Glycosylation Defects: From Transgenic Animals to Inborn or Acquired Errors of Metabolism

S01-01

CONDITIONAL TARGETING OF GLYCOSYLTRANSFERASE GENES

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To address the role of specific glycosylation pathways in mammalian development and physiology, we have generated mice with null-mutations in genes encoding glycosyltransferases that act at key points along these pathways. To better characterize the potential pleiotropic effects of such gene disruptions, we have selected an approach based on the Cre/loxP recombination system, which allows both systemic and tissue-restricted gene ablations. Mutated alleles of the genes coding for a polypeptide N-acetylgalactosaminyltransferase, which initiates the O-linked glycosylation pathway, and for α2,6-sialyltransferase, a terminal transferase acting on N-linked and O-linked glycans, have been introduced into the mouse genome. The phenotypes of mice carrying systemic and thymocyte-specific null-mutations at these genes will be described.

S01-02

MOLECULAR GENETICS OF THE HISTO-BLOOD GROUP ABO SYSTEM

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The Histo-blood group ABO system constitutes one of the major allogeneic antigen systems in man. Blood group A and B individuals express glycosyltransferases adding GalNAc or Gal to the precursor H structure resepectively, while O individuals have a defect gene thus being incapable of converting the H precursor. Within the last five years knowledge of the molecular genetic basis of these inherited changes or defects in glycosylation have been obtained through cloning of the allelic ABO genes. This presentation will update developments in our understanding of the genetic basis of the ABO polymorphisms, the ABO locus, as well as describe methods for DNA genotyping.

S01-03

GLYCOSYLATION DEFICIENCY
The role of β1,3 galactosyltransferase in the Tn-syndrome

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The Tn-syndrome is a rare acquired disease due to a deficiency of β1,3galactosyltransferase (gal-T), leading to hemolytic anemia and thrombopenia and affecting all hemopoietic lineages. In patient R.R. 93% of erythrocytes expressed Tn- or sialosyl-Tn cell surface epitopes (GalNAc-R or Neu5Ac-GalNAc-R) in contrast to only 4% of peripheral lymphocytes. These lymphocytes, which were totally lacking the fully elongated Neu5Acα(2→3)Galβ(1→3)[Neu5Acα(1→6)]GalNAcα(1→R) mucin type epitope were cloned by limiting dilution. Application of the transcriptional activators 5'-azacytidine and Na n-butyrate led to reexpression of gal-T activity and its product on Tn^+ lymphocytes. Another severe disease also showing a glycosylation deficiency is IgA nephropathy, which is characterized by an impaired uptake of IgA_1 by liver and kidney. Incomplete galactosylation of mucin type glycans in the hinge region of IgA_1 was discovered recently and attributed a pathogenetic role. This disease also focuses the interest on gal-T, an enzyme which has not been cloned yet. Work is in progress aimed at cloning this enzyme by a subtraction strategy based on cDNA libraries derived from Tn^+ and normal T-lymphocytes.

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S01-04

CARBOHYDRATE-DEFICIENT GLYCOPROTEIN (CDG) SYNDROMES.

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The CDG syndromes are genetic, multisystemic disorders characterized by deficiencies of the carbohydrate moiety of glycoproteins. We have reported the first patients in 1980 (Pediatr Res 14: 179) and as of November 1995 at least 180 patients from some 20 countries were known. Two types have been well delineated. The large majority of the patients belong to type I. We know only two families with type II. The basic defect in the latter has been identified as a Golgi localised N-acetylglucosaminyltransferase II (Arch Dis Child 1995; 71: 123) which is coded by a gene on chromosome 14q21. In type I, evidence has been presented for a defect at the level of the endoplasmic reticulum and for linkage with 16pl3.3-13.12. (Hum Molec Genet 1994; 3:2037). Very recently Van Schaftingen and Jaeken have confirmed an early glycosylation defect (in preparation).

S01-05

CLONING AND CHARACTERIZATION OF *ALG3* FROM *SACCHAROMYCES CEREVISIAE*

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The *alg3*-mutant of *S. cerevisae* is defective in the biosynthesis of lipid-linked oligosaccharides. Specifically, the defect results in an accumulation of Man5GlcNAc2-PP-Dol and the transfer of EndoH-resistant saccharides to secretory proteins. Due to lack of a selectable phenotype, the gene was refractory to cloning so far. We isolated the *ALG3* gene by complementation of an *alg3-1stt3-3* double mutant, which is temperature-sensitive at 37°C (Zufferey et al. EMBO J., 14:4949-4960, 1995). The *ALG3* gene encodes a membrane protein of 458 aa/53 kD with a C-terminal KKXX-sequence, suggesting localization in the endoplasmic reticulum. The isolated *ALG3* gene complements the defects of the *alg3-1* mutant and the disruption of the *ALG3* gene results in the *alg3-1* phenotype. Antibodies raised against a Gst-Alg3-fusion protein recognize Alg3p as a 44 kD in Westernblot experiments.

