Characterization of the N-linked high-mannose oligosaccharides of the insulin pro-receptor and mature insulin receptor subunits

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The insulin receptor is synthesized as a 190000-M_r single-chain precursor that contains exclusively asparagine-N-linked high-mannose-type carbohydrate chains. In this study we have characterized the structure of the pro-receptor oligosaccharides. IM-9 lymphocytes were pulse–chase-labelled with [³H]mannose, and the insulin pro-receptor was isolated by immunoprecipitation and SDS/polyacrylamide-gel electrophoresis. The pro-receptor oligosaccharides were removed from the protein backbone with endo-glycosidase H and analysed by h.p.l.c. Immediately after a [³H]mannose pulse the largest oligosaccharide found in the pro-receptor was $Glc_1Man_9GlcNAc_2$; this structure represented only a small fraction (3%) of the total. The predominant oligosaccharides present in the pro-receptor were $Man_9GlcNAc_2$ (25%) and $Man_8GlcNAc_2$ (48%). Smaller oligosaccharides were also detected: $Man_7GlcNAc_2$ (18%), $Man_6GlcNAc_2$ (3%) and $Man_5GlcNAc_2$ (3%). The relative distribution of the different oligosaccharides did not change at 1, 2 or 3 h after the pulse with the exception of the rapid disappearance of the $Glc_1Man_9GlcNAc_2$ component. The mature α- and β-subunits of the insulin receptor are known to contain both high-mannose-type and complex-type oligosaccharides. We have also examined here the structure of the high-mannose chains of these subunits. The predominant species in the α-subunit was $Man_8GlcNAc_2$ whereas in the β-subunit it was $Man_7GlcNAc_2$. These results demonstrate that most (approx. 75%) oligosaccharides of the insulin pro-receptor are chains of the type $Man_8GlcNAc_2$ or $Man_9GlcNAc_2$. Thus, assuming that a $Glc_3Man_9GlcNAc_2$ species is transferred co-translationally, carbohydrate processing of the pro-receptor appears to be very rapid and limited to the removal of the three glucose residues and one mannose residue. Further mannose removal does not occur until the pro-receptor has been proteolytically cleaved. In addition, the degree of mannose trimming appears to be different in the α- and β-subunits.

INTRODUCTION

The insulin receptor is a membrane glycoprotein composed of two major subunits, each of which contains N-linked high-mannose-type and complex-type carbohydrate side chains [1,2]. Both mature subunits are processed from a single polypeptide chain pro-receptor of apparent M_r 190000 that contains only high-mannose-type carbohydrate side chains [2,3] and is found predominantly in the endoplasmic reticulum and Golgi apparatus [3]. Thus processing of the pro-receptor involves: peptide cleavage, trimming of some mannose residues from high-mannose side chains, addition of the distal sugars (N-acetylglucosamine, galactose, sialic acid) to form complex carbohydrate side chains, and insertion of the mature receptor into the plasma membrane. Tunicamycin, an inhibitor of N-linked glycosylation [4], inhibits insulin pro-receptor processing [5], suggesting that the carbohydrate component plays an important role in pro-receptor processing.

Recently the entire amino acid sequence of the insulin pro-receptor has been deduced from a cDNA clone [6]. This sequence contains 21 potential N-glycosylation sites. The structure of the actual N-linked carbohydrate

chains has been characterized so far only in terms of their endoglycosidase H sensitivity and their differential labelling by radioactive monosaccharides [2,3].

In the present work we have analysed for the first time the structure of the carbohydrate chains attached to the insulin pro-receptor as well as the structure of the high-mannose oligosaccharide chains of the mature α- and β-subunits of the receptor. Our experimental approach involved labelling of the insulin receptor of human IM-9 lymphocytes with [³H]mannose followed by isolation of the labelled pro-receptor and mature subunits by immunoprecipitation and SDS/polyacryl-amide-gel electrophoresis. The high-mannose oligosaccharide chains were released from the receptor components by treatment with endoglycosidase H. Finally, the released oligosaccharides were analysed by h.p.l.c.

