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Structure and Conformation

STRUCTURAL STUDIES ON OVINE SUBMAXILLARY MUCIN

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Sheep submaxillary mucin was purified by density gradient centrifugation and gel filtration chromatography on Sephacryl S-1000. The purified mucin was polydisperse, having an average molecular weight of approximately 5.0-5.5x10^6. N-terminal analysis of intact mucin using the method of Haas and Rosenberry (Anal. Biochem. (1985) 148, 154-162) revealed the presence of 1-2 moles of N-terminal serine per mole of mucin. Smaller quantities of N-terminal glycine and alanine were also detected.

The mucin was reduced with dithiothreitol, alkylated with [^{14}C]iodoacetamide and the products chromatographed on Sephacryl S-1000. The major peak contained all of the carbohydrate and most of the incorporated label, was polydisperse with an average molecular weight of approximately 1.6-1.7x10^6. A minor peak of radioactivity appeared at the included volume; SDS gel electrophoresis of the minor peak suggested a molecular weight of approximately 200 kDa. The major peak was digested with trypsin and the products chromatographed on Sephacryl S-200. Two peaks containing both label and carbohydrate eluted near the void volume while a third peak containing radioactivity but little carbohydrate eluted at the included volume. In contrast to the high degree of polydispersity seen for both the intact and reduced mucins, these peaks appeared to be relatively homogenous. (Supported by NIH Grant DK-27551 and Grants from the Cystic Fibrosis Foundation)

ANALYSIS OF THE CARBOHYDRATE STRUCTURES ON THE ALPHA SUBUNIT OF HUMAN CHORIOGONADOTROPIN.

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HCG is a glycoprotein hormone composed of two subunits, α and β. The intact hormone as well as its free α subunit are secreted during pregnancy and also by a variety of malignant cells. While there is general agreement that the hCGα subunit contains two asparagine-linked oligosaccharide units, the structure of these units remains controversial. It is known, however, that the carbohydrate on the hCGα subunit is important for the adenylate cyclase stimulating activity of the intact hormone. We have purified both the hCGα and the free α subunits from pregnancy urine and compared their carbohydrate moieties. HCGα subunit contains primarily monoantennary oligosaccharides and the sialic acid content of the molecule is consistent with such structures. In addition, DEAE analysis of oligosaccharides released from hCGα demonstrated that most of these oligosaccharides contained only one sialic acid residue. A portion of the hCGα oligosaccharides were sensitive to release by endoglycosidase H. The released oligosaccharides appeared to be hybrid structures, based on DEAE binding properties and carbohydrate compositional analysis. The endo H-resistant oligosaccharides on hCGα were released using glycopeptidase A. These structures appeared to contain a mixture of truncated hybrid structures and biantennary oligosaccharides which could be separated by DEAE binding. In contrast, the free α subunit does not contain any endo H sensitive material. Most of the oligosaccharides on free α are biantennary and triantennary structures, many of which are fucosylated. We propose that combination of the hCGα subunit with hCGβ results in steric hindrance of oligosaccharide processing on hCGα, while the glycosylation sites on the unhindered free α are processed to more highly branched forms.

COUNTERION DEPENDENCE OF HYALURONATE SEGMENT ASSOCIATION. D.M. Hittner, J.S. Kim, and M.K. Cowman. Department of Chemistry, Polytechnic University, Brooklyn, NY 11201.

In previous studies, we demonstrated that sodium hyaluronate (NaHA) segments can self-associate in 0.15 M NaCl solution (Turner, Lin, and Cowman [1988] Arch. Biochem. Biophys., in press). In the present work, we have compared the relative tendencies of KCl and NaCl solutions to induce self-association, and have examined nmr properties of the resulting samples.

HA segments averaging 25 disaccharides in length (range ca. 17-35) were isolated from HA polymer by limited testicular hyaluronidase digestion and fractionation of the resulting mixture by high performance gel permeation chromatography. The average chain length was established by sensitivity-enhanced polyacrylamide gel electrophoresis. Low angle laser light scattering studies of NaHA and KHA segments in 0.15 M NaCl and 0.15 M KCl, respectively, indicated marked concentration-dependent association only in the NaCl solvent (in agreement with the HA polymer studies of Sheehan et al. [1983] Int. J. Biol. Macromol. 5, 222). ^{1}H-nmr of the amide proton resonances in the same aqueous salt solutions showed no significant differences in either case relative to salt-free solution. ^{13}C-nmr, however showed changes in chemical shift for several carbons, most notably the resonances tentatively assigned to C5 and C6 of the uronic acid residue. The changes were greater for the NaCl case than for KCl.

These results are interpreted as indicative of ion specificity in the self-association process. The associated form does not appear to be stabilized by hydrogen bonds involving the amide proton.

(Supported by NIH Grant EY 04804.)

GASTRIC MUCIN HYDROPHOBICITY: EFFECTS OF ASSOCIATED AND COVALENTLY BOUND LIPIDS, PROTEOLYSIS, AND REDUCTION. A. Slomiany, H. Nishikawa, K. Okazaki and B.L. Slomiany. Dental Res. Ctr., NJ Dental School, UMDNJ, Newark, NJ 07103-2425.

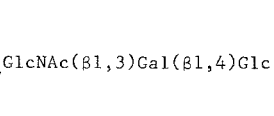
Among the factors contributing to the preservation of gastric mucosal integrity is the hydrophobicity of the mucosal surface. As mucus layer constitutes the first line of mucosal defense and the component primarily responsible for its functional qualities is mucus glycoprotein, we have employed fluorescent probe to examine the hydrophobic characteristics of this glycoprotein. The undergraded mucus glycoprotein polymer, isolated from pig gastric mucus, was subjected to removal of associated and covalently bound lipids, degradation with pepsin, and disulfide bridge reduction. Analyses of the titration data revealed the presence of 55 hydrophobic binding sites in the intact mucin molecule, 71 binding sites in the glycoprotein devoid of associated lipids, and 53 binding sites in the glycoprotein devoid of associated lipids and covalently bound fatty acids. Proteolytic destruction of the nonglycosylated regions of the mucin molecule with pepsin essentially abolished the probe binding, while reduction of disulfide bridges resulted in glycoprotein subunits whose combined number of binding sites was about 3 times greater than that of the mucin polymer. The binding of the probe to mucus glycoprotein varied with the pH of the medium, being highest at pH 2.0 and lowest at pH 9.0. The results indicate that lipids contribute to the hydrophobic character of gastric mucus glycoprotein, that hydrophobic binding sites reside on the nonglycosylated regions of the glycoprotein polymer buried within its core, and that this property of mucus glycoprotein is pH dependent. (Supported by USPHS, NIH Grants AA#05858-06, HL#32553-4 and DK#21684-12).

EFFECT OF ETHANOL AND PROSTAGLANDIN ON THE ENZYMATIC SULFATION OF SALIVARY MUCIN. A.Slomiany, Y.H. Liau, J. Piotrowski, and B.L. Slomiany. Dental Res. Ctr., NJ Dental School, UMDNJ, Newark, NJ 07103-2425.

We report here on the in vitro sulfation of salivary mucin in submandibular glands, and the effects of ethanol and prostaglandin E2 on this process. Subcellular fractionation studies revealed that the enzyme which catalyzes the transfer of sulfate ester group from PAPS to mucus glycoprotein is associated with Golgi-rich membrane fraction. The enzyme exhibited optimum activity at pH 6.8 in the presence of Triton X-100, $MgCl_2$ and NaF. The Golgi enzyme also catalyzed the sulfation of galactosylceramide, but the sulfation of mucus glycoprotein was not affected by the presence of this glycolipid. The sulfotransferase activity was inhibited by ethanol and stimulated by prostaglandin. The rate of inhibition was proportional to the concentration of ethanol up to 0.4M, at which concentration a 39% reduction in the sulfotransferase activity occurred. The enhancement of the glycoprotein sulfation was proportional up to $2x10^{-5}$M prostaglandin at which point a 31% increase in sulfation was attained. The synthesized glycoprotein gave on CsCl density gradient centrifugation ^{35}S-labeled peak which coincided with that of the glycoprotein. Alkaline borohydride treatment led to the liberation of the label into reduced oligosaccharide fraction. A 75.4% of the label was found incorporated in four oligosaccharides. These were identified in order of abundance as sulfated penta-, tri-, hepta- and nonasaccharides. The results of structural analyses suggested that the trisaccharide has following structure, SO3H→6GlcNAcβ1→3Galβ1→3GalNAc-ol. The pentasaccharide was characterized as SO3H→6GlcNAcβ1→3Galβ1→4GlcNAcβ1→3(NeuAcα2→6)GalNAc-ol, and the heptasaccharide as SO3H→6Galβ1→4GlcNAcβ1→6(Fucα→Galβ→GlcNAcβ→Galβ→)GalNAc-ol. (Supported by USPHS, NIH Grants AA#05858-06 and DE#05666-09)

SOME NOVEL OLIGOSACCHARIDES FROM GOAT MILK P. CHATURVEDI and C. B. SHARMA, Department of Biosciences and Biotechnology, University of Roorkee, ROORKEE-247667, INDIA.

Five novel neutral oligosaccharides have been purified from goat milk using Bio-Gel P-4 and reverse phase C-18 high performance liquid chromatography. Their structures, as determined by high field ^1H NMR, are:

1. GlcNAc(β1,6)Gal(β1,4)Glc
2. Gal(β1,4)GlcNAc(β1,6)Gal(β1,4)Glc
3. Gal(β1,3)

GlcNAc(β1,3)Gal(β1,4)Glc
Gal(β1,4)
4. Gal(β1,4)GlcNAc(β1,6)Gal(β1,4)Glc

Fuc(α1,3)
5. Gal(β1,3)GlcNAc(β1,6)Gal(β1,4)Glc

Fuc(α1,3)

Structurally, the goat milk oligosaccharides differ markedly from that of the corresponding oligosaccharides from human milk [Dua, V.K. and Bush, C.A. (1983) Anal. Biochem. 133, 1-8]. The oligosaccharide, no. 3, has an interesting structure in the sense that it has two galactose residues linked via β1→3 and β1→4 linkage, respectively, to the same GlcNAc residue at the non-reducing end. The biological functions of these oligosaccharides are unknown, however, they can serve as substrates for various glycosyltransferases involved in the synthesis of complex carbohydrates of milk. They would also be useful in characterization of related structures from glycolipids and glycoproteins.

Analytical Techniques

ANALYTICAL AND SEMIPREPARATIVE SEPARATIONS OF OLIGOSACCHARIDES USING HIGH-pH ION EXCHANGE CHROMATOGRAPHY. Louisette J. Basa and Michael W. Spellman. Medicinal and Analytical Chemistry Dept., Genentech, 460 Point San Bruno Blvd. South San Francisco, CA 94080.

We are investigating the application of high-pH anion exchange chromatography to the separation of Asn-linked oligosaccharides. A nonmetallic HPLC system (Dionex BioLC) equipped with a polymeric pellicular anion exchange column (Dionex HPIC-AS6) and a pulsed amperometric detector was used for the separations. Elution was carried out at a constant base concentration (0.1 N NaOH) using a linear gradient from 0-0.1 M NaOAc and a flow rate of 1 ml/min. Mixtures of high-mannose oligosaccharides (ranging from $Man_5GlcNAc$ to $Man_9GlcNAc$) were baseline resolved in a 40-min gradient. Three closely related biantennary oligosaccharides were also separated under these conditions. Separations of sialylated oligosaccharides were carried out under similar conditions but with a steeper gradient (0-0.2 M NaOAc in 45 min). Although these separations were dominated by the number of residues of sialic acid on each oligosaccharide, significant resolution could be obtained among different structures with the same net charge. The use of pulsed amperometry permits routine detection of underivatized oligosaccharides in the range of 0.1 -1 nmol. Because of the sensitivity of this technique, it can be used in conjunction with reversed-phase HPLC tryptic mapping to characterize the distribution of oligosaccharides at individual glycosylation sites.