S01-06

SELECTION OF GPI-MUTANTS IN YEAST *S. CEREVISIAE*

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α-agglutinin is a Glycosyl Phosphatidylinositol (GPI)-anchored glycoprotein that gets expressed at the cell surface of Mat α cells after induction with type a mating factor.

We tried to obtain mutants in GPI-biosynthesis complementation classes residing in other ones previously obtained. We mutagenized the wild type α cells first transformed with a multicopy plasmid harbouring a chimeric gene composed of the Gal7 promoter, the α-galactosidase as reporter gene, terminating with the 3' part of α-agglutinin gene encoding for the GPI-anchoring signal sequence.

Transformed cells give blue colonies on a medium containing galactose and X-α-Gal.

After mutagenesis and a first selection by immunoselection of potential GPI-mutants, we replica-plated cells on the inducing medium. White colonies were kept and analysed. After elimination of secretory mutants, we radiolabeled *in vitro* the remaining cells using one of the first substrat of the GPI-biosynthesis : the [^{3}H]-UDP-GlcNAc. Several mutants with abnormal lipid profiles were isolated.

S01-07

alg9 AND *alg10* : TWO NEW MUTANTS AFFECTING THE BIOSYNTHESIS OF DOLICHOL-LINKED OLIGOSACCHARIDES IN *SACCHAROMYCES CEREVISAE*

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N-linked glycosylation is an essential modification of proteins and follows a highly conserved pathway in eukaryotic cells. The core oligosaccharide $Glc_3Man_9GlcNAc_2$ is assembled on the lipid carrier dolichol phosphate and transferred en bloc to selected asparagine residues of nascent polypeptide chains. This transfer is catalyzed by the enzyme *N-oligosaccharyltransferase* .
The assembly of the lipid-bound core oligosaccharide occurs at the membrane of the endoplasmic reticulum. In *S. cerevisiae, alg* (asparagine-linked glycosylation) mutants deficient in the assembly of the core oligosaccharide were isolated. *ALG* genes required for the early steps in the assembly pathway are essential, whereas mutations in *ALG* genes necessary for late reactions show no detectable growth defect. However, *alg* mutations in combination with a mutation affecting the *oligosaccharyltransferase* activity have a synthetic lethal phenotype at 30°C. Based on this phenotype, we initiated a screen for novel mutations affecting the biosynthesis of the lipid-linked oligosaccharide. The red/white sectoring assay was applied. Mutations in 8 different complementation groups show glycosylation defects. They define known *ALG* loci and two novel loci: *ALG9* and *ALG 10*. *alg9* mutants accumulate dolichol-linked $Man_6GlcNAc_2$, *alg10* mutants dolichol-linked $Glc_2Man_9GlcNAc_2$. The corresponding genes were cloned by complementing the temperature sensitive phenotype of the *alg wbp1* double mutants. The *ALG9* locus encodes a putative mannosyl transfersase and deletions in this gene led to accumulation of lipd-linked $Man_6GlcNAc_2$ *in vivo* and in underglycosylation of secreted proteins. Genetic and biochemical experiments support the hypothesis that the assembly of the lipid-linked oligosaccharide in the lumen of the ER occurs in a stepwise fashion.

S01-08

OST5 IS A NOVEL SUBUNIT OF YEAST OLIGOSACCHARYL-TRANSFERASE

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N-linked glycosylation of secretory proteins is an essential and highly conserved process in all eukaryotes. The core oligosaccharide $Glc_3Man_9GlcNAc_2$ is transferred en bloc to selected asparagine residues of nascent polypeptide chains by the multimeric enzyme *N-oligosaccharyltransferase*. Thus the enzyme is located at the site of translocation ino the ER. In *S. cerevisiae* , afirst mutant (*wbp1*) with a reduced oligosaccharyltransferase activity has been described [1]. A combination of *wbp1* with individual *alg* mutations deficient in the biosynthesis of the core oligosaccharide results in a synthetic temperature sensitivity of the double mutant [2].

