytes and in detergent-solubilized insulin receptors and receptor precursors were covalently linked to ^{125}I-insulin by using the bifunctional reagent disuccinimidyl suberate (Pilch & Czech, 1980) as described in our previous studies (Deutsch et al., 1982, 1983). The ^{125}I-labeled polypeptides were immunoprecipitated and analyzed by electrophoresis and autoradiography as previously described (Deutsch et al., 1982, 1983) and outlined above for metabolically labeled insulin receptor subunits and precursors.

Insulin Binding Assays. The binding of ^{125}I-insulin to cell surface receptors on 3T3-L1 adipocytes and to Triton X-100 solubilized insulin receptors and receptor precursors was carried out according to the procedures described by Rubin et al. (1978) and Deutsch et al. (1982), respectively.

Partial Proteolytic Mapping Studies. Insulin receptor subunits were metabolically labeled by subjecting 3T3-L1 adipocytes to a 1-h pulse with [^{35}S]Met and a 4-h chase period to allow sufficient time for the relatively slow conversion of the proreceptor to mature α and β subunits to proceed (see Results). To obtain a sufficient quantity of ^{35}S-labeled, 200-kDa insulin proreceptor for analysis, the adipocytes were first preincubated with 5 μg/mL monensin for 1 h. Subsequently, the cells were subjected to a 1-h pulse with [^{35}S]Met and a 1-h chase period in the presence of the same concentration of the ionophore. A detailed description of the experimental conditions used for pulse–chase analyses is presented above. Insulin receptor subunits and the proreceptor were solubilized with Triton X-100, immunoprecipitated, and subjected to SDS–polyacrylamide gel electrophoresis and autoradiography as described above. The labeled polypeptides

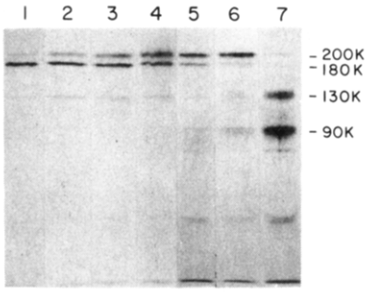

FIGURE 1: Pulse–chase analysis of the synthesis and processing of insulin receptor precursors. 3T3-L1 adipocytes were pulse labeled for 7 min with [^{35}S]Met and then were incubated in complete medium lacking radioactive Met for various periods of time as described under Experimental Procedures. Insulin receptor precursors and/or α and β subunits were solubilized with Triton X-100, immunoprecipitated, and subjected to electrophoresis on a 0.1% SDS–7.5% polyacrylamide gel as indicated under Experimental Procedures. The fluorogram presented was obtained by applying immunoprecipitated polypeptides from 1.5×10^7 cells to each lane after chase periods of 0 (lane 1), 10 (lane 2), 20 (lane 3), 40 (lane 4), 60 (lane 5), 90 (lane 6), and 300 min (lane 7).

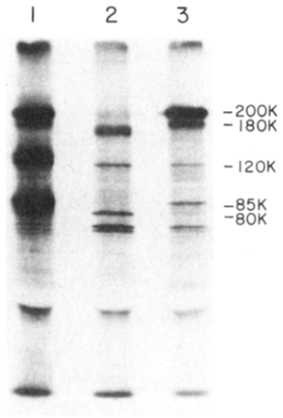

FIGURE 2: Effects of tunicamycin and monensin on the processing of insulin receptor precursors. 3T3-L1 adipocytes were continuously labeled with [^{35}S]Met for 5 h as described under Experimental Procedures. Solubilized insulin receptor subunits and precursors were immunoprecipitated and analyzed as indicated in Figure 1 and under Experimental Procedures. A fluorogram is shown. The sample applied to lane 1 was obtained from control cells; lane 2 received immunoprecipitated polypeptides from cells labeled in the presence of 0.5 μg/mL tunicamycin; lane 3 received immunoprecipitated polypeptides from cells labeled in the presence of 5 μg/mL monensin.

were excised and applied to the lanes of a 0.1% SDS–4% polyacrylamide stacking gel above a 0.1% SDS–12% polyacrylamide running gel. The gel slices were overlayered with 0, 0.2, or 1 μg of α-chymotrypsin, and proteolysis was carried out in the stacking gel according to the method of Cleveland et al. (1977). The patterns of labeled chymotryptic fragments were visualized by fluorography as previously described (Deutsch et al., 1983).

Insulin-Stimulated Phosphorylation of the Insulin Proreceptor and Receptor Subunits. Detergent-solubilized extracts of 3T3-L1 adipocyte membranes were prepared and incubated with [γ-^{32}P]ATP by using the procedure of Petruzzelli et al. (1982). Subsequently, insulin receptors and receptor precursors were immunoprecipitated and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography as described above.

Digestion of Insulin Receptor Subunits and Precursors with Endoglycosidase H. Insulin receptor subunits and precursors were labeled with [^{35}S]Met and immunoprecipitated as described above. The labeled polypeptides were released from the *S. aureus*–IgG complexes by boiling for 5 min in 80 μL of 50 mM sodium citrate buffer, pH 5.5, containing 0.25% SDS. The insoluble *S. aureus* cells were removed by centrifugation at 12000*g* for 2 min, and the supernatant solution was made 0.5 mM in phenylmethanesulfonyl fluoride, 5 μg/mL in aprotinin, and 5 μg/mL in leupeptin by the addition of 20-fold concentrated stock solutions. Each sample was divided in two, and one aliquot received 5 milliunits of endoglycosidase H. All of the samples were then incubated at 37 °C for 45 min. Digestions were terminated by the addition of 10 μL of 0.31 M Tris-HCl, pH 6.7, containing 10% SDS, 5% 2-mercaptoethanol, and 50% glycerol, and the samples were heated at 100 °C for 5 min. SDS–polyacrylamide gel electrophoresis and fluorography were performed as described above.

Results

Pulse–Chase Analysis of the Early Phase of Insulin Receptor Biogenesis. The principal polypeptide that was immunoprecipitated by anti-insulin receptor antibodies after 3T3-L1 adipocytes received a very brief pulse (7 min) of [^{35}S]Met had a molecular weight of 180000[2] (Figure 1, lane 1) after denaturation and reduction of disulfide bonds. The radioactivity in this polypeptide was chased into a 200-kDa protein over a period of 90 min (Figure 1, lanes 2–6). Fifty percent of the initial radioactivity in the 180-kDa protein shifted to a molecular weight of 200000 in 30–40 min in three experiments. In contrast, the mature α and β subunits of the receptor were not observed until much longer periods of time had elapsed (e.g., Figure 1, lane 7, 300 min). When short pulses of [^{35}S]Met were used, the earliest chase time at which the 130- and 90-kDa subunits were detected was 3 h (data not shown). After 1–2 h, precursors of α and β subunits with molecular weights of 120000–125000 and 85000, respectively, were noted (see Figure 4A and lane 3 of Figure 5, below).

