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RECENT DEVELOPMENTS IN N.M.R. OF COMPLEX CARBOHYDRATES

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To gain an insight into the biological and physicochemical functions of complex carbohydrates at the molecular level, knowledge of their complete (*i.e.*, primary and secondary) structures is a prerequisite. Structural characterization of complex carbohydrates involves determining *(i)* the type, number and primary sequence of the constituting monosaccharides (including the occurrence of branch points, and the location of appended non-carbohydrate groups such as alkyl, acyl, phosphate and sulfate groups), and *(ii)* the 3-D conformation(s) and dynamics in solution. As to *(i)*, we have designed a strategy for the *de-novo* sequencing of a carbohydrate by the combination of multiple-pulse, ^{1}H and ^{13}C NMR spectroscopic techniques. 1-D and 2-D $\{^{1}H,^{1}H\}$ TOCSY (or HOHAHA) experiments are invaluable for the complete assignment of the ^{1}H-NMR spectrum of a carbohydrate. We have implemented the DIPSI-2 sequence for isotropic mixing, and use double- and multiple-DANTE for selective inversion of resonances in the 1-D experiments. 1-D COSY with chemical-shift selective filter and $\{^{1}H,^{1}H\}$ triple-quantum spectroscopy are helpful to assign signals of exocyclic protons. ^{1}H-Detected heteronuclear multiple-quantum correlation experiments (HMQC and HMBC for scalar connectivities through one bond and multiple bonds, respectively) provide $\{^{1}H,^{13}C\}$ chemical-shift correlation maps of an oligosaccharide. Once the ^{1}H and ^{13}C spectra of a carbohydrate are completely assigned essentially by TOCSY and HMQC, the HMBC spectrum permits the sequencing of the oligosaccharide and determination of the location of most types of appended groups [1].

Solution conformational analysis of carbohydrates by NMR relies heavily on the measurement of scalar and dipolar $\{^{1}H,^{1}H\}$ couplings. The TOCSY-edited monosaccharide ^{1}H subspectra provide the scalar couplings necessary to define the ring conformations. The torsional angles around the interglycosidic bonds are defined in terms of interproton distances through the measurement of dipolar couplings. NOESY spectroscopy and its counterpart in the rotating frame (ROESY) are invaluable tools in 3-D structural analysis. We will evaluate the usefulness of both techniques for oligosaccharides of different size, for different sample temperatures and solvent systems. In addition to NMR as the experimental approach, conformational analysis of complex carbohydrates requires the theoretical evaluation of the NOE-generated internuclear distance constraints by a potential-energy minimization method such as GESA, MM2, or AMBER.

The approach of 1-D and 2-D TOCSY, 1-D COSY, HMQC, and HMBC for the sequencing of oligosaccharides, and NOESY, ROESY and GESA for their solution conformational analysis, will be illustrated for some bacterial polysaccharides [2] as well as for a number of oligosaccharide side chains of mammalian glycoproteins [3].

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This research is supported by NIH grant RR-05351 and DOE grant DE-FG09-87ER13810.

STRUCTURAL STUDIES OF THE OLIGOSACCHARIDE CHAINS OF ART v II, A GLYCOPROTEIN ALLERGEN FROM MUGWORT POLLEN

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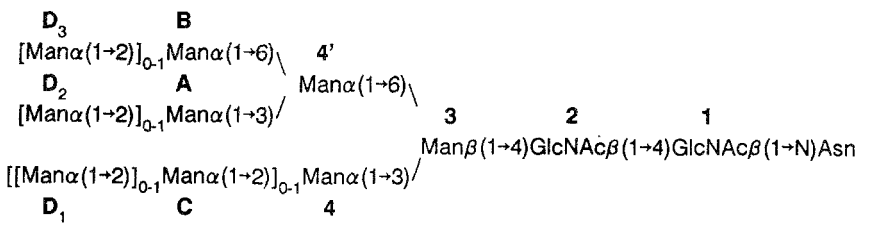
A number of allergens isolated from various plant sources have been demonstrated to be glycoproteins; their molecular weights range from 5 to 70 kDa. However, the structures of their carbohydrate moieties have not been investigated in detail.

We have isolated a Con-A binding glycoprotein allergen, Art v II, from pollen of mugwort (*Artemisia vulgaris* L.) which is one of the main causative agents of allergic airway diseases. Art v II has a molecular weight of 35 kDa; it appears to be composed of two identical subunits linked together by disulfide bridges. The pI of the allergen is between 4.10 and 4.65; the glycoprotein consists of 6 or 7 different isoforms. The carbohydrate content of Art v II is approximately 9%. Carbohydrate composition analysis showed mannose, *N*-acetylglucosamine, glucose and galactose in the ratio of 13.4:3.2:1.3:1.0.

To study the molecular structure of the carbohydrate part of this glycoprotein allergen, Art v II was treated with PNGase F (*N*-glycanase™). The released *N*-type oligosaccharides were isolated by gel-filtration on Biogel P-4 and analyzed by a combination of 500-MHz ^{1}H-NMR spectroscopy, FAB mass spectrometry, and high-pH anion-exchange chromatography (Dionex BioLC). These experiments demonstrated that the oligosaccharide chains in Art v II are of the high-mannose type. They differ in the number of constituting mannose residues, with about 2.5% of the chains being $Man_5GlcNAc_2$, 46.1% $Man_6GlcNAc_2$, 16.6% $Man_7GlcNAc_2$, 28.0% $Man_8GlcNAc_2$, and 6.8% $Man_9GlcNAc_2$. On the basis of the structures of the $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, and $Man_8GlcNAc_2$ oligosaccharides obtained from Art v II, we propose that, during the biosynthetic processing of the carbohydrate chains, the Man-D_2 residue is the first to be removed, followed by Man-D_3, Man-D_1, and Man-C.



As far as we are aware, this is the first time the structures of the oligosaccharide chains from a plant glycoprotein allergen have been fully elucidated.

This research is supported by N.I.H. Grant RR-05351.

^{252}CF-PLASMA DESORPTION MASS SPECTROMETRIC MAPPING OF A RECOMBINANT CHITINASE

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A chitinase gene from Vibrio Parahemolyticus was cloned into pUC18 in E.coli DH5a. Both the recombinant and native Vibrio chitinase were 95 KDa. The plasmid construct, pC139, has a 5.9 kb Vibrio DNA insert which was subcloned into a Bluescript plasmid, (the construct designated pBS 139) for localization and sequencing of the structural gene. Cloned inserts in plasmid DNA were sequenced directly using commercially available kits and following the protocols applied to double-stranded templates. To confirm the DNA-derived protein sequence of the chitinase gene, plasma desorption mass spectrometry was used to map the tryptic fragments of the recombinant chitinase which was purified from extracellular compartments of E. coli JM 101 harboring pBS 139. After reduction and carboxymethylation, chitinase was cleaved by CNBr and a 30 KDa fragment was isolated from CNBr fragments by DEAE-Biogel A chromatography and Sephadex G-75 gel permeation chromatography. Tryptic fragments of the 30 KDa fragment were fractionated by reversed- phase HPLC on C-8 and C-18 Columns. Plasma desorption mass spectra were obtained on a Bio-Ion Nordic Model BIN 20K mass spectrometer, equipped with a 10-μCi source of ^{252} Cf. Agreement between predicted tryptic fragment masses which we calculated from the amino acid sequences predicted by DNA data and the found masses of tryptic fragments measured by plasma desorption mass spectrometric mapping indicated that amino acid the sequence of the 30 KDa fragment near the C-terminal of the chitinase was correctly assigned. (Supported, in part by Grants # LEQSF(1989-1991)-RD-B-2 from the Louisiana 8g fund and by an NIH shared instrumental grant #RR03832A to RAL).

RAPID SEPARATION OF ASN-LINKED OLIGOSACCHARIDES ON A POLY BIS (AMINE)-SILICA COATED HPLC COLUMN. S. Hirani, C. Bartlett, M-Y.G. Chang, D. McNeilly, J. Kremsky* and R. Hamilton*. Genzyme Corporation, 75 Kneeland Street, Boston, MA 02111 and *Millipore Corporation, 75C Wiggins Avenue, Bedford, MA 01730

Asn-linked oligosaccharide separations based upon some of their structural features have become a widely used technique. To be able to perform separations rapidly and yet at the same time gather structural information is highly desirable. A particularly convenient and sensitive method of analysis involves reducing the free oligosaccharides with NaB^3H_4 or 2-aminopyridine and fractionating the oligosaccharide by hplc using an amine-bonded resin. A drawback of these columns is that with repeated chromotographic separations, the column performance deteriorates resulting in poor separations. We evaluated a recently developed chemically more stable polymer coated silica packing material to separate Asn-linked oligosaccharides. The packing material was prepared by coating silica particles with a unique bis-amine polymer. The use of a functionalized polymer coating enhances the stability of the silica based particle. Mixtures of ^3H labeled high mannose and desialylated complex oligosaccharides were separated on the hplc column under different conditions with acetonitrile and water. Separations similar to those observed previously on an amine-bonded resin were obtained. However, separations with the poly amine-silica column could be carried out within 30 minutes compared to 60 minutes with the amine-bonded column. With the same column sialylated complex type oligosaccharides could also be fractionated according to the number of sialic acid residues. Elution was carried out with Tris-HCl buffer and a linear gradient of NaCl. Thus this column provides an excellent method to carrying out rapid separations of Asn-linked oligosaccharides.

Carbohydrate Compositional Analysis in the Quality Control of mt-PA-6

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One concern in the quality control of therapeutic recombinant glycoproteins is the production of reproducibly glycosylated protein. Many analytical methods are involved in the evaluation of glycosylation, including carbohydrate compositional analysis, determination of the sites of glycosylation, mapping of the size and charge of oligosaccharides, and oligosaccharide sequencing of selected lots. These methods must be rigorously validated when used in a quality control environment. Our laboratory has validated two methods for the carbohydrate compositional analysis of a modified tissue plasminogen activator (mt-PA-6) that is expressed in Syrian hamster cell line. These methods are: 1) an assay for the neutral and amino sugar content using acid hydrolysis, and 2) the determination of neuraminic acid by enzymatic release with neuraminidase. Both methods employ high pH anion exchange chromatography with pulsed amperometric detection for the determination of the monosaccharides.

The hydrolysis conditions for the determination of neutral and amino sugars were optimized with respect to the type of acid and the temperature. Hydrolysis conditions of 2M trifluoroacetic acid at 110°C were selected after examination of the yield of released monosaccharides and the formation of decomposition products. De-acetylation of amino sugars was complete under these conditions. The release of the monosaccharides was examined also as a function of time. The maximum concentration of free monosaccharides occurred after approximately four hours hydrolysis. The relative rates of decomposition of the monosaccharides were estimated from the concentration vs. time profiles. The aminohexoses were the most stable followed by the hexoses and pentoses. Deoxyhexoses were very unstable under these hydrolysis conditions. The concentration of individual monosaccharides may be estimated at zero-time from the apparent rates of decomposition. The enzymatic release of N-acetyl-neuraminic acid was optimized for enzyme concentration and reaction time at 37°C. Two neuraminidases (*Arthrobacter ureafaciens* and *Clostridium perfringens*) that nonselectively cleave $\alpha(2,3)$ and $\alpha(2,6)$ linkages were investigated. Complete release was attained in less than one hour at enzyme concentrations ≤ 0.2 U/mL. Equivalent concentrations of N-acetylneuraminic acid were determined in mt-PA-6 following mild acid hydrolysis or enzymatic release. N-glycolylneuraminic acid was not detected in the mt-PA-6.

An interlaboratory study was performed among three laboratories using the optimized conditions described above. A protocol was developed that permitted establishment of 95% confidence limits for the composition data using a given number of replicate analyses. Equivalent results were obtained for a control sample of mt-PA-6 that was analyzed in each of the labs. This assay protocol has been used to determine the carbohydrate composition of several lots of mt-PA-6. These data are used to monitor the variation in lot-to-lot processing and are being correlated with oligosaccharide structural information.

CHARACTERIZATION OF THE ASN-LINKED OLIGOSACCHARIDES IN THE LYSOSOMAL MEMBRANE GLYCOPROTEINS, LAMP-1 AND LAMP-2 OF THE HUMAN PROMYLOCYTIC HL-60 CELLS AND DIFFERENTIATION INDUCER-RESISTANT HL-60 CELLS.

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Structures of Asn-linked oligosaccharides attached to human lysosomal membrane glycoproteins (lamp-1 and lamp-2) from wild-type, dimethylsulfoxide (DMSO)-resistant and retinoic acid (RA)-resistant HL-60 cells were studied. Cells were metabolically labeled with radioactive sugars and lamp-1 and lamp-2 were isolated by immunoprecipitation with specific antibodies. Glycopeptides were prepared and fractionated by serial lectin affinity chromatography on Concanavalin A (ConA)-Sepharose, tomato lectin-Sepharose and Datura stramonium agglutinin (DSA)-Agarose. The structures of glycopeptides bound to these lectins were analyzed by endo-β-galactosidase digestion and methylation before and after various glycosidase digestions.

The carbohydrate chains attached to lysosomal membrane glycoproteins isolated from wild-type HL-60 cells are very complex and consist of complex-type tetraantennary oligosaccharides with longer or shorter poly-*N*-acetyllactosaminyl side chains, triantennary saccharides with no N-acetyllactosaminyl repeats, and high mannose-type oligosaccharides.

Comparison of lamps from wild-type and differentiation-inducer-resistant cells reveals the following features. Lamps from both DMSO- and RA-resistant HL-60 cells have smaller apparent Mr on SDS-PAGE than those from wild-type HL-60 cells. There are 3-fold less glycopeptides bound to tomato lectin-Sepharose for both mutant cells than wild-type HL-60 cells. These indicate lamps from both mutant cells contain less polylactosaminoglycan than from wild-type cells. The RA-resistant cells contain at least 5-fold less sialic acid residues on lamp glycopeptides compared to wild-type HL-60 cells. In contrast, DMSO-resistant cells has similar amount of sialic acid residues on glycopeptides as wild-type HL-60 cells. These results indicate that differentiation-resistant HL-60 cells are invariably deficient in polylactosaminoglycan synthesis. (Supported by CA48737).

N-GLYCOSYLATION OF HUMAN SERUM AMYLOID P COMPONENT AND OF C-REACTIVE PROTEIN FROM *LIMULUS POLYPHEMUS*

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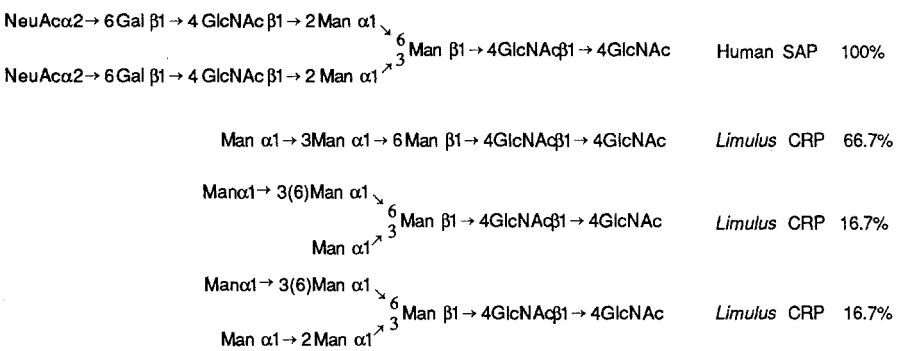
The N-linked oligosaccharides present on human serum amyloid P component (SAP) and on C-reactive protein (CRP) from horseshoe crab, *Limulus polyphemus*, an arachnid, have been analysed. SAP is a member of the pentraxin superfamily of vertebrate plasma proteins, which includes CRP, and which typically have a pentameric or decameric subunit structure. Although *Limulus* CRP has 12 subunits, it shares ligand specificity and some amino acid sequence homology with vertebrate CRPs, and is the most phylogenetically distant known protein related to the pentraxins.

Each subunit in both glycoproteins contains one N-glycosylation site from which oligosaccharides were released by hydrazinolysis and tritium-labelled. The oligosaccharides were isolated by gel-filtration, ion-exchange chromatography, or high voltage electrophoresis and the structures were determined by a combination of methods including NMR, GC-MS, sequencial exoglycosidase digestions and acetolysis.The structures of the N-linked oligosaccharides in SAP and CRP are illustrated below.



SAP contained only biantennary complex type oligosaccharides with two sialic acids attatched to the non-reducing end galactose residues. This is the first naturally occuring mammalian circulating glycoprotein to contain no carbohydrate heterogeneity. Furthermore, analysis of SAP present in malignant effusion fluids still showed no carbohydrate heterogeneity. This is in constrast with the changes observed in other liver derived glycoproteins during an acute phase response. Carbohydrate analysis is at present being performed on paired samples of amyloid P component from serum and from amyloid deposits in patients with amyloidosis.

The majority of the N-linked oligosaccharides in *Limulus* CRP consists of a linear trimannosyl chitobiose structure. In addition, two minor components containing 4 or 5 mannosyl residues linked to the chitobiose core were found. The molar ratio of these three components is 4:1:1 implying that carbohydrate may have some role in the quaternary structure of the glycoprotein. The structures found on *Limulus* CRP are biantennary and monoantennary oligomannose type oligosaccharides similar to those recently found in Leshmania GP63 [Olafson, R. W. *et. al.*, (1990) *J. Biol. Chem.*, in press].

STRUCTURES OF THE ASPARAGINE-LINKED SUGAR CHAINS OF HUMAN CHORIONIC GONADOTROPIN PURIFIED FROM THE URINE OF A PATIENT WITH EXTRAGONADAL GERM CELL TUMOR

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Human chorionic gonadotropin (hCG) highly purified from the urine of a male patient with extragonadal germ cell tumor contained four asparagine-linked sugar chains in one molecule. The sugar chains were quantitatively released from the polypeptide moiety by hydrazinolysis and recovered as oligosaccharides after N-acetylation. The oligosaccharide mixture was separated into a neutral (N) and three acidic (A1, A2, and A3) fractions by anion-exchange column chromatography. By combination of sequential exoglycosidase digestion, methylation analysis, and lectin column chromatography, the structures of these oligosaccharides were elucidated to be the same as those of female gestational choriocarcinoma hCGs. Both contain eight kinds of sugar chains: triantennary, abnormal and normal biantennary, and monoantennary complex-type sugar chains with or without fucosylated core portion. Whether the same structural changes of the sugar chains were induced in the glycohormone of hCG producing tumors, either gestational or non-gestational, gonadal or extragonadal, male or female, remains to be established.

ASPARAGINE-LINKED OLIGOSACCHARIDES CONTAINING TERMINAL α-GALACTOSYL RESIDUES IN COBRA VENOM FACTOR.

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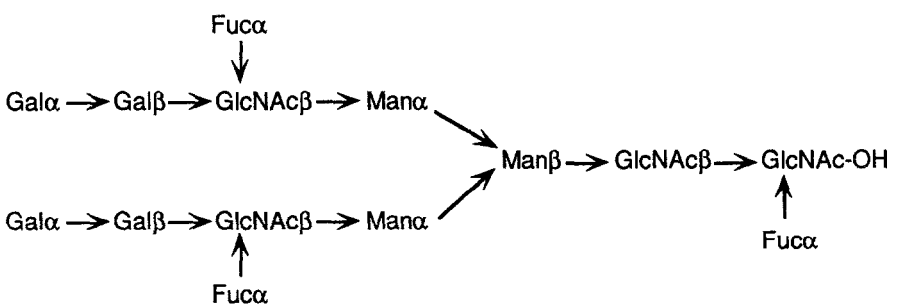
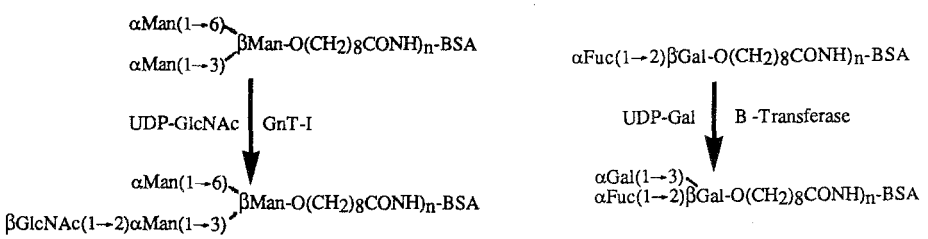
Cobra venom factor (CVF) is a 136 kDa molecular mass glycoprotein that is capable of activating the complement system via the alternate pathway. It contains 4 to 5 *N*-linked oligosaccharide chains per molecule and is devoid of *O*-linked saccharides and sialic acid. Previous studies indicated that the carbohydrate moieties are essential for its function. In the present study, the structural features of the *N*-linked oligosaccharides of CVF from *Naja naja siamensis* were studied by lectin affinity chromatography of radiolabeled glycopeptides, obtained by pronase digestion and *N*-acetylation with [^{3}H]acetic anhydride. About 80% of the oligosaccharides are biantennary complex-type, 10 to 15% triantennary complex-type, and 5 to 10% high mannose-type structures. The majority of the biantennary (86%) and triantennary (>50%) oligosaccharides terminate with α-galactosyl residues, and contain fucose residues linked to the proximal *N*-acetylglucosamine of the chitobiose core. At least 50% of the triantennary oligosaccharide chains appear to contain C-2 and C-6 disubstituted core α-mannosyl residues. A major biantennary oligosaccharide, comprising about 45% of the carbohydrate present in CVF, was purified to homogeneity after hydrazinolysis followed by reduction with NaB[^{3}H]$_4$. Its sugar sequence as established by exoglycosidase treatment and analysis of the products by Bio-Gel P-4 chromatography and MicroPak AX-5 HPLC is:



(Supported by NIH Grants AI 26821 and CA 01039)

ELISA ASSAYS FOR GLYCOSYLTRANSFERASES. L. Keshvara, E. Newton, S. C. Crawley, O. Hindsgaul and M. M. Palcic.*Departments of Chemistry and Food Science, University of Alberta, Edmonton, Alberta, Canada T6G 2P5.

