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IN VITRO BIOSYNTHESIS OF TWO HUMAN GALACTOSYLTRANSFERASE POLYPEPTIDES

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SUMMARY - HeLa cell galactosyltransferase is synthesized as two precursor polypeptides of M_r=45,000 and M_r=47,000. The enzyme is present in the Golgi complex as a (mature) M_r=54,000 glycoprotein. If cells are treated with tunicamycin, two precursor polypeptides are synthesized without N-linked oligosaccharides with molecular weights of 42,000 and 44,000, respectively. To investigate whether the two precursor polypeptides are synthesized on different mRNAs total RNA from HeLa cells was translated in a wheat germ cell-free system. Galactosyltransferase polypeptides were isolated by immunoprecipitation and compared to the polypeptides synthesized *in vivo* in the presence of tunicamycin. The two *in vitro* translated polypeptides co-migrate exactly with the polypeptides made in the cells in the presence of tunicamycin, indicating two different mRNAs for galactosyltransferase. The results also indicate that translocation of galactosyltransferase through the membrane of the rough endoplasmic reticulum is not followed by signal peptide cleavage.

INTRODUCTION - Study of the biosynthesis and metabolism of galactosyltransferase is a first step to the understanding of the question as to why galactosyltransferase (E.C. 2.4.1.22) is mainly present in the Golgi complex. At present there are no data available which provide a comprehensive explanation for the mechanisms that determine whether a membrane protein is retained at a specific subcompartment along the secretory pathway, or expressed at the cell surface. The biosynthesis of the Golgi enzyme galactosyltransferase is of interest as it is usually not only present in the trans cisternae of the Golgi complex, where it is involved in galactosylation of proteins in transit, but also a constituent of most body fluids (1, for review see 2). Intracellularly it is a resident membrane protein embedded in the membranes of the Golgi complex from where it is released as a soluble protein, after losing its hydrophobic character (3,4). Detailed biochemical studies have shown that galactosyltransferase is synthesized as two polypeptides of different apparent molecular weights, but of almost identical primary structure (5). Both polypep-

ABBREVIATIONS - ER, endoplasmic reticulum; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate.

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tides are provided with N- and O-linked oligosaccharides (6). Their topology has not been elucidated yet. Some inference on the mechanism of translocation and topology may be obtained by investigating as to whether translocation is followed by a processing step referred to as cleavage of a signal sequence (for review see 7). In order to address this problem we have examined the products of cell-free translation of HeLa cell RNA after immunoprecipitation, we have compared them with the precursor peptides synthesized in HeLa cells after inhibiting the N-glycosylation, and we demonstrate absence of signal peptide cleavage for galactosyltransferase.

MATERIALS AND METHODS - Nearly confluent HeLa cells grown on 60 mm culture dishes were pulse labeled with [^{35}S]methionine (60-70 μC/ml) (800-1200 Ci/mmol, The Radiochemical Centre, Amersham), chased in the presence of regular minimal essential medium containing unlabeled methionine, and lysed in 1% Triton X-100, 0.1 mM phenylmethanesulfonyl fluoride in phosphate buffered saline. The solution was clarified by centrifugation and prepared for immunoprecipitation.

Total RNA was isolated from HeLa and HepG2 cells as described by Chirgwin (8). Wheat germ extract was prepared according to Erickson and Blobel (9). The *in vitro* translation reactions were performed using an RNA-dependent wheat germ extract (10,11). Translation reactions (0.5 ml) were incubated for 90 min at 25°C and terminated by placing on ice.

Immunoprecipitations were performed as previously described (3). The antiserum was raised against soluble human milk galactosyltransferase. The enzyme was purified by affinity chromatography on α-lactalbumin- and acetylglucosamine-sepharose columns (11) and the antiserum was shown to be monospecific for galactosyltransferase (1). Aliquots of the Triton X-100 soluble material were immunoprecipitated with either normal rabbit IgG (control) or rabbit anti-human galactosyltransferase. The immune precipitates were isolated following centrifugation and washing at 4°C and the antigen and antibody were released from the Sepharose beads (Pharmacia) by boiling in SDS-PAGE sample buffer. In all instances a sufficient amount of antiserum was added to quantitatively immunoprecipitate the antigen.

Gel electrophoresis was performed in 10% polyacrylamide gels in the presence of sodium dodecylsulphate (SDS-PAGE). After electrophoresis the gels were fluorographed (12) and the fluorograms were scanned with a microdensitometer (E.C. Apparatus, FL) within the linear range of the film and the microdensitometer.