Based on this synthetic phenotype, we have employed a mutagenesis screen to identify new mutants that are synthetic lethal with *alg* and affect oligosaccharyltransferase function *in vivo*. Seven complementation groups with glycosylation defects were isolated. One of them is complemented by *OST5*, encoding a small , non-essential subunit of the transferase complex. Although Δ*ost5* results in a comparatively weak underglycosylation phenotype, its synthetic effect in combination with *alg* is rather strong. *OST5* seems to promote binding of the lipid-linked oligosaccharide substrate to the enzyme.

[1] teHeesen, S. et al. (1992) EMBO J., 11, 2071-2075
[2] Stagljar, I. et al. (1994) Proc.Natl.Acad.Sci.U.S.A., 91, 5977-5981

S01-09

LOCALIZATION OF ANTICOAGULANT HEPARAN SULFATE PROTEOGLYCANS IN RAT OVARIAN FOLLICLES

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At ovulation coagulation enzymes are activated around ovulatory follicles, while the follicular wall undergoes proteolytic breakdown. These activities are tightly regulated in time and space and heparin-activated protease inhibitors are present in the follicle. We have characterized anticoagulant heparan sulfate proteoglycans (aHSPGs) in cultured ovarian granulosa cells (GC). aHSPGs bind and activate anthithrombin III (AT) conferring antithrombotic properties to the vascular wall. In this study we have localized GC aHPSGs in rat ovary cryosections at different stages of natural and gonadotropin-induced cycles. Sections were incubated with ^{125}I-AT, which binds specifically aHSPGs, and labeling was revealed by autoradiography. aHSPGs were detected on GC from all antral follicles but not in preantral follicles. During the ovarian cycle the follicles recruited to ovulate display increasing amounts of aHSPGs on GC with maximum ^{125}I-AT-labeling during the peri-ovulatory period. These data indicate that aHSPGs are upregulated along the ovarian cycle; they might be critically located to modulate proteolytic activities at ovulation and prevent inappropriate clotting of follicular fluid before expulsion of the oocyte.

S01-10

Characterization of β-secretase cleavage of the amyloid precursor protein in human HEK-293 cells. *G. Schrader-Fischer and P. A. Paganetti. Sandoz Research Institute. CH-3001 Berne.*

β-amyloid (βA4) secretion occurs upon cleavage of the amyloid precursor protein (APP) by β-secretase (N-terminus of βA4) and γ-secretase (C-terminus). When APP was truncated at the N-term. of βA4, a robust cellular secretion of βA4 was observed. Truncation after amino acid ßA4-40, -42 or -43 (at the C-term. of βA4) abolished βA4 secretion. Thus C99, the C-terminal metabolite of APP produced by β-secretase, is likely to be the processing intermediate of βA4. The role of the endosomal/lysosomal pathway in βA4 generation was investigated with a) agents that raise the pH in acidic compartments and b) APP molecules lacking endocytosis consensus sequences. Our data indicate that the formation of βA4 from APP carrying the Swedish mutation occurs in an acidic compartment along the secretory pathway. In sharp contrast to this, βA4 generation from wtAPP occurs also in the endosomal/lysosomal compartment. The role of lysosomes was further studied with APP molecules carrying signals for lysosomal targeting. We obtained evidence that βA4 may be produced in lysosomes but that, in these organelles, it is rapidly degraded. Therefore, lysosomes may not contribute to βA4 accumulation in the brain.

S01-11

REMODELLING OF GPI-ANCHORS ON PROTEINS IN YEAST *SACCHAROMYCES CEREVISIAE*

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The GPI-anchors in yeast *Saccharomyces cerevisiae* have two different lipid moieties: diacylglycerol (DAG) and ceramide (Cer). These two moieties are found in the GPI-anchors bound to the proteins, but the biosynthetic precursors have only DAG moieties.
We used [^{3}H]-radiolabeled dihydrosphingosine, the precursor of ceramides, to radiolabel proteins. This radiolabeling is specific for GPI-anchored proteins, since after PI-PLC or GPI-PLD treatement no protein remains radiolabeled. Inhibiting protein translation with cycloheximide, we could empty the ER of GPI-anchored proteins and demonstrate that the remodelling of GPI-anchor lipid moieties takes place in the Golgi. Similary, if we blocked the vesicular traffic between ER and Golgi, GPI-anchored proteins couldn't get remodelled because the putative substrate proteins couldn't reach the Golgi.

S01-12

ALTERATIONS OF PLASMA PROTEINS IN ALCOHOLIC PATIENTS ANALYZED BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

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Several reports have shown that excessive consumption of ethanol interferes with protein synthesis, secretion and glycosylation. This study was undertaken to identify the plasma protein alterations in alcoholic patients. By two-dimensional gel electrophoresis combined with silver staining of proteins, we identified the carbohydrate-deficient transferrin, 2 new glycoprotein alterations and an immunoglobulin modification. The glycosylation modifications of the plasma proteins were caracterized by lectin blotting followed by chemiluminescence detection.