Enzyme-linked immunosorbent assays (ELISA) have been developed for *N*-acetylglucosaminyltransferase I (GnT-I) and for the α(1→3)galactosyltransferase (blood group B transferase). Microtiter plates coated with the bovine serum albumin conjugate of a specific synthetic acceptor saccharide (Scheme 1) are incubated with enzyme and appropriate nucleotide donor. The resulting immobilized products are detected and quantitated with either polyclonal or monoclonal antibodies that are selected not to cross-react with the substrate structures. Product formation is proportional to time of incubation and enzyme concentration. Less than a pmol of product can be detected for a one hour incubation with a microliter of human serum as the enzyme source. A similar approach does not appear to be succesful for monitoring α(1→2)fucosyltransferase acting on βGal(1→4)βGlcNAc-BSA conjugates.



DOLICHOL KINASE ACTIVITY IN MICROSOMES FROM ETIOLATED SHOOTS OF SECALE CEREALE.

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Dolichyl phosphate (Dol-P) is an obligatory intermediate in the biosynthesis of N-linked glycoproteins in both plants and animals. However, seeds of several monocots of the Gramineae family contain both dolichol (with a saturated α isoprene unit) and polyprenol (with an unsaturated α isoprene). Microsomes isolated from etiolated rye shoots (Secale cereale) also contain dolichol and polyprenol in a ratio of 1.5 to 1. With such a large proportion of polyprenol present, it is of interest to know whether polyprenol may participate in the reactions of the dolichol cycle. A kinase for dolichol has been observed in rye microsomes. It appears to have pH optima at 6.5 and 8 with the activity being nearly 2-fold higher at pH 8. Triton X-100 (0.38%) was required for optimal activity. Exogenous phosphate donors were not required as both CTP and ATP inhibited Dol-P formation. Ca^{+2} was only slightly stimulatory. A high concentration of AMP (225 mM) enhanced activity by about 2-fold. Studies are currently in progress to determine whether this kinase may also act on polyprenol. (Supported by the Natural Sciences and Engineering Research Council of Canada)

CONTROL OF GLYCOPROTEIN FUCOSYLATION R. L. Giuntoli, D. R. B. Gillies and M. C. Glick Department of Pediatrics, University of Pennsylvania Medical School and The Children's Hospital of Philadelphia, Philadelphia, PA 19104

Human erythroleukemic (HEL) cell glycoproteins/glycolipids have been shown previously with the use of defined antibodies to express fucosyl residues in α1→2 linkage to Gal but not α1→3 to GlcNAc (1). We confirmed these results using an H-2 specific antibody and almond α-*L*-fucosidase specific for Fucα1→3/4GlcNAc (2). HEL cells should therefore be a good source of α1→2fucosyltransferase (FucT) but not α1→3FucT. Surprisingly, HEL cell extracts contained significant activity of α1→3FucT even though the product of the enzyme reaction was not expressed either on the membrane or cell glycopeptides. In order to demonstrate that the conditions of the assay and the methods of detection (3) could separate the products of both FucTs, human serum was used as a positive control with Galβ1→4GlcNAc as substrate. Under similar conditions only one major product was formed by the HEL cell extract whereas two products were formed by serum. The HEL cell product was chromatographically comparable to Galβ1→4(Fucα1→3)GlcNAc, prepared with a purified α1→3FucT , and not Fucα1→2Galβ1→4GlcNAc prepared with serum. In addition, Fuc was released (90%) from the HEL cell product by almond α1→3/4fucosidase. α1→2FucT activity was detected in the HEL cell extract with phenyl-β-*D*-galactoside (3) as substrate. The presence of available substrate for α1→3FucT among the HEL cell glycoproteins was demonstrated by the H-2 antibody since the antibody was known to react with Fucαl→2Galß1→4GlcNAc (4). Thus, terminal fucosylation of HEL cell glycoproteins is not controlled by lack of the substrate (5) or by the mRNA level (6) for the enzyme. Moreover, the control may not be by competition (7) among the FucTs since *in vitro* α1→3FucT competes successfully for the substrate and will fucosylate 2'-fucosyllactose. The HEL cells represent a system to investigate control mechanisms involved in fucosylation of cell and membrane glycoproteins since the proposed mechanisms do not appear applicable to these cells. Supported by CA 37853.

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PRELIMINARY CHARACTERIZATION OF UNUSUAL GLYCANS IN THE SLIME MOLD *DICTYOSTELIUM DISCOIDEUM.*

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We study the structure and function of glycans in the slime mold *Dictyostelium discoideum*. In previous studies, we used N-glycanase digestion to release, identify and characterize N-linked oligosaccharides in this organism. However, only about 5-10% of the cellular [^{3}H]-GlcN label is released by this enzyme. Moreover, the great majority is insensitive to base/borohydride treatment (>75%) or digestion with Proteinase K (85%) when analyzed by S-200 gel filtration chromatography. Nearly all (90-95%) of the incorporated [^{3}H]GlcN, but only 2% of the ^{35}S-Met partitions into the aqueous phase of a butanol/water two phase system. Analysis of the [^{3}H]GlcN-labeled material by SDS-PAGE shows distinct protein bands on a smeared background covering most of the gel. The material is not digested by either endo-β-galactosidase or endo-glycoceramidase treatments. Its synthesis is blocked concomitant with the addition of cycloheximide, showing that all of the material is protein associated. More than 60% of total [^{3}H]GlcN label binds to Phenyl-Sepharose, and nitrous acid deamination at pH 4.0 reduces this to 15%, suggesting the presence of a non-acetylated GlcN residue in the structure. Furthermore, about 10% of the bound material is sensitive to PI-PLC digestion, suggesting the presence of a glycophospholipid anchor.

In contrast, [^{3}H]GlcN-labeled secreted macromolecules appear as discreet bands by SDS-PAGE, with little binding to Phenyl-Sepharose. Approximately 75% of the total label appears to occur as a phosphodiester with a half-life of 3-5 min at pH 2 at 95°C. HPLC analysis of the released products indicates the presence of mono- and di-saccharides. It is likely that these components are derived from the previously described GlcNAc-P-Ser structure found on several *Dictyostelium* glycoproteins. Similar material is also found in the cells, where it accounts for only about 20% of the [^{3}H]GlcN label. (Supported by GM32485 and Established Investigator of the American Heart Association Award to HF.)

SUBUNIT STRUCTURE OF CANINE SUBMAXILLARY MUCIN

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Purified Canine submaxillary mucin was fractionated by size on Sephacryl S-1000. The highest molecular weight fraction eluted near the void volume and had a molecular weight of ca. 10^7 kDa. It was reduced with dithiothreitol, alkylated with [^{14}C]iodoacetamide and the products chromatographed on a Sephacryl S-1000. The major peak (subunit peak) had a molecular weight of ca. $2X10^6$ kDa and contained all of the carbohydrate and all of the incorporated label. SDS gel electrophoresis of the included volume (link protein) gave a major band of ca. 150 kDa, suggesting that the subunits were linked by disulfide bonds to the link protein. The subunit peak was treated with trypsin and the products separated by gel filtration on Sephacryl S-500. Two peaks were observed. The first peak (glycosylated domains) had a molecular weight of ca. 300 kDa. and contained no radioactivity but had a carbohydrate composition similar to that of intact mucin. The second peak contained all the radioactivity but no carbohydrate and consisted of very small peptides.

The data suggest that Canine submaxillary mucin contains subunits and linker protein. The subunits are composed of glycosylated and nonglycosylated domains. Thus the structure of Canine submaxillary mucin appears to be similar to that described for salivary, cervical, gastrointestinal and tracheobronchial mucins suggesting that all of these mucins have a similar architecture.

Supported by NIH grants DK 39918 and DK 27651 and by the Cystic Fibrosis Foundation, Rainbow Chapter.

EXPRESSION OF RAT SUBMANDIBULAR GLAND GLUTAMINE-GLUTAMIC ACID RICH PROTEIN AND HUMAN GLYCOPHORIN-A IN CHO CELLS.

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O-glycosylation occurs on selected hydroxyamino acids of a protein, and moreover, only some proteins undergo this modification. To date, no particular sequence or structure is known to facilitate the process. CHO cells are capable of initiating O-glycosylation of heterologous glycoproteins with fidelity (1). We are using this model to study the effect of flanking sequences on the glycosylation of residues, in proteins that normally are O-glycosylated (Human Glycophorin A-GPA), and in those that are not glycosylated (Rat SMG Glutamine-glutamic acid rich protein-GRP). Using cDNAs for GPA (obtained through the courtesy of Dr. M. Fukuda) and GRP, we have expressed the proteins in DG44 CHO cells (by courtesy of Dr. L. Chasin) with the vector pMT2, which contains the DHFR selectable marker. A number of positive colonies were picked for characterization. A peptide was made by solid phase synthesis corresponding to amino acids 26-36 of GPA, which does not contain O-linked glycans. The peptide was linked to thyroglobulin and a polyclonal antiserum was raised. This antiserum immunoprecipitates material from GPA-transfected cells that has an electrophoretic mobility the same as that of human glycophorin, suggesting that glycosylation has occurred in a similar manner.

We are currently amplifying expression of GPA and GRP using methotrexate, to provide enough recombinant protein for structural characterization. In particular, we are interested in defining the fidelity of GPA glycosylation and confirming that GRP fails to glycosylate.

This work was supported in part by NIH grants DE-08108, K16-DE00159 (B.C.O)

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VARIATION IN SIALYLATION OF AN ANTI-CEA MONOCLONAL ANTIBODY, ZCE025/IMPACT ON PERFORMANCE

Lana Rittmann-Grauer and Mark Guido.

ZCE025 is a mouse monoclonal antibody (IGg_1) which recognizes carcinoembryonic antigen in human tissues and tumors. The antibody is the reagent which is currently utilized in Hybritech's 111-indium diagnostic imaging kit for colon carcinoma called Hybri-CEAker TM. Since changes in glycosylation of an antibody can potentially impact biodistribution of the antibody in patients as well as alter immunoreactivity and Ka we initiated a study of the carbohydrate compostion of this antibody. The antibody is glycosylated on its heavy and light chains. The carbohydrate composition of the antibody has been determined using anion exchange chromatography followed by pulsed amperometric detection (Dionex Carbopac AS-6). The predominant form of sialic acid seen in both the in vitro and ascites derived antibody is N-glycolylneuramic acid although minor amounts of N-acetylneuramic acid were also detected. Sialic acid was removed from the antibody by neuraminidase treatment and the effect on affinity and immunoreactivity was determined. Stability of the terminal sialic acid moieties were measured over time and lot to lot variation in sialic acid content of ZCE025 was also examined. In summary, we have studied the carbohydrate compostion of ZCE025 which is currently in clinical trials to ascertain whether glycosylation modifications occur from lot to lot and to determine if modifications of sialic acid could impact the performance of antibody by altering the Ka or immunoreactivty of the antibody.

BIOSYNTHESIS OF SIALO-Le^x AND Le^x GLYCOLIPID BY FucT-3 (GDP-Fuc:SA-nLc4 or nLc4 α1-3FUCOSYLTRANSFERASE) FROM EMBRYONIC CHICKEN BRAIN. J.W. Hawes, M. Basu, B. Geraghty, S. Ghosh and S. Basu. Dept. of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556.

There is increasing evidence that sialo-Le^x (NeuAcα2-3Galß1-4(Fucα1-3)GlcNAcß1-3Galß1-4Glc-Cer) and Le^x (Galß1-4(Fucα1-3)GlcNAcß-1-3Galß1-4Glc-Cer) occur on the surfaces of carcino-embryonic cells of non-neuronal as well as neuronal origin. We previously reported the characterization of FucT-2 (GDP-Fuc:nLc4 α1-2FucT) and FucT-3 (GDP-Fuc:nLc5,nLc4 or Lc3 α1-3FucT) from human neuroblastoma IMR-32 (Proc. Natl. Acad. Sci. USA (1978) 75, 289-293) and human colon carcinoma Colo-205 cells (Ind. J. Biochem. Biophys. (1988) 25, 112-118). The present report is concerned with the characterization of these two activities in embryonic (or developing) chicken brains (7 to 19 day).
In addition to FucT-2 and FucT-3 activities, ECB also contains FucT-2' (GDP-Fuc: GM1 α1-2 FucT) activity. FucT activities have been solubilized with TDC (90-100% recovery) and can be resolved by differential adsorption to an acid-washed chitin affinity column. The solubilized, partially purified FucT-3 and FucT-2' have pH optima of 7.0 and 6.5, respectively. The reaction rate for FucT-3 remains constant for 2 hours, whereas FucT-2' is perhaps stable up to 30 minutes at 37°C in an incubation mixture containing G-3634A detergent. It also appears that ECB FucT-3 is active with Galß1-4GlcNAc, but nearly inactive with Galß1-3GlcNAc. LM1 (NeuAcα2-3Galß1-4GlcNAcß1-3Galß1-4Glc-Cer) , nLcOse4Cer (Galß1-4GlcNAcß1-3Galß1-4Glc-Cer) and nLcOse5Cer (Galα1-3nLcOse4Cer) are active substrates for FucT-3 of embryonic chicken brain (ECB) or of embryonic pig brain (EPB) origin. On the other hand, FucT-2' activity is relatively low in these two sources. The [^{14}C]-fucose-containing (Le^x) product (3000 cpm) obtained from nLcOse4Cer was treated with purified E. coli ß-galactosidase (25u) and the cleaved [^{14}C]-product (60%) migrated on TLC with authentic tetraglycosylceramide. The [^{14}C]-fucose-containing product obtained from nLcOse5Cer was treated with purified α- and ß- galactosidases (papaya) and the cleaved, radiolabeled product co-migrated with tetraglycosylceramide on TLC ($CHCl_3$: MeOH : H_2O = 60 : 35 : 8). The results suggest that ECB FucT-3 catalyzed [^{14}C]-product from nLcOse5Cer contains an internal GlcNAc-linked [^{14}C]-fucose. Further characterization of the enzymatic products and the purification of these three FucT activities (FucT-3, FucT-2 and FucT-2') are underway.
(Supported by NIH grant CA-14764 and NS-18005 to S. Basu)

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CODING AND CLASSIFICATION OF Gal/GalNAc SPECIFIC LECTINS*

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Grouping of lectin binding properties, based on determinant structure rather than monosaccharide inhibition pattern, should facilitate the selection of lectins as structural probes for glycans as well as for the interpretation of the distribution and the properties of the carbohydrate chains on the cell surface.

Based on the binding specificities studied with glycan by precipitin-inhibition, competitive-binding and hemagglutinin-inhibition assays, twenty Gal and/or GalNAc specific lectins have been divided into six classes according to their specificity for the disaccharide as all or part of the determinants and GalNAcα1→Ser(Thr) of the peptide chain. A scheme of classification is shown as follows.

(1) **F** specific lectins (GalNAcα1→3GalNAc, Forssman specific disaccharide) - *Dolichos biflorus* (DBL), *Helix pomatia* (HPL), Hog peanut (ABL, *Amphicarpaea bracteata*), and *Wistaria floribunda* (WFL) lectins.

(2) **A** specific lectins (GalNAcα1→3Gal, Blood Group A specific disaccharide) - *Griffonia(Bandeiraea) simplicifolia*-A_4 (GSI-A_4), Lima bean (LBL), soy bean (SBL), *Vicia villosa (VVL)*, *Wistaria floribunda* (WFL) lectins, *Dolichos biflorus* (DBL) and *Helix pomatia (HPL)*.

(3) **Tn** specific lectins (GalNAcα1→ to Ser(Thr) of the protein core) - *Vicia villosa* B_4 (VVL-B_4), *Salvia sclarea* (SSL), *Maclura pomifera* (MPL), *Bauhinia purpurea alba* (BPL), HPL and WFL.

(4) **T** specific lectins (Galβ1→3GalNAc, the mucin type sugar sequences on human erythrocyte membrane, T antigen or the disaccharides at the terminal nonreducing end of the gangliosides) - Peanut (PNA), *Bauhinia purpurea alba* (BPL), *Maclura pomifera* (MPL), *Sophora japonica* (SJL), *Artocarpus integrifolia* (Jacalin, AIL), and *Artocarpus lakoocha* (Artocarpin) lectins.

(5) Type **I** and **II** specific lectins (Galβ1→3(4)GlcNAc - the disaccharide residues at nonreducing end of the carbohydrate chains derived from either *N*-glycosidic or *O*-glycosidic linkages) - *Ricinus communis* agglutinin (RCA1), *Datura stramonium* (TAL, Thorn apple), *Erythrina cristagalli* (ECL, Coral tree), and *Geodia cydonium* (GCL).

(6) **B** specific lectin (Galα1→3Galβ1, Human blood group B specific disaccharide) - *Griffonia(Bandeiraea) simplicifolia* B_4(GSI-B_4).

Many other GalNAc and/or Gal specific lectins that can be used as tools are described.

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*This work aided by grants from the Chang Gung Medical Research Plan (CMRP) N0.293, Kwei-san, Tao-yuan, Taiwan, and the Kleberg Foundation, Texas, U.S.A.

REGULATION OF THE EXPRESSION OF GALα1-3GALβ1-4GLCNAC-GLYCOSPHINGOLIPIDS IN KIDNEY. Bruce A. Macher, Stephen P. Hendricks, Pingren He, and Cheryl L. M. Stults. Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, CA 94132.

Previous studies have established that there is a unique evolutionary distribution of glycoconjugates carrying the Galα1-3Galβ1-4GlcNAc- epitope. These glycoconjugates are expressed by cells from New World monkeys and non-primate mammals, but not by cells from humans, Old World monkeys,or apes. The lack of expression of this epitope in the latter species appears to result from the suppression of gene expression for the enzyme α1-3-galactosyltransferase (α1-3GalT).

Although many non-primate species are known to express this carbohydrate epitope, the nature (i.e. glycoprotein or glycosphingolipid) of the glycoconjugate carrying this epitope is only known for a few tissues, in a few animal species. Furthermore, it is not known whether all animal species express this epitope in the same tissues. We have investigated these questions by analyzing the glycosphingolipids in kidney from several non-primate animal species. Immunostained thin layer chromatograms of glycosphingolipids from sheep, pig, rabbit, cow and rat kidney with the Galα1-3Galβ1-4GlcNAc- glycosphingolipid specific monoclonal antibody, Gal-13, demonstrated that kidney from all of these species except rat contained Galα1-3Galβ1-4GlcNAc- neutral glycosphingolipids. To evaluate the enzymatic basis for the lack of expression of Galα1-3Galβ1-4GlcNAc- glycosphingolipids in rat, we measured the level of activity of three glycosphingolipid glycosyltransferases (α1-3GalT, β1-4-galactosyltransferase and β1-3-N-acetylglucosaminyltransferase) using an ELISA-based assay system and carbohydrate sequence-specific monoclonal antibodies. The lack of expression of these glycosphingolipids in rat is not due to a lack of expression of α1-3GalT, but rather appears to result from the lack of synthesis of neolacto glycosphingolipids. Thus, Galα1-3Galβ1-4GlcNAc- glycosphingolipids are not expressed in rat kidney because the precursors in the pathway leading to the synthesis of Galα1-3Galβ1-4GlcNAc- glycosphingolipids are not made.

DEMONSTRATION OF STRUCTURAL DIFERENCES BETWEEN CHITINS FROM VARIOUS ANIMALS. Maria L. Bade, Boston College, Chestnut Hill, MA 02167. The impression that chitin is chitin is chitin regardless of the origin of the chitin is almost universally held. Close perusal of the literature shows that this view rests on a few decades-old observations which were interpreted with an enthusiasm not warranted by the results. Problems also persist with obliteration of native fine structure of chitins isolated by methods still widely in vogue; such interference with microstructure continues to plague both basic research in the chitin field as well as a multitude of possible practical applications. This laboratory has previously reported that methods widely utilized in chitin purification result in obliteration of chitin fine structure, but that a method has been developed in which native fine structure of this glycoprotein is preserved and covalently stabilized in products obtained from a variety of animal sources. These isolates include fibrous chitin from insect larvae, horseshoe crabs, Red crabs, Dungeness crabs, lobsters, and several shrimp species. Structural differences between chitins from these species have been demonstrated by several criteria. For example:

1. Shrimp and lobster chitins differ in width and length of isolated fibers, and insect larval chitins differ from either.
2. Fibrous chitins differ in linear rates at which the identical enzyme degrades them, although larval chitins from two orders of insects appear to be degraded at the same rate.
3. Homologous chitin is degraded at a markedly greater rate by a specific enzyme than chitin of heterologous origin; during the same time interval, none of the fibrous chitins is attacked by lysozyme.
4. Horseshoe crab chitin resembles insect chitin more than it does crustacean chitin, but exhibits a greater tendency to self-associate than does insect chitin.
5. Chitins differ markedly in their ability to withstand contact with acids.
7. Fibrous chitins are more active substrates for specific chitinases than the substrates in common use for measuring "chitinase" activity; some of the latter are probably lysozymes rather than true chitinases.

Since microstructure is important for mechanical properties as well as characterization of enzyme mechanisms, these findings have an important bearing on future research in this field.

LIPOARABINOMANNAN OF MYCOBACTERIA: ITS STRUCTURE AND FUNCTIONS.

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Within species of the Mycobacterium genus, there exists a major carbohydrate antigen termed lipoarabinomannan (LAM) which is anchored in the plasma membrane of the bacteria and also excreted by growing bacteria. Many of its biological properties are similar to those of the O-antigenic lipopolysaccharides, such as inhibition of γ-interferon activation of macrophages, induction of the release of tumor necrosis factor and a generalized inhibition of antigen presentation by antigen presenting cells. Earlier work on the composition of this polysaccharide has established that arabinomannan consists of a backbone of $\alpha(1\rightarrow6)$-linked D-Manp units to which are attached short chains of $\alpha(1\rightarrow2)$-linked D-Manp residues and immunodominant $\alpha(1\rightarrow5)$-linked D-Araf residues. We have recently isolated LAM in its native state and have shown that at its reducing end, the mannan core is attached to a phosphatidylinositol residue. More recently, by applying a protracted process involving permethylation, partial acid hydrolysis, sodium borodeuteride reduction, ethylation and subsequent HPLC and capillary GC/MS of oligofuranosyl alditols, we have established that the arabinan region of LAM of M. tuberculosis is comprised of four major structural motifs. One of these three structural motifs is composed of linear 5-linked-α-D-arabinofuranosyl (Araf) residues. A second motif consists of a branched 3,5-linked α-D-Araf unit substituted at both positions 3- and 5- with linear 5-linked-α-D-Araf. A third motif contains 3,5-Araf substituted at both position 3- and 5- with a disaccharide β-D-Araf-$(1\rightarrow2)$-α-D-Araf. The fourth structural domain is composed of another nonreducing terminal region characterized by a 5-linked-α-D-Araf residue substituted at C-5 with the disaccharide β-D-Araf-$(1\rightarrow2)$-α-D-Araf. ^{13}C-NMR spectroscopy on the native LAM substantiated the presence of these structural motifs and confirmed the presence of terminal Araf residues in the β configuration. Thus the structural elucidation of both reducing and nonreducing ends of LAM is nearing completion and should help in understanding the role of this molecule in the pathogenesis of mycobacteria.