Effects of Tunicamycin and Monensin on the Appearance and Processing of the 200-kDa Proreceptor. To determine whether co- and posttranslational glycosylation reactions were involved in the production of the 180- and 200-kDa insulin receptor precursors[3] 3T3-L1 adipocytes were continuously labeled with [^{35}S]Met for 5 h in the presence of either tunicamycin or monensin. Tunicamycin blocks N-linked protein glycosylation by inhibiting the assembly of a lipid-linked precursor oligosaccharide, which is often transferred to nascent membrane and secreted polypeptides during translation (Hubbard & Ivatt, 1981). Monensin interferes with glycoprotein processing in the distal elements of the Golgi apparatus (Tartakoff & Vassalli, 1977, 1978; Tartakoff et al., 1981; Pesonen & Kaariainen, 1982; Ledger et al., 1983). The effects of this Na^+, K^+ ionophore include alterations in the rate or

[2] The molecular weights reported for insulin receptor precursors and subunits are apparent molecular weight values estimated from the mobilities of these polypeptides relative to myosin, RNA polymerase subunits, β-galactosidase, and phosphorylase standards on a 0.1% SDS–7.5% polyacrylamide gel. Since the receptor precursors and α and β subunits are glycoproteins, their relative mobilities may not be closely correlated with the log of their actual molecular weights. A precise determination of these values will require amino acid and oligosaccharide sequence data. While the limitation in assigning a true molecular weight value is recognized, it should also be noted that the apparent molecular weights observed are highly reproducible and comparable to those obtained by other laboratories.

[3] We have designated the 180-kDa polypeptide as the initial precursor and refer to the 200-kDa protein as the insulin proreceptor.

pathway of the intracellular translocation of plasma membrane proteins and the inhibition of the addition of terminal sugars (e.g., galactose, fucose, sialic acid) to complex glycoproteins (Strous & Lodish, 1980; Tartakoff & Vassalli, 1977, 1978; Tartakoff et al., 1981; Pesonen & Kaariainen, 1982; Ledger et al., 1983).

Since the continuous labeling period exceeded the minimum time required for complete processing of the receptor (see above), the two large precursors and the mature α and β subunits were readily observed in control cells (Figure 2, lane 1). When 3T3-L1 adipocytes were treated with tunicamycin, neither the large precursors nor the α and β subunits were detected. The largest polypeptide that was specifically immunoprecipitated had a molecular weight of 170 000. This may be a minimally glycosylated or nonglycosylated form of the insulin proreceptor (Figure 2, lane 2). A portion of this precursor was apparently cleaved into polypeptides with molecular weights of 120 000 and 80 000. In contrast, monensin treatment (5 μg/mL) had no effect on the synthesis and accumulation of the 180- and 200-kDa forms of the proreceptor (Figure 2, lane 3). However, the specific proteolytic cleavage that is necessary for the generation of the α and β subunits normally found in the plasma membrane was highly inhibited by the ionophore in the 5-h experiment. Inhibition was not complete because small amounts of 120- and 85-kDa polypeptides were identified by immunoprecipitation. In these experiments, tunicamycin and monensin inhibited total protein synthesis by only 18% and 7%, respectively. Unlike the larger polypeptides, the polypeptides smaller than 80 kDa were observed in samples immunoprecipitated with nonimmune serum, and they were not studied further.

Effect of Low Concentrations of Monensin on the Cleavage, Late Processing, and Translocation of the 200-kDa Insulin Receptor Precursor and Its Products. High concentrations of monensin alter multiple steps in pathways of protein glycosylation (Strous & Lodish, 1981; Tartakoff & Vassalli, 1977, 1978; Tartakoff et al., 1981; Pesonen & Kaariainen, 1982; Ledger et al., 1983; Johnson & Spear, 1983). If the putative Golgi protease that catalyzes the cleavage of the proreceptor were less sensitive to changes in ionic (or proton) gradients than other enzymes in the pathway, then lower doses of the drug might selectively disrupt other processing steps and provide further insights on the complex maturation of the α and β subunits. This possibility was explored by subjecting 3T3-L1 adipocytes, which were incubated with 0.2–5 μg/mL monensin, to a 1-h pulse with [^{35}S]Met and a 4-h chase period with nonradioactive Met. Labeled subunits and precursors were then solubilized with Triton X-100 and immunoprecipitated with anti-receptor antibodies. Radioactively labeled polypeptides in the immune complexes were resolved and analyzed by SDS–polyacrylamide gel electrophoresis and fluorography.

In control cells, most of the immunoprecipitated ^{35}S radioactivity was found in the mature 130-kDa α and 90-kDa β subunits (Figure 3, lane 5) as anticipated from the results in Figures 1 and 2. Some residual 200-kDa proreceptor was also apparent at the termination of the chase period. At 5 μg/mL, monensin was a potent inhibitor of the posttranslational cleavage of the insulin proreceptor (Figure 3, lane 1), but lower concentrations of the ionophore did not block the conversion of the 200-kDa polypeptide into two smaller subunits (Figure 2, lanes 2–4). The subunits generated in the presence of monensin were apparently smaller than the α and β subunits synthesized in untreated cells (compare lanes 2–4 with lane 5 of Figure 3): the larger and smaller polypeptides,

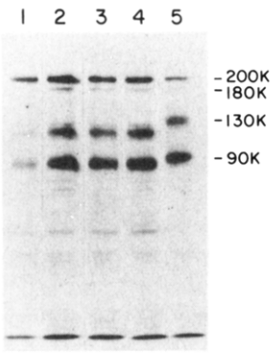

FIGURE 3: Effects of high and low monensin concentrations on the cleavage and processing of 200-kDa proreceptor. 3T3-L1 adipocytes were subjected to a 1-h pulse with [^{35}S]Met and a 4-h chase period as described under Experimental Procedures. Immunoprecipitated insulin receptor subunits and the proreceptor were analyzed as indicated in the legends to Figures 1 and 2 and under Experimental Procedures. A fluorogram is shown. ^{35}S-Labeled polypeptides were isolated from cells exposed to 5 μg/mL monensin (lane 1), 2 μg/mL monensin (lane 2), 0.5 μg/mL monensin (lane 3), 0.2 μg/mL monensin (lane 4), and no monensin (lane 5).