The predominant oligosaccharides in the insulin pro-receptor are $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$. At a very early stage, in the newly translated pro-receptor, $Glc_1Man_9GlcNAc_2$ is also present. In addition, we have found differences in the extent of oligosaccharide processing between the mature α- and β-subunits of the receptor.

MATERIALS AND METHODS

Cell culture and radiolabelling

IM-9 lymphocytes were grown in RPMI 1640 medium (Biofluids, Rockville, MD, U.S.A.) supplemented with 10% (v/v) foetal bovine serum (Biofluids). After they reached a stationary phase of growth, the cells were incubated with D-[2-^{3}H]mannose (14 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) for either short periods (pulse-labelling) or overnight (continuous labelling) as previously described [1,2]. After the pulse-labelling experiments the cells were either immediately processed or transferred to fresh medium containing unlabelled mannose (2 mM) and incubated for various periods of time (chase).

Isolation of insulin receptors

The radiolabelled cells were harvested, solubilized in 1% (v/v) Triton X-100, and the insulin receptors were immunoprecipitated by using anti-receptor antibodies as previously described [1,2]. The immunoprecipitates were subjected to SDS/polyacrylamide-gel electrophoresis. The ^{3}H-labelled receptors were detected by fluorography [1,2] and the receptor bands were located by superimposing fluorographs on the dried gels. As shown in Fig. 1, the insulin receptor components are fully separated by this procedure from other radiolabelled glycoproteins.

Extraction of endoglycosidase H-sensitive N-linked oligosaccharides from the insulin receptor subunits

The areas of the gels that contained the insulin pro-receptor or the mature receptor subunits were excised, washed in 20% (v/v) methanol and dried. After rehydration with 0.15 M-citrate buffer, pH 5.5, the gel slices were incubated in the same buffer containing endoglycosidase H (endo-β-N-acetylglucosaminidase H from Streptomyces plicatus; Miles Research Laboratories, Naperville, IL, U.S.A.) at a final concentration of 0.2–0.5 unit/ml at 37 °C for 18 h. No differences in enzymic efficiency of ^{3}H removal were observed within this range of enzyme activities. After centrifugation, the gel slices were removed and the supernatants were diluted in acetonitrile (h.p.l.c. grade; Burdick and Jackson Laboratories, Muskegon, MI, U.S.A.) before h.p.l.c.

H.p.l.c.

The chromatography was carried out with an h.p.l.c. system comprising a model 720 systems controller, an M6000A pump, an M45 pump and a model U6K injection valve (Waters Associates, Milford, MA, U.S.A.). Fractionation was performed with a μBondapak-NH$_2$ column (3.9 mm × 300 mm) (Waters Associates), which before application of sample was washed with 100 ml of propan-2-ol (h.p.l.c. grade; Burdick and Jackson) and then equilibrated with water/acetonitrile (35:65, v/v). After injection, the samples were eluted isocratically with water/acetonitrile (35:65, v/v) for 20 min followed by a linear gradient to water/acetonitrile (50:50, v/v) over 80 min, at a flow rate of 1 ml/min and at ambient temperature. Fractions (1 ml) were collected, mixed with 5 ml of ACS scintillation fluid (Amersham Corporation, Arlington Heights, IL, U.S.A.) and their radioactivities counted with a scintillation counter.

The columns were calibrated with ^{3}H or ^{14}C standards generously donated by Dr. S. Kornfeld of Washington University School of Medicine (St. Louis, MO, U.S.A.) and Dr. S. J. Turco of the University of Kentucky (Lexington, KY, U.S.A.). Standards were run adjacent to all unknown samples.

Control experiments

To ensure that the labelling and extraction procedures were reproducible, we performed certain control experiments. In the first of these experiments, portions of an immunoprecipitated sample of labelled receptor were electrophoresed in separate lanes. Thereafter each lane was treated with endoglycosidase H and extracted separately. After h.p.l.c. fractionation, the chromatographic profiles of these lanes were identical, demonstrating reproducibility in endoglycosidase H digestion and oligosaccharide extraction. In a second experiment, to show that the extraction procedure gave uniform elution of the labelled oligosaccharides from the gel pieces, a re-extraction was performed. This revealed a similar ratio of labelled oligosaccharide components after h.p.l.c. fractionation as was obtained in the initial extraction. The extraction efficiency was 75–80% of the radioactivity for the pro-receptor and 50–60% for the α-and β-subunits; that this latter value is lower is probably due to the presence of [^{3}H]mannose incorporated into endoglycosidase H-resistant complex sugars that were not released. Immunoprecipitation with either of the anti-receptor sera B-2 and B-d gave similar results.