S01-13

IMMUNODETECTION OF FucT-VI IN TRANSFECTED COS CELLS AND DETECTION OF A CROSSREACTIVE ANTIGEN IN WEIBEL-PALADE BODIES IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVECS)

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Cell surface α1,3- and α1,4- fucosylated glycans play an important role in adhesion to vascular endothelia during the inflammatory process. α1,3 fucosyltransferases (FucTs) are responsible for the final step of synthesis of sialyl-Lewis^x [sLe^x] determinant. From a family of 5 different Fuc-Ts, cloned till now, we concentrated our interests on FucT-VI, FucT-V. Antibodies were raised against two non-conserved peptide stretches for FucT-VI. These antibodies were characterized by ELISA, immunofluorescence, and immunoprecipitation of transiently transfected COS cells with FucT-VI. One of them, PEP6B antibody, recognizes specifically FucT-VI. No crossreactivity with FucT-III or FucT-VII was observed, but partial crossreactivity with FucT-V. In transfected COS cells, specific FucT-VI staining was confined to the ER, but no Golgi staining was observed. The same cells expressed surface sLe^x as revealed by double staining. The antibody immunoprecipitated a 48 kD glycoprotein from FucT-VI-transfected but not mock transfected COS cells, compatible with the size of FucT-VI. On the basis of published results on the presence of FucT-VI transcripts in HUVEC cells, these cells were stained with PEP6B antibody. Unexpectedly, bright staining of Weibel-Palade bodies was detected and confirmed by co-localization with P-selectin and von Willebrand factor using laser scanning fluorescence microscopy.

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S01-14

COMPARATIVE LOCALISATION OF THREE LATE ACTING GLYCOSYLTRANSFERASES

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The Golgi apparatus consists of at least four different compartments: *cis*, medial, *trans* and *trans* Golgi network (TGN). Soluble and cell surface glycoproteins pass the Golgi apparatus in a *cis* to *trans* direction where they acquire their sugar moieties. Early acting glycosyltransferases were shown to predominate in *cis* cisternae whereas late acting enzymes are mainly found in *trans* Golgi/TGN. The TGN probably consists of functionally different subdomains. The α 2,3 N-Sialyltransferase (ST3N) is another late acting enzyme in the synthesis of N linked oligosaccharides. It forms the terminal NeuAc α-2,3 Gal β-1,3(4) GlcNAc carbohydrate group of glycoproteins and glycolipids. A pool of mRNA from different cell lines was used to amplify by specific primers the ST3N sequence. A fragment of the PCR product was cloned into the expression vector pEx2, expressed in *E. coli* as a β-Galactosidase-ST3N fusion protein, purified and used for the immunization of a rabbit. The antibody we obtained was characterized by ELISA, by immunofluorescence on ST3N transfected COS cells and by western blot. This antibody, together with previously described antibodies against two other late acting enzymes, namely β1,4-galactosyltransferase (GT) and α-2,6 sialyltransferase (2,6ST) were used for comparative localisation studies in double immunofluorescence microscopy. ST3N co-localizes with GT and 2,6ST in a compact, juxtanuclear organelle typical for the Golgi apparatus in several human, simian and rat cell lines. The antibody will also be used for investigation of cell- and tissue-specific expression on human and rat tissues.

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S01-15

ASSESSMENT OF β1,4 GALACTOSYLTRANSFERASE AND α2,6 SIALYLTRANSFERASE ENZYME EXPRESSION IN THE GOLGI APPARATUS OF B-LYMPHOCYTES BY FLOW CYTOMETRY

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Up to now, quantification of Golgi glycosyltransferase expression has been estimated by measurement of specific activity or densitometry of western blots. These methods refer to a protein lysate of the investigated cells. Using flow cytometry, it is possible to quantify the expression of intracellular antigens on a single cell event level. Using permeabilized Epstein-Barr virus transformed B lymphoblastoid cells as a model cell line for native B-lymphocytes, we established a flow cytometric method for the measurement of Golgi β1,4 galactosyltransferase (GT) and α2,6 sialyltransferase (ST) enzyme expression. Measurements of intracellular GT using either mAb GT2/36/118 to human milk GT or affinity purified polyclonal F(ab)$_2$ fragment of a polyclonal protein specific antiserum to GT or ST, resp., showed a monomodal and reproducible mean fluorescence intensity (MFI) increase over the corresponding negative controls. Furthermore, mixing of defined portions of unlabeled cells with mAb-labeled cells resulted in expected bimodal distributions of fluorescent cells confirming the Golgi as carrier of the fluorescent signal. Staining of the Golgi apparatus was ascertained by conventional immunofluorescence microscopy and confocal laser scanning microscopy. Simultaneous detection of GT and ST using two color flow cytometry has been performed. Further work is aimed at using this method to investigate α2,6 sialyltransferase enzyme upregulation upon stimulation of peripheral blood B-lymphocytes by various factors as well as possible upregulation of other glycosyltransferases.

