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2-Deoxy-2-fluoro-D-galactose protein *N*-glycosylation

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2-Deoxy-2-fluoro-D-galactose (dGalF), added to the medium of primary cultured rat hepatocytes, inhibited *N*-glycosylation of membrane (gp 120) and secretory glycoproteins (α_1-macroglobulin) in a concentration dependent manner. Complete inhibition of *N*-glycosylation was achieved at concentrations of 1 mM and above. At identical concentrations, 2-deoxy-2-fluoro-D-glucose (dGlcF) caused only incomplete inhibition of *N*-glycosylation. dGalF reduced incorporation of D-[2,6-^{3}H]mannose into lipid-linked oligosaccharides indicating interference with their assembly in the dolichol cycle.

Inhibition of *N*-glycosylation; Glycoprotein; Tunicamycin; 2-Deoxy-2-fluoro-D-galactose; 2-Deoxy-2-fluoro-D-glucose; Hepatocyte

1. INTRODUCTION

N-Glycosylation of proteins involves three steps: (1) the assembly of a common lipid-linked oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$, in the endoplasmic reticulum; (2) its transfer to nascent or newly synthesized polypeptides at Asn-X-Ser/Thr-sequences; and (3) processing of the protein-bound oligosaccharide to yield either high-mannose-type, hybrid-type or complex-type oligosaccharides on the mature glycoproteins. Processing includes removal of glucose and mannose residues by glucosidases and mannosidases in the endoplasmic reticulum and in the Golgi apparatus, and transfer of peripheral sugars GlcNAc, Gal, Fuc and NeuAc by specific glycosyltransferases in the Golgi apparatus and the trans-Golgi-network [1].

Different inhibitors of *N*-glycosylation have been described that affect either the assembly of dolichol-pyrophosphate-linked oligosaccharides or processing of the protein-bound oligosaccharides [2,3]. The most prominent inhibitor of lipid-bound saccharide formation, tunicamycin, inhibits the first step of the dolichol cycle, i.e. transfer of GlcNAc-1-P from UDP-GlcNAc to Dol-P, and may thus block *N*-glycosylation of newly synthesized proteins completely. Furthermore, metabolites of 2-deoxy-D-glucose [4] and of fluoro-deoxy-sugars, e.g. 2-deoxy-2-fluoro-D-glucose (dGlcF) [5], 2-deoxy-2-fluoro-D-mannose [6], and 4-deoxy-4-fluoro-D-mannose [7] have been shown to interfere with dolichol-pyrophosphate-linked oligosaccharide assembly. Tunicamycin and the various sugar analogues provided valuable tools for studying the function of glycoprotein glycans [8,9]. These observations prompted us to investigate the effects of dGalF on *N*-glycosylation. Evidence is provided that dGalF inhibits *N*-glycosylation of both membrane and secretory glycoproteins, to a similar extent as tunicamycin.

Abbreviations: dGalF, 2-deoxy-2-fluoro-D-galactose; dGlcF, 2-deoxy-2-fluoro-D-glucose; Dol-P, dolichyl monophosphate; Dol-PP, dolichyl pyrophosphate; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; SDS, sodium dodecylsulfate.

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2. MATERIALS AND METHODS

2.1. *Materials*

Constituents of tissue culture media were obtained from Biochrom (Berlin, Germany); other materials for tissue culture were purchased from Falcon (Heidelberg, Germany) or Nunc (Wiesbaden, Germany). Dulbecco's minimal essential medium dry powder (DMEM) and horse serum were obtained from Gibco (Berlin, Germany). DMEM, without L-methionine and D-glucose, but containing 10 mM pyruvate, was prepared according to DMEM formula. DGalF was synthesized as described previously [10] using gaseous F_2 and tri-*O*-acetyl-D-galactal as substrate and further purified to homogeneity by HPLC on a preparative Eurokat H column (Eurochrom Säulentechnik, Berlin, Germany). dGlcF was obtained from Sigma (München, Germany) and tunicamycin was purchased from Calbiochem (Frankfurt am Main, Germany). Protein A-Sepharose was obtained from Pharmacia (Freiburg, Germany). L-[^{35}S]Methionine (>30 TBq/mmol) and D-[2,6-^{3}H]mannose (2.6–3.1 TBq/mmol) were purchased from Amersham Buchler (Braunschweig, Germany). The monoclonal antibody 47.2, directed against a rat liver membrane glycoprotein (manuscript in preparation), and monoclonal antibody 11.1, recognizing α_1-macroglobulin [11], were purified from the respective hybridoma cell culture supernatants by established procedures.