PARTIAL PURIFICATION AND CHARACTERIZATION OF TRANSVERSE TUBULAR MEMBRANE 85KD GLYCOPROTEIN

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The transverse tubule (TT) of chicken skeletal muscle contains a very active (~250 umol/hr/mg) MgATPase. The enzyme exhibits a host of distinguishing features, including: 1) unusual kinetic properties, 2) insensitivity to FITC and vanadate, and 3) modulation by diacylglycerols and phorbol esters. An important characteristic of the chicken T-tubule MgATPase is its ability to be stimulated by lectins that can bind to the inner core of complex-type oligosaccharide chains; these include ConA, PHA-E, Lentil and WGA. In purified chicken T-tubule preparations a low abundance glycoprotein of Mr 85 kD (85 kD-GP) is reactive to stimulatory lectins on Western blots. To date, the only polypeptide that can be associated with the MgATPase is the 85 kD glycoprotein. Partial purification of the 85 kD-GP has thus far been accomplished using the non-ionic detergent Triton X-114. Integral membrane proteins have been shown to partition preferentially into the detergent-rich phase while soluble proteins partition into the aqueous phase. The 85 kD glycoprotein of purified chicken T-tubules is highly enriched in the detergent-rich phase as well as the insoluble phase, indicating a possible integral membrane origin. Under these conditions the lectin-protected ATPase co-purifies with the 85 kD-GP. Deglycosylation with Endo H reduced the 85kD-GP to Mr 75,000. N-Glycanase reduced the 85 kD-GP to Mr 65,000, consistant with results obtained by complete chemical deglycosylation with TFMSA. O-Glycanase treatment had no effect. These preliminary results indicate that the 85 kD-GP contains both high mannose/hybrid and complex N-linked oligosaccharides and no O-linked oligosaccharides. (Supported in part by NSF DMB 8613881, NSF INT 8515846, The California Metabolic Research Foundation).

ISOLATION AND CHARACTERIZATION OF THIOL-INDEPENDENT LEUCOCYTE LECTINS.
Ashu Sharma[1], Richard A. DiCioccio[2], and Howard J. Allen[1], Dept. of Surgical Oncology[1] and Gynecologic Oncology[2], Roswell Park Memorial Institute, Buffalo, NY 14263. We have carried out studies to identify and characterize thiol-independent lectins present in human leucocytes. Spleen tissue was homogenized in the presence of Triton X-100/Ca++/Mg++/saccharide. The dialyzed extract was applied to a sham affinity column and the effluent was passed through a column containing the corresponding saccharide linked to Sepharose via divinyl sulfone. Putative lectin was eluted with the corresponding saccharide. In some cases, lectin was also eluted with 10 mM EDTA. The eluted polypeptides were analyzed following reductive alkylation, by SDS-PAGE. Lactose, a β-galactoside, yielded four major polypeptides of molecular weight 15, 17, 21 and 31 kDa. Melibiose, an α-galactoside, yielded a major polypeptide of molecular weight 53 kDa; minor polypeptides were obtained corresponding in mass to the lactose-eluted polypeptides. Mannose, GlcNac and L-Fuc yielded identical polypeptide patterns. Two major polypeptides of molecular weight 97 and 110 kDa and minor polypeptides of molecular weight 30, 31 and 55 kDa were obtained.
None of the above polypeptides reacted with anti-[human splenic galaptin] serum, anti-C reactive protein serum, anti-amyloid P component serum and anti-epidermal keratin serum when assayed by Western blot analyses. Anti-[human core-specific lectin] serum reacted strongly with the 55 kDa and 30, 31 kDa polypeptides eluted from the Man, L-Fuc, and GlcNAc affinity columns. The 53 kDa melibiose-eluted polypeptide gave a very weak reaction with anti-core-specific lectin. All other polypeptides gave negative reactions with this antiserum. The results of Western blot experiments revealed that the 15, 17, 21 and 31 kDa lactose-eluted polypeptides were prominently reactive with anti-[murine CBP-35] serum. The 15 and 17 kDa lactose-eluted polypeptides reacted strongly with anti-[rat lung lectin 18] serum. Anti-[rat lung lectin 29] serum reacted strongly with the lactose-eluted 31 kDa polypeptide. All of the polypeptides that were eluted from the different affinity columns were detected by immunostaining of Western blots with anti-[human buffy coat] serum. B lymphoblastoid cells were labelled with [35-S]-methionine/[3-H]-leucine and lectins were isolated as for the splenic thiol-independent lactose-eluted polypeptides. The molecular mass of the radioactive polypeptides detected, corresponded to those polypeptides isolated from spleen. The lactose-binding polypeptides described here appear to be present in spleen, buffy coat cells and B lymphoblastoid cells, and hence, are not simply serum lectins present in the splenic extracts. Our current data permit primarily speculation concerning the precise nature and importance of the newly described leucocyte lectins. Of interest is the identification of a major melibiose-binding lectin (subunit molecular weight 53 kDa) absent from the lactose-binding lectin(s) and present as only a minor component in the Man/Fuc/GlcNAc-binding lectins. This lectin may have relevance to the lymphoid and myeloid glycosphingolipids that bear non-reducing terminal α-galactoside residues and/or to the presence of terminal α-galactoside residues in laminin. Supported by CA42584 (HJA) and DK32161 (RAD).

A MONOCLONAL ANTIBODY TO AMPHOMYCIN BLOCKS ITS INHIBITORY ACTION.

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Amphomycin, a straight chain undecepeptide with either 3-isododecenoic or 3-anteisotridecenoic acid attached to the NH_2-terminal aspartic acid found to be a potent inhibitor of the enzymatic transfer of mannose, glucose, and GlcNAc-1P to Dol-P in eukaryotic cells. The inhibitory effect of the lipopeptide when analyzed on mannosylphosphoryldolichol (Man-P-Dol) biosynthesis in calf brain rough endoplasmic reticulum, the K_m for GDP-mannose did not change (1.37 µM in the absence and 1.08 µM in the presence of amphomycin) whereas the V_{max} was reduced to 0.17 pmol/mg protein/min as compared to 1.86 pmol/mg protein/min in its absence. On the other hand, when Man-P-Dol synthase activity was measured in the presence of amphomycin and as a function of Dol-P concentrations, the shape of the substrate velocity curve changed from a retangular hyperbola to a sigmoid. The Hill coefficients (n) were 2.02 and 1.22 in the presence and absence of amphomycin and the corresponding K_m values for Dol-P were 333 and 47.3 µM, respectively. In seperate experiments, when [^{3}H]amphomycin was reacted with Dol-P a complex was formed. The complex formation was dependent upon the presence of Ca^{2+} in the reaction mixture as well as the fatty acid residue on the antibiotic. Similar complex formation was also observed with undecaprenylmonophosphate but not with dolichylpyrophosphate, or undecaprenylpyrophosphate, or with their free alcohols indicating that it is highly selective for Dol-P. To understand the molecular details of this inhibitory process we have isolated two antibody producing clones (4C and $4C_9$) for amphomycin. Characterization of these monoclonal antibodies suggested that they belong to IgG + IgM subclass. Further studies indicated that the antibody $4C_9$ recognizes both native as well as acid-hydrolyzed amphomycin with equal potencies. Neutralizing effect of the monoclonal antibody on amphomycin-mediated inhibition of Man-P-Dol synthase activity was studied by pre-incubating the lipopeptide and the antibody at 4°C for 30 min followed by its addition into the Man-P-Dol synthase assay mixture. The obtained results indicated that the monoclonal antibody blocked the inhibitory effect of amphomycin.

Supported by USPHS Grant No. SO6RR08224.

MUCOUS GLYCOPROTEIN SECRETED BY CULTURED BOVINE TRACHEAL EPITHELIAL CELLS. P.-W. Cheng, T.F. Boat, J. Carson. Departments of Pediatrics and Biochemistry, University of North Carolina, Chapel Hill, NC, 27599-7220.

The objective of this study was to establish a tracheal epithelial cell culture system which secretes mucin. Pronase (0.1%)-dissociated bovine tracheal epithelial cells were plated on collagen (0.22%)-coated culture dishes and fed with F-12 medium supplemented with insulin (10 μg/ml), hydrocortisone (1 μM), cholera toxin (10 ng/ml), epidermal growth factor (25 ng/ml), endothelial cell growth supplement (7.5 ng/ml), T3 (0.03 nM), transferrin (5 ng/ml), and retinol (1 μM) for 3 days and then with the same medium containing 50% DME medium conditioned with 3T3 cells for 3-5 days. The cultured cells exhibited differentiated morphology, i.e. presence of cilia or secretory granules. [H-3]GlcN and [S-35]sulfate were employed to label newly synthesized high M.W. glycoconjugates, which were isolated on Sepharose CL-6B following treatment of harvested medium with testicular hyaluronidase. Purified glycoconjugates had a peak buoyant density of 1.59 g/ml and alkaline labile, GalNAc-linked carbohydrate chains, and contains 77.7% carbohydrate, 6.9% sulfate, and 15.4% peptide. Carbohydrate moieties detected were GalNAc (11.4%), GlcNAc (27.7%), Gal (25.0%), Fuc (6.6%), Sialic acid (3.1%), and small amounts (3.9%) of Man, but not uronic acids. The peptide is rich in ser (18 mole%), thr (16.1%), gly (10.9%), glu (10.6%), ala (7.2%), and pro (6.3%). The physicochemical properties of this glycoprotein are similar to those of tracheobronchial mucins. Analysis of recovery data indicated that mucins were the predominant high M.W. glycoconjugate secreted. We conclude that bovine tracheal epithelial cells cultured under the described conditions exhibit differentiated characteristics and secrete mucins. This culture system should facilitate the study of modulation of mucin secretion by secretory cells of the surface epithelium. (Supported by NIH grant HL-34322)

MOLECULAR CLONING OF cDNAs DERIVED FROM A SECOND HUMAN INTESTINAL MUCIN GENE

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Human small intestinal mucin is heterogeneous and may consist of glycoconjugates with more than one type of polypeptide backbone. To examine this, we purified mucin from normal human small intestine and compared it to the previously characterized acidic threonine- and proline-rich mucin from LS174T colon cancer xenografts. The small intestinal mucin was found to be less acidic on DEAE, to have a different lectin-binding pattern, and to have a higher content of Fuc, Gal, GlcNAc, and Ser, but a lower content of Sia and Pro. The small intestinal mucin was deglycosylated with anhydrous HF and polyclonal antibody (anti-SIB) was produced in rabbits. Antibody to deglycosylated xenograft mucin bound to its immunogen much more than to SIB, but anti-SIB bound to both deglycosylated mucins. Anti-SIB was then used to screen a human small intestinal λ gt11 cDNA library and fourteen immunoreactive clones were isolated. Six of these hybridized to SMUC 41, a previously isolated threonine and proline-rich intestinal mucin cDNA (Gum et al., J. Biol. Chem. 264:6480-6487, 1989) and were therefore not further characterized. Of the remaining eight clones however, at least four appear to be derived from a different mucin gene. These include two partial cDNA clones, SIB 124 and SIB 139, that contain 51 nucleotide tandem repeats which encode a seventeen amino acid threonine and serine-rich repetitive peptide. SIB 139 hybridized to messages produced by small intestine, colon, colonic tumors and also by high mucin variant LS174T colon cancer cells. Genomic DNA blot analysis with twelve different restriction enzymes was used to examine the SIB-type mucin gene. Ten of the twelve enzymes produced multiple bands. Only two of the enzymes tested produced a single hybridizing fragment (SphI and XbaI) and in both cases the band observed was larger than 23 KB. Hence, the SIB-type mucin gene (proposed name MUC 3) appears to be quite large. The gene from which cDNAs SIB 124 and SIB 139 are derived is polymorphic within the human population and maps to chromosome 7, distinct from other known human mucin genes. Thus, cDNAs SIB 124 and SIB 139 define the tandem repeats of a novel threonine and serine-rich human intestinal mucin.

EFFECT OF ISOPROTERENOL TREATMENT ON THE COMPOSITION AND STRUCTURE OF RAT SUBMANDIBULAR GLAND MUCIN-GLYCOPROTEIN. B.C. VanWuyckhuyse and L.A. Tabak. Departments of Dental Research and Biochemistry, University of Rochester, Rochester NY, 14642.

Rat submandibular gland (RSMG) mucin is secreted in response to ß-adrenergic stimuli (1). Several studies have suggested that glycosylation is regulated by neurotransmitters (2). To determine the extent to which RSMG mucin glycosylation and exocytosis are coupled events, we have compared mucins derived from saline- (control) and isoproterenol-treated (0.033 g/kg, once daily, intraperitoneally for 11 d) male Wistar-strain rats. Mucins were purified from aqueous extracts of RSMG or from cannulated RSMG saliva elicited by a single intraperitoneal injection of isoproterenol (0.033 g/kg), using a modification of methods previously described (3). Briefly, glandular extract or saliva was fractionated sequentially on columns of Sepharose CL-6B and Sephacryl S-200 using buffers made 6M with respect to urea. Mucin-containing fractions were applied to columns of DEAE-cellulose and the fall-through peak was subjected to reductive methylation to dissociate low molecular weight peptides from the mucin (3). Final purification was achieved by gel filtration on columns of 300 SW and 200 SW linked in tandem. The relative molecular weight of both glandular and secreted mucins derived from saline-treated animals (Mr 150K) was greater than that of isoproterenol-treated animals (Mr 142K). No significant differences in amino acid composition were observed as a result of isoproterenol treatment. However, the GalNAc/Ser+Thr ratio was reduced 2 (salivary mucins) - 3 (glandular mucins) fold in samples isolated from the isoproterenol-treated animals. Quantitative enzyme-linked immunoassay using a monoclonal antibody which recognizes the type 3 blood group A RSMG mucin oligosaccharides (4) indicated antigenic similarity among the different samples. More glandular mucin derived from isoproterenol-treated animals (130 ng mucin peptide) was needed to reach half maximal binding with this antibody than the glandular mucin isolated from control animals (93 ng mucin peptide). Collectively, these data indicate that systemic exposure to isoproterenol influences the glycosylation of the RSMG mucin, suggesting that glycosylation and exocytosis may be coupled at the secretagogue level.

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Supported, in part, by NIH Grant DE08108.

CHARACTERIZATION OF MUCIN FAMILIES IN THE HUMAN COLON. C. Richard Boland, Wesley L. Rigot, and Eugene R. Kraus. Department of Medicine, VA Medical Center and University of Michigan School of Medicine, Ann Arbor, MI.

Mucins are high molecular weight glycoproteins with an extraordinary degree of microheterogeneity. A clear understanding of the structural basis of this polydispersity has been difficult. Over 30 different oligosaccharide structures have been isolated from colonic mucins alone, several different apomucin genes have been identified, and other post-translational modifications such as sulfation and fatty acylation may add to the molecular complexity.

Mucins were purified from fresh specimens of human colon using saline extraction and sequential Sepharose 4B chromatography, nuclease digestion, and CsCl density gradient ultracentrifugation. Rechromatography of purified mucin on Sepharose 4B yielded a single high molecular weight peak. The purified mucin was chromatographed using a 1 x 100 cm Sepharose 2B column, and three distinct peaks were produced. All three peaks were recognized by polyclonal antibodies raised in our laboratory against purified mucins. The totally excluded peak (V_0, or peak I), and the high molecular weight, included peak (peak II) were both mucins by compositional analysis, but the very low molecular weight peak (peak III) did not contain monosaccharides characteristic of whole mucins. Treatment of normal colonic mucin with protease eliminated peak I, partially degraded peak II, shifted the radioactivity into peak III, and destroyed most of the immunoreactivity in all three peaks. Peak I contained high density, fucose-rich mucin with longer mean oligosaccharide chain length, whereas the lower molecular weight mucin in peak II was of slightly lower density, had shorter side chains, and was richer in sialic acid.

Radiolabeled mucins from a xenograft raised using the human colon cancer cell line LS174t were subjected to ion-exchange chromatography on an FPLC system using a continuous ascending salt gradient, and at least two distinct radioactive peaks were identified. The early-eluting ion-exchange peak corresponded to the lower density, sialic acid-rich mucin in peak II described above, and contained unique immunodeterminants not present in the later-eluting ion-exhange peak. The early-eluting peak from colonic cancers carried all of the tumor-associated antigens including immunoreactivity with monoclonal antibodies B72.3 (to sialosyl Tn), G-9 (from D. Gold: a colon-specific antibody that recognizes a cancer-associated epitope by immunohistochemistry), and three monoclonal antibodies raised in our laboratory against mucin purified from LS174t xenografts: GK-2, GK-3, and GK-9. The early-eluting peak from normal colonic mucins did not carry the tumor-associated antigens. The late-eluting ion-exchange peak from both normal colon and colon cancers corresponded to the high density, fucose-rich peak I described above, and bound the monoclonal antibody 91.9H (from T. Irimura, specific for sulfated oligosaccharides), suggesting it is the sulfomucin fraction.

Colonic mucins may be fractionated into at least two "families" that differ with regard to molecular weight, density, charge, carbohydrate content, sulfation, and the presence of tumor antigens. These families may correspond to the sialomucin and sulfomucin classes that have been defined by traditional histochemistry. The functional significance of these two families of colonic mucins is of some interest, but remains to be elucidated.

STRUCTURE AND FUNCTION OF A LOW MOLECULAR WEIGHT SALIVARY MUCIN

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Human submandibular-sublingual saliva (HSMSL) contains high and low molecular weight mucins, designated MG1 and MG2, respectively. MG2 (120-140 kDa) has a single polypeptide chain with O- and N-glycosidically linked oligosaccharides comprising ~70% of the mucin's weight. The major di- and trisaccharides of MG2, Galβ1,3GalNAc and NeuAcα2,3Galβ1,3GalNAc, interact with oral bacteria containing galactose or sialic acid binding adhesins and thereby help regulate microbial clearance or adherence in the oral cavity.

This study was done to further define the structure and biological properties of MG2. 2D-PAGE of HSMSL followed by Western transfer revealed charge heterogeneity within MG2. MG2 was purified from HSMSL (O blood type) (Prakobphol et al. Carbohydr. Res. 108: 111, 1982) and two species, designated, MG2a and MG2b, were separated by DEAE Sepharose CL-6B chromatography. These differed in their ratios of sialic acid:fucose; 1.0:2.3 for MG2a and 1.0:0.5 for MG2b. Proteolysis of MG2a with TPCK-treated trypsin followed by gel filtration on Sephacryl S-300 yielded two fragments, MG2a-1(100 kDa) and MG2a-2 (40 kDa). Similarly MG2b yielded MG2b-1 (105 kDa) and MG2b-2 (37 kDa). Sequence analysis of MG2a-1 and MG2b-1 indicated that the first 25 residues were identical. Most of the mannose (83%) was located in MG2a-2 suggesting that the N-glycosidically linked units are localized at one end of the mucin. The GalNac:Ser/Thr content of the tryptic peptides indicated that a higher percentage of O-linked units were localized in MG2a-1 and MG2b-1.

MG2-bacterial interactions were examined by both solid and solution phase assays. In a solid phase assay, Streptococcus gordonii G9B and Streptococcus oralis KS32AR bound both MG2a and MG2b but not their asialo-derivatives nor their tryptic glycopeptides. In a solution phase assay, Streptococcus gordonii G9B, Staphylococcus aureus 15334, and Pseudomonas aeruginosa 1244 grown to log phase were incubated with unlabeled HSMSL, MG2a and MG2b. Following centrifugation, bound salivary components were extracted from the bacterial surface with SDS, and examined by SDS-PAGE. With HSMSL, MG2 as well as several other salivary components, bound to bacteria. Purified MG2a and MG2b did not bind. Similar results were obtained when bacteria were incubated with either [^{125}I]-MG2a or [^{125}I]-MG2b. The solution phase assay was repeated employing HSMSL fractions at different stages of MG2 purification. Reductive methylation or reduction and alkylation diminished MG2 binding to these bacteria. Collectively, these results indicate that MG2 bacterial interactions may be predicated upon mucin conformation and/or mucin interaction with other salivary molecules.

Supported by USPHS Grants AI27401, DE00158, DE08240 and DE07585.

EFFECT OF AN INHIBITOR OF GLUCOSYLCERAMIDE SYNTHESIS ON MUSCLE CELL DIFFERENTIATION IN VITRO. L.D. Cambron and K.C. Leskawa. Department of Anatomical Sciences and Neurobiology, University of Louisville, Louisville, KY 40292

We have previously reported that upon examination of normal (E63) and fusion-defective (fu-1) rat muscle cells there was a transient increase in total glycosphingolipid synthesis at the time of myoblast contact and membrane fusion, but that fusion-defective myoblasts did not show this response. During this same period of myogenesis there was a dramatic increase in LacCer synthase (GalT-2) activity, and was also absent in fu-1 cells.

Recently we have obtained direct evidence for the involvement of membrane glycosphingolipids in myogenesis by analyzing the effect of PDMP on E63 cells. D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) is a ceramide analog which inhibits GlcCer synthase (GlcT). Since all glycosphingolipids undergo degradation, inhibiting GlcCer synthesis ultimately leads to decreased levels of all glycosphingolipids derived from this compound.

When PDMP was added to growth media (25 μM) myotube formation by E63 cells was inhibited (20% fusion compared to 95% in controls). When exogenous LacCer or ganglioside GM3 (in mixed micelles with phosphatidylcholine) were added to cultures concurrently with PDMP the ability to form myotubes was restored (80% fusion).

These effects are specifically attributable to alterations of glycosphingolipids. At the same concentration PDMP did not influence the synthesis of total DNA, protein or phospholipids. In addition, the distribution of ^{32}P among phospholipid molecular forms was not different between control and treated cells. When incorporation of ^{3}H-serine into sphingolipids was examined, neutral glycosphingolipid and ganglioside labeling was reduced 50% and 80% respectfully.