RESULTS

Pro-receptor

To examine the N-linked carbohydrates attached to the insulin pro-receptor, IM-9 lymphocytes were pulse-labelled with [^{3}H]mannose and then incubated with non-radioactive mannose for various periods of time (chase). The cells were solubilized and the labelled insulin pro-receptor and receptor were immunoprecipitated with anti-receptor sera and subjected to SDS/polyacrylamide-gel electrophoresis. The pro-receptor was located by fluorography (Fig. 1), and the N-linked high-mannose-type oligosaccharides were removed enzymically with endoglycosidase H and subjected to h.p.l.c. Fig. 2 shows the profile obtained from cells that were pulse-labelled for 30 min and not subjected to a chase incubation. The predominant peaks are of oligosaccharides containing 1 N-acetylglucosamine residue and 8 mannose residues (Man 8) or 1 N-acetylglucosamine and 9 mannose residues (Man 9). A small peak of an oligosaccharide containing 1 N-acetylglucosamine, 9 mannose and 1 glucose residues (Glc 1) is also detected. In addition, there are also components containing 1 N-acetylglucosamine and 7, 6 or 5 mannose residues (Man 7, Man 6 or Man 5).

Shorter pulse periods resulted in the incorporation of less radioactivity into the pro-receptor, but the general distribution of oligosaccharides removed from the pro-receptor was unaltered. In particular, no species containing 3 or 2 glucose residues with 9 mannose residues and 1 N-acetylglucosamine residue were identified with the use of shorter pulse periods.

When the pro-receptor oligosaccharides were examined after various chase periods, the relative distribution of oligosaccharides was essentially unchanged. However, the peak of the oligosaccharide containing one glucose residue (Glc 1) was no longer detected during the chase.
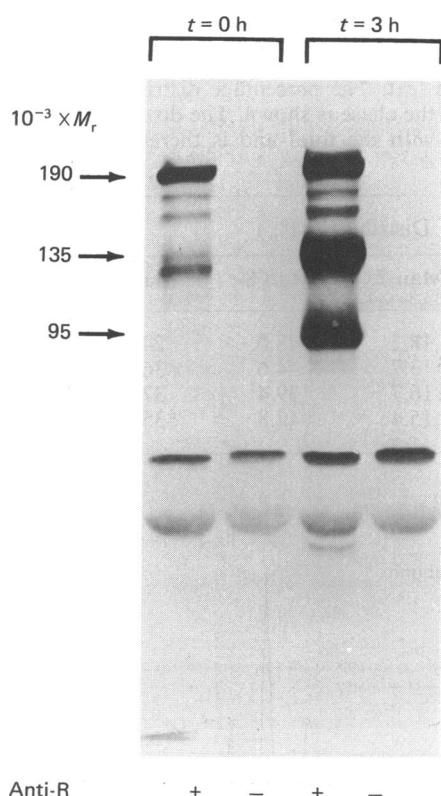
Fig. 1. [³H]Mannose labelling of the insulin pro-receptor and mature receptor subunits

IM-9 lymphocytes were grown to stationary phase, concentrated and incubated with [³H]mannose for 30 min at 37 °C. After they had been washed, one portion of cells was processed immediately ($t = 0$ h) and another portion returned to incubation medium supplemented with 2 mM non-radioactive mannose for 3 h ($t = 3$ h). The cells were harvested and solubilized in 1% (v/v) Triton, and the pro-receptor and receptor were immunoprecipitated with anti-receptor sera and subjected to SDS/polyacrylamide-gel electrophoresis. A fluorograph is shown. Immuno-precipitation with anti-receptor serum (anti-R) is indicated by + at the foot of each lane; − indicates immuno-precipitation with control serum.

Table 1 shows the relative distribution of the oligo-saccharides after chase periods of 0–3 h. No evidence of trimming, i.e. sequential removal of mannose residues, is seen over this 3 h period.