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2.2. *Cell culture*

Primary hepatocytes were isolated according to the protocol of Seglen [12] with a minimum viability of 85–90%, as evidenced by Trypan blue exclusion test, and plated on Collagen I-coated plastic dishes. Cells were maintained in DMEM supplemented with antibiotics (penicillin and streptomycin), 10^{-9} M insulin, 10^{-8} M dexamethason and 5% complement-inactivated horse serum in a humidified atmosphere with 5% CO_2 at 37°C. Cells were allowed to adhere overnight.

2.3. *Metabolic labelling of cells*

The obtained monolayers were washed twice with PBS and were pre-incubated with inhibitors for 60 min in DMEM without L-methionine and D-glucose, but supplemented with 10 mM pyruvate, 0.5 mM uridine and 1 mM phosphate. Hepatocytes were metabolically labelled by addition of either 1.85 MBq L-[^{35}S]methionine or D-[2,6-^{3}H]mannose per dish. Inhibitor concentrations were maintained throughout the labelling period.

2.4. *Isolation of lipid-bound oligosaccharides*

After labelling of cells with D-[2,6-^{3}H]manose for 4 h, medium was removed by aspiration, cells were washed twice with ice-cold PBS and harvested in PBS by scraping with a rubber policeman. An aliquot was taken for protein determination. Lipid-bound oligosaccharides were extracted according to Schmitt and Elbein [13]. Briefly, the lipid-bound saccharides were extracted from cell pellets with chloroform/methanol/water (1:1:1, by vol.). Lipid-bound oligosaccharides were extracted with chloroform/methanol/water (10:10:3, by vol.). The incorporated radioactivity was determined by liquid scintillation counting.

2.5. *Immunoadsorption and SDS-polyacrylamide gel electrophoresis*

All steps were carried out at 0–4°C. After metabolic labelling cells were harvested as detailed above. For detergent extraction cells were resuspended in 5 ml lysis buffer A (0.15 M NaCl, 0.01 M Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM phenylmethylsulfonylfluoride, 1% NP-40) and further kept on ice for 2 h. Detergent-insoluble material was removed by centrifugation (105 000 × g, 30 min). The supernatants were pre-cleared by shaking end-over-end with 100 mg Sepharose 4B for at least 2 h. Sepharose 4B was pelleted by centrifugation. Supernatants were removed and 10 μg of monoclonal antibody (47.2 or 11.1) coupled to 7 mg protein A-Sepharose were added as suspension in lysis buffer A. The suspension was shaken end-over-end at 4°C for at least 4 h. Immunocomplexes bound to protein A-Sepharose were pelleted by centrifugation and washed 5-times with washing buffer B (0.5 M NaCl, 0.01 M Tris-HCl, pH 8.0, 1 mM EDTA, 1% NP-40). For a final washing step PBS was used. Immunocomplexes were eluted by boiling for 3 min in 20 μl SDS-electrophoresis sample buffer and were separated in 7.5% acrylamide gels in the presence of 0.1% SDS as described by Laemmli [14]. Fluorography was performed as described by Bonner and Laskey [15].

2.6. *Glycopeptidase F-treatment*

Digestion of immunopurified gp 120 with PNGase F was essentially performed as recently described [16].

3. RESULTS AND DISCUSSION

dGalF inhibited *N*-glycosylation of membrane and secretory glycoproteins of primary cultured rat hepato-