Working with B16 melanoma cells others have reported that the major action of PDMP was to inhibit attachment to laminin or type IV collagen, but not to fibronectin. In addition, cells preattached to laminin or collagen were induced to round up by addition of PDMP (Inokuchi et al., J. Cell. Physiol. 141:573, 1989). In our studies, PDMP did not inhibit attachment of E63 cells to tissue culture plastic, nor were major changes in morphology observed. This was also true of PDMP-treated fu-1 cells. At higher concentrations (50 μM), however, we did observe an accumulation of intracellular granules near the nucleus, which may reflect segregation of accumulated precursors (ceramide and sphingosine).

These results demonstrate a role for glycosphingolipids in myogenesis which may not be due to altered cell-substratum interactions.

(PDMP was kindly provided by Dr. N.S. Radin, University of Michigan. Supported by NINDS grant NS21057.)

α1→3-GALACTOSYLTRANSFERASE: THE USE OF RECOMBINANT ENZYME FOR THE SYNTHESIS OF α-GALACTOSYLATED GLYCOCONJUGATES

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We have reported the isolation and characterization of a bovine cDNA clone containing the complete coding sequence for UDP-Gal:Galβ1→4GlcNAc-α1→3-galactosyltransferase (Joziasse, D.H. *et al.*, (1989) J. Biol. Chem. 264, 14290-14297). Insertion of this cDNA clone into the genome of Autographa californica nuclear polyhedrosis virus (AcNPV) and subsequent infection of Sf9 insect cells with recombinant virus, resulted in high-level expression of enzymatically active α1→3-galactosyltransferase. The recombinant α1→3-galactosyltransferase could be readily detergent solubilized and subsequently purified by affinity-chromatography on UDP-hexanolamine-Sepharose. The recombinant α1→3-galactosyltransferase showed the expected preference for the acceptor substrate N-acetyllactosamine (Galβ1→4GlcNAc), and demonstrated enzyme kinetics identical to those previously reported for affinity-purified calf thymus α1→3-galactosyltransferase.

The recombinant enzyme was examined for the ability to synthesize α1→3-galactosylated oligosaccharides, glycolipids and glycoproteins. By a combination of ^{1}H-NMR, methylation analysis, HPLC, and exoglycosidase digestion it was established that, for each of the model compounds, the product of galactose transfer had the anticipated terminal structure, Galα1→3Galβ1→4-R. Our results demonstrate that catalysis by recombinant α1→3-galactosyltransferase can be used to obtain preparative quantities of various α1→3-galactosylated glycoconjugates. Therefore, enzymatic synthesis using the recombinant enzyme is an effective alternative to the chemical synthesis of these biologically relevant compounds.

This work was supported in part by a NATO Grant CRG-890490 (D.H.J.) and by Grant GM-38310 (J.H.S.) from the National Institutes of Health.

HUMAN α1→3-GALACTOSYLTRANSFERASE: CHARACTERIZATION OF A PROCESSED PSEUDOGENE

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UDP-Gal:Galβ1→4GlcNAc α1→3-galactosyltransferase is a terminal glycosyltransferase that is widely expressed in a variety of mammalian species, with the notable exception of man, apes and Old World monkeys. We recently reported the isolation of a bovine cDNA clone that contains the complete coding sequence for this enzyme [Joziasse, D.H., Shaper, J.H., Van den Eijnden, D.H., Van Tunen, A.J., and Shaper, N.L. (1989) J. Biol. Chem. 264: 14290-14297]. Using this cDNA as a probe, we have demonstrated that, although transcripts cannot be detected in a variety of established human cell lines by Northern blot analysis, homologous sequences are present in human genomic DNA. To establish that these sequences represent a human homologue of α1→3-galactosyltransferase, we have used the bovine cDNA as a probe to isolate two non-overlapping clones (HGT-2 and HGT-10) from a human genomic DNA library. Clone HGT-2 contains a 1.5kb uninterrupted linear sequence similar to bovine α1→3-galactosyltransferase that is organized as a processed pseudogene. This sequence, flanked by direct repeats, contains a complete recognizable coding region that is 81% similar at the nucleotide level to bovine α1→3-galactosyltransferase. Multiple frameshift- and nonsense mutations are present in this sequence which precludes the synthesis of a functional enzyme. The α1→3-galactosyltransferase sequence in clone HGT-10 is organized quite differently. It is distributed over ~12 kb and is interrupted by at least two introns. By analysis of a panel of human-hamster somatic cell hybrids we have established that the sequences contained in HGT-2 and HGT-10 are located on different human chromosomes. Interestingly, comparison of the predicted amino acid sequence of human α1→3-galactosyltransferase, with the corresponding region of the human blood group A and B glycosyltransferases [Yamamoto, F. *et al.*, (1990) Nature 345:229-233] reveals a significant similarity, which suggests that α1→3-galactosyltransferase and the A/B enzymes may have arisen from the same ancestral gene as a result of gene duplication and subsequent divergence.

This work was supported in part by a NATO Grant CRG-890490 (D.H.J.) and by Grant GM-38310 (J.H.S.) from the National Institutes of Health.

CLONING AND SEQUENCE ANALYSIS OF A cDNA ENCODING HUMAN PLACENTA GLYCOSYLASPARAGINSE. Krishna J. Fisher, Ole K. Tollersrud, and Nathan N. Aronson, Jr. Department of Molecular and Cell Biology, Althouse Laboratory, The Pennsylvania State University, University Park, PA 16802 USA

Glycosylasparaginase (EC 3.5.1.26) is a lysosomal amidase that hydrolyzes the GlcNAc-Asn bond which joins oligosaccharides to the peptide of asparagine-linked glycoproteins. Physiological and biochemical importance of asparaginase in the ordered, bi-directional degradation of asparagine-linked glycoproteins is evidenced by the occurrence of the inborn lysosomal storage disease aspartlyglucosaminuria (AGU) when there is a deficiency of the enzyme. We recently purified asparaginase to homogeneity from rat liver and found it to have a native molecular mass of 49 kDa and to be a heterodimer consisting of an α (24 kDa) and β (20 kDa) subunit [Tollersrud, O.K. and Aronson, N.N. (1989) *Biochem. J.* **260**, 101-108]. Polyclonal antibodies against the α and β subunits of rat liver asparaginase have shown that the human enzyme is similar in structure to the rat enzyme. Using amino acid sequence data from amino terminal analysis and a CNBr peptide of the rat β subunit, we PCR amplified a 221-bp fragment containing rat liver asparaginase gene sequences. This partial rat asparaginase cDNA was subsequently used to isolate a full-length 1.2 kb human placenta asparaginase cDNA clone designated HPAsn.6. The deduced amino acid sequence from HPAsn.6 encoded a 34.6 kDa polypeptide that showed sequence identity to amino acid sequence from both the α and β subunits of the rat enzyme, suggesting the human enzyme (and likely the rat as well) is post-translationally processed to generate the two subunits. The predicted site of cleavage is a stretch of 11 hydrophilic residues immediately upstream of the sequence that corresponds to the amino terminus of the β subunit of rat asparaginase. Of the 131 amino acids that overlap between rat asparaginase protein/cDNA sequence and the human asparaginase clone HPAsn.6, 86% of the residues are identical. Fibroblasts from Finnish AGU patients are presently being used to characterize the mutation(s) responsible for the disease.

CHARACTERIZATION AND MOLECULAR CLONING OF GLYCOSYLASPARAGINASE. Q.K. Tollersrud and N.N. Aronson, Jr. Department of Molecular and Cell Biology, PENN STATE UNIVERSITY, University Park, PA 16802.

The lysosomal enzyme, glycosylasparaginase, cleaves the amide bond between Asn and GlcNAc in Asn-linked glycoproteins. The enzyme requires a free α-amino and carboxyl group on the Asn and any α-L-fucose at the 6 position of the GlcNAc must be removed prior to GlcNAc-Asn hydrolysis. This substrate specificity ensures that the enzyme is acting at a late stage of lysosomal glycoprotein degradation. The rat liver glycosylasparaginase is a heterodimer of 24 kDa(α-subunit) and 20 kDa(β-subunit) joined by strong noncovalent forces. Antisera directed against each of the rat enzyme subunits crossreacted to the corresponding subunit peptides of glycosylasparaginase in a number of other mammals, including human, hog and mouse. Subsequent studies on the native and subunit molecular weights of the enzyme from these species and studies on various physical properties including pH and temperature curves and SDS-stabilities revealed a high degree of structural conservation. We have generated an ordered CnBr-peptide map of both the rat enzyme subunits, and obtained sequences from three CnBr peptides in addition to N-terminal sequences from each subunit. These data account for about 20% of the total peptide sequence. The sequences have been used to obtain oligonucleotide primers for PCR and the subsequent cloning of a complete cDNA of the human placenta enzyme. We are presently in the progress of cloning the rat enzyme using this cDNA as a probe.

ISOLATION OF 13 AND 15 KILOBASE HUMAN GENOMIC DNA CLONES CONTAINING THE GENE FOR UDP-*N*-ACETYLGLUCOSAMINE:α-3-D-MANNOSIDE β-1,2-*N*-ACETYLGLUCOSAMINYLTRANSFERASE I

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UDP-GlcNAc:α-3-D-mannoside β2-GlcNAc-transferase I (GnT I) catalyzes an essential first step in the conversion of high mannose to hybrid and complex N-glycans, i.e., UDP-GlcNAc + (Manα1-6[Manα1-3]Manα1-6)(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn-X to (Manα1-6[Manα1-3]Manα1-6)(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn-X + UDP. Rabbit liver GnT I has been highly purified (Y.Nishikawa *et al.*, J.Biol.Chem. 263, 8270, 1988) and a 2.5 kb cDNA has been cloned from a rabbit liver cDNA λgt10 library (M.Sarkar *et al*, these proceedings, and Proc.Xth International Symp.Glycoconjugates, Jerusalem, 1989, pg.127, abstract 80). We used the polymerase chain reaction (PCR) to obtain a 0.5 kb ds-cDNA representing the carboxy-terminal half of the rabbit liver GnT I coding sequence and labelled this DNA fragment by the random primer technique. The probe was used to screen 10^6 plaques from an amplified human genomic DNA library in λEMBL3 prepared from chromosomal DNA from chronic myeloid leukemia cells (kindly provided by Dr.G.Grosveld, Erasmus Univ., Rotterdam, The Netherlands). Positive plaques (23) were purified and phage DNA was subjected to restriction enzyme analysis using the 0.5 kb rabbit cDNA as probe. All 23 preparations gave the same *Sau*3A 0.4 kb fragment. This fragment showed 87% base similarity and 90% amino acid sequence similarity to the rabbit GnT I carboxy-terminal sequence. Inserts of 13 and 15 kb were cut from two of the human genomic DNA clones with *Sal*I and subcloned into plasmid pGEM-5zf(+) (Promega). Restriction maps of the two inserts show that they represent an overlapping 18 kb DNA sequence. No introns were present in the carboxy-terminal 40% of the human GnT I coding region. The remaining coding sequence was located in a 4.0 kb fragment of human genomic DNA by screening restriction maps with a probe containing the entire coding region of the rabbit GnT I cDNA. This 4.0 kb DNA fragment is being sequenced. Preliminary results indicate that transfection of the gene into Lec 1 Chinese hamster ovary cell mutants (which lack GnT I activity) results in the expression of GnT I activity. (Supported by the MRC of Canada).

CLONING OF A SIALYLTRANSFERASE INVOLVED IN BIOSYNTHESIS OF O-LINKED CARBOHYDRATE GROUPS

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Sialyltransferases are a family of glycosyltransferases which catalyze the transfer of sialic acid to terminal positions on the carbohydrate groups of glycoproteins and glycolipids. At least 10-12 different sialyltransferases are required to synthesize all the sialyloligosaccharide sequences known since a distinct enzyme is required for formation of each type of sequence. To date, four of these enzymes have been purified to homogeneity, and a full length cDNA of a β-galactoside α2,6 sialyltransferase has been cloned (JBC, 262, 17735). Purification of two molecular weight forms of the β-galactoside α2,3 sialyltransferase from porcine liver and subsequent N-terminal sequencing yielded two seperate regions of amino acid sequence. Based on one of these regions, a unique 53 bp oligonucleotide probe was synthesized and used to screen a porcine submaxillary gland cDNA library. Two overlapping clones were obtained that encode a complete open reading frame whose translated amino acid sequence contains both of the peptide sequences derived from the purified protein. The two clones have been completely sequenced and are identical except for a single 120 bp. insert in one of the clones which maintains the open reading frame. Based on our analysis of the α2,6 sialyltransferase, which has been shown to undergo alternate splicing, this provides evidence that alternately spliced forms of the α2,3 sialyltransferase may exist. Additionally, a 45 residue region of the amino acid sequence was found to have 62% identity to a portion of the α 2,6 sialyltransferase. This provides evidence that sialyltransferases may comprise a unique gene family. (supported by NIH grants GM27904 and GM08042)

ALTERED LOCALIZATION OF RAT LIVER β-GALACTOSIDE α2,6 SIALYLTRANSFERASE EXPRESSED IN CHINESE HAMSTER OVARY CELLS

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The β-galactoside α2,6 sialyltransferase (Galα2,6ST) was cloned from rat liver and sequenced (Weinstein, et. al. *J. Biol. Chem.* **262**, 17735, 1987). The cDNA encoding the entire amino acid sequence was expressed in Chinese hamster ovary (CHO) cells using the pMT010/A+ expression vector (Choo, et. al. *DNA* **5**, 529, 1986). This vector features dihydrofolate reductase amplification as well as the inducible metallothionein promoter. Wild type CHO cells normally produce N-linked carbohydrates terminating in SAα2,3Gal. By expressing the Galα2,6ST, transfected CHO cells will prduce carbohydrates with SAα2,6Gal which are not normally synthesized. Transfected CHO cell lines resistant to increasing levels of methotrexate express increasing levels of enzymatic activity in cellular membranes with coordinately increasing mRNA levels. In addition, immuno-electron microscopy localized the recombinant enzyme to the Golgi apparatus at low levels of expression; however, upon over expression of the Galα2,6ST, the membrane bound enzyme gradually exhibited less restricted localization, appearing throughout the Golgi apparatus, the endoplasmic reticulum, the nuclear envelope, and post-Golgi vesicles, but not on the cell surface. Time course of induction of Galα2,6ST using the heavy metal, zinc, revealed protein over expression by 20 hours. Supported by NIH grant GM-27904 and *Swiss National Science Foundation Grant 31-26273.89.

ISOLATION OF cDNAs ENCODING PORCINE N-ACETYLGALACTOSAMINIDE α2,6-SIALYLTRANSFERASE.

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The N-acetylgalactosaminide α2,6-sialyltransferase (α2,6-ST(*O*)) forms the linkage NeuAcα2,6GalNAc found in *O*-linked carbohydrate groups. Polyclonal antibodies to the α2,6-ST(*O*) protein purified from porcine submaxillary glands (PSG) was raised in rabbits and characterized. This antibody was shown to contain strong blood group type A activity in addition to the reactivity to purified α2,6-ST(*O*). The antibody was subjected to PSG-mucin-coupled Sepharose 4B column chromatography to remove the blood group type A activity. Approximately 5 X 10^5 recombinants from a PSG λZAPII cDNA library were screened for expression of α2,6-ST(*O*) protein in *E. coli* using the purified antibody. One immunopositive clone was isolated and characterized. The immuno-reactivity was diminished by preincubation of the antibody with the purified α2,6-ST(*O*) protein. Subsequently, the same library was screened by nucleotide hybridization using the cDNA insert as a probe. Five independent clones were isolated. Nucleotide sequence analysis showed that these clones carried cDNAs encoding a 1,304-residue protein missing the N-terminal portion. This putative protein was shown to have significant homology with a porcine α2,3-sialyltransferase (ST) forming the *O*-linked NeuAcα2,3Gal linkage for which we have recently cloned a gene. Taken together, we propose these cDNAs encode α2,6-ST(*O*). Furthermore, in this putative α2,6-ST(*O*) sequence, we found at least 11 repeats of EGF(epidertmal growth factor)-like domains just upstream of the region sharing homology with α2,3-ST. From recent studies on the function of EGF domains, it is expected the EGF repeats may be involved in acceptor-protein recognition. (Supported by NIH grant GM27904).

MOLECULAR ANALYSIS OF THE HEX β- SUBUNIT GENE IN A PATIENT WITH INHERITED MOTOR NEURON DISEASE

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GM2 ganglioside storage can result from mutations in either Hex A, Hex B or Hex activator protein (AP) genes, since the active Hex complex is [$\alpha\beta_2$.AP]. Typical adult/chronic GM2 gangliosidosis results from ^{269}Gly→Ser [Navon & Proia (1989) 243, 1471], creating an α subunit which fails to associate with the β-chain thus causing motor neuron disease. We have previously reported a patient (KL) with a similar motor neuron disease in which Hex A appeared normal and Hex B was <5%, suggesting a β-subunit defect. Northern blot analysis of RNA from cultured fibroblasts obtained from the patient revealed normal amounts of α- and β- chain message, suggesting that both β-chain alleles were transcribed into normal length mRNA. However, pulse-chase labeling studies and Western blot analysis revealed that only half the normal amount of β-chain was present in cells in contrast to normal levels of α-chain. Although this β-chain could combine with an α-chain to produce a Hex A which could hydrolyze synthetic substrates, hydrolysis of the natural GM2-ganglioside was only 10% of normal. Total RNA from the patient's cells was reverse transcribed into cDNA which was then PCR-amplified using synthetic 20-base oligonucleotide primers specific for β-chain. Following ligation of the 1.5 Kb β-chain cDNA into plasmid inserts and amplification, sequencing revealed two types of single missense mutation. In Type I, mutation of ^{619}A to G converted a ^{207}isoleucine to valine in a highly conserved region associated with activator protein binding which could result in an unstable polypeptide. In Type II, an ^{362}A→G mutation converted ^{121}lysine into arginine at the putative cleavage site of the b and c subunits. We therefore propose that the patient is a compound heterozygote in which a combination of unstable β-chain and impaired activator protein binding result in a partial β-Hex deficiency and slow accumulation of GM2 ganglioside, primarily in motor neurons. (Supported by UPHS Grant HD-06426 and the Muscular Dystrophy Association)

PURIFICATION AND MOLECULAR CLONING STUDIES OF RAT LIVER LYSOSOMAL β-D-GALACTOSIDASE. H. Park and N.N. Aronson, Jr. Department of Molecular and Cell Biology, PENN STATE UNIVERSITY, University Park, PA 16802.

The lysosomal glycosidase β-D-galactosidase was purified to homogeneity from rat liver using acid extraction and ammonium sulfate precipitation followed by column chromatography on CM-cellulose, DEAE-cellulose, ACA 54, ConA, and an affinity matrix p-aminophenyl-1-thio-β-D-galactopyranoside agarose. The purified enzyme was a single band on SDS-PAGE with a molecular weight of 64 kDa. N-terminal amino acid sequencing yielded the primary peptide structure:

QRTFELDYKRDRFLKDGQPFRYI...

This sequence is over 80% identical to the sequence of the human galactosidase either deduced from molecular cloning of the enzyme or direct amino acid sequencing by three other laboratories. A monospecific polyclonal antiserum was made against rat β-D-galactosidase in a rabbit. Several studies on the biosynthesis and processing of β-D-galactosidase have shown that the initial precursors detected in pulse-chase experiments have an approx. M_r of 85 kDa with the M_r of mature enzyme within lysosomes being about 64 kDa for the human and mouse protein which is the same size as we have found for the rat enzyme. The human galactosidase cDNA predicted a molecular weight of 75 kDa from the deduced 677 amino acid structure. This included a putative 23 amino acid signal peptide which after removal by signal peptidase in the endoplasmic reticulum would leave an inital naked polypeptide precursor of approx. 73.5 kDa for the enzyme prior to its arrival within lysosomes. Approx. 11.5 kDa(85-73.5 kDa) of the earliest form of the enzyme observed in pulse-chase experiments resulted from high mannose oligosaccharides on the protein that are added cotranslationally. Since only a few amino acids were missing from the rat enzyme in comparison to the deduced N-terminus from these human clones, substantial processing of galactosidase must take place at its C-terminus upon reaching the lysosomes(85 kDa ——> approx. 64 kDa). There is an 18% loss of the initial translation product after removal of the signal peptide. This calculation takes into account possible oligosaccharides being present on five remaining consensus Asn-X-Ser/Thr glycosylation sequences. We are now using the antibody against rat galactosidase and a synthesized oligonucleotide deduced from its N-terminal sequence to screen a rat liver λgt11 library for a complete clone of rat β-D-galactosidase to be compared to the human enzyme.

Sialic acid analogs define regions of sialic acid involved in binding to the influenza virus hemagglutinin

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The initial step of influenza infection is binding of the virus particles via their hemagglutinin to cell surface sialic acids. Recently, X-ray crystallographic studies have revealed the exact location of the sialic acid residue in the binding pocket of the hemagglutinin (Weis et al. 1988, Nature **332**,426-431). From this information hydrogen bonds, hydrophobic or ionic interactions between amino acid residues in the binding pocket and the sialic acid molecule can be proposed. However, we do not know which of these interactions are important for the binding. In order to address this question, synthetic sialic acid analogs were tested in a virus adsorption inhibition assay for their inhibitory potency. These analogs differ from sialic acid at only one or two positions in the sugar molecule. These modifications were made in three regions of the sialic acid molecule, the carboxyl group, the acetamido group, and the glycerol side chain. The data obtained show that the negative charge and orientation of the carboxyl group is essential for the binding of sialic acid to the hemagglutinin. Therefore, it is very likely that the hydrogen bond and/or the ionic interaction of this group are involved in binding as predicted from the crystallographic studies. At C_5, acylamide residues other than acetamido are not permitted in the binding pocket. It can be concluded that hydrophobic interactions of the methyl group with a tryptophane in the binding pocket are essential for binding. In the glycerol side chain, a hydrogen bond to the hydroxyl group at C_8 is important for binding, whereas the hydroxyl at C_9 is not involved, although amino acids of the hemagglutinin are close enough to form hydrogen bonds with the hydroxyl group at C_9 as known from the X-ray crystallographic analysis. This information will allow us to develop novel compounds which may enhance or alter sialic acid-hemagglutinin interactions, leading to sialic acid analogs which can bind more avidly and more specifically to the influenza virus hemagglutinin. This strategy may contribute to the design of new anti-influenza drugs.