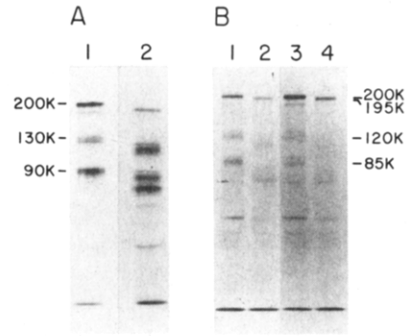

FIGURE 4: Transport of the insulin proreceptor and receptor subunits to the cell surface in the presence and absence of monensin. 3T3-L1 adipocytes were labeled with [^{35}S]Met for 1 h as described under Experimental Procedures. At the end of the labeling period, one set of cells was treated with external chymotrypsin according to the method of Deutsch et al. (1982). Other batches of cells were incubated in standard medium for a 3-h chase period and then were either digested with external chymotrypsin or used as controls. After proteolysis, solubilized insulin receptor polypeptides and the proreceptor were isolated by lectin chromatography and immunoprecipitation and were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography. (A) Immunoprecipitated polypeptides were obtained from control cells immediately either after the 1-h pulse (lane 1) or after a 3-h chase period and chymotrypsinization (lane 2). (B) Immunoprecipitated polypeptides were isolated from 3T3-L1 adipocytes exposed to 0.2 μg/mL monensin (lanes 1 and 2) and 5 μg/mL monensin (lanes 3 and 4). The samples applied to lanes 1 and 3 were prepared from control (unproteolyzed) cells after 1-h labeling and 3-h chase periods; samples applied to lanes 2 and 4 were derived from chymotrypsinized cells which had been incubated for a 3-h chase period after pulse labeling.

designated α' and β', exhibited apparent molecular weights of 120 000 and 85 000, respectively. In contrast, the size of the proreceptor (200 kDa) was identical under all conditions (Figure 3).

The hypothesis that the cleavage of the insulin proreceptor into subunits and/or late processing events involved in the maturation of α and β subunits are necessary signals for accomplishing the export of the insulin receptor from internal membranes to the cell surface was tested. Intact 3T3-L1 adipocytes were treated with external chymotrypsin under conditions that preserve cell integrity, viability, and the capacity for synthesizing and processing macromolecules (Deutsch et al., 1982). Adipocytes that received a 1-h pulse

of [^{35}S]Met contained insulin receptor and subunit precursors that were resistant to the external protease (Figure 4A, lane 1). When an aliquot of the same cells was sequentially subjected to a chase period of 3 h and then external chymotrypsin, the entire populations of α and β subunits were partially degraded, and immunoprecipitable polypeptides with molecular weights of 115 000, 85 000, and 75 000 were observed (Figure 4A, lane 2). The small amount of 200-kDa proreceptor remaining after the chase period was also reproducibly proteolyzed to yield a 195-kDa protein (Figure 4A). Parallel results were found in monensin-treated 3T3-L1 adipocytes, where a much larger portion of the ^{35}S radioactivity appears in the 200-kDa proreceptor (Figure 4B). In cells treated with 0.2 μg/mL monensin, the 200-, 120-, and 85-kDa polypeptides all reached the cell surface within 4 h (Figure 4B, lane 2). When a high monensin concentration (5 μg/mL) was employed, the principal event observed was the conversion of the proreceptor to a 195-kDa form, i.e., the externalization of the proreceptor (Figure 4B, lane 4). The same results were obtained with chymotrypsin treatment at 37 and 4 °C, suggesting that an obligatory role for internalization of the protease is unlikely. The preceding experiments indicate that the translocation of insulin proreceptor and α and β subunits is not dependent on the proteolytic and late processing steps in the pathway of biogenesis.

The 200-kDa Proreceptor Is a High Mannose Glycoprotein. Both the 180-kDa precursor and the 200-kDa proreceptor appear to be N-linked glycoproteins since both forms bind to wheat germ agglutinin–agarose and neither appears in the presence of tunicamycin (Figure 2). Endo-β-*N*-acetylglucosaminidase (endo H) was used as a probe to examine the relationship between these two glycoproteins. Endo H eliminates N-linked oligosaccharide side chains from high mannose glycoproteins by cleaving the bond between the two *N*-acetylglucosamine residues immediately proximal to the modified Asn (Tarentino & Maley, 1974; Robbins et al., 1977). Glycoproteins that contain the further processed, complex type oligosaccharide chains are not substrates for endo H (Tarentino & Maley, 1974; Robbins et al., 1977). Sensitivity to endo H is generally detected by an increase in polypeptide mobility (decrease in molecular weight) on SDS–polyacrylamide gels.

The size of the initial insulin receptor precursor (M_r 180 000) was not altered by endoglycosidase H treatment (Figure 5, lanes 1 and 2), suggesting that only a very limited amount (if any) of N-linked oligosaccharide is added cotranslationally. However, the 200-kDa insulin proreceptor from untreated adipocytes served as a good substrate for endo H, and its molecular weight was reduced to 180 000 (Figure 5, lanes 3 and 4). This indicates that the rapid, posttranslational conversion of the 180-kDa precursor to the 200-kDa proreceptor (Figure 1) is accomplished by the addition of high mannose oligosaccharides. Even when the cells were subjected to an extended chase period to permit the conversion of the bulk of the proreceptor into mature subunits, the residual 200-kDa polypeptide was cleaved by endo H to generate a 180-kDa species (Figure 5, lanes 5 and 6). Similarly, the 200-kDa polypeptide that accumulates in 3T3-L1 adipocytes exposed to low or high concentrations of monensin was also sensitive to endo H treatment, yielding a polypeptide identical in apparent size with the initial insulin receptor precursor (180 kDa) (Figure 5, lanes 1, 2, and 7–10). Thus, the insulin proreceptor, the 200-kDa polypeptide that is inserted into the plasma membrane (see above and Figure 4), and the 200-kDa polypeptide that accumulates in the presence of the ionophore are high mannose glycoproteins that are indistinguishable on the basis of either apparent size or high mannose oligosaccharide content.

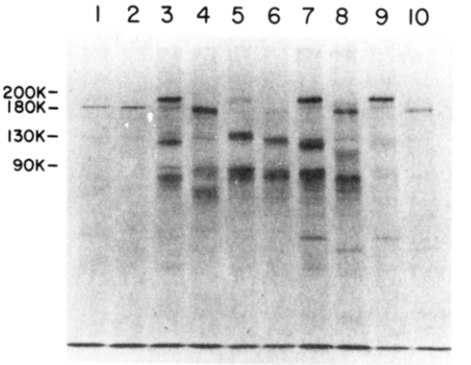

FIGURE 5: Effects of endo H on insulin receptor precursors and subunits. Cells were metabolically labeled with [^{35}S]Met and, in some cases, chased for 4 h to label predominantly one form of the insulin receptor or its precursors. Insulin receptor precursors and subunits were then solubilized and immunoprecipitated as described under Experimental Procedures and Figures 1–4. Antigen–antibody complexes were disrupted by heating at 100 °C in 0.25% SDS for 5 min. Half of each sample was then incubated with 5 milliunits of endo H in 50 mM sodium citrate buffer, pH 5.5 at 37 °C, for 45 min (for details, see Experimental Procedures). After endo H digestion, the samples were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography. Immunoprecipitated polypeptides were isolated from 3T3-L1 adipocytes that were treated as follows: 20-min pulse label (lanes 1 and 2); 60-min pulse label and 60-min chase (lanes 3 and 4); 60-min pulse label and 4-h chase (lanes 5 and 6); 60-min pulse label and a 4-h chase in the presence of 0.2 μg/mL monensin (lanes 7 and 8); 60-min label and a 4-h chase in the presence of 5 μg/mL monensin (lanes 9 and 10). The cells used for lanes 1–6 were not treated with the ionophore. The samples applied to lanes 2, 4, 6, 8, and 10 were digested with endo H; the samples in lanes 1, 3, 5, 7, and 9 did not receive endo H.