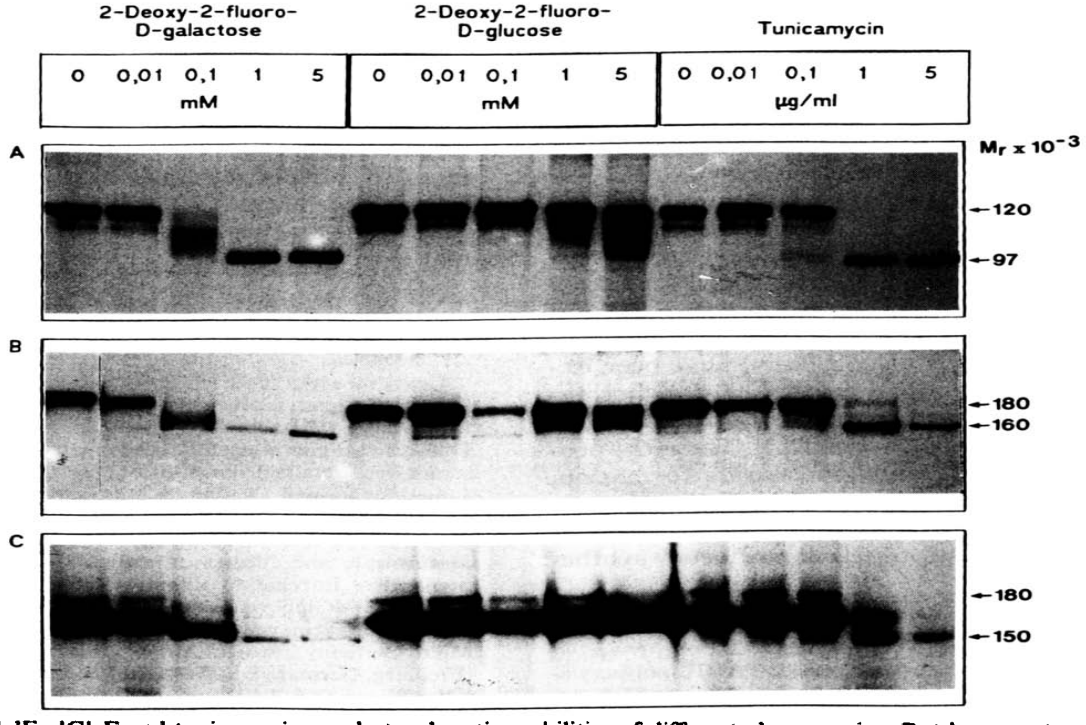

Fig. 1. Influence of dGalF, dGlcF and tunicamycin on electrophoretic mobilities of different glycoproteins. Rat hepatocytes were labelled with 1.85 MBq/ml L-[^{35}S]methionine in the presence of increasing concentrations of dGalF, dGlcF and tunicamycin for 10 h. Cells were detergent-extracted as described in section 2 and different antigens were immunoprecipitated either from cell extracts or from cell culture supernatants. Immunoprecipitation of: (A) gp 120 from cell extract; (B) α_1-macroglobulin from cell extract; (C) secreted α_1-macroglobulin from cell culture supernatants. Arrows indicate M_r of polypeptide bands determined by comparison to M_r marker proteins.

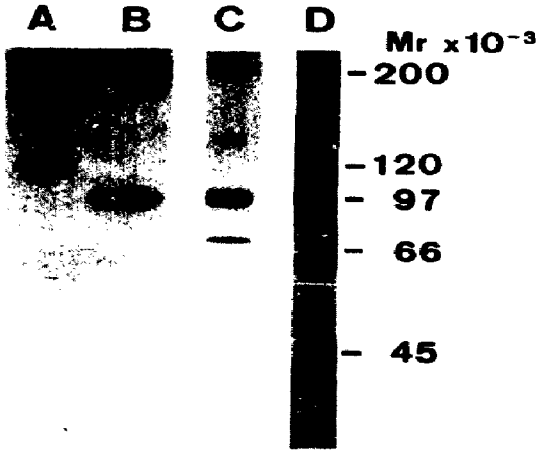

Fig. 2. Digestion of gp 120 immunoprecipitated from rat hepatocytes with PNGase F. Gp 120 was immunoadsorbed from hepatocytes after metabolic labelling with 1.85 MBq/ml for 10 h, and was subsequently either mock-treated (A) or treated with PNGase F (B). (C) gp 120, immunoadsorbed from detergent-extracts of hepatocytes metabolically labelled in the presence of 2 μg/ml tunicamycin. (D) gp 120 immunoisolated from hepatocytes treated with 1 mM dGalF.

cytes in a dose-dependent manner, as shown by comparing the effect of dGalF to that of the well-characterized glycosylation inhibitor tunicamycin. Hepatocytes were treated with increasing concentrations of dGalF and metabolically labelled with L-[^{35}S]methionine. dGalF, at concentrations of 1 mM and above, caused a reduction in the M_r of the membrane glycoprotein gp 120 and of the secretory glycoprotein α_1-macroglobulin, as shown by SDS-PAGE, that was indiscernible from M_r shifts obtained after treatment of cells with 1 and 5 μg/ml of tunicamycin (Fig. 1). At these concentrations tunicamycin completely blocks protein *N*-glycosylation in rat hepatocytes [17,18]. As shown for the membrane glycoprotein gp 120, the same shift in M_r was obtained by enzymatic *N*-deglycosylation with PNGase F (Fig. 2), cleaving oligosaccharides of both complex and high-mannose-type glycans [19,20]. At concentrations in between (0.1 mM and 1 mM) dGalF caused the formation of glycoproteins with M_r in between that of the completely glycosylated proteins and that of the non-*N*-glycosylated variants, indicating an incomplete inhibition of *N*-glycosylation (Fig. 1).