N-ACETYLNEURAMINYLLACTOSAMINE SYNTHESIS BY A GENERAL ENZYMATIC METHOD FOR PRODUCING OLIGOSACCHARIDES. Thomas Schaal*, Debra Vail, Edward J. McGuire, and S. Roth. Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, and * Department of Cell and Molecular Biology, Harvard University, Cambridge, MA.

Oligosaccharides have important functions in intermolecular and intercellular recognition, and the number of different structures is growing almost daily. Nevertheless, the study and utility of these compounds are hampered by the absence of a general method for their synthesis. We here suggest a completely enzymatic method that could yield large amounts of any oligosaccharide composed of linkages that exist in nature. The technique relies on the sequential activity of the same glycosyltransferases that synthesize *in vivo* the desired linkages. The newly glycosylated product of each enzyme cycle is used (a) as an affinity substrate to obtain the next enzyme, and (b) as the next acceptor substrate for the enzyme obtained.

To synthesize the trisaccharide N-acetylneuraminyllactosamine (sialyllactosamine), for example, we partly purified from human colostrum a β1-4 galactosyltransferase and an α 2-3 sialyltransferase. The galactosyltransferase produces N-acetyllactosamine from GlcNAc and UDPgalactose. The sialyltransferase then produces the trisaccharide from the N-acetyllactosamine and CMP-[^{14}C]-NAN. In batch preparations, with decreased overall efficiency, the formation of the trisaccharide required UDPgalactose, CMPNAN, GlcNAc, and Mn^{++}, and did not occur at 0°C. The product was characterized by its electrophoretic mobility on borate-impregnated paper. The enzymes can be used in their soluble forms or immobilized, which greatly facilitates their re-utilization. To immobilize the enzymes, we have used poly(acrylamide-*co*-*N*-acryloxysuccinimide) as described by Pollak et al. (1980, J. Am. Chem. Soc., 102, 6324-6336), or oxirane acrylic beads (Eupergit; Accurate Chemical and Scientific Co., NY, NY).

In one batch experiment, using 25 µl each of the partly purified, soluble enzymes (each at about 4 µg protein/ml), in a total incubation volume of 95 µl, we synthesized 0.001 nanomoles of trisaccharide in one hour under conditions that were sub-optimal for the sialyltransferase. Under identical conditions, sequential steps are about 100 times more efficient, yielding 0.1 nMoles of the trisaccharide. Scaled-up syntheses of this and other oligosaccharides are under way. The method should also be useful for specifically glycosylating lipids, proteins, and any other aglycones.

T-LYMPHOCYTIC LEUKEMIA EXPRESS COMPLEX, BRANCHED O-LINKED OLIGOSACCHARIDES ON A MAJOR SIALOGLYCOPROTEIN, LEUKOSIALIN.

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We showed previously that normal T-lymphocytes express on leukosialin the tetrasaccharides NeuNAcα2→3Galβ1→3(NeuNAcα2→6)GalNAc as the major oligosaccharides, while activated T-lymphocytes express more complex hexasaccharides, NeuNAcα2→3Galβ1→3 (NeuNAcα2→3Galβ1→4GlcNAcβ1→6)GalNAc (J. Biol. Chem. 263, 15146-15150, 1988). In the present study, we have analyzed structures of O-glycans attached to leukosialin present in various T-lymphocytic leukemia cells.

T-lymphoid cells from patients with acute T-lymphocytic leukemia express a large amount of more complex branched hexasaccharides, NeuNAcα2→3Galβ1→3 (NeuNAcα2→3Galβ1→4GlcNAcβ1→6)GalNAc. T-lymphoid cells from patients with chronic T-lymphocytic leukemia, on the other hand, express mainly the tetrasaccharides, NeuNAcα2→3Galβ1→3(NeuNAcα2→6) GalNAc, although they also express a small but significant amount of the hexasaccharides. The same hexasaccharides can be detected in thymocytes but are present at negligible levels in peripheral T-lymphocytes of normal persons. The increased amount of the hexasaccharides is associated with the increased activity of β1→6GlcNAc-transferase, a key enzyme to form the hexasaccharides. Western blotting study of leukosialin was performed, using anti-leukosialin protein antibodies and monoclonal antibody T305 specific for the hexasaccharides attached to leukosialin. The increased amount of the hexasaccharides was found to be associated with strong reactivity of T-305 and the apparent molecular weight of leukosialin increases when the branched hexasaccharides are attached to leukosialin.

These findings indicate that the hexasaccharides can be regarded as onco-differentiation antigen, and can be a useful marker of leukemic cells of T-cell origin. (Supported by CA33895).

EXPRESSION OF ABERRANT O-GLYCANS ATTACHED TO LEUKOSIALIN IN DIFFERENTIATION-INDUCER-RESISTANT HL-60 CELLS.

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The promyelocytic leukemia HL-60 cells can be induced to differentiate into granulocytic cells by various agents including retinoic acid (RA), dimethylsulfoxide (DMSO) and 6-thioguanine (6-TG). Although the induced cells are no longer capable of proliferation, a few cells continue to divide in the presence of inducers, and these cells are resistant to terminal differentiation by these inducers (Gallagher et.al., Blood 68, 1402-1406, 1986).

When leukosialin, a major sialoglycoprotein of HL-60 cells, was analyzed by SDS-polyacrylamide gel electrophoresis, leukosialin from RA-resistant and 6-TG resistant HL-60 sublines migrated much slower than those from wild-type HL-60 cells. DMSO-resistant HL-60 subline, on the other hand, express leukosialin with Mr similar to wild-type HL-60 cells. We have analyzed *O*-glycans attached to leukosialin in these HL-60 cells and the following was found. RA-resistant and 6-TG-resistant HL-60 sublines produce a significant amount of *O*-glycans that contain no sialic acid residues such as Galβ1→3(Galβ1→4GlcNAcβ1→6) GalNAc, while wild-type and DMSO-resistant HL-60 cells express mainly disialosylhexasaccharide. Wild-type HL-60 cells treated with the inducers for 4 days were found to express the same saccharides as found in untreated wild-type HL-60 cells.

To understand better the mechanisms underlying the differences in *O*-glycans, we have measured three sialyltransferases; Galβ1→3GalNAc α2→3sialyltransferase, Galβ1→3(4)GlcNAc α2→3sialyltransferase and GalNAc α2→6sialyltransferase. Among them, Galβ1→3GalNAc α2→3sialyl transferase was much lower in RA-resistant and 6-TG-resistant HL-60 sublines than in wild-type HL-60 cells. These findings indicate that the difference in *O*-glycans are due to the difference in α2→3sialyltransferase activity.

These results strongly suggest that *O*-glycans associated with leukosialin play some role in HL-60 cell differentiation. (Supported by CA33895).

DEFECTIVE O-LINKED GLYCOSYLATION IN LYMPHOCYTES FROM PATIENTS WITH THE WISKOTT-ALDRICH SYNDROME. **E.A. HIGGINS*, D.L. ZHUANG, K.A. SIMINOVITCH, I. BROCKHAUSEN+ AND J.W. DENNIS*.** Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, 600 University Ave., Toronto, CANADA M5G 1X5 and *Dept of Medical Genetics, U. of Toronto. +Hospital for Sick Children, Toronto.

The Wiskott-Aldrich syndrome (WAS) is an X-linked, recessive, immunodeficiency in which lymphocyte proliferation is defective. Leukosialin (CD43), a glycoprotein heavily glycosylated in O-linked structures, has been shown to have an abnormal molecular weight in lymphocytes from patients with WAS. To determine whether O-linked glycosylation in the lymphocytes of these patients is defective, we assayed glycosyltransferase activities in circulating lymphocytes and in Epstein-Barr Virus (EBV) immortalized B cell lines established from normal individuals and from WAS patients.

The UDP-GlcNAc:Galβ1-3GalNAc-R (GlcNAc to GalNAc) β6-N-acetylglucosaminyltransferase (core 2 GlcNAc-T) activity in cell lysates of normal resting lymphocytes was low (0.7 nmol/mg/h, n=8) compared to that found in activated lymphocytes (2.2 nmol/mg/h, n=8). A similar increase in core 2 GlcNAc-T was observed by Piller et al. (JBC 263:15146). We have found that in lymphocytes from WAS patients, the regulation of the core 2 GlcNAc-T activity is reversed. Unstimulated lymphocytes from WAS patients showed high levels of core 2 GlcNAc-T activity (2.3 nmol/mg/h, n=7) when compared to stimulated cultures (0.9 nmol/mg/h, n=7). In contrast, the activity of UDP-Gal:GalNAc-R β3-galactosyltransferase was similar in resting and stimulated lymphocytes from normal and WAS individuals.

Activated B cells from WAS patients showed a similar deficiency in core 2 GlcNAc-T activity. In EBV-immortalized B cell lines (activated B lymphocytes), core 2 GlcNAc-T activity was 2.4 nmol/mg/h (n=4) in lines from normals and <0.3 nmol/mg/h (n=4) in cell lines from WAS patients. Since decreased CMP-NANA:sialylα2-3Galβ1-3GalNAc-R (sialic acid to GalNAc) α6 sialyltransferase II activity has been shown to be associated with T cell activation we assayed this enzyme and found that it was also aberrant in WAS lymphocytes. WAS EBV-B cell lines have 2.1 nmol/mg/h of α6SA-TII activity while normal EBV-B cell lines have an α6SA-TII activity of <0.3 nmol/mg/h. Eight other glycosyltransferases were assayed and their activities were found to be the same in WAS and normal cell lines.

Finally, we have shown that the amount of polylactosamine associated with O-linked oligosaccharides in WAS EBV-immortalized B cell lines is decreased compared to EBV-immortalized B cell lines from normal individuals. This suggests that the decrease in core 2 GlcNAc-T activity in WAS cells results in a loss of β1-6 linked lactosamine antennae, an acceptor for polylactosamine addition. This reduction in O-linked associated polylactosamine appears to be compensated for by an increase in polylactosamine associated with N-linked structures.

The results show that in lymphocytes from WAS patients, activation-dependent regulation of core 2 GlcNAc-T and α6SA-TII, is abnormal. Work is in progress to determine whether defective glycosylation of specific glycoproteins is associated with abnormal lymphocyte function in WAS patients.

BIOSYNTHESIS OF O-GLYCANS IN LEUKEMIA: INCREASE IN UDP-GLCNAC: GALβ3GALNACα-R (GLCNAC TO GALNAC) β6-GLCNAC-TRANSFERASE IN CML AND AML.*

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We have studied the biosynthesis of altered O-glycan structures on leukocytes from patients with chronic myelogenous leukemia (CML) and with acute myeloblastic leukemia (AML). We have previously shown that the activity of CMP-NeuAc: Galβ1-3GalNAcα-R (sialic acid to Gal) α(2-3)-sialyltransferase (Baker, Kanani, Brockhausen, Schachter, Hindenburg and Taub, 1987 Cancer Res.47, 2763-2766) is increased in leukocytes from patients with CML and AML. This increased activity may in part be responsible for the hypersialylation observed in leukemic leukocytes. Hypersialylation may also be due to changes in underlying O-glycan structures. To test this hypothesis, we have investigated in normal human granulocytes and leukemic leukocytes several glycosyltransferases involved in the synthesis and elongation of the four common O-glycan cores. HPLC was used to separate and identify enzyme products. UDP-GlcNAc:Galβ1-3GalNAc-R (GlcNAc to GalNAc) β(1-6)-GlcNAc-transferase, which synthesizes O-glycan core 2 (GlcNAcβ1-6[Galβ1-3]GalNAcα), is significantly elevated in CML granulocytes (four-fold) and AML blast cells (eighteen-fold) relative to normal human granulocytes. UDP-Gal:GalNAc-R β(1-3)-Gal-transferase and the elongation enzyme UDP-Gal:GlcNAc-R β(1-4)-Gal-transferase are moderately elevated in leukocytes from CML and AML patients. Neither normal nor leukemic cells show detectable activities of UDP-GlcNAc:GalNAc-R β(1-3)-GlcNAc-transferase or UDP-GlcNAc:GlcNAcβ1-3GalNAc-R (GlcNAc to GalNAc) β(1-6)-GlcNAc-transferase or UDP-GlcNAc:GlcNAcβ1-3Galβ-R (GlcNAc to Gal) β(1-6)-GlcNAc-transferase. UDP-GlcNAc:Galβ1-3(R1β1-6)GalNAc-R2 (GlcNAc to Gal) β(1-3)-GlcNAc-transferase, an enzyme which elongates core 1 and core 2, was found at low levels in normal granulocytes but was absent from leukemic cells. UDP-GlcNAc:Galβ1-4GlcNAc-R (GlcNAc to Gal) β(1-3)-GlcNAc-transferase, an enzyme required for poly-N-acetyllactosamine synthesis, was present in all samples, but was increased in AML blast cells. These enzymatic changes observed in leukemia may significantly affect the backbone structures of O-glycans and may thus contribute to altered sialylation and the pathophysiology of leukemic cells.

IMMUNOFLUORESCENCE FLOW CYTOMETRIC ANALYSIS OF EPITECTIN ON THE H. EP. 2 CELL SURFACE.

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Epitectin is a mucin-type glycoprotein expressed on a wide range of human tumors and certain specialized normal epithelia and has been thought to play a role in tumorigenicity of the H. Ep. 2 cells. Epitectin is detected on the H. Ep. 2 cell surface by the Ca2 monoclonal antibody. In this study we have used immunofluorescence flow cytometric analysis with the Ca2 antibody to examine the mode of association of epitectin with the cell membrane. It was found that the intensity of fluorescence was not altered by treatment of the cells with 0.1% NP-40, 0.1% deoxycholate, 0.5 M urea, or phosphatidylinositol-specific phospholipase C. Fluorescence intensity was reduced when cells were treated with trypsin. These results, as well as the requirement of detergents to extract the glycoprotein from cells, indicate that epitectin is an intrinsic glycoprotein which is anchored to the membrane, probably via a hydrophobic peptide segment. It was observed through the course of these studies that the fluorescence intensity decreased with increasing passage number of the cultured cells. When injected into athymic mice, both low and high passage number cells induce tumor growth. This finding suggests that the presence of the glycoprotein on the cell surface does not modulate the tumorigenicity of the H. Ep. 2 cells, but rather that expression of epitectin may be induced by the environment within the tumor. This is consistent with the previously proposed function of epitectin in specialized normal epithelia, namely that of shielding the epithelial cells from toxic agents. [Supported by NIH grant CA38797.]

SYNTHESIS AND RELEASE OF CELL SURFACE-DERIVED AND SULFATED LACTOSAMINOGLYCANS BY HUMAN OVARIAN CARCINOMA CELLS. Howard J. Allen[a], Marie Gamarra[b], M. Steven Piver[c], Ashu Sharma[a], Hafiz Ahmed[a] and Edward A. Z. Johnson[d], Departments of Surgical Oncology[a], Pathology[b], Gynecologic Oncology[c], and Diagnostic Immunology and Biochemistry[d], Roswell Park Memorial Institute, Buffalo, N.Y. 14263. Ovarian carcinoma cell clusters were isolated from patient effusions. Cell surface glycoconjugates were radiolabelled by a galactose oxidase-borotritide method. The surface-labelled glycoconjugates and metabolically labelled glycoconjugates released to culture medium were characterized. The surface-derived glycoconjugates were highly heterodisperse and had the same molecular weight distribution as the metabolically labelled components as assessed by tube gel and slab gel SDS-PAGE. Lectin precipitation assays showed that both classes of glycoconjugates contained N-linked oligosaccharides bearing N-acetyllactosamine moieties. A121 ovarian carcinoma cells also synthesized and released a heterodisperse array of glycoconjugates to culture medium. Ricinus communis agglutinin I (RCAI) precipitated metabolically labelled glycoconjugates of MW >100 kDa for both A121 cells and cells from effusions. Galaptin, an endogenous β-galactoside-binding lectin, also precipitated high molecular weight glycoconjugates. Cells of different ovarian carcinoma histology yielded similar results. Metabolic labelling experiments with $^{35}SO_4$ showed that the RCAI-binding and galaptin-binding glycoconjugates released by A121 cells were sulfated, as determined by lectin affinity chromatography. Competitive galaptin binding inhibition assays revealed the presence of galaptin inhibitor(s) in normal sera, effusions, and carcinoma patient sera. The levels of inhibitor(s) in effusions were up to 8 fold greater than normal sera. Five out of 8 sera from colorectal carcinoma patients and 7 out of nine sera from ovarian carcinoma patients had elevated levels of inhibitor(s). Four out of 8 sera from other gynecologic cancer patient sera had elevated levels of galaptin inhibitor(s). The sulfated lactosaminoglycans may be associated with malignant transformation and/or metastasis since similar components were not produced by mesothelial cells isolated from effusions [Allen, H. J., M. Gamarra, M. S. Piver and E. A. Z. Johnson (1989). Cancer Biochem. Biophys. 10, 219-226]. Supported by CA14854, CA42584, NIH BRSG funds, ACS IRG funds and Elsa U. Pardee Foundation.

CHARACTERIZATION OF GLYCOCONJUGATES RELEASED IN VITRO BY HUMAN OVARIAN CARCINOMA CELLS ISOLATED FROM EFFUSIONS. Howard J. Allen[a], Marie Gamarra[b], M. Steven Piver[c] and Edward A. Z. Johnson[d], Departments of Surgical Oncology[a], Pathology[b], Gynecologic Oncology[c] and Diagnostic Immunology and Biochemistry[d], Roswell Park Memorial Institute, Buffalo, N.Y. 14263. Ovarian carcinoma cell clusters were isolated from patient effusions. The glycoconjugates released to culture medium *in vitro* were characterized by electrophoretic, immunoassay and gel filtration procedures. Metabolically radiolabelled glycoconjugates were heterodisperse with respect to molecular weight and this heterodispersity was independent of incubation time *in vitro*. This heterodispersity was also characteristic of mixed Mullerian tumor cells of endometrial origin whereas mesothelial cells released a discrete glycoconjugate of MW 65-70 kDa. Multiple Coomassie blue-stained polypeptides were released by the carcinoma cells. These polypeptides were not adsorbed serum components as assessed by immunodiffusion analyses. Periodic acid-Schiff-reactive macromolecules appeared only at the top of electrophoresis gels. The high molecular weight glycoconjugates synthesized by ovarian carcinoma cells precipitated with an effusion globulin fraction at low ionic strength but the low molecular weight components (40-70 kDa) were soluble. Immunoprecipitation with anti-Ig failed to precipitate carcinoma glycoconjugates. Antisera raised against the released carcinoma macromolecules precipitated carcinoma glycoconjugates and normal ovarian polypeptides. Antisera raised against normal ovarian macromolecules precipitated ovarian polypeptides but reacted only slightly with carcinoma glycoconjugates. Immunodiffusion analyses showed the presence of α_1-acid glycoprotein and carcinoembryonic antigen (CEA)-like components in the carcinoma glycoconjugates. The presence of CEA-like glycoconjugates was confirmed by immunoprecipitation. The antigens and antisera for different histologic types of ovarian carcinoma were cross-reactive. The presence of β_2-microglobulin suggested that some of the glycoconjugates were shed from the cell surface. The role that these glycoconjugates play in the biology of ovarian carcinoma remain to be elucidated. Some of the glycoconjugates described here may yield new antigenic specificities to supplement those currently in use for carcinoma diagnosis and prognosis. Supported by CA14854, NIH BRSG funds, ACS IRG funds, and Elsa U. Pardee Foundation.

SUBSTRATE REQUIREMENTS FOR PURIFIED α1→3FUCOSYLTRANSFERASE FROM HUMAN NEUROBLASTOMA. D.R.B. Gillies, C.S. Foster and M.C. Glick, Department of Pediatrics, School of Medicine, University of Pennsylvania and The Children's Hospital of Philadelphia, Philadelphia, PA 19104.

Fucosyl residues in α1→3linkage to *N*-acetylglucosamine (Fucα1→3GlcNAc) on the oligosaccharide branch of glycoproteins have been described on select groups of human tumors including neuroblastoma (1). The characterization of GDP-*L*-Fuc: *N*-acetyl-β-*D*-glucosaminide α1→3fucosyltransferase (α1→3fucosyltransferase) from neuroblastoma is a necessary prerequisite to determining how the enzyme regulates the expression of these fucosyl residues in tumor cell glycoproteins. α1→3Fucosyltransferase was affinity purified to near electrophoretic homogeneity from human neuroblastoma cells, CHP 134. The enzyme had a specific activity of approximately 1 Unit (μmol/min/mg) under saturating conditions for GDP-fucose. The apparent $K_{GDP\text{-}Fuc}$ was 43 μM and $K_{Gal\beta1\rightarrow4GlcNAc}$ was 0.4 mM. Further examination of the substrate requirements of the purified enzyme showed that Fucα1→2Galβ1→4GlcNAc had a relative activity to Galβ1→4GlcNAc of 140%. However, the purified α1→3fucosyltransferase was unable to transfer to either NeuNAcα2→3Galβ1→4GlcNAc or NeuNAcα2→6Galβ1→4GlcNAc. Moreover, the enzyme did not transfer to NeuNAcα2→3 or α2→6Galβ1→4Glc whereas a transfer was made to 2'fucosyllactose with 270% of the relative activity of lactose. $K_{Gal\beta1\rightarrow4Glc}$ was 5.4 mM and $K_{Fuc\alpha\rightarrow2Gal\beta1\rightarrow4Glc}$ was 1.0 mM. Therefore, in contrast to α1→3 fucosyltransferases reported from other human tumor cells (2, 3) neuroblastoma α1→3fucosyltransferase will not fucosylate a sialylated substrate. This substrate requirement is shared with that of the purified enzyme from chronic granulocytic leukemic cells (4) and human milk (5). Whether or not the neuroblastoma sialyltransferases will sialylate fucosylated substrates has yet to be determined, however, these results support the prediction that Fucα1→3GlcNAc resides on an oligosaccharide branch not containing NeuNAc residues (1). The substrate specificity leads to the suggestion that the neuroblastoma α1→3fucosyltranferase may represent a unique fucosyltransferase of certain human tumors. It remains to be resolved if this is a reexpression of the fetal enzyme or activation in the tumor cells of a hitherto silent gene.