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S01-16

RECOMBINANT β1,4 GALACTOSYLTRANSFERASES EXPRESSED IN *S. CEREVISIAE*: PURIFICATION, CHARACTERIZATION AND COMPARISON WITH HUMAN ENZYME

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β1,4 galactosyltransferase (gal-T, E.C.2.4.1.38) transfers Gal from UDP-Gal to GlcNac or GlcNac-R. Soluble gal-T, purified from human breast milk, was shown to be very heterogeneous by isoelectrofocusing (IEF). In order to produce sufficient homogeneous enzyme for 3D analysis, the human gal-T (hgal-T) has been expressed in *S. cerevisiae*. Scaling-up produced 187 U of recombinant gal-T (rgal-T) in a 270 l fermentation. rgal-T has been purified to homogeneity. Analysis on SDS-PAGE revealed hyperglycosylated forms of rgal-T. The N-linked mannan prevented recognition by antibodies. Analysis on IEF revealed considerable heterogeneity of rgal-T. The N-glycan could be removed by Endoglycosidase H treatment. The N-deglycosylated form of rgal-T retained full activity and showed only 3 isoforms by IEF analysis. Next we abolished the single N-glycosylation consensus sequence by site-directed mutagenesis. The soluble mutated enzyme (Ndrgal-T) was expressed in *S. cerevisiae* and scaled up to 60 U. Using the same purification protocol as described above, Ndrgal-T has been purified to homogeneity. Ndrgal-T was resolved in 2 bands by IEF analysis. The kinetic parameters of Ndrgal-T were shown not to differ to any significant extent from those of the hgal-T and no significant changes in CD spectra were observed. Light scattering analysis revealed dimerisation of both enzymes. These data indicate that Ndrgal-T was correctly folded and thus suitable for 3D studies.

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S01-17

THE TRANSMEMBRANE DOMAIN OF HUMAN MEPRIN α IS INVOLVED IN ITS ER RETENTION

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Human Meprin (EC 3.4.24.18) is a Zn-metallo-endopeptidase. It is a type I transmembrane protein consisting of differentially processed glycosylated α and β subunits. In order to investigate the possible role of the transmembrane and/or the C-terminal domain of the α and β subunits we exchanged these domains by recombinant PCR. On the basis of enzymatic activity, misfolding of the mutant proteins could be excluded. Biosynthesis and posttranslational processing of these mutants were investigated in transiently transfected COS-1 cells. When PPH β subunit was expressed alone in COS-1 cells we observed a complex glycosylated form after 30 min. With the β/α tailswitch mutant a complex glycosylated form was detected only after 5 h. These data suggest a possible role of the α tail in ER-retention. This potential effect was further investigated in chimeras of secreted proteins and the α subunit TM domain.

S01-18

ANTICOAGULANT HEPARAN SULFATE PROTEOGLYCANS ARE SYNTHESIZED BY OVARIAN GRANULOSA CELLS

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Anticoagulant heparan sulfate proteoglycans (aHSPGs), like heparin bind and activate antithrombin III (AT). aHSPGs are synthesized by endothelial cells and they endow the vascular endothelium wall with anticoagulant properties. Rat ovarian granulosa cells constitute an avascular compartment in the ovarian follicle and produce aHSPGs. To investigate the nature and function of these species outside the vascular bed we have characterized granulosa cell aHSPGs heparan sulfate chains (aHS) and have studied the impact of FSH stimulation on granulosa cell aHSPGs. Affinity fractionation of metabolically labeled ^{35}S-HS chains revealed that aHS constitute about 6.5% of the total amount of granulosa cell HS. Disaccharide analysis of aHS chains showed that they contained 13% of 3-O-sulfated disaccharides which are markers of the AT binding site of heparin. Purified aHS from granulosa cells were biologically active as they bound to AT and accelerated the formation of thrombin-AT complexes. Stimulation of granulosa cells in culture by the gonadotropin FSH resulted in the redistribution of aHSPGs from the cell layers to the culture medium. In conclusion, we have demonstrated that rat ovarian granulosa cells synthesize and secrete aHSPGs under gonadotropin control.