The mature subunits derived from the proreceptor in the presence and absence of monensin were also sensitive to endo H digestion. The α subunit (M_r 130 000) from untreated cells had an apparent molecular weight of 125 000 after being exposed to endo H (Figure 5, lane 6), while the apparent molecular weight of α' in ionophore-treated cells declines from 120 000 to 115 000 (Figure 5, lanes 7 and 8) upon enzymic digestion. Parallel, but smaller changes were observed in the β and β' polypeptides. The results in Figure 5 also suggest that α' and β' subunits (see also Figure 3) are produced in ionophore-treated 3T3-L1 adipocytes because monensin inhibits a late processing step(s) that occur(s) after the proteolytic conversion of the proreceptor to two subunit chains.

Although protease inhibitors were routinely included during endo H digestions (See Experimental Procedures), further experiments were performed to ensure that possible contaminating proteolytic enzymes did not contribute to our observations. Cytosol and Triton X-100 solubilized membrane proteins (minus the immunoprecipitated insulin receptor) were prepared from 3T3-L1 adipocytes that were metabolically labeled with [^{35}S]Met, and samples of the two subcellular fractions were digested with endo H under the conditions employed to probe the insulin receptor precursors and subunits (see above). If endo H were contaminated with proteases, then decreases in the sizes of a large number of polypeptides would be expected in both samples. Endo H had no effect on the mobilities of 32 cytosolic proteins that could be resolved by SDS–polyacrylamide gels and elicited a shift in mobility in only 3 out of 35 detectable membrane proteins (data not shown). (Presumably, the three endo H sensitive membrane proteins contain significant amounts of N-linked high mannose oligosaccharide.) These findings strongly suggest that con-

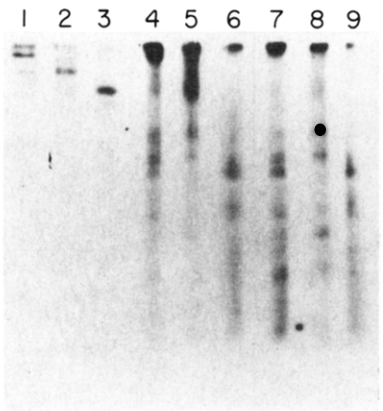

FIGURE 6: Partial proteolytic digests of the insulin proreceptor and the mature α and β subunits. The α and β subunits of the insulin receptor were metabolically labeled by incubating 3T3-L1 adipocytes with [^{35}S]Met for 1 h and then further incubating the cells for a 4-h chase period in the absence of radioactive amino acids to allow processing to occur. Samples derived from cells treated in this manner appear in lanes 2, 5, and 8 (α) and lanes 3, 6, and 9 (β). To label the 200-kDa proreceptor, the cells were subjected to a 1-h pulse with [^{35}S]Met and a 1-h chase period in the presence of 5 μg/mL monensin. Samples derived from these cells were applied to lanes 1, 4, and 7. After labeling and chase periods, the receptor subunits and the proreceptor were solubilized, immunoprecipitated, and purified on a 0.1% SDS–7.5% polyacrylamide gel (see Experimental Procedures). The ^{35}S-labeled polypeptides were identified by autoradiography, excised, and subjected to partial proteolytic digestion in a second gel according to the method of Cleveland et al. (1977) as indicated under Experimental Procedures. Gel slices in lanes 4–6 were overlayered with 0.2 μg of chymotrypsin; gel slices in lanes 7–9 were overlayered with 1 μg of chymotrypsin; gel slices in lanes 1–3 were overlayered with buffer that did not contain the protease. The material at the top of the lanes did not penetrate the running gel. The amount of sample used for lanes 1–3 was 33% of that used in lanes 4–9.

taminating proteases did not contribute to the results shown in Figure 5.

Comparison of the 200-kDa Polypeptide with the α and β Subunits of the Insulin Receptor by Partial Proteolytic Mapping. The relationship among the 200-kDa precursor and the α and β subunits of the insulin receptor was further assessed by partial proteolytic mapping analyses. ^{35}S-Labeled subunits from control 3T3-L1 adipocytes and the ^{35}S-labeled 200-kDa polypeptide from monensin-treated cells were solubilized with Triton X-100 and then isolated and purified by lectin affinity chromatography, immunoprecipitation, and SDS–polyacrylamide gel electrophoresis. Gel slices containing each of the three polypeptides were digested with two concentrations of chymotrypsin as described by Cleveland et al. (1977). The patterns of peptide fragments obtained from the α and β subunits of the receptor were distinct (Figure 6, lanes 5, 6, 8, and 9), and each peptide exhibited a different mobility. Six or seven peptide fragments were derived from the 200-kDa protein (Figure 6, lanes 4 and 7). Each of these peptides appeared to be aligned with a chymotryptic peptide of similar molecular weight obtained from either the α or the β subunit of the receptor (Figure 6, lanes 5, 6, 8, and 9). In addition, only one peptide fragment derived from the subunits did not match with a peptide of similar size in the chymotryptic digest of the 200-kDa protein (Figure 6). These findings are consistent with the idea that the 200-kDa polypeptide contains sequences corresponding to both the α and β subunits.

Functional Properties of the Insulin Proreceptor and the α' and β' Subunits Produced in the Presence of Monensin. To determine whether the proteolytic cleavage and late processing steps are essential for the expression of normal insulin receptor functions, cells containing a homogeneous population of α' and β' receptor subunits, instead of the larger α and β subunits, were produced. 3T3-L1 adipocytes were incubated with 0.2 μg/mL monensin for 4 h to allow newly synthesized normal insulin receptors to translocate to the cell surface. More than 90% of the externalized receptors were than inactivated by chymotrypsin treatment as previously described (Deutsch et al., 1982, 1983). After removal of the protease, the cells were maintained in 0.2 μg/mL monensin to allow the accumulation of the $\alpha'_2\beta'_2$ receptors and an increased level of the 200-kDa proreceptor (see Figure 3). Adipocytes exposed to 5 μg/mL monensin were treated in a similar manner. Cells maintained in monensin for 28 h were fully viable and exhibited a normal ability to synthesize new proteins.[4]

In a typical experiment, 10^6 control cells bound 12 fmol of ^{125}I-insulin at equilibrium after incubation with 3×10^{-10} M hormone. Under the same assay conditions, 3T3-L1 adipocytes exposed to low and high concentrations of monensin (see above) for 24 h bound 5 and 4 fmol of ^{125}I-insulin/10^6 cells, respectively. Scatchard (1949) analyses of equilibrium binding data obtained by using 2×10^{-11} to 2×10^{-7} M insulin disclosed that the cell surface insulin receptors produced in the presence of the ionophore exhibited the same affinity for the hormone as the receptors in control cells and confirmed that the number of sites per cell declined to 33–40% of the normal value after monensin treatment (data not shown).