Mature receptor α- and β-subunits

The mature α-subunit (M_r 135000) and β-subunit (M_r 95000) of the insulin receptor can be clearly identified after a [³H]mannose pulse-labelling and a 2–3 h period of chase incubation (Fig. 1). Both subunits are known to contain high-mannose-type (endoglycosidase H-sensitive) and complex-type (endoglycosidase H-resistant) oligo-saccharide chains [2,3]. The bands corresponding to the α-and β-subunits were excised, and digested with endoglycosidase H in a similar manner to that followed with the pro-receptor. The released high-mannose oligosaccharides were examined by h.p.l.c. and the profiles obtained after 3 h chase are shown in Fig. 3. In the α-subunit the predominant type of oligosaccharide is Man 8, followed by Man 9. This pattern is very similar to that observed for the pro-receptor except for a
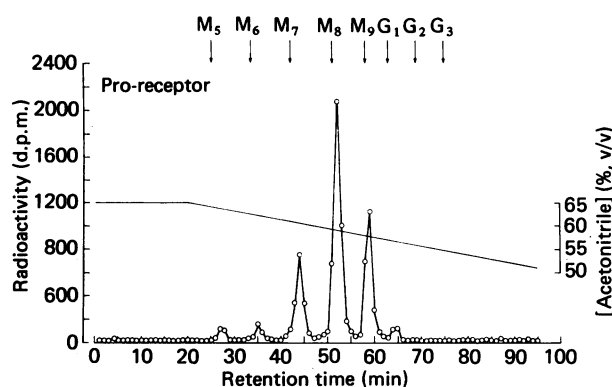

Fig. 2. H.p.l.c. profile of the oligosaccharides attached to the insulin pro-receptor after a short pulse-labelling period

IM-9 lymphocytes were processed as described in Fig. 1 legend with no chase period ($t = 0$ h). A fluorograph was used to locate the pro-receptor on the gel. The gel band containing the pro-receptor was excised and the high-mannose oligosaccharides were released with endo-glycosidase H. The released oligosaccharides were examined by h.p.l.c. A ³H elution profile is shown. The column was standardized with primary standards (G_x, $Glc_xMan_9GlcNAc_1$; M_y, $Man_yGlcNAc_1$) both before and after examination of the unknown.

noticeable increase in the proportions of Man 7 and Man 6 in the α-subunit. In contrast, the predominant oligosaccharide in the β-subunit is Man 7, followed by similar relative amounts of Man 8 and Man 6 and smaller contributions of Man 9 and Man 5. Thus there is a clear difference in the composition of the endoglycosidase H-releasable oligosaccharides of the α- and β-subunits. This difference in oligosaccharide content between the α- and β-subunits is suggestive of more extensive mannose trimming in the β-subunit compared with the α-subunit. Nevertheless, it is also conceivable that the mannose trimming had occurred in the pro-receptor and that the differences observed represent the uncovering of the heterogeneity of the oligosaccharide chains present in the α- and β-subunit portions of the pro-receptor. In order to distinguish between these two possibilities the relative distribution of the different peaks from the pro-receptor was compared with those of the combined α- and β-subunits obtained at the same time point of the chase (Table 2). A clear shift towards smaller oligosaccharide units in the combined α- and β-subunits as compared with the pro-receptor is apparent. Similarly, a shift towards smaller oligosaccharide units is observed in the combined α- and β-subunits at 3 h as compared with 2 h, whereas, as seen previously (Table 1), no shift is observed for the pro-receptor oligosaccharide distribution over this time period. Thus the differences observed in the α- and β-subunits appear to be the result of a different extent of trimming undergone by the oligosaccharides attached to each subunit. Further, this trimming seems to occur after proteolytic cleavage of the pro-receptor.

When the cells were continuously labelled for 18 h in the presence of [³H]mannose, the h.p.l.c. profiles of the high-mannose oligosaccharides from the α- and β-subunits were very similar to that obtained at 3 h of the pulse–chase study. Thus no further processing of the high-mannose chains of the receptor subunits seems to occur 1–2 h after their synthesis.