Table I

Metabolic products of dGalF in liver

Metabolite	Content (μmol/g)
dGalF-1-P	1.21 ± 0.44
UDP-dGalF	0.85 ± 0.17
UDP-dGlcF	1.64 ± 0.16

The contents of dGalF metabolites were analyzed by enzymatic analysis in combination with anion-exchange HPLC of the nucleotides [21] 5 h after injection of dGalF at a dose of 1 mmol/kg body weight. Mean values from 5 livers ± SD. The metabolite identification was confirmed by ^{19}F-NMR spectroscopy [21].

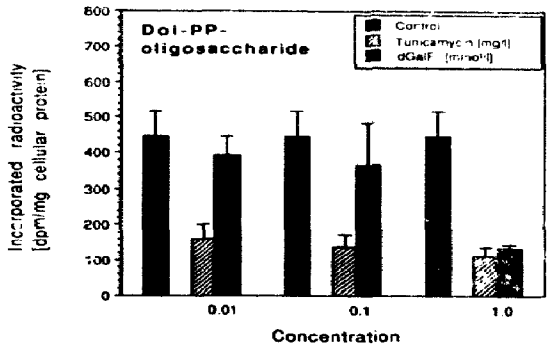

Fig. 3. Influence of tunicamycin and dGalF on the incorporation of D-[2,6-^{3}H]mannose into lipid-bound oligosaccharides of rat hepatocytes. Cells seeded semiconfluently in 6-well dishes were pre-incubated with different amounts of tunicamycin or dGalF for 1 h, and were labelled with 1.85 MBq/well D-[2,6-^{3}H]mannose for 6 h in the presence of the inhibitor. Cells were washed and lipid-bound saccharides were extracted as described in section 2. Radioactivity (dpm) per mg of cell protein. Data are means ± SEM for triplicate determinations.

In rat hepatocytes dGalF is metabolized like D-galactose via the Leloir pathway to dGalF-1-phosphate, UDP-dGalF and UDP-dGlcF (Table I and [21]). Therefore, the inhibitory effect of dGalF was compared to that of dGlcF, which was also shown to interfere with protein *N*-glycosylation [22]. In order to rule out that different effects on *N*-glycosylation reflect competition phenomena in cellular uptake of dGlcF, D-glucose was omitted from labelling media in these experiments. In contrast to dGalF, only partial inhibition of *N*-glycosylation was obtained with dGlcF at concentrations of 1 mM and at even higher concentrations (Fig. 1). Further evidence that dGalF, and not its conversion to dGlcF, is essential for the inhibitory effect on *N*-glycosylation was provided by experiments in which the epimerization of dGalF to dGlcF was inhibited. Inhibition of UDP-Glc/UDP-Gal-4′-epimerase by addition of 20 mM ethanol to the labelling medium according to [23] did not affect inhibition of *N*-glycosylation by dGalF (data not shown).

All known inhibitors of *N*-glycosylation, tunicamycin, 2-deoxy-D-glucose, 2-deoxy-2-fluoro-D-glucose, 4-deoxy-4-fluoro-D-mannose and 2-deoxy-2-fluoro-D-mannose have been shown to interfere with the assembly of the lipid-linked oligosaccharides in the dolichol cycle [3,6,7,21]. In an attempt to characterize the inhibitory mechanism of dGalF, its effect on the incorporation of D-[2,6-^{3}H]mannose into lipid-linked oligosaccharides was studied in hepatocytes and compared to the effect of tunicamycin. Both tunicamycin and dGalF significantly decreased incorporation of mannose into Dol-PP-linked oligosaccharides (Fig. 3). This indicates that dGalF may interfere either with the formation of the mannose donors GDP-Man or Dol-P-Man, or with

the transfer of mannose residues to Dol-PP-linked oligosaccharides. GDP-4dManF, the metabolite of another fluorosugar, 4-deoxy-4-fluoro-D-mannose (4dManF), inhibits oligosaccharide assembly by blocking the formation of Dol-PP-$GlcNAc_2Man_2$ [24], whereas tunicamycin is known to block the first step of the dolichol cycle, i.e. formation of Dol-PP-GlcNAc by inhibition of GlcNAc-1P-transferase [25]. The use of dGalF in conjunction with other inhibitors could be used to further characterize distinct steps in the formation of dolichol-linked oligosaccharides.

Additionally, dGalF as a potent inhibitor of *N*-glycosylation of secretory and membrane glycoproteins may be a useful tool to study the biological roles of protein *N*-glycosylation.

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