Relatively short-term labeling experiments (see Figures 3 and 4) revealed that cells treated with a low concentration of monensin synthesized α' and β' subunits while the 200-kDa proreceptor appeared to accumulate when a high concentration of the drug was employed. Do the α' subunit and the 200-kDa polypeptide account for the high-affinity hormone binding activity at the cell surface? This issue was addressed by covalently cross-linking ^{125}I-insulin to binding sites on the cell surface by means of the bifunctional reagent disuccinimidyl suberate (Pilch & Czech, 1980). After solubilization with Triton X-100, immunoprecipitation, and SDS–polyacrylamide gel electrophoresis, the hormone binding polypeptides were visualized by autoradiography (Figure 7). In control cells, the insulin binding site on the 130-kDa α subunit was readily labeled (Figure 7, lane 1). Insulin binding activity on the surface of adipocytes exposed to both 0.2 and 5 μg/mL monensin was located in a 120-kDa subunit (α'), and no ^{125}I radioactivity appeared in the region of the gel corresponding to a molecular weight of 200 000. The relative intensities of the labeled polypeptides in Figure 7 were consistent with the determinations of the numbers of insulin binding sites in control and monensin-treated cells described above. These results suggest that the inhibition of the proteolytic cleavage of 200-kDa proreceptor observed in the presence of high monensin concentration (Figure 2–4) is a kinetic phenomenon. Either the adipocytes convert the proreceptor to α' and β' subunits very slowly and reach a new steady-state level over an extended period of time or α' and β' subunits are generated at similar rates in adipocytes treated with high and low levels of monensin, but a lag period (e.g., 4–5 h) is introduced in the former case.

The insulin binding activity of the proreceptor was disclosed when the particulate fraction of 3T3-L1 adipocytes was extracted with Triton X-100 and the solubilized proteins were subjected to affinity cross-linking with ^{125}I-insulin and disuccinimidyl suberate. The 130-kDa α subunit was heavily labeled in extracts of control adipocytes, and a substantial amount of radioactivity also appeared in the 200-kDa precursor

[4] A. Salzman and C. S. Rubin, unpublished results.

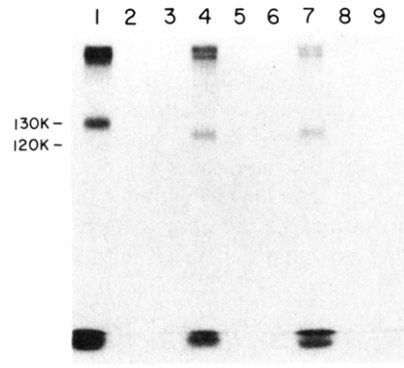

FIGURE 7: Cross-linking of cell surface insulin receptors in control and monensin-treated 3T3-L1 adipocytes. Cells containing homogeneous populations of smaller insulin receptor subunits were produced by a combination of chymotrypsin pretreatment and prolonged incubation with monensin as described in the text. Cell surface receptors on control and ionophore-treated cells were covalently cross-linked to ^{125}I-insulin with disuccinimidyl suberate as previously described (Deutsch et al., 1982). Subsequently, ^{125}I-labeled polypeptides were solubilized, immunoprecipitated, and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography (see Experimental Procedures). Immunoprecipitated polypeptides applied to lanes 1–3 were from control cells; lanes 4–6 received immunoprecipitated polypeptides from cells incubated with 0.2 μg/mL monensin; lanes 7–9 received immunoprecipitated polypeptides from adipocytes incubated with 5 μg/mL monensin. Insulin binding polypeptides in lanes 1, 4, and 7 were cross-linked with ^{125}I-insulin and were complexed with anti-insulin receptor antibodies; the samples applied to lanes 2, 5, and 8 were cross-linked with ^{125}I-insulin and precipitated with nonimmune serum; the samples applied to lanes 3, 6, and 9 were cross-linked to ^{125}I-insulin in the presence of 10 μM nonradioactive insulin and were precipitated with anti-insulin receptor antibodies.

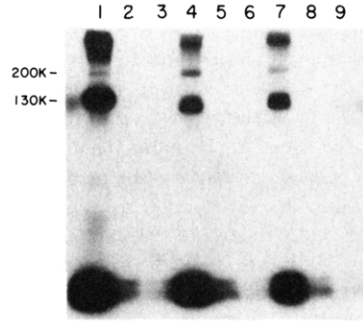

FIGURE 8: Cross-linking of Triton X-100 solubilized insulin binding polypeptides from control and monensin-treated 3T3-L1 adipocytes. Insulin receptors and receptor precursors were extracted from the particulate fractions of duplicate batches of the cells described in Figure 7 with 1% Triton X-100 as delineated under Experimental Procedures. After partial purification by affinity chromatography on wheat germ agglutinin–agarose (see Experimental Procedures), insulin binding proteins were cross-linked to ^{125}I-insulin with disuccinimidyl suberate as outlined by Deutsch et al. (1983). Further analyses were performed as described in the legend for Figure 7. The monensin concentrations, the sources of the labeled polypeptides (control and monensin-treated adipocytes), the conditions and reagents used for cross-linking (±10 μM insulin) and immunoprecipitation (immune, nonimmune IgGs), and the numbering of the samples are the same as in Figure 7.

(Figure 8, lane 1). The α' subunit and the 200-kDa proreceptor were covalently labeled in extracts of cells exposed to the ionophore (Figure 8, lanes 4 and 7). Scanning densitometry showed that the ratio of ^{125}I radioactivity in the proreceptor to ^{125}I radioactivity in the α' subunit was considerably higher (0.15) than the corresponding ratio (0.02) in the control cells.

The β subunit of the insulin receptor undergoes hormone-dependent autophosphorylation in vitro (Kasuga et al., 1982b,c; Petruzzelli et al., 1982; Avruch et al., 1982; Roth & Cassell, 1983; Van Obberhen et al., 1983; Shia et al., 1983; Roth et al., 1983). Since the α' subunit and the proreceptor avidly bound insulin (see above), it was possible to determine whether proreceptor cleavage and/or late processing affect the activation of the intrinsic protein kinase activity. A high level of insulin-stimulated phosphorylation of the 90-kDa β subunit was observed in detergent-solubilized extracts of control 3T3-L1 adipocytes (Figure 9, lanes 1 and 2). The smaller β' subunit synthesized in the presence of monensin appeared to be similarly susceptible to hormone-stimulated phosphorylation (Figure 9, lanes 3–6). Scanning densitometry showed that the relative enhancement of ^{32}P incorporation by insulin was 7-fold in both the control and experimental samples. Moreover, insulin also increased the phosphorylation of the 200-kDa proreceptor 5-fold in the monensin-treated adipocytes (Figure 9, lanes 3–6). A lesser amount of hormone-activated phosphorylation of the proreceptor was seen in control extracts (Figure 9, lanes 1 and 2).