Table 1. Percentage distribution pattern of the N-linked oligosaccharides from the insulin pro-receptor

The insulin pro-receptor oligosaccharides were analysed as described in the text. The percentage distribution of recovered radioactivity in each of the oligosaccharide species at different time points of the chase is shown. The distribution is based on the percentage of radioactivity associated with each fraction as compared with the total and is therefore biased towards structures with higher mannose content.

Time of chase (h)	Oligosaccharide . . .	Distribution (%)					
		Man 5	Man 6	Man 7	Man 8	Man 9	Glc 1
0		2.8	3.7	18.2	48.0	24.7	2.6
1		2.2	2.7	13.7	44.6	36.7	—
2		2.3	3.6	16.7	39.4	37.9	—
3		2.2	3.7	15.4	42.8	35.8	—
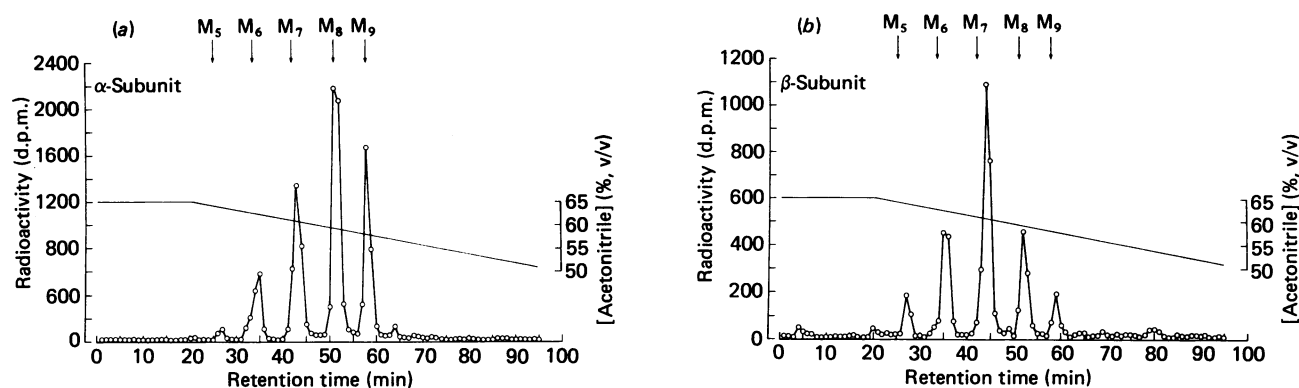
Fig. 3. H.p.l.c. profiles of the high-mannose-type oligosaccharides attached to the mature insulin receptor subunits

IM-9 lymphocytes were processed as described in Fig. 1 legend except that the chase period was 3 h. The high-mannose oligosaccharides were released from the mature subunits of the insulin receptor as described in Fig. 2 legend. The released oligosaccharides were examined by h.p.l.c. A ^{3}H elution profile is shown for both the α-subunit (a) and the β-subunit (b).

Table 2. Comparison between the percentage distributions of oligosaccharides from the insulin pro-receptor and the mature β-subunits

The oligosaccharides from the insulin pro-receptor and mature subunits were analysed as described in the text. The percentage distribution of recovered radioactivity in each of the oligosaccharide species of the insulin pro-receptor at 2 and 3 h of the chase was compared with the combined values obtained for the α- and β-subunits at the same time points.

Oligosaccharide . . .	Distribution (%)				
	Man 5	Man 6	Man 7	Man 8	Man 9
(a) 2 h chase					
Pro-receptor	2.3	3.6	16.7	39.4	37.9
Mature α- and β-subunits	2.8	8.7	25.8	37.4	25.3
(b) 3 h chase					
Pro-receptor	2.2	3.7	15.4	42.8	35.8
Mature α- and β-subunits	2.9	14.3	31.0	33.4	18.3

DISCUSSION

These data identify and characterize the N-linked carbohydrate attached to the insulin pro-receptor, all of which we have previously shown to be of the high-mannose endoglycosidase H-sensitive type [2,3]. The pattern of transfer of the N-linked high-mannose oligosaccharides from the dolichol intermediate to the protein backbone is thought to occur via a general mechanism in animal cells (reviewed in [7]), with the exception of some minor variations in certain cell types

during glucose deprivation [7–11]. The monosaccharides are assembled in a dolichol-linked complex, and a $Glc_3Man_9GlcNAc_2$ structure is formed. This oligosaccharide is transferred to asparagine residues in the nascent protein, and attached via the N-acetylglucosamine moiety while the protein is being secreted in the endoplasmic-reticulum lumen. Thereafter sequential removal of glucose and mannose residues results in an $Man_5GlcNAc_2$ structure. At this point an external N-acetylglucosamine moiety is added, and further mannose trimming combined with addition of other

sugars can form a large variety of complex-type side chains.