Supported by NIH CA 37853.

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ALTERED GLYCOSYLATION IS ASSOCIATED WITH EXPRESSION OF LOW-AFFINITY RGD-DEPENDENT FIBRONECTIN RECEPTORS ON A MOUSE MELANOMA MUTANT WITH REDUCED SPREADING ON FIBRONECTIN AND REDUCED METASTATIC POTENTIAL, Orhan Oz and Tien-wen Tao, Stanford University School of Medicine, Stanford, CA 94305.

The acquisition of resistance to wheat-germ-agglutinin (WGA) by a WGA-resistant (WGA-R) mutant selected from B16 mouse melanoma cells is characterized by altered N-linked oligosaccharide structures, including decreased terminal sialic acid and the expression of the X-antigenic structure, (Galß1 →4[Fucα1→3]GlcNac) (Kawano, et al,.manuscript in preparation). Accompanying the glycosylation change are greatly reduced metastatic potential and altered adhesive properties. While its homotypic adhesiveness increases, it shows reduced adhesion and spreading on extracellular matrix components such as fibronectin and laminin. To test the hypothesis that the alteration in the carbohydrate structure of the adhesion receptor(s) affects the function of the receptor, the fibronectin receptor (FNR) was studied. Cell-binding-domain of fibronectin (CBD) immobilized on Sepharose was used to compare the structure and the binding affinity of the receptors isolated from the detergent extracts of cells surface labeled with ^{125}I. Two distinct RGD-dependent $\alpha\beta_1$ heterodimers with different binding affinities were identified, namely high affinity $\alpha_5\beta_1$ and low affinity $\alpha_x\beta_1$ as reflected by the difference in GRGDS concentrations needed for elution. The glycan portion of the integrin subunits of the mutant cells was structurally altered and appeared to contain the X-antigenic structure (Takasaki, Kobata, personal communication). While the parental cells appear to use both $\alpha\beta_1$ integrins as receptor for CBD, the mutant cells use predominantly only the low affinity $\alpha_x\beta_1$. More than 80% of the mutant receptors bound to CBD could be eluted at 1µg GRGDS/ml, whereas GRGDS concentrations at greater than 100µg/ml was necessary to elute the bound parental receptors. The decreased affinity of the mutant receptors for the ligand could be causally related to the reduced adhesive interaction of the cells with fibronectin.

CASTANOSPERMINE ANALOGS AS POTENTIAL ANTIMETASTATIC AGENTS.

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We have previously shown that castanospermine (CAST), an inhibitor of glycoprotein processing, has antimetastatic activities in murine metastatic models. The objective of the present study was to synthesize analogs of CAST with better potency as glucosidase I inhibitors and antimetastatic agents. Inhibition of glucosidase I was evaluated by determining the accumulation of Glc_3 Man_9 $GlcNAc_2$ ($G_3M_9N_2$) oligosaccharide containing glycoproteins in treated cells. Antimetastatic activity was determined by the inhibition of pulmonary colonization of *in vitro* treated B_{16} F_{10} melanoma cells upon i.v. injection in mice. A good relationship between inhibition of glucosidase I and lung colonization by CAST analogs was observed. The IC_{50} values of these inhibitors against the enzyme and metastasis ranged from 0.3 to 10 µg/ml, 6-octanoate analog being the most potent with an IC_{50} of 0.3 µg/ml as compared to 10 µg/ml for CAST. Two of the active analogs were further evaluated for *in vivo* effect on experimental metastasis of B_{16} F_{10} cells. The tumor cells ($1x10^5$ B_{16} F_{10} cells) were administered by tail-vein injection into C_{57} BL/6 mice and animals were treated with different doses ranging from 25-250 mg/kg i.p., once daily from day 1 to 14. Pulmonary metastatic foci were determined at the end of 14 days. The results indicated 75 and 77% inhibition of metastasis with 6-octanoate and 6-butyrate analogs, respectively, compared to 48% inhibition with CAST when the compounds were administered at 50 mg/kg, i.p. once daily from day 1 to 14. These studies suggest that derivatization of CAST resulted in more potent antimetastatic agents.

GLYCOSYLATION DOES NOT AFFECT SPECIFIC ACTIVITY OF MURINE IL-3. Hermann J. Ziltener, The Biomedical Research Centre, 2222 Health Sciences Mall, UBC, Vancouver, B. C. V6T 1W5

Murine IL-3 is secreted by activated T cells in three major molecular forms which differ from one another in the extent of their N-linked glycosylation. These IL-3 forms, with Mr values of 22K, 28K and 34-36K, have been purified using antibody affinity chromatography and SDS-PAGE. Portions of these samples were then enzymatically deglycosylated to test whether the removal of carbohydrate influenced specific activity as measured in a proliferation assay. Removal of N-linked carbohydrate did not change the specific activity of IL-3 and all three major glycosylated forms had identical specific activity. Many different cell types respond to IL-3, these cells have potentially heterogeneity in IL-3 receptors for example in glycosylation. We also tested whether the differently glycosylated IL-3 forms might differ in their interaction with IL-3 receptors, found on various cell lines. No difference in responsiveness of the cell lines tested was found.

PURIFICATION AND PHOTOAFFINITY LABELING OF GLUCOSYLTRANSFERASE OF DOLICHOL CYCLE FROM RAT MAMMARY GLAND.

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UDP-Glc: Dol-P glucosyltransferase catalyzes the synthesis of Glc-P-Dol, an important intermediate in the dolichol-linked assembly of oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$, of *N*-linked glycoproteins.

Attempts to purify the membrane-bound glycosyltransferases have been severely hampered due to extreme instability of these enzymes after solubilization with detergents. To date, the UDP-GlcNAc: Dol-P GlcNAc-1-P-transferase from bovine mammary tissue and the GDP-Man: Dol-P mannosyltransferase from yeast represent the only glycosyltransferases of the dolichol cycle that have been purified to homogeneity and characterized. We have earlier demonstrated that several glycosyltransferases of the dolichol cycle, including glucosyltransferase, and glucosidase I are developmentally regulated in the mammary tissue. Thus, a study of these enzymes becomes significant in understanding the regulation of protein *N*-glycosylation in hormonally responsive tissue such as mammary gland. In this investigation, we have partially purified glucosyltransferase by a combination of $(NH_4)_2SO_4$ fractionation, gel filtration, ion-exchange chromatography on DEAE-TSK followed by affinity chromatography on UDP-glucuronic acid-Sepharose. The partially purified enzyme exhibited a few minor and a 35 kDa protein bands on 10% SDS-PAGE under reducing conditions. Photoaffinity labeling of the partially purified enzyme with 5-azido-[β-^{32}P]-UDP-Glc identified a 35 kDa polypeptide band. Labeling of solubilized enzyme from crude and stripped microsomes also revealed the similar protein band. Photoinsertion of the probe in this polypeptide is enhanced by the presence of dolichol phosphate and $MgCl_2$. The intensity of the photolabeling of 35 kDa band coincided with the enzyme activity in the peak fractions of DEAE-TSK column. In order to find out the specificity of the photolabeling, UDP-Glc, the glucosyl donor for glucosyltransferase, was used as a competitor in the photolysis reaction. It inhibited the photolabeling of 35 kDa band in a dose dependent manner. Similar inhibition in the photoinsertion of the probe was also observed when UDP-glucuronic acid was used as competitor. Inhibition of photoaffinity labeling of 35 kDa band by several sugar nucleotides provided evidence to validate the specific labeling of 35 kDa band. These results suggested that this protein band is involved in the synthesis of Glc-P-Dol in rat mammary tissue. The possibility that this protein may represent glucosyltransferase has been discussed.

Supported by NIH grant DK-19682.

SYNTHESIS AND APPLICATIONS OF N-GLYCYL GLYCOSYLAMINES

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Incubation of reducing sugars in ammonium bicarbonate (1,2) was found to be a simple procedure for the formation of β-D-glycosylamines of purified complex oligosaccharides in 70-80% yield. These provide valuable intermediates for the synthesis of a wide range of oligosaccharide probes and derivatives by acylation of the 1-amino function.

A study of the properties of the 1-amino function indicated that it showed different rates of reactivity with different reagents. In general, the interactions with large ring systems such as the fluorophores Dansyl chloride and FITC gave 10-20% yields of products which consisted of mixtures of both anomeric forms, whereas smaller acylating reagents gave practically quantitative yields of the desired β-D-derivatives. The differences in the reactivity of the glycosylamine towards different reagents can be rationalised on steric grounds.

This observation was used as the basis for an adaptation of the method to incorporate a linker which retains the amine functional group. N-chloroacetamido derivatives could be obtained in high yield with retention of the β-anomeric configuration. Formation of the N-glycyl derivative by ammonolysis of the haloacetamido function was found to produce synthetic intermediates with several useful properties. Firstly, the sugar is fixed in the β-anomeric configuration by the formation of an amide bond to the linker. Secondly, N-glycyl derivatives are more stable than the glycosylamine, both for storage purposes and in handling during coupling. Thirdly, they should show uniform reactivity with a range of reagents. In particular, they show improved reactivity with complex aromatic fluorophores compared with the free glycosylamine. Finally, the linker is structurally analogous to the biological N-glycosidic linkage between the carbohydrate and the asparagine side chain. It therefore retains all of the available biological "information" in the structure, and should be both stable under physiological conditions and non-immunogenic. This modification enabled fluorophore, biotin and lipid conjugates to be obtained in high yields. β-N-haloacetylation can also provide other interesting synthetic alternatives (such as thiol-reactive glycoconjugates).

In contrast to derivatives formed by reductive amination, biotinylated and lipid-linked derivatives of a fucosylated biantennary oligosaccharide formed via N-glycyl intermediates were effective in binding to lentil lectin. High affinity interactions with the lectin were therefore shown to be dependent upon the integrity of the reducing terminus (3). This illustrates the importance of retention of this biological "information", and indicates that despite its popularity, reductive amination of oligosaccharides as a technique for formation of glycoconjugates should be used with caution. The chemical simplicity of the procedure outlined here suggests that this represents both a viable alternative to the use of reductive amination and an opportunity to obtain a wide range of oligosaccharide probes.

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METHYL 3,4-O-ISOPROPYLIDENE-2,6-DI-O-(4-METHOXYBENZYL)-1-THIO-ß-D-GALACTOPYRANOSIDE AND PENTENYL 3,4-O-ISOPROPYLIDENE-2,6-DI-O-(4-METHOXYBENZYL)-ß-D-GALACTOPYRANOSIDE- TWO GLYCOSYLATING REAGENTS FOR CONVENIENT SYNTHESIS OF α-D-GALACTOPYRANOSYL OLIGOSACCHARIDES

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In the last few years, we have become interested in the development of new glycosylating reagents for the stereoselective synthesis of α-D-glycosidically linked oligosaccharides. As a general rule in the synthesis of α-D-glycosidic linkages, the requirement for 2-O-benzyl as a non-participating protecting group was critical and consequently necessitated hydrogenolysis for removal. As a result the synthesis of oligosaccharides containing 4-nitrophenyl or benzyl as an aglycon were prohibited. We have developed two glycosylating reagents which overcome this problem and provide for the efficient synthesis of chromogenic, α-linked oligosaccharides. For the synthesis of α-D-linked galactose oligosaccharides, the title glycosyl donors were utilized and their stereoselectivity was examined by employing 4-nitrophenyl 2-acetamido-2-deoxy-4,6-O-isopropylidene-ß-D-glucopyranoside, 4-nitrophenyl 2,3-di-O-acetyl-ß-D-glucopyranoside, 4-nitrophenyl 2,3,-di-O-acetyl-ß-D-galactopyranoside and 1,2,3,4-di-O-isopropylidene-α-D-galactopyranose as acceptors. Their results will be presented.

These investigations were jointly supported by Grant No. CA35329 awarded by the National Cancer Institute and by American Cancer Society Grant No. CH419.

SYNTHETIC ANTIGENS: SYNTHESIS OF β-D-GlcpNAc-(1→6)-α-D-Manp-(1→6)-β-D-Manp→$OC_6H_4NO_2$(4) And RELATED TRISACCHARIDES.

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N-acetylglucosaminyltransferase V (GnT-V, EC 2.4.1.155) is thought to be responsible for the transfer of a 2-acetamido-2-deoxy-β-D-glucopyranosyl group to O-6 of the (1→6)-linked α-D-mannosyl residue that forms part of the trimannopyranosyl core of asparagine-linked N-glycans. This enzyme has attracted a great deal of interest as a potential tumor marker because of its increased expression in cells transformed by tumor viruses or oncogenes. Furthermore, recent work of Dennis and coworkers (Science, 236 (1987) 582-585) has suggested that an increase in intracellular activity of GnT-V is directly related with metastatic potential of certain tumor cell lines. In addition, these authors (Cancer Res., 49 (1989) 945-950) have also reported that an increased expression of GnT-V activity and resulting cell surface structures (patricularly increased β-D-GlcpNAc-(1→6)-α-D-Manp-(1→6)-β-D-Manp- branching in Asn-linked oligosaccharides) are associated with a number of human breast carcinomas compared to nonmalignant tissues. Since clinical symptoms appear usually late when the disease is in a comparatively advanced stage, it is of paramount significance to characterize these bio- and immunological tumor markers. For the past few years we have been actively engaged with the synthesis of acceptor substrates for GnT-V. Now we have centered our attention upon the synthesis of oligosaccharide tumor markers associated with this particular enzyme, and on studies of antibodies raised against these structures. In inauguration of this program we have synthesized three trisaccharides, namely, β-D-GlcpNAc-(1→6)-α-D-Manp-(1→6)-β-D-Manp→$OC_6H_4NO_2$(4), β-D-GlcpNAc-(1→6)-α-D-Manp-(1→6)-β-D-Glcp→$OC_6H_4NO_2$(4), and β-D-GlcpNAc-(1→6)-α-D-Manp-(1→6)-α-D-Manp→$OC_6H_4NO_2$(4). These trisaccharides, after reduction of their nitro groups and subsequent coupling of the resulting amino groups (as their diazonium salts) to BSA can be used for the preparation of antibodies which in turn can recognize the original carbohydrate structure. These antibodies can serve as diagnostic markers.

This investigation was supported by Grant No. CH-419 awarded by the American Cancer Society

PENTENYL GLYCOSIDE AS A GLYCOSYLATING REAGENT--CONVENIENT SYNTHESIS OF OLIGOSACCHARIDES CONTAINING Fucα1→3GlcNAc→ AND Galβ1→3GlcNAc→ AS TERMINAL UNITS

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Two disaccharide glycosylating reagents were efficiently prepared. Pentenyl 4,6-O-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranoside (1) upon glycosylation with 2,3,4-tri-O-benzyl-α-L-fucopyranosyl bromide (2) and 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl bromide (3) provided, respectively, pentenyl 4,6-O-benzylidene-3-O-(2,3,4-tri-O-benzyl-α-L-fucopyranosyl)-2-deoxy-2-phthalimido-β-D-glucopranoside (4) and pentenyl 4,6-O-benzylidene-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-2-deoxy-2-phthalimido-β-D-glucopyranoside (5). The effective glycosylating capability of these two glycosyl donors was established with a variety of sugar alcohols. The successful synthesis of several oligosaccharides containing either Fucα1→3GlcNAc or Galβ1→3GlcNAc as terminal units were accomplished. These will be presented. It is noteworthy that methyl-1-thio-2-p-methoxybenzyl-3,4-O-isopropylidene-α-L-fucopyranoside was not a suitable glycosyl donor and did not react with pentenyl 4,6-O-isopropylidene-2-deoxy-2-phthalimido-β-D-glucopyranoside to yield the desired disaccharide agent. Such possible limitations to pentenyl glycosides are discussed. Attempts towards the synthesis of glycolipid possessing X-determinant, Galβ1→4[Fucα1→3]GlcNAcβ→, via fucopyranosyl fluoride glycosylation and Fucα1→3GlcNAc→SPh are in progress.

These investigations were supported by Grant No. DMB87 15954 awarded by the National Science Foundation and by American Cancer Society Grant No. CH419.

SYNTHESIS OF SOME SULFATED OLIGOSACCHARIDES RELATED TO SULFATED GLYCOLIPID--USE OF PHENYL 2,4,6-TRI-O-BENZYL-3-O-PIVALOYL-1-SULFOXIDE-D-GALACTOPYRANOSE AS A GLYCOSYLATING AGENT

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A variety of glycoconjugates having a sulfate group at O-3 of GalNAc or Gal residues have been reported from human tissues. For the production of such oligosaccharides, we have developed the synthesis of Phenyl 2,4,6-tri-O-benzyl-3-O-pivaloyl-1-sulfoxide-D-galactopyranose as a novel glycosyl donor which allows for 3-O-sulfation or chain extension after removal of the pivaloyl group. This donor was prepared by the oxidation of its corresponding 1-thiophenyl compound. In order to demonstrate its utility we now describe some of the synthetic targets we have hitherto accomplished. Glycosylation of Benzyl 4-O-(2,3,6-tri-O-benzyl-ß-D-galactopyranosyl)-2,3,6-tri-O-benzyl-ß-D-glucopyranoside with this donor afforded a fully protected trisaccharide derivative which after the removal of the pivaloyl group followed by GalNAc oxazoline reaction provided a tetrasaccharide compound. This intermediate upon deacetylation followed by 4,6-O-benzylidene formation on the GalNAc residue left the 3-OH group available for further reaction. Sulfation of this 3-hydroxy intermediate by SO_3-pyridine complex followed by hydrogenolysis produced the sulfated tetrasaccharide 3-O-SO_3Na-ß-D-GalNac(1→3)-α-D-Gal(1→4)-ß-D-Gal(1→4)-D-Glc. We have also likewise accomplished the synthesis of 3-O-SO_3Na-ß-D-GalNAc(1→3)-α-D-Gal-OMe and 3-O-SO_3Na-α-D-GalNAc-OMe. Strategy towards the synthesis of these compounds will be presented.

These investigations were jointly supported by Grant No. DMB87 15954 awarded by the National Science Foundation and by Grant No. CA42584 awarded by the National Cancer Institute.

SYNTHESIS OF POLYLACTOSAMINE OLIGOMERS BY CONDENSATION POLYMERIZATION.

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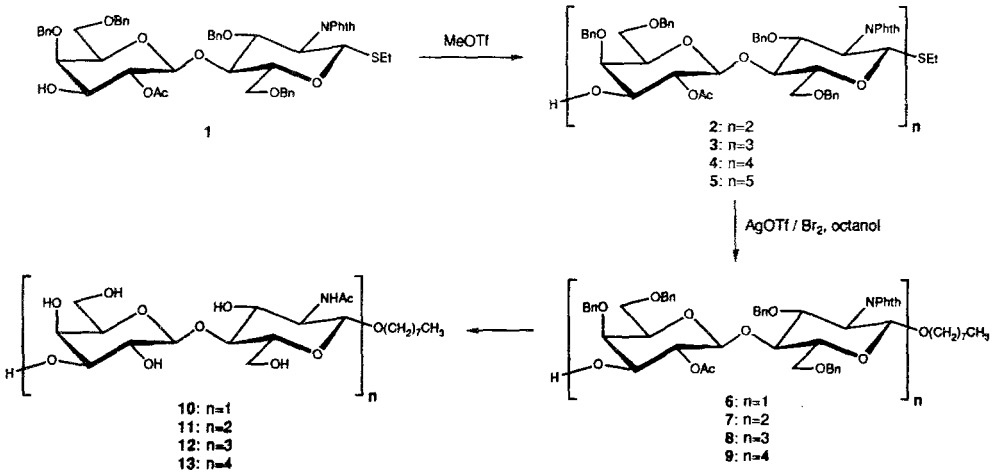
Addition of methyl triflate to disaccharide **1** (1.1 mmol) in dichloromethane (8 mL) at -15° resulted in its controlled polymerization to provide the protected polylactosamine oligomers **2** - **5** as their thioethyl glycosides. When the reaction time was kept to 24 h, oligosaccharides **2** - **5** could be isolated in yields of 20, 9, 4 and 2%, respectively, after chromataography on Iatrobeads. Unreacted disaccharide 1 (30%) was aslo recovered. Longer reaction times resulted in the formation of larger polymers which have not yet been characterized.

The protected oligomers **1**- **5** were designed to serve as glycosyl-donors for the preparation of biologically active polylactosamine glycoconjugates. Reaction of **1** - **4** with n-octanol, promoted by silver triflate/bromine, produced the β-linked glycosides in yields of 70, 71, 40 and 27%, respectively. On deprotection, the hydrophobic lactosamine oligomers **10** - **13**, useful as substrates in the assay of glycosyltransferase activites, are obtained. These compounds can also be enzymatically homologated to polymeric Lewis-X oligosaccharides on a multi-milligram scale.



FATTY ACID REMODELING OF TRYPANOSOME GLYCOSYL PHOSPHATIDYLINOSITOLS

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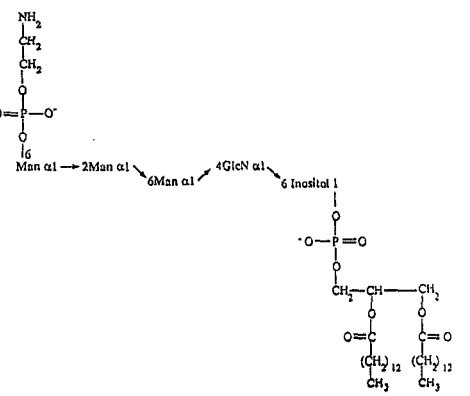
Glycosyl phosphatidylinositols (GPIs) anchor diverse proteins to the plasma membranes of organisms ranging from yeast to mammals. The best characterized GPI anchor is that of the variant surface glycoprotein (VSG) of *Trypanosoma brucei* (a protozoan parasite). VSG is abundant and straightforward to purify, and has therefore been the subject of intensive efforts directed at elucidation of GPI structure and biosynthesis. The GPI anchor is preconstructed as a compound termed glycolipid A (see structure below), it is linked to the VSG protein, and then both anchor and protein are further processed during transport to the cell surface. The VSG anchor differs from mammalian GPIs in that it contains exclusively myristic acid in its diacyl glycerol portion.