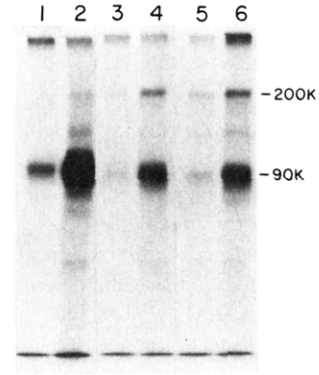

FIGURE 9: Insulin-stimulated phosphorylation of insulin receptor subunits and the proreceptor from control and monensin-treated 3T3-L1 adipocytes. Insulin receptors and receptor precursors from control cells and cells containing receptors generated in the presence of monensin (see legend to Figure 7 and text) were solubilized with Triton X-100 and were partially purified by affinity chromatography on wheat germ agglutinin–agarose (see Experimental Procedures). The enriched fractions were pooled and dialyzed against 500 volumes of 20 mM Hepes buffer, pH 7.4, containing 0.15 M NaCl to remove *N*-acetylglucosamine. Aliquots of the dialyzed samples were then incubated with [γ-^{32}P]ATP using the conditions described by Petruzzelli et al. (1982). Phosphorylated insulin receptors and insulin proreceptors were precipitated with anti-insulin receptor IgG. ^{32}P-Labeled polypeptides were resolved by SDS–polyacrylamide gel electrophoresis and were visualized by autoradiography (see Experimental Procedures). Lanes 1 and 2 received immunoprecipitated polypeptides derived from control cells; lanes 3 and 4 received immunoprecipitated polypeptides from cells exposed to 0.2 μg/mL monensin; lanes 5 and 6 received immunoprecipitated polypeptides from cells exposed to 5 μg/mL monensin. Samples for lanes 1, 3, and 5 were phosphorylated in the absence of insulin; samples for lanes 2, 4, and 6 were phosphorylated in the presence of 5 μg/mL insulin.

Discussion

The biogenesis of the cell surface insulin receptor in hormone-responsive 3T3-L1 adipocytes proceeds through a complex series of steps over a period of several hours. Overall, the α and β subunits of the plasma membrane receptor appear to be derived from a single precursor via a specific proteolytic cleavage reaction (Figure 1). Therefore, both subunits of the receptor are the products of a single gene.

The principal polypeptide that was immunoprecipitated by anti-insulin receptor antibodies after the cells were briefly pulsed (7–15 min) with [^{35}S]Met had a molecular weight of 180000 [Figure 1; see also Deutsch et al. (1983)]. This initial receptor precursor was characterized as a glycoprotein by virtue of its ability to bind to wheat germ agglutinin–agarose and the inhibition of its appearance by tunicamycin (Figure

2). The accumulation of a smaller polypeptide (M_r 170000) in the presence of the drug (Figure 2) suggests that the initial 180-kDa precursor had undergone cotranslational, N-linked glycosylation. Although the addition of high mannose oligosaccharide chains is typical during protein translation (Hubbard & Ivatt, 1981), the molecular weight of the initial precursor was not altered by endo H treatment (Figure 5). Two possible explanations for this result are either that a very limited number of oligosaccharide moieties (e.g., one or two) are removed by the enzyme but the resolving power of the polyacrylamide gel system is not sufficient to separate the two species or that the cotranslationally added N-linked core oligosaccharides are inaccessible to endo H because of subsequent changes in protein structure (e.g., folding, disulfide bond formation, etc.) that are maintained under the relatively harsh conditions of endo H digestion.

The 180-kDa precursor is rapidly converted to a 200-kDa polypeptide which we have designated the insulin proreceptor (Figure 1). The increase in size of the precursor might be due to the addition of Asn-linked high mannose oligosaccharides because the 180-kDa polypeptide can be regenerated by endo H digestion (Figure 5). The posttranslational addition of N-linked core oligosaccharides is atypical with respect to the biogenesis of many other plasma membrane and secreted glycoproteins, where cotranslational glycosylation seems to be the general rule (Hubbard & Ivatt, 1981). However, there are precedents for our findings in reports on the posttranslational addition of Asn-linked oligosaccharides to certain immunoglobulin heavy and light chains (Bergman & Kuehl, 1978), α_1-fetoprotein (Belanger et al., 1979), and α_1-acid glycoprotein (Jamieson, 1977). The results obtained from the endo H digestion experiments do not exclude the involvement of other posttranslational modifications in the conversion of the initial precursor to the 200-kDa proreceptor. For example, the phosphorylation or sulfation of carbohydrate and/or amino acid residues could contribute significantly to the generation of the proreceptor.

Our observations are also consistent with previous studies on the effects of tunicamycin on insulin receptors in 3T3-L1 adipocytes (Rosen et al., 1979; Reed et al., 1981). Upon prolonged treatment with the drug, insulin binding activity at the cell surface decays with a $t_{1/2}$ that agrees with its normal degradation rate (Reed et al., 1981). Ronnett & Lane (1981) have shown that insulin binding activity can be restored in the absence of protein synthesis after removal of the drug. The 170-kDa polypeptide identified in Figure 2 may correspond to the inactive precursor implied in the cited study, and the posttranslational glycosylation step documented in Figures 1 and 5 provides a potential mechanism for channeling the inactive precursor back into the normal biosynthetic pathway in the absence of protein synthesis.

The 200-kDa proreceptor is subsequently cleaved proteolytically to yield two dissimilar subunits (Figure 1). This processing step first becomes apparent approximately 3 h after the synthesis of the 180-kDa precursor. Specific proteolytic cleavage probably takes place in the distal portion of the Golgi apparatus because the reaction is strongly inhibited by monensin (Figures 2 and 4) which is known to exert its effects at this intracellular locus (Tartakoff & Vassalli, 1977; Tartakoff et al., 1979; Uchida et al., 1979; Griffiths et al., 1983). Lane and colleagues (Reed et al., 1981a,b; Ronnett et al., 1983) observed that there was a 3-h delay between the initiation of new insulin receptor biosynthesis and the appearance of new insulin binding activity in density-shift labeling experiments performed on 3T3-L1 adipocytes. These investigators concluded that this lag period represented the transit time for newly synthesized insulin receptors to travel from polysomes to the cell surface (Reed et al., 1981a,b; Ronnett et al., 1983). Our studies indicate that the addition of high mannose oligosaccharide chains marks the beginning of the transit period while proreceptor cleavage, the key processing event that is essential for the production of functional insulin receptor (see below), occurs during the transit period and precedes the rapid late processing and export of the α and β subunits to the cell surface.