For semipreparative applications we have used on-line desalting with an anionic micromembrane suppressor (AMMS) cartridge. Within the AMMS cartridge, the column effluent is passed along one face of a polyanionic membrane, which is permeable to cations but impermeable to anions. Sodium ions in the effluent stream are exchanged for protons from aqueous sulfuric acid on the other side of the membrane, thus converting the NaOH and NaOAc to water and acetic acid, respectively. Salt-free oligosaccharides can then be recovered after lyophilization of column fractions. The AMMS cartridge can effectively desalt column effluents containing up to 0.2 M sodium ions at a flow rate of 1 ml/min. The low dead volume of the cartridge causes minimal peak broadening. We obtained approximately 60% recovery of a neutral disaccharide (maltose) using the AMMS cartridge, which indicates that leakage of larger oligosaccharides past the membrane is not likely to be a significant problem.

SEPARATION OF REDUCING OLIGOSACCHARIDES DERIVED FROM GLYCOPROTEINS ON STABLE POLYMERIC HPLC PACKINGS.

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N-linked oligosaccharides released either by hydrazinolysis or by enzymatic treatment (EndoH or N-Glycanase®) from glycoproteins have been analyzed by a new family of polymeric columns. Glyco-Pak™ DEAE columns have been used for the purification of acidic oligosaccharides using either isocratic or gradient elution with various buffers. Glyco-Pak™ N columns have been used for the purification of Neutral oligosaccharides using eluents comprised of mixtures of acetonitrile and water.

The retention of oligosaccharides on the Glyco-Pak N column has been investigated with various acetonitrile/water mixtures using standard oligosaccharides having between 2-11 monosaccharides residues. Oligosaccharides differing by one monosaccharide in size have been readily separated on the Glyco-Pak N column. Resolution of structural isomers and/or linkage isomers have been obtained with both columns.

The use of these two columns, as the basis of a general multicolumn separation approach for the purification of oligosaccharides prior to structure elucidation analysis, has been investigated for standard glycoproteins.

PROGRESS IN THE PICOMOLE LEVEL CHARACTERIZATION OF INTACT CARBOHYDRATES BY MASS SPECTROMETRY. A.L. Burlingame, Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143-0446.

In order to eventually optimize the structural information obtainable on picomole quantities of intact carbohydrates using liquid matrix secondary ion based mass spectrometric strategies, a knowledge of how to manipulate the parameters required to produce high quality mass spectra is required. In the case of the oligosaccharides, these factors include the hydrophobicity/hydrophilicity ratio, control of the site of charge localization, control of vibronic energy content, and minimization or elimination of matrix-associated chemical noise. Instrumental factors include control of the sputtering process, minimization or elimination of the chemical noise, control of vibronic energy, and optimization of overall sensitivity including the use of electro optical multichannel array detection on both double focusing instruments and tandem double focusing instruments.

In this laboratory, considerable effort is being directed toward development of mass spectrometric methods for structural characterization of intact N-linked oligosaccharides obtained from glycoproteins by enzymic cleavage using PNGaseF (Gillece-Castro, 1987). We have demonstrated the utility of reductive coupling of p-aminobenzoic acid ethyl ester (ABEE) with the PNGaseF liberated free oligosaccharide reducing terminus for HPLC separation, charge localization and induction of an abundant fragmentation pattern in the negative ion mode (Gillece-Castro, 1987; Webb, 1988). More recently, we have shown that the sensitivity of these analyses may be increased significantly by increasing the hydrophobicity of this derivative (Poulter, 1988) and by using multichannel array detection on both two-sector (Poulter, in press) and tandem four sector double focusing instruments.

Financial support was provided by the Division of Research Resources, NIH and the NSF Biological Instrumentation Program.

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COMPARISON OF DETECTION TECHNIQUES FOR LIQUID CHROMATOGRAPHIC ANALYSIS OF NEUTRAL AND ACIDIC CARBOHYDRATES.

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Classically, the detection methods employed in HPLC analysis of carbohydrates have been limited to off-line colorimetry or scintillation counting or on-line UV or RI.

Off-line methodology is generally avoided for reasons of sensitivity, reliability and efficiency of time.

Both low wavelength UV (190-210 nm) and refractive index (RI) detection suffer from poor sensitivity and general gradient incompatibility.

Recently, the technique of pulsed amperometry has been shown to be extremely sensitive (low picomole levels) for non-derivatized carbohydrates as well as being gradient compatible.

Chemically suppressed conductivity is a well established technique for sensitive detection of ionic species and gives picomole sensitivity for acidic carbohydrates (e.g., uronic acids, phosphorylated carbohydrates), again with gradient compatibility.

A comparison of several detection modes will be made for both isocratic and gradient analysis of neutral and acidic sugars.

STRUCTURE ELUCIDATION OF THE HAPTENIC OLIGOSACCHARIDES OF THE GLYCOLIPID ANTIGENS OF THE Mycobacterium avium COMPLEX. D. Chatterjee and P. J. Brennan. Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523.

A renewed interest in "atypical" mycobacteria stems from their occurrence as opportunistic pathogens in many patients with acquired immunodeficiency syndrome. Most important among these mycobacteria are serovars of the Mycobacterium avium complex, about 30 in number. The surface antigens which differentiate these serovars are glycopeptidolipids (GPLs), composed of a common glycosylated lipopeptide "core", fatty acyl-D-Phe-D-alloThr-D-Ala-L-alaninyl-O-3,4-di-O-methyl-6-deoxyhexose, to which a haptenic oligosaccharide is linked at the threonine substituent and which is the source of type specificity. We have been exploring the chemistry of these haptenic oligosaccharides using NMR (2D and 1D experiments), FAB/MS, and Californium desorption mass spectrometry. A wide diversity in structure is revealed, notably at the non-reducing end of the oligosaccharide chain, marked by the presence of new branched-chain sugars, amino sugars and acidic sugars. The detailed structures of the oligosaccharide haptens of the GPL antigens from 12 of the 31-serovar complex have been characterized in detail, and the sugar epitopes that distinguish the remainder have been characterized by gas chromatography/ mass spectrometry. This information and its applications will be presented.

SEQUENCING OF POLYSACCHARIDES CONTAINING AMINOSUGAR RESIDUES BY THE REDUCTIVE-CLEAVAGE METHOD. A.J. D'Ambra and Gary R. Gray. Department of Chemistry, University of Minnesota, Minneapolis, MN 55455.

Previous studies in this laboratory (Bennek *et al.*, *Carbohydr. Res.*, 157 (1986) 125-137) have demonstrated the behavior of simple glycosides of *N*-acyl and *N*-alkyl aminosugars under the conditions of reductive cleavage. Specifically, a 1,2-*trans* *N*-acyl aminosugar undergoes simple hydrolysis to give the free hemiacetal via an oxazolinium-ion intermediate. No reaction occurs with 1,2-*cis* *N*-acyl aminosugars or with either anomeric form of the *N*-alkyl aminosugar (i.e., after reduction of the *N*-acyl group). The present work takes advantage of this selective reactivity in sequencing a polysaccharide containing aminosugar residues by the reductive-cleavage method.

The O-antigenic polysaccharide of *Pseudomonas aeruginosa* ATCC 33358 (Habs O:11) (Dmitriev *et al.*, *Eur. J. Biochem.*,106 (1980) 643-651) was chosen as a model system. The permethylated polysaccharide was subjected to reductive cleavage in the presence of $Me_3SiOSO_2CF_3$, with subsequent benzoylation *in situ*. The products were isolated by HPLC and characterized by NMR and FABMS. The expected products are a disaccharide, i.e., the permethylated, 3-*O*-benzoyl derivative of α-L-FucNAc-(1→3)-D-FucNAc, and 1,5-anhydro-2-*O*-benzoyl-3,4,6-tri-*O*-methylglucitol in a ratio of 1:1. Characterization of these products should establish the correct sequence of the polysaccharide's repeating unit.

This investigation was supported by Grant GM34710 awarded by the U.S. Department of Health and Human Services.

RAPID RADIOLECTIN PROBE METHOD FOR MICRO GELS. Connie M. David, Catherine R. Adams-Burton & Roger A. Laine. Louisiana State University, Baton Rouge, LA, 70803

Polyacrylamide gel electrophoresis on a "PhastSystem" (Pharmacia Co.) was followed by fixation and direct probing with ^{125}I - labeled Concanavalin A. After 2 hours of washing, gels could be analyzed by autoradiography or could be cut into strips for gamma counting or scintillation spectrometry. Sensitivity was high, and the limit for detection of a band of ovalbumin ($43000M_r$) was 10 ng (approximately 1 picomole) with 48 hours of exposure. The total time from beginning of experimental gel preparation to beginning of autoradiography was 5 1/2 hours.

COLLISION ACTIVATED DISSOCIATION TANDEM MASS SPECTROMETRY and MOLECULAR MODELLING IN SUGAR LINKAGE POSITION DETERMINATION

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Six trisaccharides were synthesized having identical reducing end disaccharides differing only by the position of linkage and sugar entity at the non-reducing terminus. Their structures are as follows:

Fuc(a1->3)GlcNAc(B1-3)Gal-OMe Fuc(a1->4)GlcNAc(B1-3)Gal-OMe

Fuc(a1->6)GlcNAc(B1-3)Gal-OMe Gal(a1->3)GlcNAc(B1-3)Gal-OMe

Gal(a1->4)GlcNAc(B1-3)Gal-OMe Gal(a1->6)GlcNAc(B1-3)Gal-OMe

Fast Atom Bombardment ionization (FAB) tandem mass spectrometry (MS-MS) using collisional activated dissociation (CAD) and molecular modelling demonstrated that linkage position on the non-reducing terminal sugar could be ascertained by stability of the bond to CAD at a threshold collisional cross section. Molecular modelling showed that the stability was related to density of states at the transition state for bond breakage, related to the degree of freedom of motion in the glycosidic bond. (Supported by NIH grants GM35755, DRR02803 and NIDDK24594 to R.A.L.)

OLIGOSACCHARIDE MONOMER SEQUENCE DETERMINATION USING FAST-ATOM BOMBARDMENT MASS SPECTROMETRY OF PERIODATE OXIDIZED ACETATE ESTER DERIVATIVES. R.S. Pappas, B. Sweetman, S. Ray, C.G. Hellerqvist. Vanderbilt University School of Medicine, Department of Biochemistry, Nashville, TN 37232.

A derivatization method for sequencing monomer linkages in oligosaccharides using positive ion FAB-MS, provides sequence information by directing fragmentation to both sides of glycosidic oxygens, and in some cases, between glycosidic carbons and ring oxygens. This modification of the method introduced by Angel, Lindh, and Nilsson[1], consists of acetylation rather than methylation after periodate oxidation and borohydride reduction. Data obtained on model compounds, using 3-nitrobenzyl alcohol as matrix for FAB-MS, demonstrated direction of fragmentation to both sides of the glycosidic oxygen and between the glycosidic carbon and "ring oxygen" in oxidized residues. Thus, oligosaccharides can be sequenced by observing fragmentation from both the reducing and non-reducing ends of the molecule. Because of its redundancy, this fragmentation pattern provides precise structural information about the molecule, as well as possibly extending the length of an oligomer which can be sequenced, since overlapping information from each end can be combined to obtain structure.

The repeating units of two Salmonella lipopolysaccharides, derivatized by this procedure, were partially hydrolyzed with trifluoroacetic acid and acetylated with acetic anhydride. The HPLC purified oligosaccharide derivatives, when analyzed by FAB-MS, demonstrated the applicability of the technique to branched structures, and that this technique combined with linkage analysis can readily resolve complex polysaccharide structures.

A computer program is introduced which assists in determining sequence from ion mass input.

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AUTOMATED COMPUTER ANALYSIS OF DATA FROM OLIGOSACCHARIDE FAST-ATOM-BOMBARDMENT MASS SPECTROMETRY. Stuart Ray, Steve Pappas, and Carl G. Hellerqvist. Vanderbilt University, School of Medicine, Department of Biochemistry, Nashville, TN 37212.

A computer program is under development which generates possible linkages when given peak data from a positive ion fast-atom-bombardment - mass spectrum of a periodate - oxidized, borodeuteride - reduced, acetylated oligosaccharide. The method for deriving structural information is based on work by Angel, Lindh, and Nilsson[1], with modifications developed in this laboratory.

Written in Turbo Pascal (Borland), this program is an expert system, meaning that it was designed to perform a task using an algorithm modelled after that used by a human expert performing the same task. As such, it is capable of indefinite refinement as the rules of the analysis become more clear to the experts.