RESULTS AND DISCUSSION - To investigate how the precursor polypeptide(s) is synthesized *in vitro* total RNA was isolated from HeLa cells and added to a translation system prepared from wheat germ. The total reaction mixture was incubated at 25°C for 90 min galactosyltransferase was isolated from the cell-free translation mixture by immunoprecipitation and analyzed by SDS-PAGE. As apparent from Fig. 1 (lane "TL") two radioactive bands are visible with apparent molecular weights of 44,000 and 42,000 (indicated with P). The two radioactive bands at higher M_r are non-specific and are also present if a pre-immune serum was used for immunoprecipitation. As the cell-free system is unable to glycosylate proteins, it is expected that the galactosyltransferase polypeptides synthesized in the cell-free system lack oligosaccharides. Therefore, we compared the cell-free translation products to [^{35}S]methionine labeled galactosyltransferase polypeptides isolated from HeLa cells cultured

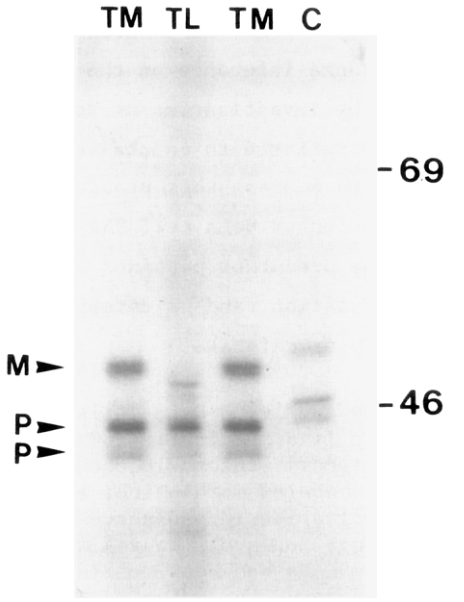

Fig. 1. Immunoprecipitations of galactosyltransferase from HeLa cells and the wheat germ cell-free system. Cells were labeled in medium containing 60 μCi/ml [^{35}S]methionine in the presence of 5 μg/ml tunicamycin (TM) or in the absence (C) for 40 min. The cells were then lysed and galactosyltransferase was isolated from the lysates by immunoprecipitation. Lane indicated "C": galactosyltransferase polypeptides isolated from control cells; lanes indicated with "TM": galactosyltransferase polypeptides synthesized in the presence of tunicamycin. Total RNA from HeLa and HepG2 cells was translated in a wheat germ extract. Cell-free synthesized galactosyltransferase is electrophoresed in the lane indicated with "TL". Markers on the right denote the position of albumin (69,000), and ovalbumin (46,000). The mature glycoprotein band migrating at M_r=52,000 in case of tunicamycin is indicated with "M"; the precursor bands (M_r=44,000 and 42,000, respectively) are indicated with "P". To judge the position of the bands precisely the "TM" lane is present at both sides of the translation products.

in the presence of 5 μg/ml tunicamycin (lanes "TM"). To obtain both labeled "mature" and precursor galactosyltransferase polypeptides the cells were labeled for 40 min, then lysed in detergent and galactosyltransferase polypeptides were immunoprecipitated. Two conclusions can be drawn from Fig. 1: first, the precursor polypeptides synthesized in HeLa cells have exactly the same apparent molecular weights as the cell-free translation products and second, HeLa cells contain two different messenger RNAs for galactosyltransferase. The two bands also occurred in the same relative amounts in both the *in vivo* and *in vitro* system (the 44,000 band always occurred in excess over the 42,000 band).

As expected, in HeLa cells the precursor polypeptides are converted to "mature" galactosyltransferase when they reach the Golgi complex (about 20 min after biosynthesis in the rough endoplasmic reticulum (ER)). During this transport galactosyltransferase is O-glycosylated causing an increase in molecular weight (6). As tunicamycin was present during the labeling the mature protein lacks N-linked oligosaccharides. This results in a somewhat