The judicious use of the cation ionophore monensin in combination with pulse–chase labeling, immunoprecipitation, affinity cross-linking, and external proteolysis has provided insights on several aspects of the receptor biogenesis pathway in 3T3-L1 cells. In short-term (5 h) experiments carried out using 5 μg/mL monensin, the 200-kDa proreceptor accumulates and is translocated to the cell surface (Figures 2–4). The limited amount of proreceptor that is not converted to α and β subunits in untreated cells also migrates to the plasma membranes (Figure 4). When the smaller α' and β' subunits are produced in the presence of lower concentrations of monensin, the receptor becomes accessible to both external insulin and chymotrypsin (Figures 3, 4, and 7). Thus, the structural determinant that specifies the plasma membrane as the ultimate location of the insulin receptor is present in the proreceptor, and receptor translocation to the cell surface is not dependent on the proteolytic cleavage or a late processing step.

The size of the proreceptor is invariant in control 3T3-L1 adipocytes and cells exposed to various concentrations of monensin (Figures 3 and 4), yet the cells treated with the ionophore produce subunits that are apparently smaller than the normal α and β polypeptides (Figure 3). These results indicate that the late processing is not required for the proteolytic generation of receptor subunits and further suggest that the late processing steps occur after proreceptor cleavage. The late processing steps may involve complex oligosaccharide formation by the addition of fucose, galactose, *N*-acetylglucosamine, and sialic acid to trimmed and remodeled high mannose oligosaccharide chains and/or the addition of *N*-acetylgalactosamine and other sugars to Ser/Thr residues to produce O-linked oligosaccharides. The addition of terminal sugars to N-linked complex oligosaccharides and the elongation of O-linked chains proceed simultaneously in the trans region of the Golgi apparatus (Johnson & Spear, 1983; Hanover et al., 1982). Moreover, several studies indicate that both of these processes occur immediately before the secretion of glycoproteins and that the mature N- and O-glycosylated proteins do not accumulate in intracellular compartments (Johnson & Spear, 1983; Hanover et al., 1982). Further experiments will be required to determine the relative contributions of N-linked and O-linked modifications to the late processing steps in insulin receptor biogenesis.

The insulin receptor with α' and β' subunits that are produced in the presence of monensin is transported to the cell surface (Figure 4). This form of the receptor binds external insulin at the plasma membrane (Figure 7, text), and its phosphorylation is stimulated by the hormone (Figure 9), suggesting that the expression of the functional properties of the subunits is not dependent upon late processing steps. We have also demonstrated that the Triton X-100 solubilized 200-kDa proreceptor undergoes insulin-stimulated phosphorylation (Figure 9) and contains a high-affinity insulin binding site [see Figure 8 and Deutsch et al. (1983)]. Therefore, cleavage of the 200-kDa precursor is not necessary for the

expression of these functional properties in solution. However, the proreceptor does not bind external insulin at the plasma membrane in intact 3T3-L1 adipocytes (Figure 7) or in membrane fractions derived from these cells[4] despite its efficient translocation to that site, as indicated by accessibility to external chymotrypsin (Figure 4). Thus, the proteolytic conversion of the proreceptor into subunits is essential for the appearance of cell surface insulin binding activity and insulin responsiveness in 3T3-L1 adipocytes. Apparently the disulfide-linked α and β subunits can assume a conformation and/or orientation in the plasma membrane bilayer that is highly unfavorable when the two subunits are also linked by a peptide bond. When cleavage does not occur, the proreceptor is transported to the plasma membrane, but its hormone binding site is sterically or otherwise occluded or, perhaps, is embedded within the bilayer. Disruption of the membrane with nonionic detergent relieves the constraints on the proreceptor, and its abilities to bind insulin and undergo hormone-stimulated phosphorylation become manifest.

Some steps in insulin receptor biogenesis differ substantially from the mode of synthesis and processing of other, "typical" membrane and secreted proteins (Hubbard & Ivatt, 1981) but are strikingly similar to aspects of the biosynthesis and intracellular transport of the Friend erythroleukemia virus glycoprotein gp 70. Fitting & Kabat (1982) have demonstrated that this 70-kDa protein is derived from a 90-kDa polyprotein precursor via proteolytic cleavage. Furthermore proteolysis coincides with the termination of a slow processing step, and pulse-labeled gp 70 accumulates slowly on the cell surface over chase periods of several hours. After gp 70 is generated, late processing and export to the cell surface occur rapidly. Like the insulin proreceptor, a mutant form of the gp 70 precursor that is not cleaved is also translocated to the cell surface (Machida & Kabat, 1982). It is possible that gp 70 and the insulin receptor contain analogous (though not necessarily identical) "signals" in their structures that dictate their processing through an alternative, more complex, and kinetically slower pathway than that used by the majority of membrane and secreted proteins studied to date. The slow appearance of newly synthesized insulin receptors at the surface of 3T3-L1 adipocytes over several hours is consistent with the random exit of receptors from a relatively large intracellular pool as defined in equations developed by Fitting & Kabat (1982).

The biosynthesis and glycosylation of the insulin receptor have also been studied in detail in human IM-9 lymphocytes. Using pulse–chase labeling with ^{3}H-labeled sugars, lactoperoxidase-catalyzed labeling with ^{125}I, and immunoprecipitation with anti-human insulin receptor antibodies as their principal methodology, Hedo et al. (1983) and Kasuga et al. (1982a) reported kinetic and structural evidence indicating that the α and β subunits are both derived from a large precursor. In addition, putative receptor precursors with molecular weights of 190 000 and 210 000 were identified (Kasuga et al., 1982a; Hedo et al., 1983). Thus, an obligatory cleavage of a proreceptor into subunits is a conserved element of insulin receptor biogenesis in transformed human lymphocytes and insulin-responsive murine 3T3-L1 adipocytes. However, a comparison of the data on these two systems revealed significant differences in receptor processing. The 180-kDa initial precursor observed in 3T3-L1 adipocytes was highly labeled in pulses as short as 7 min, 50% of its label was chased into a 200-kDa species in 30–40 min, and it was resistant to endo H treatment. A precursor with these properties was not found in IM-9 cells. The 200-kDa polypeptide observed in the adipocytes shares common properties with the 190-kDa precursor of Hedo et al. (1983). Both are high mannose glycoproteins, and their conversion to subunits was potently inhibited by monensin. Nevertheless, these two precursors differed in their derivation and fate. The 200-kDa adipocyte proreceptor arose by an atypical, posttranslational addition of high mannose chains, and some of this protein ultimately reached the cell surface. The 190-kDa lymphocyte protein, which was cotranslationally glycosylated, appeared to be restricted to intracellular compartments (Hedo et al., 1983). In independent studies of IM-9 lymphocytes, Jacobs and colleagues (Jacobs et al., 1983) described a similar protein that accumulated in the presence of monensin, although a lower molecular weight (180 000) was assigned. In contrast to the adipocyte proreceptor, this glycoprotein exhibited insulin binding activity at the cell surface after translocation (Jacobs et al., 1983). A very small amount of this precursor was converted to subunits with molecular weights of 89 000 and 115 000, and the latter also bound hormone at the cell surface. The sensitivity of this receptor precursor to endo H and its ability to undergo insulin-stimulated phosphorylation were not studied. Jacobs et al. (1983) did not report glycoprotein precursors analogous to either the 180-kDa adipocyte polypeptide or the 210-kDa protein described in IM-9 lymphocytes by Hedo et al. (1983) in control or treated cells. The 210-kDa lymphocyte polypeptide that is complexed by anti-insulin receptor IgGs contains polypeptide sequences corresponding to α and β subunits as well as the complex type of oligosaccharide chains (Hedo et al., 1983). This precursor is apparently resistant to proteolytic conversion to subunits and is transported to the plasma membrane without further modification. A polypeptide analogous to the 210-kDa protein was not seen in 3T3-L1 adipocytes. Further comparison among the adipocyte and lymphocyte glycoprotein precursors will become possible when studies on functional properties of the 190- and 210-kDa proteins and the role of cleavage in determining cell surface hormone binding activity in IM-9 cells are performed.