The program utilizes a database which can be tailored at run-time to prior knowledge about the sample. Multiple databases, consisting of residues and their expected fragmentation patterns, may be developed for different classes of molecules. Based on such a database, a list of all solutions is generated and then stratified by relative merit. A list of putative sources for each of the peaks is also generated, yielding useful information about fragmentation patterns.

This program provides the user with a distinct advantage over human interpretation of spectra by finding all possible solutions for such spectra (in the context of its database and rules), in a short amount of time (about five seconds). The stratified list of possible solutions provides the intelligent user with useful structural information.

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PAIRED ION CHROMATOGRAPHIC SEPARATION OF SIALYLATED TRYPTIC GLYCOPEPTIDES FROM BOVINE FETUIN. KEVIN G. RICE and YUAN C. LEE
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Sialylated glycopeptides, derived from the tryptic digestion of bovine fetuin, containing from fifteen to thirty amino acids have been resolved in a single chromatogram, on the basis of peptide sequence and degree of sialylation using an octyl HPLC-RP column eluted at pH 7 with an ion pairing reagent composed of triethylamine and boric acid. In the preparative isolation of glycopeptides, as much as 75 mgs of glycopeptide could be injected onto a 2 x 25 cm octyl HPLC-RP column. Intact sialylated glycopeptides were obtained after efficient removal of the ion pairing reagent after repeated solvent evaporation and methanol dissolution cycle.

Sialylated glycopeptides from each glycosylation site were converted to shorter asialo glycopeptides by digestion with neuraminidase and pronase. The isolated asialo glycopeptides demonstrated a high degree of purity, >95% by proton NMR and by chromatography on high performance anion exchange (HPAE) at alkaline pH with pulsed amperometric detection. Resolution of galβ(1-3)- and galβ(1-4)-triantennary positional isomers occurred predominantly during the paired ion separation due to a difference in the degree of sialylation of these two triantennary isomers.

STRUCTURAL ANALYSIS OF NEUTRAL OLIGOSACCHARIDE-ALDITOLS FROM RESPIRATORY MUCINS OF A NON-SECRETOR (Le^{a+b-}) PATIENT SUFFERING FROM CHRONIC BRONCHITIS.

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The oligosaccharide chains of respiratory mucins from a patient (War.) suffering from chronic bronchitis have been examined. Alkaline borohydride reductive cleavage was used to prepare oligosaccharide-alditols. These were purified by ion-exchange chromatography (Dowex AG1X2), gel filtration (Bio-Gel P4) and HPLC (Lichrosorb-NH_2) [1]. Here we report on the detailed investigation of the HPLC-purified smaller-size neutral oligosaccharide-alditols.

To date, by a combination of monosaccharide composition analysis and 500-MHz ^{1}H-NMR spectroscopy, twenty-three structures have been identified, seven of which are previously unreported for respiratory mucin oligosaccharides. In keeping with the non-secretor (Le^{a+b-}) status of the patient, no structures were found which contained the Fucα(1→2)Galβ(1→•) moiety. However, several of the structures identified expressed either the Le^a [Galβ(1→3){Fucα(1→4)}-GlcNAcβ(1→•)] or the X [Galβ(1→4){Fucα(1→3)}GlcNAcβ(1→•)] determinant. An example of one such structure is:

Galβ(1→3)GlcNAcβ(1→3)Galβ(1→3)GalNAc-ol
Fucα(1→4)/

Of those HPLC fractions investigated, many contained a mixture of two or three components. Nevertheless, their structures could be determined by 500-MHz ^{1}H-NMR spectroscopy when at least one had been identified previously. This illustrates the power of this non-destructive technique.

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QUANTITATIVE ANALYSIS OF SIALYLATED OLIGOSACCHARIDE MIXTURES USING HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION. R. R. Townsend, M. R. Hardy, B. Bendiak, D. A. Cumming and J. P. Carver. Department of Biology, Johns Hopkins Univ., Baltimore, MD 21218, Department of Biochemistry, Sick Children's Hospital, Toronto M5G 1X8, and Department of Medical Genetics and Medical Biophysics, Univ. of Toronto, Toronto, Ontario M5S 1A8 CANADA.

The sialylated oligosaccharides released from bovine fetuin using N-glycanase were resolved into 20 major components (≃ 90% of the total) and 15 minor oligosaccharides (≃ 10% of the total) using high-performance anion-exchange chromatography under alkaline conditions. Electrochemical detection was used to quantify these underivatized oligosaccharides in the pmol range (20-100 pmols). The relative electrochemical response factor for ten characterized tri-, tetra-, and penta-sialylated oligosaccharides from bovine fetuin was 5.1 ± 16% using glucose as an external standard. The similarity in electrochemical response among these related compounds permitted direct determination of the relative amounts of these characterized oligosaccharides in the mixture of oligosaccharides released from fetuin by N-glycanase. Features of the separation of branched sialylated oligosaccharides using this technique included distinct separation among bi-, tri-, tetra-, and penta-sialylated compounds and baseline resolution of most linkage and branch positional isomers.

STRUCTURAL CHARACTERIZATION OF THE CARBOHYDRATE MOIETIES OF THE THREE ISOZYMES OF HUMAN NEUTROPHIL ELASTASE. *HERMAN VAN HALBEEK*[1,2], *Wieslaw Watorek*[2,3] *and James Travis*[2]. [1]Complex Carbohydrate Research Center and [2]Department of Biochemistry, University of Georgia, Athens GA [1]30613 and [2]30602, USA; [3]Institute of Biochemistry, Wroclaw University, 50-137 Wroclaw, Poland.

Human neutrophil elastase (HNE) is a serine protease capable of degrading elastin, collagen and proteoglycans, as well as foreign proteins, during phagocytosis. HNE has, therefore, been suggested to play an important role in the development of connective tissue disorders, including emphysema and rheumatoid arthritis. Here we report on the structural characterization of the carbohydrate moieties of the enzyme.

HNE was obtained as a mixture of three isozymes by Sepharose-trasylol chromatography of granule extracts. The three forms (HNE-1, HNE-2 and HNE-3) were separated by CM-cellulose chromatography. They were found to be identical in their amino acid sequence [1], but their carbohydrate contents differed significantly: 9% for HNE-1, 7% for HNE-2, and 4% for HNE-3. The monosaccharide compositions of HNE-1, -2, and -3 suggested that the carbohydrate chains are *N*-linked to Asn in the polypeptide. Glycopeptides were prepared from each of the three forms of HNE by pepsin/pronase digestion. The structures of the carbohydrate and the peptide portions (and thus the glycosylation sites to which the carbohydrates in the HNE forms are attached) were determined by the combination of 500-MHz ^{1}H-NMR spectroscopy, sugar and amino-acid analyses. HNE-3, the quantitatively most abundant isozyme form, has only one of three potential glycosylation sites occupied, namely, Asn-95. The structure of the oligosaccharide chain of HNE-3 was determined to be:

$$\begin{matrix} \text{Man}\alpha(1\rightarrow6)\diagdown & & [\text{Fuc}\alpha(1\rightarrow6)]_{0\text{-}1}\diagdown \\ & \text{Man}\beta(1\rightarrow4)\text{GlcNAc}\beta(1\rightarrow4)\text{GlcNAc}\beta(1\rightarrow\text{N})\text{Asn}^{95}. & \end{matrix}$$

Both HNE-1 and HNE-2 also have a second site (Asn-144) glycosylated where the structures are of the di-antennary *N*-acetyllactosamine type; the branches end mostly in $\beta(1\rightarrow4)$-galactosyl, but occasionally in $\alpha(2\rightarrow6)$-sialyl residues. In neither of the three isozyme forms of HNE was the third site, Asn-59, found to be glycosylated.

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STRUCTURAL ANALYSIS OF POLYSACCHARIDES CONTAINING PYRUVATE KETAL SUBSTITUENTS BY THE REDUCTIVE-CLEAVAGE METHOD. Sam Zeller and Gary R. Gray. Department of Chemistry, University of Minnesota, Minneapolis, MN 55455.

A series of investigations were carried out using compounds that model naturally occurring 4,6-*O*-(1-carboxyethylidine) derivatives of carbohydrates in order to establish conditions under which reductive cleavage could be accomplished. Methyl 4,6-*O*-[(**R**)- and (**S**)-(1-methoxycarbonylethylidine)]- 2,3-di-*O*-methyl-α-**D**-glucopyranoside was reductively cleaved using a mixture of triethylsilane, boron trifluoride etherate and trimethylsilyl methanesulfonate to give 1,5-anhydro-4,6-*O*-[(**S**)-(1-methoxycarbonylethylidine)]-2,3-di-*O*-methyl-**D**-glucitol. Using information obtained through model studies, a general approach was developed for the analysis of complex polysaccharides that contain a pyruvate ketal substituent. Permethylated gum xanthan, a well characterized polysaccharide having approximately one-half of the terminal **D**-mannopyranosyl groups substituted with acetal-linked pyruvic acid, was reductively cleaved to demonstrate this approach. The expected anhydroalditols were isolated, including 1,5-anhydro-4,6-*O*-[(**S**)-(1- methoxycarbonyl-ethylidine)]-2,3-di-*O*-methyl- **D**-mannitol. Reductive cleavage was then carried out on the permethylated polysaccharide after reduction of the ester groups and mild acid hydrolysis of the newly formed hydroxyisopropylidine substituent. The expected anhydroalditols, including 4,6-di-*O*-acetyl-1,5-anhydro-2,3-di-*O*- methyl-**D**-mannitol, were again isolated. The presence of the latter anhydroalditol is thus indicative of the original position of attachment of the pyruvate ketal moiety.

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Immunological Techniques

MONOCLONAL ANTIBODIES PROVIDE EVIDENCE FOR TWO NONIDENTICAL SUBUNITS OF HUMAN LIVER α-L-FUCOSIDASE. J.A. Alhadeff[1], R.-F. Wang[2], N. Bitto[1], S. Piesecki[1], and I. Damjanov[2]. [1]Department of Chemistry, Lehigh University, Bethlehem, PA 18015 and [2]Department of Pathology, Jefferson Medical College, Philadelphia, PA 19107.

α-L-Fucosidase (α-L-F) (fucoside fucohydrolase, EC 3.2.1.51) is a ubiquitous lysosomal glycosidase involved in the degradation of a diverse group of fucoglycoconjugates. Absence or gross deficiency of α-L-F activity results in the neurovisceral storage disease fucosidosis. Human liver α-L-F is a tetrameric glycoprotein with an apparent M_r of 226,000 (1). In the present investigation, monoclonal antibodies (MAb's) prepared against purified human liver α-L-F have been used to provide evidence that the enzyme is composed of two nonidentical subunits.

α-L-Fucosidase was purified to apparent homogeneity from human liver by affinity chromatography (1) and used to immunize rats. Hybridomas were produced using spleen cells from the immunized rats and mouse myeloma cells (P3-X63-Ag-8.653) employing the fusion protocol and limiting dilution cloning method of Oi and Herzenberg (2). Four hybridoma clones positive for α-L-F were mass produced in ascites of Nude mice. Isotyping indicated that two MAb's (72,116) were IgG's and two MAb's (40,100) were IgM's. The four MAb's were used for blotting after α-L-F was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to nitrocellulose. SDS-PAGE indicated the presence of two protein bands with apparent M_r's of 56 and 51 kDa's. Western blotting indicated that two MAb's (72,100) recognized only the 56 kDa band and the other two MAb's (40,116) recognized only the 51 kDa band. These results suggest that human liver α-L-F contains two nonidentical subunits.

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This investigation was supported in part by NIH grant DK 33532 (to JAA).

USE OF POLYCLONAL RABBIT ANTI-GBS TOXIN IgG FOR LOCALIZATION OF A POSSIBLE TOXIN BINDING SITE IN SHEEP LUNG TISSUE. B. Russell, R. Pappas, J. Brandt, H. Sundell, C.G. Hellerqvist. Vanderbilt University School of Medicine, Department of Biochemistry, Nashville, TN 37232.