We have employed a cell free system to elucidate the biosynthetic pathway of glycolipid A (Masterson *et al*, Cell 56, 793-800; Doering *et al*, JBC 264, 11168-11173). First, GlcNAc is transferred from UDP-GlcNAc to endogenous PI. This product is deacetylated (to form GlcN-PI), and sequentially mannosylated to form Man_3GlcN-PI. Phosphoethanolamine is then added to the terminal mannose at the nonreducing end of the linear glycan; the VSG C-terminus is ultimately amide linked to this ethanolamine. Prior to this point in biosynthesis the GPI intermediates contain no myristate; the final steps in biosynthesis are fatty acid remodeling, ensuring that the final anchor contains only myristate.

The fatty acid remodeling sequence begins from a glycolipid called A', which has the same glycan as glycolipid A but with both fatty acids more hydrophobic than myristate (probably stearate at position *sn-1*). A GPI-specific phospholipase A_2 removes one fatty acid from glycerol at position *sn-2*, which is subsequently replaced by myristate from myristoyl-CoA. Finally, the other fatty acid is replaced by myristate to form glycolipid A (Masterson *et al*, Cell *in press*). Details of this remodeling pathway are under investigation.

We are currently employing fatty acid analogs (containing an oxygen heteroatom in the alkyl chain) to further study the incorporation of myristate into the VSG anchor. These analogs are incorporated into glycolipid A and VSG, and one analog is toxic to trypanosomes in culture.

GLYCOLIPID A



GENETIC EVIDENCE SUGGESTS THAT MANNOSYLPHOSPHORYLDOLICHOL SYNTHASE IS ACTIVATED BY A PROTEIN PHOSPHORYLATION SIGNAL. Dipak K. Banerjee, Department of Biochemistry & Nutrition, School of Medicine, University of Puerto Rico, San Juan, PR 00936-5067, USA.

Activation of mannosylphosphoryldolichol synthase (GDP-mannose: dolichylphosphate-O-ß-D-mannosyltransferase, EC.2.4.1.83) in rat parotid acinar cells by cAMP-mediated protein phosphorylation suggested that the cAMP-dependent protein kinase plays an obligatory role in regulating asparagine-linked (N-linked) protein glycosylation during transmembrane signaling of ß-adrenoreceptor stimulation. This conclusion was further supported by the enhanced Man-P-Dol synthesis with partially purified Man-P-Dol synthase from rat liver phosphorylated *in vitro*. To understand this activation process, the relationship between the expression of cAMP-dependent protein kinase gene and the regulation of N-linked protein glycosylation has been examined. Treatment of capillary endothelial cells, human fibroblasts, C_6-glioma and wild type Chinese hamster ovary (CHO) cells with isoproterenol (10^{-5} M) or 8 Br-cAMP (20 mM) exhibited 60-120% increase in glycosylation over the untreated controls. This was also confirmed by the 2-fold increase in glycosylation of a cpillary endothelial cell specific glycoprotein, Factor VIII:C in the presence of isoproterenol (10^{-7} M). In contrast, cAMP-dependent protein kinase deficient CHO mutants showed only 7-23% increase in protein glycosylation in the presence of 8 Br-cAMP. This was highly correlated with the reduced rate of [^{3}H]mannosylated-PP-Dol synthesis in these mutants. Man-P-Dol synthase, a 'key' enzyme in the oligosaccharide-PP-Dol biosynthetic pathway has been found to be altered in these mutants when examined in rough endoplasmic reticulum preparations. A 3-4 fold higher K_m for GDP-mannose for Man-P-Dol synthase in mutant 10248 with altered type I regulatory subunit which binds cAMP poorly as well as in mutant 10260 with lowest level of total kinase activity supports the above alteration. *In vitro* phosphorylated rough endoplasmic reticulum membranes from rat parotid acinar cells exhibited a major phosphoprotein of M_r 32,000 dalton on 10% SDS-PAGE. Phosphorylation of the 32 Kd protein was greatest in the presence of Mg^{2+}, comparatively less in the presence of Mn^{2+} and none in the presence of Ca^{2+}. Reduced phosphorylation in the presence of Mn^{2+} correlated well with the sub-optimal activation of the synthase and provided strong evidence that the 32 Kd protein is indeed Man-P-Dol synthase. The results thus indicated a role for cAMP-mediated protein phosphorylation as an intracellular signal, which in responsive cells promotes protein N-glycosylation. Supported by USPHS Grant No. SO6RR08224.

GLUCOSIDASE I IS AN ENDOPLASMIC RETICULAR TRANSMEMBRANE GLYCOPROTEIN WITH A LUMINAL CATALYTIC DOMAIN AND A CYTOPLASMIC TAIL.

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Processing of the protein-linked oligosaccharide is initiated by removal of the outermost α 1,2 glucose by glucosidase I, followed by trimming of the remaining two α 1,3 glucosyl residues by glucosidase II. With the exception of RER resident proteins, the newly synthesized glycoproteins are transferred to the golgi complex where they are extensively processed and targeted to their destination. The available information indicates that the early steps of the glycoprotein processing take place in RER. We have earlier demonstrated that this enzyme is developmentally regulated in this tissue. It was also observed that lactogenic hormones such as hydrocortisone, insulin and prolactin were involved not only in the regulation of glucosidase I synthesis but also played an important role in the glycosylation of α-lactalbumin. This would suggest that glucosidase I may have a crucial role in the overall regulation of protein *N*-glycosylation.

Glucosidase I was purified from rat mammary tissue and polyclonal antibodies against the enzyme have been prepared. The purified enzyme exhibited a single band of 85 kDa on 7.5% SDS-PAGE, whereas on 12.5% Tricine-SDS-PAGE, it resolved into two bands of 82 & 84 kDa. The peptide mapping of these two bands revealed identical fragments, suggesting the similarities in the primary structure of these two protein bands. Endoglycosidase digestions indicated that glucosidase I is a high mannose type *N*-linked glycoprotein. Biotinylated-lectin binding assays ruled out the possibility of the presence of *O*-linked sugar chains. Deglycosylation of glucosidase I with endoglycosidase H did not affect the catalytic activity significantly. The tryptic digestion of the enzyme also did not affect the enzymatic activity. Amongst the peptide bands generated by trypsin, a band of 69 kDa appeared to be enzymatically more active than the original 85 kDa band. The tryptic peptides of as low as 39 kDa also appeared to be enzymatically active. The membrane topology experiments with purified and intact RER suggested that glucosidase I is a transmembrane protein with a luminal catalytic domain and a cytoplasmic tail. Phase separation with Triton X-114 indicated that this enzyme is amphipathic in nature. The possible orientation of glucosidase I in RER membrane is also discussed.

Supported by NIH grant DK-19682.

Properties of highly purified human lymphoblast N-acetylglucosamine-1-phosphotransferase. K. Zhao, R. Yeh, A. L. Miller. Dept of Neurosciences, UCSD School of Medicine, La Jolla, CA 92093.

N-acetylglucosamine-1-phosphotransferase facilitates the transfer of N-acetylglucosamine-1-phosphate to high-mannose oligosaccharide chains on lysosomal enzymes. The resulting mannose-6-phosphate serves as a recognition marker for targeting these enzymes to lysosomes. A significant reduction or absence of this phosphotransferase activity gives rise to I-cell disease or pseudo-Hurler polydystrophy. We have partially purified this phosphotransferase from cultured human lymphoblasts using conventional and affinity chromatography methodology, and carried out characterization of selected properties. A non-ionic detergent, Tergitol is required for the solubilization and purification of the phosphotransferase. The stability of the enzyme could be maintained by ß-mercaptoethanol, glycerol, and α-methyl-D-mannoside. The enzyme demonstrated optimal activity at pH 7.0 to 7.5, and in the presence of 15 mM Mn^{2+} or Mg^{2+}. Kinetic analysis revealed an apparent K_m of 24 μM and 117 mM for its substrate and artificial acceptor, UDP-N-acetylglucosamine and α-methyl-D-mannoside, respectively. The phosphotransferase activity was inhibited by inorganic phosphate, EDTA, AMP, ADP, ATP, UDP, UDP-glucose, guanidine hydrochloride, urea, phosphatidylglycerol, phosphatidylserine, and phosphatidic acid, while incubation with sucrose, phosphatidylcholine, and phosphatidylethanolamine stimulated the enzyme's activity. (Supported by NIH NINDS/NS12138).

PHOSPHORYLATION OF ALPHA-L-FUCOSIDASE IN I-CELL DISEASE LYMPHOID CELLS. R.A. DiCioccio[1] and A.L. Miller[2]. [1]Dept. Gynecologic Oncology, Roswell Park Cancer Institute, Buffalo, NY, [2]Dept. of Neurosciences, UCSD, LaJolla, CA

We reported that I-cell disease lymphoid cell lines synthesized and secreted increased fucosidase protein relative to control cells but retained normal levels of intracellular enzyme protein (Glycoconjugate J. 6:405, 1989). The deficiency of UDP-N-acetylglucosamine phosphotransferase in I-cell lymphoid cultures indicates that these cells do not synthesize the mannose 6-phosphate (M6P) recognition marker found on most lysosomal enzymes. This marker is used for the proper targeting of many acid hydrolases to lysosomes and its absence would explain the increased secretion of fucosidase from I-cell lymphoid cultures. However, the maintenance of normal intracellular levels of fucosidase by I-cell lymphoid cells suggests the existence of an independent mechanism for intracellular retention of fucosidase in these cells. Thus, both M6P dependent and independent mechanisms may be involved in routing fucosidase to lysosomes in lymphoid cells. The present study examined the presence of M6P residues in fucosidase of lymphoid cells. Phosphorylation of fucosidase in I-cell and control lymphoid cultures was observed by measuring incorporation of ^{32}Pi into immunoprecipitable enzyme protein. In control cells, less than 1% of the ^{32}Pi incorporated into fucosidase was associated with carbohydrate chains and more than 99% with polypeptide chains. In I-cell disease lymphoid cells, the ^{32}Pi incorporated into fucosidase was associated solely with polypeptide chains. A qualitative analysis of phosphorylated residues identified the presence of phosphoserine in fucosidase from control and I-cell lymphoid cells, and M6P in fucosidase from only control cells. These results, in conjunction with the presence of normal intralysosomal levels of other lysosomal enzymes in I-cell lymphoid cells (J. Cell Biol. 107:341A, 1988), are consistent with the proposal that I-cell lymphoid cells may have a M6P independent mechanism for routing fucosidase. Furthermore, the data support the co-existence of M6P dependent and independent mechanisms in control lymphoid cells. Current investigations may reveal other structural feature(s) involved in targeting acid hydrolases to lysosomes (Supported by DK32161 and NS12138).

SYNTHESIS AND PROCESSING OF α-L-FUCOSIDASE IN RAT SPERM.

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The acid hydrolase α-L-fucosidase is one of a number of glycosidases expressed by mature rat sperm; it has been proposed that many if not all of these enzymes may participate in fertilization via their action on the cumulus oophorus and zona pellucida of the oocyte. To date, however, the synthesis, processing, and targeting of sperm-associated acid glycosidases has not been assessed.

We have utilized a polyclonal antibody to α-L-fucosidase isolated from rat liver (generously provided by Dr. N. Aronson, Penn State U.), in conjunction with metabolic labeling of immature testicular germ cells and immunoblot analysis of epididymal sperm, to define the developmental stage at which α-L-fucosidase is expressed in sperm and to determine the post-translational modifications which sperm-associated enzyme undergoes in comparison to the lysosomal α-L-fucosidase of somatic cells (cultured Clone 9 cells, a liver-derived rat cell line).

Immunoprecipitation of pachytene spermatocytes and round spermatids metabolically labeled with (^{35}S)methionine gave a labeled polypeptide of M_r 54,000; there was no apparent processing as assessed by SDS-PAGE. In contrast, condensing spermatids labeled under the same conditions showed no immunoprecipitable α-L-fucosidase polypeptide. Immunoblot analysis of round spermatids also showed a polypeptide of M_r 54,000, consistent with the metabolic labeling data; similar analysis of caput epididymal sperm revealed a polypeptide of M_r 52,000, suggesting the possibility of post-translational processing during transit from the testis to the epididymis. Metabolic labeling of Clone 9 cells with (^{35}S)methionine revealed a time-dependent processing of immunoprecipitable polypeptide from a precursor of M_r 54,000 to a mature polypeptide of M_r 52,000, with processing complete within 16 hours of labeling.

Finally, analysis of enzyme activity in highly purified populations of testicular germ cells and caput epididymal sperm revealed an increase in specific activity of α-L-fucosidase (expressed per cell) of at least two-fold during transit from the testis to the epididymis. It remains to be determined whether the post-translational modifications of rat α-L-fucosidase are the result of proteolysis or carbohydrate modification, and whether the post-translational modifications of α-L-fucosidase observed in Clone 9 cells are identical to those of the sperm-associated enzyme. It is also of immediate interest to determine how post-translational processing in sperm may be delayed until arrival in the epididymis, and whether the increase in the specific activity of α-L-fucosidase observed in caput epididymal sperm is related to processing of the enzyme.

The detailed analysis of the synthesis and processing of α-L-fucosidase, as afforded by the methods described above, may serve as a model for other hydrolytic enzymes of sperm.

Supported by NIH Grant DK-38593 and the Minnesota Medical Foundation.

N-LINKED OLIGOSACCHARIDES ON FREE ALPHA INTERFERE WITH ITS ABILITY TO COMBINE WITH HCG-BETA SUBUNIT.

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Human chorionic gonadotropin (hCG) is a glycoprotein hormone that is essential for maintenance of pregnancy. HCG has also been associated with a wide variety of pathologic conditions including trophoblastic diseases as well as nontrophoblastic malignancies. Free α molecules are also produced in abundance during pregnancy as well as most pathologic conditions associated with hCG. Intact hCG can be dissociated into its α and β subunits and these subunits will readily recombine to form active hormone. The free α molecules produced in pregnancy and in JEG choriocarcinoma cells are unable to combine with hCG-β to form intact hormone. Since the free α molecule has the same polypeptide sequence as the hCG-α subunit obtained by dissociation of hCG, it has been speculated that the lack of combining ability of free α may be due to its carbohydrate structures.

Both free α and hCG-α contain two N-linked oligosaccharides. The following experiments were designed to examine the role of these N-linked units in preventing combination of free α with hCG-β. JEG-cells were cultured in the presence or absence of Swainsonine. Swainsonine interferes with glycosylation by inhibiting α-mannosidase II, thus preventing the formation of biantennary or more highly branched structures. The predominant oligosaccharide predicted to be formed in the presence of Swainsonine is a hybrid structure (one complex antenna and a high mannose [man_5] core). This hybrid structure is similar to the structure found on dissociated hCG-α.

Culture media from JEG cells incubated in the presence or absence of Swainsonine were filtered on Sephadex G-100 and free α was identified by RIA. Approximately 50% of the free α from Swainsonine-treated cells (Swainsonine-free α) had an apparent molecular size that was smaller than that of free α from control cells. On the basis of this size difference, the Swainsonine-free α molecules were divided into pools 1 and 2. Both pools of Swainsonine-free α and control-free α molecules were incubated with a molar excess of hCG-β subunit, followed by chromatography on Sephadex G-100. Each fraction was assayed by RIA for intact hCG and for α immunoreactivity. Only 9% of total control free α molecules could combine with hCG-β subunit. In contrast to control free α, combination of Swainsonine-free α with hCG-β was markedly increased; 54% of Swainsonine α-pool 2 and 40% of Swainsonine α-pool 1 combined with hCG-β to form intact hCG. Thus, modulation of N-linked oligosaccharide processing converted free α to forms that can combine with hCG-β. This result indicates that the inability of a substantial portion of control free α molecules to combine with hCG-β is due to the presence of N-linked oligosaccharide structures that interfere with combination.

GRANULOCYTIC DIFFERENTIATION OF HL-60 CELLS IS ASSOCIATED WITH INCREASE OF POLY-N-ACETYLLACTOSAMINE IN ASN-LINKED OLIGOSACCHARIDES ATTACHED TO HUMAN LYSOSOMAL MEMBRANE GLYCOPROTEINS.

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The HL-60 cell line, dervied from a patient with acute promylocytic leukemia, can be induced to differentiate to granulocyte-like cells by dimethylsulfoxide (DMSO). Since it was shown that lamps purified from HL-60 are major glycoproteins carrying polylactosaminoglycans, lamp-1 and lamp-2 serve as good models for understanding polylactosaminoglycan formation during cell differentiation. Comparison of Asn-linked oligosaccharides from undifferentiated HL-60 cells and differentiated HL-60 cells reveals the following features. First, the number of Asn-linked oligosaccharides containing poly-*N*-acetyllactosaminyl side chains increases dramatically with a concomitant decrease in less complex Asn-linked oligosaccharides after differentiation. Secondly, the number of poly-*N*-acetyllactosaminyl side chains per Asn-linked oligosaccharides increases significantly. These increases in poly-*N*-acetyllactosamine were associated with increased activity of β1,3N-acetylglucosaminyltransferase "extension enzyme", a key enzyme in the formation of poly-*N*-acetyllactosamines. Further, the increased amount of poly-*N*-acetyllactosamines in lamp-1 and lamp-2 resulted in longer half-lives of lamp-1 and lamp-2 in differentiated HL-60 cells. These results suggest that the differentiation of HL-60 cells into more phagocytic cells is associated with an increase in the complexity of Asn-linked oligosaccharides attached to lysosomal membrane glycoproteins, which in turn stabilizes lysosomes.

In order to further understand the role of polylactosaminoglycan in the processing of lamps, HL-60 cells were labeled with [^{3}H]galactose at 37° C or 21° C for 6 hours, and lamp-1 and lamp-2 were immunoprecipitated. Lamps from cells after incubation at 21° C exhibit much broader and slightly higher bands upon examination by SDS-PAGE than after incubation at 37° C. This difference was shown to be due to the increased amount of polylactosaminoglycan by the analysis of glycopeptides with endo-β-galactosidase digestion. The results clearly indicate that longer association with Golgi compartments at a lower temperature allows lamps to acquire more polylactosaminoglycan. (Supported by CA48737).

EFFECT OF DIFFERENTIATION ON THE GLYCOSYLATION OF LAMP-1 AND LAMP-2 IN F9 TERATOCARCINOMA CELLS. P.A. Romero and A. Herscovics, McGill Cancer Centre, McGill University, Montreal, P.Q., Canada, H3G 1Y6.

It has been demonstrated that during differentiation of CaCo-2 human colonic adenocarcinoma cells there is a decrease in polylactosaminoglycans associated with the lysosomal membrane glycoprotein lamp-1 (Youakim et al (1989) Cancer Res. 49, 6889-6895). Since it was found that in F9 cells about 45% of the [^{3}H]glucosamine-labeled glycopeptides obtained after Pronase digestion are excluded from Bio-Gel P-6 and contain relatively large polylactosaminoglycans, the effects of differentiation on lamp glycosylation was studied in these cells. F9 and retinoic acid (RA)-treated F9 cells were incubated with [^{3}H]glucosamine and labeled glycoproteins extracted with NP-40 were immunoprecipitated with anti-m-lamp-1 and anti-m-lamp-2 monoclonal antibodies obtained from Dr. J.T. August. SDS-PAGE of the immunoprecipitates showed a broad band of about 90 kDa in both control and RA-treated F9 cells. Between 3-7% of the radioactivity associated with glycoproteins was immunoprecipitated with anti-lamp-1 and only 0.5-1% with anti-lamp-2. The immunoprecipitated glycoproteins were exhaustively digested with Pronase and the glycopeptides were fractionated on Bio-Gel P-6. The proportion of large glycopeptides excluded from the column accounted for about 4% of the labeled lamp-1 glycopeptides and about 35% of the lamp-2 glycopeptides. No major difference in the proportion of these glycopeptides was observed between F9 and RA-treated F9 cells. When the lamp-1 glycopeptides included in Bio-Gel P-6 were chromatographed on ConA-Sepharose most of the labeled glycopeptides (75-80%) did not bind in both control and RA-treated cells. The unbound material was further fractionated on Datura Stramonium (DSA)-agarose which binds polylactosaminoglycans and complex oligosaccharides containing lactosamine ß-1,6- and ß-1,2-linked to mannose. About 19% of the glycopeptides from RA-treated F9 cells were specifically bound to DSA-agarose whereas only 2% of the glycopeptides from control F9 cells were bound. These results indicate that, although F9 cells synthesize large polylactosaminoglycans excluded from Bio-Gel P-6, lamp-1 does not acquire a significant amount of these oligosaccharides. The results obtained with DSA-agarose suggest that lamp-1 of F9 cells contains tri- and tetra-antennary oligosaccharides and possibly relatively short polylactosaminoglycans, and that these oligosaccharides increase with differentiation.

Supported by the Medical Research Council of Canada.

FUNCTIONAL ROLES OF SUGAR CHAINS ON THE BIOLOGICAL ACTIVITY OF HUMAN RECOMBINANT ERYTHROPOIETIN

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Human erythropoietin (EPO) is a glycoprotein hormone with three Asn-linked and one mucin-type sugar chains. Although the sugar chain structure of recombinant EPO produced in Chinese hamster ovary(CHO) cells were determined in detail[1], the roles of sugar chains have not yet been clarified. To address this problem, we have examined the biological activity of a series of EPO-glycoforms.

We obtained EPO samples comprising different populations of glycoforms from subclones of recombinant CHO cells, and compared the biological activity of each by the *in vivo* assay using exhypoxic polycythemic mice[2]. Our results were as follows:

1) The branching structure of Asn-linked sugar chains strongly co-relates with the *in vivo* activity. EPO that has the higher tetra/bi-antennary ratio has the greater activity.

2) EPO rich in biantennary-type chain appears to lose its ability to target correctly *in vivo*, since it has very low *in vivo* activity and yet maintains high *in vitro* activity. The loss of *in vivo* activity is not due to the hepatic clearance, since they have enough sialic acids to escape from the hepatic asialoglycoprotein binding lectin.

From these results together with previous findings, we postulate that the Asn-linked sugar chains of EPO are composed of three functional units: Terminal (Neu5Ac, Gal, and N-acetyllactos-amine repeating unit), branching (GlcNAc's linked to the core part), and core part (Man_3•GlcNAc•±Fuc•GlcNAc). The terminal part acts as the recognition site for clearance from blood circulation, and the branching part may be the signal for targeting to bone marrow. The function of the core part is not understood. We are now investigating this point using a sugar chain-remodeling technique.