When this paper was in the final stages of preparation, Ronnett et al. (1984) reported studies relevant to the data presented in Figure 1 and portions of Figures 4 (lanes 1 and 2) and 5 (lanes 1–6) and our previous investigations (Deutsch et al., 1983). Our current and previous studies are in accord with the results of Ronnett and co-workers (Ronnett et al., 1984) with respect to the occurrence of two multipeptide precursors for receptor subunits, the kinetics of appearance of the proreceptor and mature subunits, the sensitivity of the proreceptor to endo H, and the synthesis of a smaller, nonglycosylated precursor in the presence of tunicamycin. In contrast to their observations, we found that the initial precursor is resistant to endo H, suggesting that a single set of of posttranslational glycosylations is the primary signal for entry of the receptor into the processing pathway and that the 200-kDa proreceptor (as well as both the α and β subunits) is transported to and exposed on the cell surface. The reason for the former difference in the two studies is not known, but the latter results may be a function of the choice of external protease. The observations of Ronnett et al. (1984) are consistent with the very limited ability of trypsin to cleave plasma membrane-associated proreceptor.[4] Treatment with chymotrypsin revealed that the monensin-generated or residual proreceptor and both receptor subunits are exposed at the cell surface (Figure 4). The effects of monensin on receptor synthesis and processing, the roles of proteolytic cleavage and later processing in transit and the development of hormone responsiveness, and the structural and functional properties

of the the proreceptor in 3T3-L1 adipocytes were not addressed in the studies of Ronnett et al. (1984).

Ultimately, a comprehensive understanding of insulin receptor biogenesis in 3T3-L1 cells will require a determination of the number and nature of oligosaccharide moieties attached to the α and β subunits, the identification of the intracellular sites of early and late oligosaccharide processing, the elucidation of the structural signals in the receptor precursors that direct their entry into the longer processing pathway and specify transport to the cell surface, and the determination of the location and mode of regulation of the proteolytic cleavage step that is essential for generating cell surface insulin binding activity and hormone responsiveness.

Registry No. Insulin, 9004-10-8.

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Structure of the Capsid of Kilham Rat Virus from Small-Angle Neutron Scattering†

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ABSTRACT: The structure of empty capsids of Kilham rat virus, an autonomous parvovirus with icosahedral symmetry, was investigated by small-angle neutron scattering. From the forward scatter, the molecular weight was determined to be 4.0×10^6, and from the Guinier region, the radius of gyration was found to be 105 Å in D_2O and 104 Å in H_2O. On the basis of the capsid molecular weight and the molecular weights and relative abundances of the capsid proteins, we propose that the capsid has a triangulation number of 1. Extended scattering curves and mathematical modeling revealed that the capsid consists of two shells of protein, the inner shell extending from 58 to 91 Å in D_2O and from 50 to 91 Å in H_2O and containing 11% of the capsid scattering mass, and the outer shell extending to 121 Å in H_2O and D_2O. The inner shell appears to have a higher content of basic amino acids than the outer shell, based on its lower scattering density in D_2O than in H_2O. We propose that all three capsid proteins contribute to the inner shell and that this basic region serves DNA binding and partial charge neutralization functions.

The mammalian parvoviruses are among the smallest animal viruses known with a linear single-stranded (ss)[1] DNA genome of approximately 5000 bases and an icosahedral capsid of 22-nm diameter. They are classified in two subgroups, the defective parvoviruses (including adeno-associated virus), which require coinfection by helper virus for replication, and the nondefective or autonomous parvoviruses (including KRV, H-1, and MVM), which have no helper requirement. In general, the autonomous parvoviruses mentioned share very similar properties in terms of capsid structure and composition (Peterson et al., 1978; Tattersall & Ward, 1978).

The capsid of KRV consists of three proteins—A, B, and C—with apparent masses of 80, 64, and 59 kilodaltons, respectively (Mitra et al., 1982). In MVM, these three proteins appear to share extensive sequence homology on the basis of two-dimensional peptide mapping (Tattersall et al., 1977). This is consistent with other experimental evidence indicating that (i) messages for polypeptides A and B are transcribed from overlapping regions of the viral genome in H-1 (Rhode & Paradiso, 1983) and (ii) protein C is generated by proteolytic cleavage of protein B during the maturation of DNA-containing, full virions (Clinton & Hayashi, 1975, 1976). The cleavage of protein B is accompanied by a decrease in the virion's buoyant density in CsCl and an increase in infectivity.

The A protein is particularly interesting for two reasons. First, it is present in both empty and full capsids in roughly a constant proportion relative to protein B and C (see below) (Tattersall et al., 1976). Moreover, A does not undergo proteolytic cleavage, unlike B, despite the fact that the B sequence is contained within A (Tattersall et al., 1977). Second, the region of the A protein that does not overlap with B or C is known to contain highly basic peptides that give A a much more basic p*I* than B or C (Mitra et al., 1982; Tattersall et al., 1976). Together, these observations suggest a conformation-specific functional role for protein A in the virus capsid, possibly in DNA binding and partial charge neutralization.

To date, little work has been done on the three-dimensional structure of parvoviruses. Early electron microscope observations of KRV and H-1 (Vasquez & Brailousky, 1965; Karasaki, 1966) indicated that the capsid is an icosahedron with 32 capsomers, and therefore had a triangulation number of 3. Later investigations (Tattersall et al., 1976) concluded that there are about 64 protein molecules per capsid, and hence the capsomeres are dimeric. On the basis of the apparent molar ratios of the proteins, it was proposed that empty capsids contain 8 or 9 molecules of A and 53–57 molecules of B. Full particles were found to contain 8–9 molecules of protein A and total of 53–57 molecules of B plus C proteins combined (Tattersall et al., 1976).

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‡ Supported by NIH Training Grant 7438.

[1] Abbreviations: ss, single stranded; AAV, adeno-associated virus; MVM, minute virus of mice; KRV, Kilham rat virus; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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