Type III group B streptococcus (GBS) causes early onset disease in newborns resulting in pulmonary edema, increased pulmonary arterial pressure and vascular permeability[1]. We have previously demonstrated that an exotoxin, GBS toxin, can be isolated from the supernatant of GBS cultures originated from infants who died of early onset disease[2]. When infused in sheep, GBS toxin induces the pathophysiology characteristic of early onset disease[1]. Extensive analysis of GBS toxin indicates the presence of specific teichoic acid derivatives. These pathogenic fractions were used to produce polyclonal rabbit anti-GBS toxin IgG. GBS toxin passed through a protein-A affinity column with absorbed anti-GBS toxin IgG's was shown to produce a reduced pathophysiologic response in sheep relative to toxin samples passed through a protein A column with absorbed preimmune IgG.

Rabbit anti-GBS toxin polyclonal antibodies were also used for immunoperoxidase localization of toxin binding sites in sheep lung. Photomicrographs taken at 1000x magnification showed marked staining of smooth muscle subjacent to pulmonary endothelium relative to control sections which were incubated with preimmune IgG, or polyclonal IgG in the absence of GBS toxin. Thus, this data suggests that a GBS toxin/receptor complex is involved in the pathogenicity of early onset disease.

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ISOANTIBODIES DIRECTED AT A GLYCOCONJUGATE OF THIO-MANNOSE AND SERUM BOVINE ALBUMIN. J. H. Pazur and B. Liu., Biochemistry, The Pennsylvania State University, University Park, PA 16802.

A glycoconjugate (Man-s-BSA) of thio-mannose (Man-s) and bovine serum albumin (BSA) has been prepared from a mannose thioglycoside and BSA (Lee et al., Biochemistry, 15, 3956, 1976) and has now been used to immunize rabbits by a subcutaneous multi-site injection procedure (Pazur, Carbohydrate Res., 107, 243, 1982). The serum from rabbits immunized for a 5 week period contained antibodies which reacted with the glycoconjugate in several types of immunological tests. The antibodies in the immune serum were isolated by affinity chromatography on an adsorbent of antigen and AH-Sepharose 4B. Results of gel electrophoresis, agar diffusion and density gradient centrifugation showed that the purified antibodies were immunoglobulins of the IGg type, possessed a molecular weight of 1.5×10^5 and yielded an antibody-antigen precipitin reaction with Man-s-BSA. The antibodies did not react with BSA whereas anti-BSA antibodies reacted with both BSA and Man-s-BSA. On the basis of these results it was concluded that the new antibodies were specific for the carbohydrate portion of the glycoconjugate. The specificity of the antibodies for carbohydrate residues was confirmed by periodate oxidation experiments which showed that periodate oxidized glycoconjugate was no longer reactive with anti-Man-s-BSA antibodies but was still reactive with anti-BSA antibodies. Inhibition tests with mannose and several derivatives of mannose were consistent with the carbohydrate specificity of the antibodies. In the latter experiments it was found that the antigen-antibody reaction was inhibited by p-nitrophenol 1-thio-α-mannoside but not by mannose or methyl α-mannoside. Isoelectric focusing of the purified antibodies coupled with an agar diffusion test showed that the purified antibody preparation consisted of isomeric proteins which have been designated as isoantibodies. Ten isoantibodies were present in the antibody preparation as revealed by electrophoresis, agar diffusion and a standard protein stain. All of the protein components exhibited antibody activity with Man-s-BSA. Studies on the isolation of individually pure isoantibodies, the determination of structural differences and the elucidation of biological functions of the isoantibodies are in progress.

ANTIBODIES AGAINST G_{T3} GANGLIOSIDE IN THE SERA OF PATIENTS WITH TYPE I DIABETES MELLITUS. B.K. Gillard, J.W. Thomas, L.J Nell and D.M. Marcus. Departments of Medicine, Microbiology and Immunology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030

Type I diabetes mellitus is thought to result from destruction of pancreatic islet cells by an autoimmune process. It has been suggested that some human autoantibodies are directed against gangliosides of islet cells. The rat insulinoma cell line RINm5F is used to detect islet cell antibodies (Abs) in human sera, and subclones of the RIN clones vary in their ability to bind these Abs. The binding of human Abs to RIN subclones is paralleled by the binding of monoclonal Ab (mAb) A2B5, which binds to gangliosides G_{Q1c}, G_{T3} and other polysialogangliosides. Our previous studies of RIN subclones indicated that their reactivity with antibodies in diabetic sera correlated with their ganglioside content and composition, and that A2B5 and R2D6, an anti-islet mAb, reacted most strongly with RIN cell ganglioside G_{T3}. In this study we tested diabetic and control sera Abs directed against RIN gangliosides and to G_{T3}. By means of an ELISA technique we demonstrated that 13/31 diabetic sera contained anti-G_{T3}, and that the Abs were predominantly of the IgG isotype. Antibodies against G_{M3}, the most abundant ganglioside of RIN cells, were not detected. Antibodies against G_{M2} were detected in comparable titers in normal and diabetic sera. Antibodies against RIN gangliosides or G_{T3} could not be demonstrated by means of a liposome lysis technique, nor by a TLC immunostaining procedure, although antibodies against other glycolipids could be demonstrated by the latter procedure. These results indicate that antibodies against G_{T3} ganglioside are found in approximately 42% of newly diagnosed patients with type I diabetes mellitus, but the role of this antigen in destruction of islet cells remains to be determined.

COLON CARCINOMA SIALOMUCIN PRODUCTION STIMULATED BY MUCOMODULIN AND ASSOCIATION WITH INCREASED IMMUNE EFFECTOR RESISTANCE. Tatsuro Irimura, Debora A. Carlson, Masato Yagita, Elizabeth A. Grimm and David M. Ota, Departments of Tumor Biology and General Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030.

Human colon carcinoma HT-29 cells growing *in vitro* produce three high molecular weight mucin-like sialoglycoproteins (Sialomucins; M_r 900,000, 740,000 and 450,000), and a greater amount of the M_r 900,000 component was associated with a metastatic subline. The amount of sialomucins produced by HT-29 cells growing *in vivo* appeared to be greater than *in vitro*. The synthesis of all three sialomucins *in vitro* was enhanced by supplementing a conditioned medium from human colon organ culture. These changes were detected by polyacrylamide gel electrophoresis of [^{3}H]-glucosamine-labeled or non-labeled cell lysates on 3% gels, followed by fluorography or staining with ^{125}I-wheat germ agglutinin. Incorporated [^{3}H]-glucosamine was released by alkaline reduction from the glycoproteins recovered from the gels indicating that the sugar chains are O-linked. There was no change detected in the protein profiles, growth rates and morphology of the cells treated with colon conditioned medium. Dissection of colon tissues into different portions showed that the connective tissues produced this soluble factor. The factor apparently is a protein having an approximate M_r 150,000, and stable at 56°C for 60 min. but inactivated by heating at 80°C. We tentatively named the factor as mucomodulin. The mucomodulin-treated human colon carcinoma cells, expressing a greater amount of sialomucins, were less sensitive to the cytolytic effects of recombinant interleukin-2 activated human peripheral blood lymphocytes (LAK-cells). The difference in the killing appeared to result from decreased binding of LAK cells to tumor cells as determined by the conjugation method. Increased cell surface sialomucins induced by host tissue factors probably facilitated the escape of colon carcinoma cells from cytolytic effects of nonspecific host immune effectors and rendered these cells more metastatic during tumor growth at the primary sites, dissemination and metastasis. (Ref.: Irimura et al., Cancer Res., 48: 2353, 1988; supported by USPHS Grant RO1-CA39319)

Glycolipids

GLYCOLIPIDS OF CARDIAC MUSCLE: AN INTERSPECIES COMPARISON. Kenneth C. Leskawa and Craig S. Short. Department of Anatomical Sciences and Neurobiology, University of Louisville, Louisville, KY 40292

Glycosphingolipid (GSL) content and composition of hearts from a number of species was examined, since little is known of GSLs in non-neuronal, electrically excitable tissues. Atria and adipose tissue were removed from cardiac muscle for glycolipid extraction. Gangliosides were separated into mono-, di-, and oligosialosyl forms by step gradient elution from DEAE-Sephadex columns.

The major gangliosides in most species were GM3 and GD3, with several other minor, complex structures. The highest content of di- and oligosialosyl gangliosides was found in the hearts of sheep, horse and pig; values from human heart were slightly lower. Such complex gangliosides were much lower in eel, tuna and turtle hearts and lowest in rodent hearts (mouse, guinea pig, hamster and rat). This phylogenetic distribution is in sharp contrast to that found in brains, where lower vertebrates contain a higher content of complex, oligosialosyl gangliosides.

Neutral glycolipid content was widely divergent, with the highest content found in hearts from eel, rat and pig, and the lowest content in hearts from tuna, turtle, steer and human. The major neutral glycolipids in most species, especially mammals, were GbOse4Cer, GbOse3Cer and LacCer. Hearts from tuna, eel and turtle contained mostly LacCer. The Forssman hapten glycolipid was dominant in hearts from guinea pig, cat, horse and chicken, but was minor or undetected in hearts from other species. GlcCer and GalCer were undetectable in hearts from all species except mouse and hamster, where they were minor components.

While calf heart contained numerous complex structures, adult bovine hearts contained primarily GM3 (99.3% of total gangliosides). With age, there was also a loss of nLcOse5Cer and a reduction in GbOse4Cer and total neutral glycolipid content. This simplification of cardiac glycolipids with aging may not apply to all species, since young and mature rabbit hearts displayed only slight pattern and content changes.

The remarkable species differences in cardiac glycolipids suggests a role in heart function and/or development. (Am. Heart Assoc., KY Affil.)

STUDIES ON FUCOLIPID BIOSYNTHESIS IN MOUSE LYMPHOMA AND HUMAN COLON CARCINOMA M.Basu,F.A.Khan,K.K.Das,B.J.Zhang and S.Basu. Department of Chemistry, Biochemistry, Biophysics, Molecular Biology Program, Univ. of Notre Dame, Notre Dame, IN 46556.

Different tumor cell-associated glycolipid:fucosyltransferase activities have been reported by several laboratories including ours(Basu,M. et al. (1987) Methods Enzymol.,138,575-607). These fucosyltransferases catalyze the transfer of fucose from GDP-fucose to either C-2 position of the terminal galactose moiety of nLcOse4Cer(FucT-2; Basu,S. et al.(1975) J.Biol.Chem.,250, 2956-2962) or to the C-3 position of the penultimate N-acetylglucosamine unit of nLcOse4Cer(FucT-3; Presper,K.A. et al.(1978) Proc.Natl.Acad.Sci.,USA,75, 289-293) to form blood group H or Le^x glycolipids, respectively. Recent reports of other fucosyltransferases which catalyze the formation of Fuc(α1-2)Gg4 in rat hepatoma cells and of cancer-associated Le^a antigen in SW-1116 cells suggest the possible existence of either tumor-specific or species-specific fucosyltransferase gene expression. From our previous studies it appears that the level of FucT-2 activity is considerably higher in mouse B-lymphocytic tumors(ABLS-140 and TEPC-15) than that present in T-lymphocytic tumor L-4946(Basu,M. et al.(1980) ACS Symp.Series,128, 188-212). We have also established recently that P-1798, another T-lymphocytic tumor, contains no detectable amounts of either FucT-2 or FucT-3 activities, while tumors of B-lymphocytic origin, ABLS-140 and TEPC-15, both have reasonable levels of FucT-3 activities in addition to FucT-2. Substrate specificity studies with partially purified soluble FucT-3' from Colo 205(Basu,M. et al. (1988) Ind.J.Biochem.Biophys.,25,112-118) indicates that the enzyme has a very stringent requirement for sialylated-lactosamine(NeuAc or NeuGcα2-3Gal-GlcNAc) terminal-containing substrates and the Km values are reduced to one-fifth compared to those of neutral substrates. Whether sialylation precedes fucosylation in mouse lymphoreticular systems is under investigation.

Both LcOse4Cer(Galβ1-3R) and nLcOse4Cer(Galβ1-4R) are active substartes with Colo 205 FucT-3'. However,Colo 205 cell surfaces most probably contain only Type-1 chain-containing glycolipids. The enzymatic control of Type-1 and Type-2 chain-containing fuco-glycolipids in Colo 205 and mouse lymphoma is our present interest. (Supported by CA 33751 and CA 14764).