1) M. Takeuchi *et al.* (1988) *J. Biol. Chem.*, **263**, 3657
2) M. Takeuchi *et al.* (1989) *Proc. Natle. Acad. Sci. U. S. A.*, **86**, 7819

THE IMPORTANCE OF THE CARBOHYDRATE CHAINS OF Gal β1-4 GlcNAc α2-6 SIALYLTRANSFERASE FOR ENZYME ACTIVITY. BY DARREN G. FAST AND JAMES C. JAMIESON. DEPARTMENT OF CHEMISTRY, UNIVERSITY OF MANITOBA, WINNIPEG, CANADA

Gal β1-4 GlcNAc α2-6 Sialyltransferase (ST) is a rat liver Golgi enzyme responsible for the addition of terminal sialic acid residues to carbohydrate chains of glycoproteins. The enzyme is synthesized in the rough endoplasmic reticulum and passes to the Golgi complex where it expresses its catalytic activity. The enzyme contains three potential sites for glycosylation (Weinstein *et al.* 1987, J. Biol Chem. **262**: 17735-17743) and it has been shown to be a glycoprotein. In this study the importance of the carbohydrate chains for catalytic activity was studied. Treatment of native ST with N-glycanase™ resulted in loss of about 80% of enzyme activity after 6 hours of incubation; controls incubated in the absence of N-glycanase showed little loss of activity under the same conditions. It was found that the presence of up to 4% methanol or ethanol was essential for efficient removal of carbohydrate chains from native ST. Immunoblot analysis of N-glycanase treated ST showed three bands. One corresponding to the native enzyme with M_r about 43,000, a second corresponded to a completely deglycosylated form of the enzyme with M_r about 40,000 and a third band was intermediate between the two. The completely deglycosylated ST was the main form of the enzyme found after treatment of the native or denatured enzyme with N-glycanase for 18 hours. The results show that catalytic activity of ST is dependent on the presence of the carbohydrate chains which presumably influence the conformation of the enzyme. The work also suggests that the presence of carbohydrate chains of the correct structure on ST may influence the expression of the catalytic activity of the enzyme in the Golgi complex.

Supported by the Natural Sciences and Engineering Research Council of Canada

CLEARANCE OF GLYCOSYLATED AND NON-GLYCOSYLATED C-REACTIVE PROTEINS BY RAT LIVER AND ISOLATED HEPATOCYTES

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C-reactive proteins (CRP's) are present in most mammalian and invertebrate species. CRP's are thought to have evolved from a common ancestral gene. Through out evolution these proteins have retained their pentraxin structure, and the highly conserved property to bind to the phosphorylcholine ligand. The biological activities of CRP are those associated with non-specific host defence responses. In humans and rabbits, CRP is a non-glycosylated typical acute phase protein. In rats, CRP is glycosylated (18% carbohydrate of the complex type) and is present in high concentration (0.5-0.6 mg/ml serum). There is a 70% amino acid homology between rat and rabbit CRP. The purpose of this investigation was to compare the clearance of glycosylated and non-glycosylated CRP's using whole animal, perfused liver and hepatocytes. Asialo α_1-acid glycoprotein was used as control. Labeled asialo rat CRP was cleared rapidly from plasma in vivo, and by perfused liver through the Gal/GalNAc specific asialo receptor. Most of the radioactive label was recovered in the liver, and to a small extent in the lungs. Non-glycosylated rabbit CRP remained in circulation and was not cleared by the asialo receptor pathway. The radioactivity associated with rabbit CRP was found mainly in the lungs. Results of the binding studies with isolated hepatocytes were at variance with our in vivo observations. The binding of asialo rat CRP to hepatocytes was not mediated through the asialo receptor, and could not be inhibited by GalNAc. Instead, the binding occurred through a phosphorylcholine ligand on the hepatocyte surface and involved the phosphorylcholine binding domain of rat CRP. This binding could be inhibited by phosphorylcholine. Our results suggest that the binding sites involving the phosphorylcholine ligand are generated on isolated hepatocytes due to hepatocyte surface alteration, either during cell isolation or due to decreasing cell viability. These newly generated binding sites mask the asialo receptor, preventing the binding of asialo rat CRP. Unlike the binding of asialo α_1-acid glycoprotein the binding of asialo rat CRP to hepatocytes did not involve receptor-mediated endocytosis. In conclusion, rat CRP after removal of terminal sialic acid is cleared rapidly in vivo by the hepatic asialo receptor pathway. In contrast, non-glycosylated CRP is not cleared by the hepatic pathway. Our results suggest that the lungs may be the principle organ involved in the clearance of non-glycosylated CRP and to some extent glycosylated CRP. The diverse clearance pathways of rat and rabbit CRP's could be attributed to the glycosyl moiety of rat CRP and as a consequence these two proteins may have distinct physiological functions.
(Supported by the Canadian Heart Foundation and the Medical Research Council of Canada)

Recycling of an Ascites Tumor Cell Surface Sialomucin: Evidence for a second pathway for O-glycosylation.

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Maturation of ASGP-1, a major tumor cell surface sialomucin, involves three distinct forms: immature (I), premature (P), and mature (M). Maturation is kinetically biphasic; I is converted into P relatively rapidly ($T_{1/2}$ = 30 min), whereas P to M conversion is much slower ($T_{1/2}$ > 4 hr). Evidence is provided for the notion that during late maturation ASGP-1 recycles between the cell surface and an intracellular compartment(s). During recycling new oligosaccharides are synthesized rapidly; however, the complete glycosylation of all potential initiation sites is slow. This viewpoint is supported by the following observations: 1) ASGP-1 transport to the cell surface is more rapid than the maturation. 2) De-sialated cell surface ASGP-1 is resialylated with a half-time of 15-30 minutes. 3) Cell surface ASGP-1 leaves the cell surface, undergoes rapid glycosylation, including initiation, and returns to the cell surface. 4) Glycosylation of initiation sites during the premature to mature conversion is slow. From this evidence we propose that a second glycosylation pathway exists in which cell surface glycoproteins recycle between the cell surface and some intracellular compartment(s). Within the compartment(s) the entire spectrum of glycosyltransferases is present to permit complete assembly of new oligosaccharides. Studies are presently underway to localize this compartment(s) and establish the presence of O-glycosyltranferases within such a compartment(s).

ASSAY OF KERATAN SULFATE AS ANION EXCHANGE-BOUND HEXOSE

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Keratan sulfate (KS) is being found in an increasing variety of connective tissues. This glycosaminoglycan (GAG) is especially abundant in the several proteoglycan species of the human intervertebral disc where it occurs mixed in varying proportions with chondroitin 6-sulfate. The need for a quantitative assay in human disc led us to develop an anion exchange method for separating KS hexose from non-KS hexose. Papain digest (usually 50µl prepared from 5mg of fresh disc) is applied to a 200µl column of AG4-X4 (acetate form, Bio-Rad) and the non-KS hexose (34-81% of total) is eluted with 5 x 100µl 0.2M sodium acetate pH 5.0. Then the KS is eluted with 5 x 100µl 1.0M pyridinium sulfate pH 2.5 and assayed specifically for hexose using the anthrone reaction; 50-200 nmole galactose equivalents could be assayed with a root mean square error of 5 nmole. The recovery of KS was 99%.

Gel chromatography on Sephacryl S-200 (Pharmacia) demonstrated that the size of the pyridinium sulfate eluted fraction (PSEF) was similar to that of the GAG and that of the sodium acetate eluted fraction (SAEF) was that expected of oligosaccharides. Sulfate analysis indicated that no sulfated GAG were eluted below 0.2M sodium acetate. The PSEF was a mixture of KS and chondroitin sulfate as shown by g.l.c. of the alditol acetates and n.m.r. Less than 5% of the hexose of the PSEF was oligosaccharides as shown by n.m.r. and β-elimination followed by gel chromatography.

Galactosaminoglycuronans were recovered quantitatively with the KS. Furthermore, the method can be used to isolate GAG on a preparative scale, to concentrate GAG from dilute solutions, or to subfractionate GAG on the basis of charge density.

Supported by The Arthritis Society (Canada)

A COMPARISON OF ENDOTHELIAL CELL UPTAKE OF HEPARINS AND DEXTRAN SULFATES. Hiebert, L.M., Liu, J. and McDuffie, N.M. Dept. of Physiology, College of Medicine, University of Saskatchewan, Saskatoon, Sask., Canada.

Previously we have described the process of uptake and internalization of heparin(s) by endothelial cells in vivo and in vitro. In vitro studies demonstrated uptake and release over a protracted period with variation in heparin internalization by cells dependent on cell source, heparin source, concentration and time (Hiebert, L.M. and N.M. McDuffie, Artery, 17/2:107, 1990). Preferential intracellular accumulation of bovine heparin was observed and confirmed by biochemical, histological and radiolabel studies. We have begun a similar study of dextran sulfates of variable molecular sizes, M_r 5000, 8000 and 500,000. Endothelial cells showed extensive accumulation of metachromatically stained vesicles when dextran sulfate 500,000 M_r was placed in media at 100 μg/ml for 48 hrs. Similar internalization occurred with doses as low as 0.5 μg/ml. Morphometric evidence of dextran sulfate internalization was apparent 3 to 6 hours after drug exposure. Additional time studies suggest patterns of internalization comparable to responses obtained with the heparins. However, preliminary indications suggest that the accumulation of dextran sulfate M_r 500,000 is far greater than previously observed for bovine heparins and for lower molecular weight dextran sulfates.

SULFATE COMPOSITION OF GLYCOSAMINOGLYCANS DETERMINED BY INFRARED SPECTROSCOPY

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Anhydrous sodium sulfate (Na_2SO_4) was analyzed at varying concentrations by infrared (IR) spectroscopy. A standard curve was obtained from a linear plot of sulfate (SO_4^{2-}) concentration vs the weight of the IR band area of S=O stretching. Standard chondroitin-4-sulfate, chondroitin-6-sulfate, heparan sulfate, heparin, keratan sulfates and various dermatan sulfates isolated from human and rat skins were also studied by IR spectroscopy. The spectrum of every glycosaminoglycan (GAG) displayed an IR band around 1230 cm^{-1} which originated from S=O stretching of sulfate esters. Therefore, the weight of the latter band was employed to quantify sulfate, by using the standard curve indicated above. Sulfate was also estimated quantitatively by the gelatin/$BaCl_2$ method of K S Dodgson and R G Price (Biochem J 84, 106-110, 1962). The sulfate composition of the GAGs investigated ranged from 8.5-22.1% (w/w), irrespective of the technique followed. In the IR spectroscopy method, sulfate was determined using the polymer forms of the GAGs. After analysis, these heteropolysaccharides were recovered unaffected in a yield > 95%. The data show that the infrared spectroscopy technique, in addition to being reliable, is much more economical than the chemical procedures presently employed to quantify GAG sulfate.

Supported by Scholarly Research Award from Purdue University Calumet and XL-Summer Faculty Grant from Purdue Research Foundation.

HYALURONAN ASSOCIATES WITH LUNG ELASTIN

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Lung tissue hyaluronan (hyaluronic acid; HA) can be correlated with the water content of the lung and it is increased in interstitial fibrosis. Bronchoalveolar lavage fluid HA is elevated in many lung diseases. In order to study the role of HA in regulating lung water and in the pathogenesis of disease, it is necessary to define its molecular associations in lung tissue. In this study we have used extraction and enzymatic digestion procedures as an approach to the problem. A composite of lyophilized and pulverized hamster lung tissue was made. Aliquots were extracted with 4M guanidine.HCl, which would dissociate HA from proteoglycans, or with 0.5M NaCl. Insoluble residues were digested with Pronase as were fresh aliquots of tissue. Residues remaining after guanidine extraction were also digested sequentially with collagenase and elastase or were re-extracted with guanidine. Analyses for nanogram amounts of HA were performed on the soluble fractions by an inhibition assay based on the specificity of binding of HA to biotinylated HA-binding protein (B-HABP). HA bound to a microtiter plate was allowed to compete for B-HABP with HA in the sample. B-HABP bound to the plate was detected with streptavidin alkaline phosphatase and its substrate. (This method was developed independently in this laboratory but is very similar to that of Kongtawelert, P. and Ghosh, P. Anal. Biochem. 185: 313-318, 1990). Protease digestion was required for the efficient solubilization of lung HA. One extraction with either 0.5M NaCl or 4M guanidine.HCl solubilized two-thirds of that released by Pronase (or by three guanidine.HCl extractions). The remaining one-third could be recovered from the residues by Pronase digestion. The amount of HA solubilized by collagenase from the guanidine residues was negligible. Subsequent elastase digestion solubilized the expected HA. These data suggest that as much as one-third of lung HA is relatively tightly bound to elastin, presumably as a component of the microfibrils. Supported by the St. Luke's-Roosevelt Institute for Health Sciences.

THYROID HORMONE STIMULATION OF HYALURONATE SYNTHETASE ACTIVITY IN CULTURED CHICK CHONDROCYTES

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3,3',5-L-Triiodothyronine (T_3) can inhibit hyaluronate (HA) synthesis in cultured human skin fibroblasts (J Clin Invest 70:1066, 1982). These studies were undertaken to ascertain whether T_3 could also exert a regulatory influence on HA synthesis in primary cultured embryonic chick chondrocytes. HA synthetase activity was assessed by incubating plasma membrane preparations (20,000 x g pellet) with UDP-[^{14}C] glucUA and measuring radiolabel incorporated into streptomyces hyaluronidase-digestible material. Cultures incubated in medium supplemented with hypothyroid serum had 37% less HA synthetase activity than did cultures maintained in medium with physiological concentrations of T_3. Addition of T_3(10^{-7}M) to hypothyroid medium increased activity 1.3 - 3.5 fold (5 separate experiments) above activities in cultures incubated with hypothyroid medium without T_3. The effect was saturated at a total T_3 concentration of 10^{-9}M (ca. 10^{-11}M "free T_3") suggesting that iodothyronines may play a physiological role in the regulation of HA synthetase in chondrocytes. Moreover T_3 may influence HA synthesis in an organ-specific manner, being stimulatory in some tissues (chondrocytes) and inhibitory in others (such as fibroblasts).

BIOCHEMICAL CHARACTERIZATION AND DISTRIBUTION OF RETINAL INTERPHOTORECEPTOR MATRIX GLYCOCONJUGATES. Gregory S. Hageman, Margaret A. Kirchoff, and Don H. Anderson, Department of Ophthalmology, Bethesda Eye Institute, St. Louis University School of Medicine, St. Louis, MO and Neurosciences Research Institute, University of California, Santa Barbara, CA.

The retinal interphotoreceptor matrix (IPM) occupies the extracellular space located between the apices of the neural retinal and the retinal pigmented epithelium (RPE). Although the precise function of most IPM constituents is not known, recent studies have provided evidence that some aqueous-insoluble components mediate retinal adhesion. Recent investigations in our laboratory have been directed towards characterizing the composition and distribution of aqueous-insoluble IPM constituents using lectin cytochemistry, immunocytochemistry, Western blot analyses, and HPLC. A large proportion of the IPM is composed of insoluble glycoconjugates exhibiting both apical-basal and photoreceptor cell type-specific distributions. Chondroitin-6-sulfate antibody-/and peanut agglutinin (PNA)-binding glycoconjugates are specifically associated with "cone matrix sheaths," compartments of the IPM that ensheath cone, but not rod, photoreceptor cell outer segments and ellipsoids. In contrast, wheat germ agglutinin (WGA) and Limax flavus agglutinin (LFA) bind intensely to the IPM associated with rod photoreceptors and weakly, if at all, to cone matrix sheaths. Anti-chondroitin-4-sulfate antibody (AC4S) and Helix aspersa agglutinin (HAA) bind uniformly throughout the IPM, whereas Phaseolus vulgaris agglutinin (PHA) selectively labels the apical interphotoreceptor matrix along the RPE interface. Western blot analyses of chondroitinase-treated insoluble IPM preparations show bands of approximately 150 and 200kD that are bound by both AC6S, PNA, HAA, LFA, and WGA, indicating that at least two classes of chondroitin 6-sulfate-containing proteoglycan exist within the matrix. In addition, PNA binds intensely to a lower molecular weight band of 17kD which also binds a battery of antibodies directed against basic fibroblast growth factor (bFGF). AC4S binds only the 200kD band and PHA only the 150kD band. Following sequential exposure to neuraminidase, O-glycanase, and N-glycanase, both the 150 and 200kD bands shift to a molecular weight of 75kD. The PHA-binding glycoconjugate is insensitive to neuraminidase and N-glycanase treatment, but migrates to a band of approximately 56kD following treatment with O-glycanase.

These studies indicate that the structure and composition of the IPM is complex: at least two similar, but distinct, chondroitin sulfate-containing proteoglycans are present and proteoglycans of 150 and 200kD are associated with both rod and cone photoreceptor cells. The cone photoreceptor cell-associated molecules appear to be similar to those associated with rods, except for the type of chondroitin sulfate and the absence of terminal sialic acid residues associated with the O-linked oligosaccharides. The identification of bFGF as a component of the insoluble IPM is the first indication that bioactive peptides may be sequestered in the IPM. This finding raises the strong possibility that bFGF may regulate one or more aspects of photoreceptor, RPE, or Müller cell function in vivo. Funded by EYO6463 (GSH), EY02082 (DHA) and an unrestricted grant to Bethesda Eye Institute from Research to Prevent Blindness, Inc.

A HIGHLY SENSITIVE SEMI-QUANTITATIVE DOT-BLOT ASSAY FOR GLYCOSAMINOGLYCANS AND PROTEOGLYCANS.

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Cationic dyes such as Alcian blue, toluidine blue or 1,9-dimethylmethylene blue stain polyanions and are used in assays for the detection of proteoglycans (PGs). The limit of detection for these assays is about 2 ug/ml. Salts, urea, proteins, nucleic acids and other molecules interfere with these assays.

We developed a highly sensitive and specific dot-blot assay employing a modification of previous Alcian blue staining methods to detect nanogram quantities of glycosaminoglycans (GAGs) and/or PGs. For these studies, standard GAGs and intact vascular heparan sulfate PGs were employed. The sensitivity and specificity of this assay for GAGs and PGs was optimized by changes in the staining buffer. The optimum concentration of Alcian blue was 0.2% (w/v); concentrations >0.2% increased background staining and did not enhance binding with GAG or PG. The effect of variations in the pH and ionic strength of the staining buffer at optimal critical electrolyte concentrations of $MgCl_2$ for specific GAGs was significant. Optimal staining for chondroitin sulfate (0.3M $MgCl_2$) occured at pH 2.7. For both heparan sulfate and chondroitin sulfate GAGs (0.05M $MgCl_2$), optimal staining occured at pH 5.7. Increases in concentration of NaCl to 0.2M optimally stained GAGs while nonspecific staining of polyanions (including DNA and proteins) was reduced. Hyaluronic acid also did not stain well under these conditions. A concentration of NaCl greater than 0.2M impaired the dye binding to GAGs. The sensitivity and specificity of our assay was actually improved in the presence of urea. Finally, different membranes (Immobilon-N & -P (Millipore); Nitrocellulose and Nytran 66 (Schleicher & Schuell); Zetabind (CUNO); Zetaprobe and DEAE-cellulose (Biorad)) were studied. Only a few positively charged membranes were extremely efficient in binding GAGs and PGs (Zetabind, Zetaprobe and Immobilon-N). Nytran 66 gave good results with intact PGs (high loading capacity and high sensitivity) but not with GAGs (no binding at all). The best combination of these factors (0.2% Alcian blue, 50mM NaAcetate pH5.7, 0.2M NaCl and 50mM $MgCl_2$) allowed us to detect GAGs to a concentration of 150 ng/ml (7.5 ng/well) and PGs to a concentration in protein of 500 ng/ml (25 ng/well).

The GAGs/PGs dot-blot Alcian blue assay we have developed has proven to be very useful in monitoring the classical isolation of PGs from tissue by ionic exchange and gel filtration chromatography. The assay can be adapted as a semi-quantitative method with a scanning densitometer (reflectance mode). This method is compatible with all the buffers used in GAGs/PGs biochemistry, and can be employed in the presence of other highly negatively charged macromolecules. It is technically easier, faster and more sensitive than previously published methods.

Supported by grants from the NIH (RO1-AI24876 & P50 AG05138) and from the Florence J. Gould Foundation.

REGULATION OF TERMINAL GLYCOSYLATION: THE TISSUE SPECIFIC EXPRESSION OF THE α2,6 SIALYLTRANSFERASE GENE

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Various terminal carbohydrate structures have been shown to play important roles in development, differentiation, and cell-cell interaction. The appearance of these carbohydrate sequences is thought to be controlled by the array of glycosyltransferase activities that the cell expresses. However, little is known about the regulation of glycosyltransferase activity within the cell. Previously, we have shown that the activity of a terminal glycosyltransferase gene, β-galactoside α2,6 sialyltransferase (α2,6ST), correlates well with mRNA levels measured in various tissues, with highest levels being detected in liver. In order to understand what factors regulate the expression of α2,6ST, we have cloned the gene encoding this enzyme and have begun an initial analysis of its promoters. This gene was found to span over 55 kb and contain three promoters: a constitutive promoter, a kidney specific promoter, and a liver specific promoter. The liver specific promoter was found to contain consensus binding sites for the liver restricted transcription factors HNF-1 and DBP. Footprinting experiments reveal that these sites are indeed bound by nuclear proteins. When linked to the reporter gene luciferase, this promoter was found to be 50-fold more active in a liver derived cell line (HepG2) than in a fibroblast-like cell line (CHO). Further, deletion analysis of this promoter reveals that most of the *cis*-acting elements necessary for HepG2 specific expression lie within 133 bp of the transcriptional start site. To test the importance of HNF-1 for α2,6ST promoter activity, a *trans*-activation experiment was performed using a HNF-1 expression vector. The results indicate that HNF-1 can *trans*-activate the α2,6ST promoter up to 50 fold in Cos-1 cells. Together, these results provide evidence for the cellular regulation of glycosylation occurring at the level of transcription via tissue restricted transcription factors interacting with glycosyltransferase gene promoters. (This work was supported by NIH research grant GM-27904 and a public health service grant GM-08042 to the UCLA MSTP. P.B.C. was supported by NIH fellowship HD-07201.)