In order to release the oligosaccharides from the receptor components we have used enzymic digestion with endoglycosidase H. This enzyme removes N-linked high-mannose oligosaccharides by cleaving the linkage between the two internal N-acetylglucosamine residues. As a result the oligosaccharides that we have examined contain only one of the internal N-acetylglucosamine residues. In the insulin receptor of IM-9 lymphocytes the highest-M_r oligosaccharide identified after a 30 min pulse was a high-mannose oligosaccharide containing 1 glucose and 9 mannose residues plus the additional N-acetylglucosamine residue. The expected or predicted initial transfer oligosaccharide containing 3 glucose residues was not found. This could have been due either to different transfer products for this protein in this cell type or, as seems more likely, to rapid removal of the glucose residues after translation. In this regard it should be noted that the half-time of action of the glycosidases in other cell types is less than 2 and 5 min for the two enzymes involved [12]. Owing to the technical limitations of labelling this relatively minor protein species and the time required to process the cells, we cannot exclude the presence or absence of an oligosaccharide precursor containing 3 glucose residues in the insulin pro-receptor. Assuming that an oligosaccharide of the type $Glc_3Man_9GlcNAc_2$ is transferred to the insulin pro-receptor, it is apparent that the three glucose residues are removed shortly after translation. In fact $Glc_1Man_9GlcNAc_1$ was only detectable as a minor species immediately after the pulse; during the chase the largest oligosaccharide present at all times points was $Man_9GlcNAc_1$. The other major oligosaccharide present in the pro-receptor was $Man_8GlcNAc_1$. Actually, it was the predominant structure in most samples, and together with $Man_9GlcNAc_1$ it accounted for nearly 80% of the oligosaccharide units. These findings indicate that one mannose residue, at least in some of the carbohydrate chains, is also very rapidly removed after the synthesis of the pro-receptor. It remains to be determined whether this difference in the number of mannose residues (nine versus eight) is determined for each glycosylation site of the pro-receptor molecule or reflects chain heterogeneity occurring at the same site among the pro-receptor molecules.

Besides the removal of the glucose residues and in some chains one mannose residue, the oligosaccharides of the pro-receptor do not appear to undergo further processing. This finding is in good agreement with the sequence of processing events proposed previously; thus proteolytic cleavage of the pro-receptor yields the α- and β-subunits before completion of carbohydrate processing [3,13]. The long interval (1–3 h) observed between the generation of the pro-receptor and its cleavage represents, most probably, transport of the pro-receptor from the endoplasmic reticulum to the Golgi structure [3,13].

The mature α- and β-subunits contain both high-mannose-type and complex-type oligosaccharides [1,2].

Our experimental approach does not allow characterization of the structure of the complex-type chains. However, we did examine the high-mannose endoglycosidase H-sensitive chains. A difference was found between the oligosaccharide chains of both subunits. The predominant oligosaccharide detected in the α-subunit was $Man_8GlcNAc_2$, whereas in the β-subunit it was $Man_7GlcNAc_2$. This is suggestive of a difference in the extent of processing of the subunits. Since both disulphide-linked subunits are exported together via the same route to the plasma membrane, this finding lends support to the view that the protein structure itself is a crucial factor in determining the extent of carbohydrate processing.

Finally, the experimental approach used in this work can be used to study the structure of the pro-receptor carbohydrates in other cell types, and especially in those situations in which abnormalities of pro-receptor processing may be present [14,15]. Thus this methodology may prove useful in examining the potential role of the pro-receptor oligosaccharides in the physiology and pathophysiology of the receptor.

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