GLYCOLIPID BIOSYNTHESIS DURING DIFFERENTIATION OF NORMAL AND FUSION-DEFECTIVE CLONAL MUSCLE CELLS. Laurence D. Cambron, James E. Segal and Kenneth C. Leskawa. Department of Anatomical Sciences and Neurobiology, University of Louisville, Louisville, KY 40292

We have recently reported an extensive study of glycolipid biosynthesis during myogenesis of several different muscle cell types (Leskawa et al., Internat. Soc. Neurochem., 1987). Although all myoblasts exhibited changes in labeling patterns during differentiation, the exact nature of these changes varied with each cell type studied. That is, there was no universal increase or decrease in any one glycolipid structure which could be related to myoblast membrane fusion. The only consistent observation in this comparative study was a transient increase in the synthesis of total neutral glycolipids, gangliosides, or both, during myoblast contact and membrane fusion. These studies, however, were not aimed to discern whether transient increased glycolipid synthesis was involved in the process of membrane fusion or cell-cell recognition and contact.

To explore this, we have now examined glycolipid synthesis by two muscle cell lines which were cloned from the rat L8 myoblast line (kindly provided by S.J. Kaufman, Univ. Illinois): E63, which is a fusion-competent clone, and fu-1, which is a fusion-defective varient. Both clones were maintained in culture for 8 days, at which time over 80% of the E63 nuclei were contained within myotubes. Using [3H]-serine as a precursor, the biosynthesis of both neutral glycolipids and gangliosides was found to increase at day 5 in culture, when both cell types achieved contact and E63 myoblasts began fusing. Similar results were found when using [3H]-Gal as a precursor, although the total cellular incorporation was much lower.

The results suggest that increased glycolipid synthesis during myogenesis is important for cell-cell recognition and adhesion, but may not play a direct role in myoblast membrane fusion. Further studies of additional fusion-defective varients are needed. (Supported by the Medical School Research Committee, Univ. Louisville and NINCDS NS21057.)

SUBSTRATE SPECIFICITY AND DETERGENT REQUIREMENT OF LEECH CERAMIDE-GLYCANASE B. Zhou, Y.-T. Li and S.-C. Li. Department of Biochemistry, Tulane University School of Medicine, New Orleans, Louisiana 70112.

We have devised a simple method for the isolation of a highly purified ceramide-glycanase (CG) from a North American leech, Macrobdella decora. The method includes water extraction, acid precipitation, ammonium sulfate fractionation, octyl-Sepharose and Con A-Sepharose chromatographies. After Con A-Sepharose step, the enzyme was enriched 173-fold with 65% recovery.

The CG releases the intact glycan chain from various glycosphingolipids (GSL) in which the glycan chain is linked to the ceramide through the β-glucosyl linkage. However, DiGalCer was found to be refractory to the enzyme. This may indicate that the enzyme is not able to hydrolyze the glycan chain linked to the ceramide through the β-galactosyl linkage. The following table summarizes the substrate specificity of this enzyme.

GSL	Relative Activity	GSL	Relative Activity
GM_1	100	nLc_4Cer	38
Gg_3Cer	197	GM_3	35
Gg_4Cer	185	$LacCer^3II\text{-}SO_4$	34
GM_2	115	nLc_3Cer	31
Gb_3Cer	67	Gb_4Cer	27
Gb_5Cer	46	GT^4	22
GD_{1a}	41	LacCer	19

GM_2 was found to be the best substrate among various gangliosides, while Gg_3Cer was the best substrate among various neutral GSL tested. This enzyme also hydrolyzed deacyl GSL (lyso-GSL) such as lyso-GM_1 and lyso-LacCer. Alkyl lactosides such as octyl-lactoside and myristyl-lactoside were also hydrolyzed by the enzyme.

The CG requires the presence of a detergent to carry out its action. Among various bile salts tested, sodium cholate at a concentration of 0.5-1 μg/μl is most effective in stimulating the hydrolysis of various GSL except LacCer. For LacCer, sodium taurodeoxycholate at a concentration of 1-2 μg/μl is most effective. Tween-20, NP-40, Triton X-100 were found to inhibit the CG activity (supported by NSF DMB-8617033 and NIH NS 09626).

BIOSYNTHESIS AND TURNOVER OF A-ACTIVE GLYCOCONJUGATES IN A-431 CELLS. B. Virginia Torres and David F. Smith, Dept. of Biochemistry and Nutrition, Virginia Tech, Blacksburg, VA 24061.

Since the human epidermoid carcinoma cell line A-431 synthesizes blood group A-active glycolipids and glycoproteins, it represents a unique system to investigate the biosynthetic pathways of a single antigenic determinant found in two different cell surface glycoconjugates. Cells were metabolically labeled in separate experiments with ^{3}H-galactose and ^{3}H-glucosamine (60 µCi, 0.6 nM, in DMEM) for 4, 8, 12 and 24 hr. The Helix pomatia affinity chromatography system developed for the purification of glycolipids that carry terminal GalNAcα1-3 residues (Torres and Smith, Anal. Biochem. 170:209 1988) was used to purify the blood group A glycolipids from A-431 cells. A-431 glycopeptides with terminal GalNAcα1-3 residues were purified by classical affinity chromatography on immobilized Helix pomatia lectin. The rate of appearance of radiolabeled blood group A determinants in the A-431 cells was determined by measuring the radioactivity in the Helix pomatia affinity purified glycolipid and glycopeptide fractions as a function of time. Incorporation of the ^{3}H-monosaccharide precursors into both A-active glycoconjugates was similar and reached a steady state at 12 hr of labeling. The amount of ^{3}H-precursor incorporated in the A-active glycopeptides was twice that incorporated into the A-active glycolipids for both galactose and glucosamine. The turnover rates of A-active glycoconjugates was investigated. A-431 cells were incubated with ^{3}H-galactose (100 µCi, 1.3 nM) for 24 hr. The labeling medium was removed and the cells were chased with 1 mM galactose containing medium for 48 hr. The turnover rate of A-active glycoconjugates was estimated by measuring the radioactivity in the Helix pomatia affinity purified fractions as a function of time. ^{3}H-Galactose continued to be incorporated for 6 hr during the chase in the labeled A-active glycopeptides, followed by a decrease with a $t_{1/2}$ of approx. 12 hr. The labeled A-active glycolipids continued to accumulate ^{3}H-Gal for 12 hr, followed by a decrease with a $t_{1/2}$ similar to that of the glycopeptides.

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GLYCOLIPIDS IN MOUSE F9 TERATOCARCINOMA CELLS AND THEIR CHANGES ASSOCIATED WITH RETINOIC ACID INDUCED DIFFERENTIATION. Carlos M. Gorbea, Karen H. Tucker, and David F. Smith, Department of Biochemistry and Nutrition, Virginia Tech, Blacksburg, VA 24061.

Retinoic acid induced changes in F9 cell surface oligosaccharides, detected immunologically, include the loss of Forssman and SSEA-I. Immunological methods for oligosaccharide analysis are limited to the detection of structures for which antibodies are available and do not address the question of what metabolic changes lead to altered antigen expression. Metabolic labeling of cells with [^{3}H]monosaccharides permits the analysis of total glycoconjugates synthesized by cells during any time. We have analyzed metabolically labeled, glycolipid-derived oligosaccharides of F9 and retinoic acid treated F9 (RA/F9) cells. Labeled oligosaccharides were purified by lectin affinity chromatography and structurally characterized by exoglycosidase digestions and methylation analyses. We have demonstrated that the loss of the metabolically labeled Forssman oligosaccharide is associated with a decrease in Forssman synthetase activity in RA/F9 cells. [^{3}H]Oligosaccharides with molecular weights >5,000 accounted for approximately 20% of the [^{3}H]Gal-labeled neutral glycolipid-derived oligosaccharides in F9 cells, whereas, this fraction amounted to only 10% in RA/F9 cells. Analyses of these fractions on a column of immobilized GS-I lectin indicated a 2-fold increase in terminal α-Gal residues on glycolipids in the RA/F9 relative to the F9 cells. This observation is consistent with an increase in α-Gal transferase activity and associated 2-3 fold increase in the terminal α-Gal residues on N-linked oligosaccharides in RA/F9 relative to F9 cells (Cummings and Mattox, J.B.C. 263, 511). RA/F9 cells synthesized less higher molecular weight gangliosides than F9 controls. Whether the changes observed in the neutral and acidic glycolipids in RA/F9 cells are due to competitive reactions between the increased α-Gal transferase and other enzymes may be determined by complete structural analyses of the glycolipid-derived, metabolically labeled oligosaccharides.

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PARTIAL CHARACTERIZATION OF A CELL SURFACE PROTEIN WHICH MEDIATES THE MEMBRANE INCORPORATION OF EXOGENOUS GM1 GANGLIOSIDE. Kenneth C. Ieskawa. Department of Anatomical Sciences and Neurobiology, University of Louisville, Louisville, KY 40292

It is well established that exogenous ganglioside GM1 promotes the sprouting and extension of neurites, both in vitro and in vivo, and that a stable association with the membrane lipid bilayer is necessary. We have reported that the membrane incorporation of GM1 does not solely proceed by hydrophobic mechanisms, but is mediated by a membrane protein since: (1) protease treatment of neuro-2A cells results in a 90% inhibition of GM1 incorporation; (2) the process is pH-dependent; and (3) if protease-treated cells are resuspended in complete medium, they recover the ability to incorporate GM1 and this follows the synthesis of new cellular protein. Further, the protein-mediation of GM1 incorporation cannot be explained by the lytic release of cytosolic ganglioside transfer proteins (Ieskawa et al., Internat. Soc. Neurochem., 1987).

Recently, the specificity of this interaction was examined by analyzing the effect of possible competing molecules. Neuro-2A cells were exposed to [3H]-GM1 in the presence of various complex carbohydrates including GM1 oligosaccharide, sialyl-lactose, lactose and others. Surprisingly, none of these compounds inhibited GM1 incorporation. The most efficient inhibitor was the intact GM1 molecule. Other glycolipids (GalCer and GlcCer) at 10-fold equimolar concentration with respect to [3H]-GM1 also inhibited incorporation.

These results suggested that the protein-mediated membrane incorporation of GM1 may be due to artifactual adherence of serum proteins from the growth media. To test this possibility, cells were first incubated with antibodies to either whole bovine serum or anti-BSA prior to incubation with [3H]-GM1. Instead of inhibiting GM1 incorporation, this treatment resulted in an approx. 50% increase. It is concluded that serum proteins do coat the cell surface, but their removal results in further exposure of an intrinsic cell surface protein which is responsible for the membrane incorporation of GM1, but does not solely recognize the carbohydrate portion of GM1.

MEASUREMENT OF IN VITRO GLYCOSPHINGOLIPID SYNTHESIS AND DEGRADATION WITH CARBOHYDRATE-SEQUENCE SPECIFIC MONOCLONAL ANTIBODIES. Bruce A. Macher, Cheryl L.M. Stults and Bruce J. Wilbur, Department of Chemistry/Biochemistry, San Francisco State University, San Francisco, CA 94132.

A new method has been developed to monitor glycosyltransferase and glycosylhydrolase activities. Reaction product identification and quantification is accomplished simultaneously with an enzyme-linked immunosorbent assay (ELISA) using carbohydrate sequence-specific monoclonal antibodies. β-galactosyltransferase and α-galactosidase reactions were used to illustrate the salient features of the method. These include simple product identification and quantification, no detergent requirement, consumption of small amounts of reagents, and no use of radioisotopes. Furthermore, it is possible to measure substrate disappearance or product formation with this method. Enzyme characteristics such as K_m, V_{max}, divalent cation requirement, and pH optimum were investigated with this new method. These studies were supported by NIH grants CA 32826 and GM 40205.

CONTROL OF CELL PROLIFERATION BY GANGLIOSIDES AND THEIR DE-N-ACETYL AND LYSO-FORM DERIVATIVES. H. Nojiri, N. Hanai, G.A. Nores, T. Dohi and S. Hakomori. The Biomembrane Institute and University of Washington, Seattle, WA 98119.

We observed previously that platelet-derived growth factor (PDGF)-dependent 3T3 cell growth and epidermal growth factor (EGF)-dependent A431 cells as well as KB cells were inhibited by exogenous addition of GM_1 or GM_3, whereby tyrosine kinase activity of the PDGF or EGF receptor, respectively, was inhibited (1,2). The study has been extended in several directions:

(i) Employing A431 cell variants showing different growth responses to EGF, the receptor kinase (RK) activity was found to be greatly altered in association with the difference in GM_3 content between EGF-inhibited and -stimulated clones. GM_3 was found to be associated with the EGF receptor (3).