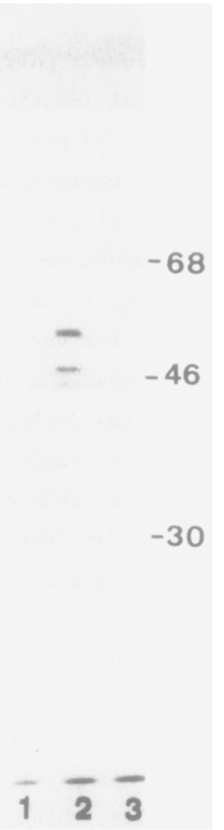

Fig. 2. Effect of soluble human milk galactosyltransferase on the immuno precipitation of galactosyltransferase polypeptides from HeLa cells. HeLa cells were labeled in the presence of [^{35}S]methionine, and the cell lysate was split in three equal aliquots. Immunoprecipitation was carried out with pre-immune serum (lane 1), with anti-serum in the absence (lane 2) and presence of 3 μg pure milk galactosyltransferase (lane 3).

lower apparent molecular weight (M_r=52,000 vs. 54,000) compared to the "mature" Golgi enzyme synthesized in control cells (compare Fig. 1, lane "TM", band marked with M and lane "C"). The band with M_r of less than 42,000 (approx. 30,000) is probably due to protein degradation. Addition of dog pancreas microsomes caused even more degradation. Together with the fact that galactosyltransferase mRNA is present in relative very low quantities this phenomenon made it impossible to study galactosyltransferase translocation through microsomal membranes. When total RNA from HepG2 cells, a human hepatoma cell line, was used, the same results were obtained after immunoprecipitation (not shown). In Fig. 2 the specificity of the immunoprecipitation reaction is demonstrated; pulse labeled HeLa cells were solubilized and used for immunoprecipitation. [^{35}S]methionine labeled galactosyltransferase polypeptides can effectively be quenched by the addition of the milk (soluble) enzyme. In addition, immunoprecipitation is solely dependent on the addition of specific antiserum.

The presence of two different precursor polypeptides is notable. Previously, we obtained some evidence for a partial identity of the two bands using limited digestion of the biosynthetically labeled precursor polypeptides (5). Additional evidence for two different galactosyltransferase polypeptides comes from biosynthetic labeling of HeLa cells, using a very short pulse (5 min) and short chase periods. No precursor-product relationship could be established, as both polypeptide chains were present in the same relative amounts just after the pulse-labeling as well as after various chase periods (not shown). The present experiments clearly show that the two precursor polypeptides of galactosyltransferase originate from two different messenger RNAs. Paquet *et al* found that the addition of galactose residues onto biantennary asparagine-linked oligosaccharides is accomplished sequentially, and that the galactose located on the $\alpha_{1,3}$-linked branch is transferred first, whereas the galactose found on the $\alpha_{1,6}$-linked branch is transferred second (13). It is tempting to speculate that two different, but closely related, enzymes are involved in sequential addition of galactose residues onto the two branches.

In order for galactosyltransferase polypeptides to reach the lumen of the rough ER a signal peptide must reside somewhere in the polypeptide. For most secretory proteins the signal peptide is present as a N-terminal hydrophobic peptide of about 20 amino acid residues, which is co-translationally removed at the luminal side of the rough ER membrane. As galactosyltransferase precursor polypeptides synthesized in tunicamycin treated HeLa cells have molecular weights identical to the *in vitro* products, it is likely that galactosyltransferase polypeptides have a non-cleavable, presumably, internal signal peptide. In several other instances it has been shown that certain membrane proteins contain internal signal sequences: the transferrin receptor (14), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (15), HLA-DR invariant chain (16), gamma-glutamyl transpeptidase (17), the glucose transporter (18), the erythrocyte anion transport protein (19) and the asialoglycoprotein receptor (20,21). Some of these membrane proteins (the glucose transporter, 3-hydroxy-3-methylglutaryl-coenzyme A reductase and opsin (22)), seem to have their uncleaved N-termini present at the extracellular membrane face, while in the case of others (the transferrin receptor, the HLA-DR invariant chain, gamma-glutamyl transpeptidase, the erythrocyte anion transport protein (21) and the asialoglycoprotein receptor) an uncleaved N-terminus protudes into the cytosolic space with a reversed orientation in the membrane, i.e. the N-terminus is at the cytoplasmic and the C-terminus at the cisternal or extracellular face of the membrane. Recently, cDNA coding for human as well bovine galactosyltransferase was isolated and cloned (23-26). The amino acid sequences derived from the cloned DNA did not contain information about the way galactosyltransferase interacts with the Golgi membrane. However, it is

apparent that it is not the C-terminus of the protein which is associated with the membrane, as the fragment contains 2 attachment sites for N-linked oligosaccharides but no hydrophobic peptide fragment (24). The available observations are not yet conclusive about the exact membrane topology of the enzyme. We speculate that the C-terminus of galactosyltransferase is at the luminal side of rough ER and Golgi complex.

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