(ii) Inhibition of RK by GM_3 was observed clearly at low detergent concentration, whereas GM_3 enhanced RK activity at high detergent concentration. I.e., GM_3 showed a bimodal regulation of RK. In contrast, lyso-GM_3 showed a consistent inhibition of RK regardless of detergent concentration. Lyso-GM_3 was detectable in A431 cells in trace quantities.

(iii) De-N-acetyl-GM_3 ($II^3NeuNH_2LacCer$) greatly stimulated RK activity, and its presence was detected by 3T3 cells, A431 cells, melanoma cells, and human colonic carcinoma tissues as demonstrated by immunoblotting with DH5 antibody (directed to de-N-acetyl-GM_3).

(iv) In striking contrast, insulin RK activity was strongly inhibited by sialylparagloboside or sialyl 2→3lactonorhexaosylceramide, but was not inhibited by GM_3 nor by disialyl lactoisooctaosylceramide (disialyl I).

References: (1) Bremer E, et al. (1984) J.B.C. 259:6818-6825. (2) Bremer E, et al. (1986) J.B.C. 261:2434-2440. (3) Hanai N, et al. (1988) J.B.C. in press. (4) Hanai N, et al. (1988) J.B.C. 263:6296-6301.

SECRETION OF BLOOD GROUP P ACTIVE GLYCOLIPIDS BY THE HEP-G2 HUMAN HEPATOMA CELL LINE. SL Spitalnik, JM Danley, SR Burger, PF Spitalnik. Department of Pathology and Laboratory Medicine, Univ. of Pennsylvania, Philadelphia, PA 19104.

Blood group active glycolipids in the ABH, Lewis, Ii, and P systems are found not only on red blood cells (RBC), but also in soluble form coupled to plasma lipoproteins. The origin of these plasma antigens, and the mechanisms by which they may transfer onto RBCs, are virtually unknown. The Hep-G2 cell line was examined as a possible model system for addressing these questions. These cells secrete many plasma proteins including lipoproteins. Glycolipids were extracted from 10 ml of Hep-G2 cells. Individual glycolipids were purified by phase partition, ion-exchange chromatography, and HPLC. Pure glycolipids were characterized by determining sugar composition, exoglycosidase susceptibility, and binding to monoclonal antibodies. These cells were found to synthesize only glucosyl ceramide, lactosyl ceramide, ceramide trihexoside, lactosyl sulfatide, and gangliosides GM3 and GM1. Ceramide trihexoside is the blood group active P^k antigen. Hep-G2 cells were then grown in serum-free media; conditioned medium was collected and separated into lipoprotein and lipoprotein-deficient fractions. Protein secretion was linear in both fractions over 40 hours. Over 90% of the total protein was found in the lipoprotein-deficient fraction but 90% of the cholesterol, triglycerides, and glycolipids were found in the lipoprotein fraction. Ceramide trihexoside secretion increased linearly and was exclusively associated with lipoproteins. This should be a useful system for studying the mechanisms and regulation of blood group glycolipid secretion and transfer.

SPECIFICITY OF GANGLIOSIDE RECEPTORS ON RAT BRAIN MEMBRANES. Michael Tiemeyer and Ronald L. Schnaar, Depts. of Pharmacology and Neuroscience, Johns Hopkins School of Medicine, Baltimore, MD 21205

We have detected a specific binding activity for gangliosides on rat brain membranes using bovine serum albumin (BSA) covalently derivatized with gangliosides as probes (Soc. Neurosci. Abstr., 13, (1987), 1396). One probe (^{125}I-(G_{T1b})$_4$BSA), having 4 ganglioside G_{T1b} molecules covalently attached per BSA molecule, revealed high affinity binding (K_D=2-4 nM, B_{max}=13-20 pmol/mg protein) to membranes prepared from rat brain but not liver. As expected, non-radioiodinated (G_{T1b})$_4$BSA inhibited the binding of its radioactive counterpart with a K_I=3-4 nM. However, 50% inhibition by soluble G_{T1b}-oligosaccharide required 10^3 times higher concentrations suggesting the importance of polyvalency for high affinity binding. The role of valency was studied using (G_{T1b})$_2$BSA and (G_{T1b})$_1$BSA to inhibit ^{125}I-(G_{T1b})$_4$BSA binding. The inhibitory potency of these conjugates decreased linearly with the log of the valency (K_I for (G_{T1b})$_2$BSA=45 nM, K_I for (G_{T1b})$_1$BSA=81 nM). Thus, the divalent neoganglioprotein was 10-fold less potent and the monovalent ligand was 20-fold less potent than the tetravalent ligand. BSA derivatized with two G_{M1} molecules per protein molecule ((G_{M1})$_2$BSA) was less than one-half as potent (K_I=110 nM) as the G_{T1b} conjugate of the same valency ((G_{T1b})$_2$BSA).

The potency of gangliosides themselves to inhibit ^{125}I-(G_{T1b})$_4$BSA binding revealed oligosaccharide structural specificity in receptor recognition. Gangliosides containing the gangliotetraose core and at least one α2-8 linked NeuAc were the most potent lipid inhibitors (IC_{50}'s: G_{D1b}, 73nM; G_{Q1b}, 77nM; and G_{T1b}, 107nM). Other gangliosides were 5 to 20-fold less potent. Among other lipids tested as inhibitors (including neutral and charged glycolipids and phospholipids), only phosphatidylglycerol, sulfatide and phosphatidylinositol (PI) demonstrated appreciable potencies but all were at least 5-fold less potent than the most potent ganglioside inhibitors. Kinetic analysis of inhibition data obtained for G_{T1b} and PI demonstrated that G_{T1b} acted as a reversible and competitive inhibitor of ^{125}I-(G_{T1b})$_4$BSA binding while PI was not competitive. Thus, these ligands have proven a useful tool for the demonstration of a specific brain receptor for gangliosides. Supp. by NIH grants HD 14010 and MH18030 (to M.T.).

INHIBITION OF PROTEIN KINASE C BY THE LIPOPHOSPHOGLYCAN OF LEISHMANIA PARASITES. T. B. MCNEELY and S. J. TURCO. Department of Biochemistry, University of Kentucky, Lexington, Ky. 40536.

The major surface glycoconjugate of Leishmania donovani parasites is lipophosphoglycan (LPG). We have proposed that this molecule plays an important role in the intracellular survival of the Leishmania parasite, as it resides within phagolysosomes of human macrophage cells. Macrophage cells undergo an oxidative burst during phagocytosis, which is believed to be mediated by protein kinase C. Therefore, ability by L. donovani to inhibit protein kinase C, and hence reduce the oxidative burst, would mean greater survival rates by the parasite in this hostile environment. The ability of LPG to inhibit isolated protein kinase C was examined. Protein kinase C purified from rat brains was inhibited by LPG using concentrations as low as 0.1 μM. The LPG had no significant effect on protein kinases A or M. LPG was fragmented and four fragments examined for inhibitory activity. Of the four fragments examined, the LPG 1-O-alkylglycerol moiety contained the most inhibitory activity, although the phosphoglycan portion also exhibited substantial inhibition of the enzyme. When competition assays were done with 1-O-alkylglycerol versus diolein, the LPG lipid was found to decrease the Vmax of PKC and to increase the Km for histone. These results are consistent with the hypothesis that protein kinase C may be a key target for the parasites to overcome within host macrophages. The ability of LPG to inhibit the oxidative burst in human monocytes is currently being examined using chemiluminescence to monitor the oxidative burst.

INOSITOL PHOSPHATE-CONTAINING LIPIDS OF LEISHMANIA MEXICANA MEXICANA PROMASTIGOTES AND TRYPANOSOMA CRUZI EPIMASTIGOTES. B.N. SINGH, C.E. Costello*, D.H. Beach and G.G. Holz, Jr. Department of Microbiology & Immunology, SUNY Health Science Center, Syracuse, NY; *Department of Chemistry, Mass Spectrometry Facility, MIT, Cambridge, MA.

Leishmania species and Trypanosoma cruzi colonize human tissue cells and the guts of insect vectors. Lectin and chemical analyses of both parasites have revealed the presence of surface glycoconjugates which may play important roles in host-parasite interactions. We have used chromatographic and mass spectrometric (MS) techniques to examine glycolipids of cultured L. mexicana mexicana MHOM/BZ/79/Woods promastigotes and T. cruzi Peru epimastigotes; i.e., vector stages. The charged, unsaponifiable fraction of those lipids was enriched with inositol phosphate-containing components which were separated and purified by HPLC before being subjected to fast atom bombardment MS and MS/MS, in both the positive and negative ion modes. Ceramides linked to inositol phosphate were found in the L. m. mexicana extracts. The predominant component had the 16:0 base and the lesser component had the 16:1 base. In both, the N-acyl group was determined to be 18:0. The MS/MS spectra of these compounds both followed the same fragmentation patterns observed for cerebrosides (Domon and Costello, Biochem. 27, 1534, 1988), but showed an additional cleavage at the inositol-phosphate ester bond. Glycosphingolipids of this nature have been reported from L. donovani (Kaneshiro et al., J. Lipid Res. 27, 1294, 1986). MS/MS spectra of two other fractions showed that they contained mono-O-alkyl and di-O-alkyl glycerol inositol phosphates. The mono alkyl compounds had either a 17:1 or an 18:1 substituent; the dialkyl compounds had 20:0 and either a 17:0 or 18:0 substituent. T. cruzi Peru contained di-O-alkyl glycerol inositol phosphates in which the substituents were 20:0 and either 15:0 or 18:0. Lyso-alkylphosphatidyl inositols have been reported as components of the lipophosphoglycan of L. donovani (Orlandi and Turco, J. Biol. Chem. 262, 10384, 1987). Preliminary chromatographic and radioisotopic analyses of two other T. cruzi components suggest that they are similar to the ceramide and mono-O-alkyl glycerol inositol phosphates of Leishmania.

Glycoproteins

EFFECT OF Ca^{++} ON THE LIGHT SCATTERING PROPERTIES OF PORCINE SUBMAXILLARY MUCIN GLYCOPROTEINS, B. K. Varma, A. Demers, A. M. Jamieson, J. Blackwell and N. Jentoft, Departments of Macromolecular Science and Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106.

Static and dynamic light scattering experiments have been carried out on solutions of porcine submaxillary mucin glycoprotein fractions to generate information on molecular weight, M_w, radius of gyration, R_g, hydrodynamic radius, R_h, and the longest relaxation time for configurational dynamics, τ_1. Addition of Ca^{++} to these solutions has no significant effect on M_w, but causes substantial decreases in R_g, R_h, and τ_1. This suggests that Ca^{++} does not change the state of aggregation of the mucin glycoprotein, but does cause a transition to a more compact macromolecular configuration. These observations may be relevant to the packaging of mucin in the secretary granules which contain high levels of Ca^{++}.

MUCUS GLYCOPROTEINS OF HUMAN ORAL MUCOSAL MUCUS COAT:DIFFERENCES IN PHYSICOCHEMICAL CHARACTERISTICS WITH CARIES. B.L. Slomiany, G. Zalesna, I.D. Mandel and A. Slomiany. Dent. Res. Ctr., NJ Dental School, UMDNJ, Newark, NJ 07103 and Ctr. Clin. Res., School of Dental and Oral Surgery, Columbia Univ., New York, NY 10032.

Oral mucosal mucus coat was isolated from epithelial surfaces of caries-resistant (CR) and caries-susceptible (CS) subjects, analyzed for the content and composition of lipids and mucus glycoproteins, and the physicochemical characteristics were evaluated. The mucus coat from CR subjects, while exhibiting protein content similar to that of CS group, displayed a higher content of carbohydrate and was lower in lipids and covalently bound fatty acid. The carbohydrate component was represented mainly by mucus glycoprotein which accounted for 28.4% of the dry weight of CR mucus and 25.3% of the dry weight of CS mucus. Both types of preparations showed the presence of high (Mr 2000kDa) and low (Mr 300kDa) molecular weight mucus glycoproteins. In CS mucus coat these two glycoproteins were present essentially in similar proportions, while the low molecular weight glycoprotein form predominated in CR mucus coat. The high molecular weight glycoprotein of CR group exhibited 60% lower content of covalently bound fatty acid, 22% lower content of associated lipids and contained 24% more sulfate and 23% more sialic acid than that of CS. The results of physical measurements revealed that the mucus coat of CR individuals differed from that of CS subjects with respect to viscosity, permeability and hydrophobicity. The CR mucus coat in comparison to that of CS coat displayed 26% lower viscosity, showed 25% reduction in glucose diffusion retardation capacity, and exhibited lower binding affinity for fluorescent hydrophobic probe. (Supported by USPHS, NIH Grant DE#05666-09.)

SALIVARY EPIDERMAL GROWTH FACTOR MAINTAINS THE PHYSICOCHEMICAL CHARACTERISTICS OF ORAL AND GASTRIC MUCUS COAT MUCIN. B.L. Slomiany, J. Sarosiek, V.L.N. Murty and A. Slomiany. Dental Res. Cent., NJ Dental School, UMDNJ, Newark, NJ 07103-2425.

The involvement of salivary epidermal growth factor (EGF) in the maintenance of oral and gastric mucosal mucus coat dimension and chemical characteristics was investigated using sialoadenectomized rats. Examination of the oral and gastric mucosal surface by phase contrast microscopy and Alcian blue uptake revealed that depravation of salivary EGF caused 31-36% reduction in mucus coat thickness and 38-43% reduction in adherent mucin content. Chemical analyses indicated that the mucus coat of sialoadenectomized group exhibited 21-28% increase in protein and 67% decrease in covalently bound fatty acids, 30% decrease in carbohydrates, and 32-37% decrease in lipids. Sialoadenectomy also evoked changes in the chemical composition of mucus glycoprotein component of oral and gastric mucus coat which were reflected in the lower content of sulfate (25-26%), associated lipids (24-25%), and covalently bound fatty acids (67-75%). Furthermore, the mucus coat from both regions contained consistently higher proportion of mucin in the low molecular weight form. The gastric mucus glycoprotein of sialoadenectomized animals contained 38% high molecular weight form, while in the oral mucus glycoprotein this form accounted for 35%. Intragastric supplementation of EGF had no effect on the physicochemical changes caused by sialoadenectomy in the oral mucosal mucus coat, but nearly complete restoration to normal characteristics occurred in the gastric mucosal mucus coat. The results suggest that salivary EGF is essential for the maintenance of mucus coat dimension and the quality of mucin needed in the protection of alimentary tract epithelium. (Supported by USPHS, NIH Grants DE#05666-09, DK#21684-12 and HL#32553-04.)

RHEOLOGIC PROPERTIES OF INTESTINAL MUCIN FROM NORMAL SUBJECTS AND PATIENTS WITH CYSTIC FIBROSIS. M. MANTLE[1], G. Stewart [1], G. Zayas[2], and M. King[2], Gastro-intestinal Research Group[1], University of Calgary & Pulmonary Defense Group[2], University of Alberta, Edmonton, Alberta, Canada.

Cystic fibrosis (CF) is characterised by thick, tenacious mucus secretions that lead to many of the respiratory, gastro-intestinal and cervical tract problems associated withthe disease. In this study, we compared the rheologic properties of purified intestinal mucin (the gel-forming glycoprotein component of mucus) from normal (N; n=7) subjects and patients with CF (n=6). Methods: Mucin was purified in the presence of proteolytic inhibitors from mucosal scrapings by equilibrium centrifugation in CsCl (twice) and gel filtration on Sepharose 2B. Polymeric mucin was harvested from the void volume fractions of the column. Gels were reconstituted in phosphate-buffered Ringers, pH 7.0, from lyophilised mucin (concn range 8-25 mg/ml) and the visco-elastic properties determined in a magnetic microrheometer (frequency range 1-100 rad/sec). G^* (dyn/cm^2) the mechanical impedance of the gel, is the stress/strain ratio, equal to the vectorial sum of elasticity and viscosity; tan δ, the loss tangent, is the ratio of viscosity/elasticity. High values of G^* with low values of tan δ are associated with 'rigid' gels. Results: One N and two CF mucins did not form gels in the concn range studied. Of the remaining N and CF mucin gels, G^* showed little concn dependence and little frequency dependence over the ranges studied. Wide variations were seen in the actual values of G^* and tan δ for individual mucins but no differences were detected between CF and N samples which were both capable of forming rigid gels.

	log G^* at 1 rad/sec		tan δ at 1 rad/sec		
CF mucin gels	2.7	(2.3-3.0)	0.18	(0.15-0.22)	values are
N mucin gels	2.3	(1.8-2.9)	0.20	(0.15-0.33)	mean (range)

Conclusions: We were unable to demonstrate a significant abnormality in the rheologic properties of gels re-constituted from purified CF mucins. Since unfractionated CF and N sputum also do not differ rheologically (King, M. (1981) Pediatr. Res. 15: 120-122), it seems likely that hypersecretion and diminished clearance account for the mucus abnormality in CF.
Supported by the Canadian Cystic Fibrosis Foundation.

THE PRESENCE OF THROMBOSPONDIN IN LIGAMENT, MENISCUS AND INTERVERTEBRAL DISC. R.R. Miller and C.A. McDevitt. Department of Musculoskeletal Research. Cleveland Clinic Foundation Research Institute, Cleveland, Ohio 44195

Thrombospondin, an adhesive glycoprotein originally identified as a constituent of platelet alpha granules, is synthesized and incorporated into the extracellular matrix by a number of cell types in vitro. The wide distribution of thrombospondin suggests that this molecule may be an important constituent of extracellular matrices. While indirect immunofluorescence studies of human tissue have suggested that thrombospondin may be a matrix component of some connective tissues, no studies to date have established this fact. We have recently demonstrated the presence of thrombospondin in articular cartilage (1). The purpose of this study was to determine if thrombospondin was present in other connective tissues such as ligament, meniscus and intervertebral disc. Using a monoclonal antibody to human platelet thrombospondin and Western blot analysis, extracts of meniscus, ligament and intervertebral disc gave a single band of immunoreactive material which co-migrated with purified thrombospondin. In addition, when ligament fibroblasts were cultured in the presence of radioactive amino acids, metabolically labeled thrombospondin was immunoprecipitated from the culture medium and cell layer extracts.

1. Miller, R.R. and McDevitt, C.A. (1988) Biochem. Biophys. Res. Commun. 153:708-714

IDENTIFICATION OF THE VACUOLAR TARGETING DOMAIN OF THE GLYCOPROTEIN PHYTOHEMAGGLUTININ, USING TRANSGENIC TOBACCO AND YEAST. M.J. CHRISPEELS, T. Voelker, B. Tague, C. Dorel and A. Sturm. Department of Biology, University of California, San Diego, La Jolla, CA 92093.

Phytohemagglutinin (PHA) is a vacuolar glycoprotein that accumulates to high levels in the protein storage vacuoles of developing bean (*Phaseolus vulgaris*) embryos. We isolated the gene (dlec2) for PHA-L and used *Agrobacterium*-mediated transformation of tobacco to study its expression and targeting. When the gene is expressed in tobacco, PHA-L accumulates in the protein-storage vacuoles of tobacco seeds, indicating organ-specific expression and correct targeting. Site-directed mutagenesis to remove the two glycosylation sites resulted in the accumulation of unglycosylated PHA-L in the protein-storage vacuoles, indicating that the glycans have no targeting information. We constructed a chimeric gene consisting of the promoter and signal peptide of PHA, and the coding sequence of a cytosolic storage protein. Expression of this chimera in tobacco resulted in the biosynthesis, glycosylation and Golgi-modification of a glycoprotein that was not transported to the vacuoles (and was most likely secreted into the periplasmic space). These experiments show that targeting to vacuoles requires a polypeptide domain needed for sorting.

Yeast was used to identify this domain. When a chimeric gene, consisting of the phosphatase promoter and the dlec2 coding sequence was introduced into yeast, PHA-L was synthesized and transported to the vacuoles of the yeast cells. Deletions from the C-terminus of PHA-L were fused with invertase to identify the domain of PHA-L which is involved in its correct targeting. We have identified a stretch of 20 amino acids close to the amino-terminus of the protein which contains the proposed targeting domain. When this domain is absent from the chimeric gene, invertase is secreted by the yeast cells. When it is present, invertase is transported to the vacuole.

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EFFECT OF N–LINKED OLIGOSACCHARIDES ON THE OLIGOMERIC STRUCTURE OF YEAST INVERTASE. V. Anthony Reddy, Robert MacColl and Frank Maley. Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201.

External invertase subunit contains 14 sequons out of which eight are fully glycosylated and five partially glycosylated to give an average of 9–10 oligosaccharides (CHO) per subunit. Endo H treatment removes about 80% of the CHO leaving the remaining CHO attached at five specific sequons (Reddy, V. A. et. al. (1988) J. Biol. Chem. 263, 6978–6986). External and Endo H–treated invertases elute on size–exclusion HPLC as an equilibrium mixture of octamer–hexamer, tetramer and dimer. Internal invertase which lacks N–linked CHO elutes as a dimer. However, at 25 mM Cl^- internal invertase forms higher oligomers, an effect that does not occur with external and Endo H–treated invertases until 150 mM and 75 mM Cl^-, respectively, is reached. Chloride ion at 0.8 M concentration promotes maximum octamer–hexamer formation of all the invertases. At 47°C the oligomers of external invertase dissociate into dimers; but 0.8 M Cl^- protects against this thermal dissociation. In comparison the oligomeric equilibrium of Endo H–treated invertase remains unaffected at 47°C, indicating that intersubunit binding is stronger in the Endo H–treated enzyme.

At pH 11.5 both external and internal invertases exist as monomers. Lowering the pH to 5.0 results in the formation of fully active internal invertase dimer, or in the case of external invertase a mixture of dimer and tetramer. Attempts to obtain heterodimer by incubation of external invertase with labelled internal invertase at pH 11.5 followed by pH–jump to 5.0 were not successful, indicating that binding between glycosylated and unglycosylated monomers may be restricted. However incubation of an oligomeric mixture of external invertase with labelled internal invertase dimer in the presence of 0.5 M Cl^- results in the formation of heterotetramer in addition to other oligomers signifying that CHO may not interfere with dimer–dimer interaction. These findings suggest that the CHO attached to invertase influences its oligomeric state. (Supported in part by a grant from NIH CA44355)

DEGLYCOSYLATION OF HUMAN COLONIC MUCIN. S.N. Bhattacharyya, J.I. Enriquez, J. S. Ramirez, and B. Manna. Departments of Clinical Investigation and Surgery, William Beaumont Army Medical Center, El Paso, Texas 79920-5001.

Human colonic mucin has been isolated from colonic mucosa. The solubilized material was subjected to Sepharose 2B column chromatography and the mucin appeared at the void volume. This preparation was further purified by cesium bromide density gradient centrifugation. Sodium dodecyl sulfate-polyacrylamide gel (5%) electrophoresis indicated high molecular weight component(s) at the top of the gel. Chemical analyses of this preparation showed a typical mucin profile of amino acids; i.e., high levels of threonine, serine, and proline and carbohydrates containing sialic acid, fucose, galactose, N-acetylglucosamine, and N-acetylgalactosamine. Chemical deglycosylation of the purified mucin preparation with trifluoromethane sulfonic acid was carried out at 20°C for different periods of time. Sialic acid, fucose, and galactose were completely removed, whereas traces of N-acetylglucosamine and N-acetylgalactosamine were detected even after 4h of hydrolysis. The reaction products were analyzed by high pressure liquid chromatography (HPLC). Six peaks were detected, of which one peak (P_1) was the most prominant. The P_1 peptide fraction was further purified by HPLC in a second solvent system. Gel filtration of the peptide in 6M guanidine hydrochloride indicated a molecular weight of about 97KDa. The amino acid profile of this peptide was dominated by high levels of threonine, serine, and proline, which combined accounted for nearly 32% of the total residues, and in most respects, the profile resembled that of native mucin. NH_2-terminal amino acid analyses, both native mucin and peptide P_1, by dansyl procedure showed a blocked N-terminus. The size and chemical compositions of this peptide are similar to a deglycosylated peptide isolated recently in our laboratory from respiratory mucin.

NOVEL HYPERGLYCOSYLATED FIBRONECTIN FROM HUMAN FETAL PLACENTA

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A previously undiscovered hyperglycosylated fraction of human term fetal placental fibronectin was found by 12 hour affinity binding to gelatin-Sepharose. This fraction is characterized by a very high N-acetylglucosamine and galactose content, diffuse, poorly Coomassie-stained streaked bands on SDS polyacrylamide gel electrophoresis and a high proportion of long polylactosamine-containing N-linked carbohydrate chains isolatable by protease digestion or hydrazinolysis of the fibronectin. Since a major fraction of this new type of fibronectin did not bind to gelatin-Sepharose in a 2-hour incubation, a lower binding affinity of the high carbohydrate form was indicated. Our previous results showed that middle length polylactosamine glycosylation mitigates the binding of fibronectin to gelatin (Zhu, B.C.R. and Laine R.A.,(1985) J.Biol. Chem,260, 4041-4045). Based on a gradient of urea used to remove fibronectin from the gelatin-Sepharose, the novel high carbohydrate fraction was bound even more weakly to gelatin. Since the standard first stage of purification for fibronectin is based on affinity to gelatin, and because the novel fraction stains poorly with Coomassie, this high carbohydrate variety of fibronectin may have been overlooked in previous work. Tomato lectin affinity chromatography was used to purify the chymotryptic gelatin binding domain (GB44) which contained the higher molecular weight carbohydrate. This report gives details of the isolation and electrophoretic properties of the novel fraction and shows that tomato lectin affinity chromatography can be used to fractionate polylactosamine chains.

REGULATION OF SYNTHESIS OF DOL-PP OLIGOSACCHARIDES. J.W. Rip, J.A. Williams and K.K. Carroll. Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1.

Germinating soybean embryos synthesize considerable quantities of N-linked glycoprotein even though their Dol-P dependent N-acetyl glucosaminyl transferase activity is very low. This suggests that Dol-PP-oligosaccharide assembly may be regulated at the level of Dol-PP-GlcNAc formation and not by the availability of free Dol-P. To test this possibility, sub-saturating (approximately physiological) amounts of [1-^{14}C] dolichol were supplied *in vitro* to soybean microsomes to enable formation of [1-^{14}C] Dol-P. The microsomes contain the phosphate donor for the kinase reaction in saturating quantities. By including saturating amounts of non-radiolabeled UDP-GlcNAc, GDP-Man, and UDP-Glc individually or in combination in the assay the extent to which newly formed rate-limiting quantities of [1-^{14}C] Dol-P are directed into [1-^{14}C] Dol-PP-GlcNAc, [1-^{14}C] Dol-PP-$(GlcNAc)_2$, [1-^{14}C] Dol-P-Man and [1-^{14}C] Dol-P-Glc could be followed. The presence of UDP-GlcNAc did not appreciably decrease the rate of accumulation of free [1-^{14}C] Dol-P, whereas GDP-Man and UDP-Glc reduced the free [1-^{14}C] Dol-P content of assays by 25 and 50% respectively. This decrease in free Dol-P was accounted for entirely by the appearance of [1-^{14}C] Dol-P-Man or [1-^{14}C] Dol-P-Glc. Even in the presence of all three nucleoside diphosphate sugars, some 40-50% of the total [1-^{14}C] Dol-P remained in the free phosphate form. This observation, and the low level of GlcNAc transferase activity detected in microsomes (when all substrates are saturating) suggests that these enzymes have a regulatory role in N-glycosylation. (Supported by the Medical Research Council of Canada.)

THE SIGNIFICANCE OF POLYPRENOL STRUCTURE FOR PROTEIN GLYCOSYLATION VIA THE DOLICHOL PATHWAY.

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In order to determine the influence of polyprenol structure on the glycosyl acceptor properties of synthetic lipid intermediates, the following compounds were synthesized: G-PP-polyprenol, G_2-PP-polyprenol, M_2G_2-PP-polyprenol; where G is GlcNAc, G_2 di-N-acetylchitobiose, M_2 is α-D-Manp-(1->6)-β-D-Manp-, and polyprenol refers to R or S Dolichol, or perhydrodolichol. The compounds were incubated with UDP-[^{14}C]GlcNAc or GDP-[^{14}C]mannose and calf pancreas microsomes. Also studied were the effects of R/S stereoisomerism and saturation on the efficiency of transfer of GlcNAc from UDP-GlcNAc to polyprenyl phosphates to yield GlcNAc-PP-Dol. In all cases the same compounds were formed irrespective of polyprenol structure. Under any given condition the nature of the polyprenol strongly affected the transfer efficiency to G-PP-polyprenol (S > R > perhydro). For the elongation of M_2G_2-PP-polyprenol, the influence of polyprenol structure diminished as the length of the glycan chain increased. Intermediate results were obtained with G_2-PP-polyprenol. Therefore, the glycosyltransferases which catalyze the above reactions discriminate strongly between acceptors with different polyprenol structures when the catalytic site is proximal to the polyprenol residue. These results are consistent with a model in which an essential step for glycosyl transfer is the formation of a complex between the enzyme, glycosyl acceptor, and phospholipid components of the microsomal membranes. [Supported by N.S.F. grant DMB 86-45896, and N.I.H. grant AM-03564].

Glycoprotein Processing in Plants. G. P. Kaushal and Alan D. Elbein University of Texas Health Science Center, San Antonio, Texas.

The biosynthesis of the oligosaccharide chains of plant N-linked glycoproteins involves a series of lipid-mediated reactions resulting in the formation of a $Glc_3Man_9(GlcNAc)_2$-pyrophosphoryl-dolichol which then donates the oligosaccharide to the growing polypeptide chain. Once this oligosaccharide has been added to the appropriate asparagine residues, the oligosaccharide undergoes a series of processing reactions which may remove certain sugars whereas other sugars may be added. We have purified glucosidase I and glucosidase II from mung bean seedlings and have studied the properties of these two enzymes. Glucosidase I removes the outermost α1,2-linked glucose from $Glc_3Man_9(GlcNAc)_2$ to give a $Glc_2Man_9(GlcNAc)_2$ structure. Glucosidase II removes the next two α1,3-linked glucoses from the $Glc_2Man_9(GlcNAc)_2$ to give a $Man_9(GlcNAc)_2$ oligosaccharide. Both of these enzymes are inhibited to about the same extent by castanospermine but 2,6-dideoxy-2,6-imino-7-O-(β-D-glucopyranosyl)-D-glycero-L-guloheptitol was a better inhibitor of glucosidase II than of glucosidase I. Mung beans also contain a number of α-mannosidases and at least two of these enzymes appear to be involved in processing the high-mannose glycoproteins. Thus, we have purified a mannosidase that appears to be specific for α1,2-linked mannose residues (mannosidase I) and a mannosidase that removes the α1,3and α1,6-linked mannoses from the $GlcNAc\text{-}Man_5(GlcNAc)_2$ structure (mannosidase II). These enzymes are similar to the animal enzymes except in their responses to some glycoprotein processing inhibitors. These activities are also present in suspension-cultured soybean cells. Polyclonal antibody prepared against glucosidase II cross reacts with a 109 KD protein from soybean cells. The biosynthesis and characterization of this protein is underway.

THE EFFECT OF DENERVATION ON ENZYMES IN THE DOLICHOL-LINKED PROTEIN GLYCOSYLATION PATHWAY. Roy W. Keenan, Robert Mueck and Takafumi Itami. University of Texas Health Science Center at San Antonio.

It is well documented that denervation results in a phenomenon called "denervation supersensitivity" in which large quantities of acetylcholine receptors and other glycoproteins are produced in skeletal muscle in response to nerve lesion. Assays were carried out to determine if this condition would affect enzymes participating in the formation of oligosaccharide by the dolichol-linked pathway.

A section of the sciatic nerve was removed from the upper thigh of young adult rats, and at various times 3 to 13 days, sarcoplasmic reticulum fractions were prepared from the denervated muscle and assayed for dolichol kinase and GDP-Man:DolP mannosyl transferase. Tissue from the contralateral leg served as a control. The specific activities of both of these enzymes was lower in the early time periods relative to the controls, but approached normal values after about two weeks following denervation. The conclusion was, that despite an increase in the production of certain types of glycoproteins, there was not a corresponding increase in these enzymes in the glycosylation pathway. (Supported by grant AQ 1109 from the Robert A. Welch Foundation)

TOPOGRAPHY OF GlcNAc-LIPID BIOSYNTHESIS: BASAL VS ACTIVATED ACTIVITIES. Edward L. Kean, Case Western Reserve University, Cleveland, Ohio 44106.

Previous studies from this laboratory have described the stimulation of the biosynthesis of GlcNAc-P-P-dolichol and $(GlcNAc)_2$-P-P-dolichol by dolichol-P-mannose and phosphatidylglycerol (Kean (1985) J.B.C., *260*, 12561-12571) using microsomes from the embryonic chick retina and other tissues. Questions surround the assignments of orientation in the microsomes of the NAc-glucosaminyl transferases which carry out the synthesis of these intermediates of the dolichol pathway. We have examined the orientation of both the basal and stimulated reactions in microsomes isolated from the livers of the 15-16 day embryonic chick. We have used the classical approach of following the susceptibility to inactivation by proteolytic enzymes to examine this problem. The integrity and native orientation of the microsomes were evaluated by measuring the latency of mannose-6-phosphatase, which in all of these studies was greater than 89%. Identification of products involved solvent partitioning of the products of the reactions, DEAE-cellulose chromatography, mild acid hydrolysis, and paper chromatography. Under all conditions, when examined under basal or stimulatory conditions, proteolysis of the intact microsomes resulted in the inhibition (80%-98%) of the formation of the chitobiosyl-lipid. The effect on the formation of GlcNAc-P-P-dolichol, however, varied with different preparations of trypsin and pronase (two preparations of each were examined): Trypsin- 0% vs 48%; Pronase- 0% vs 97%. These findings support the assignment of cytoplasmically oriented domains which influence the synthesis of $(GlcNAc)_2$-P-P-dolichol and its allosteric stimulation. We suggest that the variation in effect seen on the formation of GlcNAc-P-P-dolichol reflects the sequestration of the transferase within the bilayer which some preparations of proteolytic enzymes can penetrate, while still not disrupting the microsome as indicated by the retention of the luminal orientation of mannose-6-phosphatase. Supported in part by EY00393 and EY03685.

DEHYDRODOLICHYL DIPHOSPHATE SYNTHASE IN SPERMATOGENIC AND SERTOLI CELLS OF PREPUBERAL RATS. Zhong Chen, Charles M. Allen, and Lynn J. Romrell, Departments of Biochemistry and Anatomy, University of Florida, Gainesville, FL 32610, USA

It is proposed that the level of dolichyl phosphate (Dol P), a carbohydrate carrier in glycoprotein biosynthesis, is regulated during spermatogenesis. Temporal expression of seminiferous tubular dehydrodolichyl diphosphate synthase has been shown previously to correlate well with the increase in Dol P during early stages of differentiation (7 to 23 days). The cellular localization of this increased synthase activity was of interest because of the multicellular nature of the tubule. The specific activity of synthase in homogenates of protease treated seminiferous tubules, cell fractions enriched in spermatogenic cells or Sertoli cells peaked in rats aged 23 days, as shown with non-protease treated cells. Homogenates of cell fractions enriched in pachytene spermatocytes, spermatids or Sertoli cells had higher synthase activity than a whole testicular homogenate or a mixture of cells prepared by protease treatment of tubules. Enzymatic activity in pachytene spermatocytes expressed per mg protein, was about 1.7 fold higher than in spermatids, about 8.3 fold higher than in spermatozoa and at least 5.3 fold higher than spermatogonia. The increase in germ cell synthase before day 23 can be accounted for by the appearance of the pachytene spermatocytes. Little net increase in enzyme occurred during or after meiotic cell division of pachytene spermatocytes into spermatids. Enzymatic activity decreased remarkably after the differentiation of spermatids into spermatozoa. Enzymatic activity in the enriched Sertoli cells was 1.5 to 1.7 fold higher than in the enriched germ cells. The increase in synthase activity in germ cells and Sertoli cells indicates that both are contributing to changes in the enzymatic activity in seminiferous tubules. This increase may be important in regulating the availability of Dol P for glycoprotein synthesis during early stages of differentiation. (Supported in part by HD 18509)

EFFECT OF TUNICAMYCIN ON SIALOMUCIN AND NK RESISTANCE OF RAT MAMMARY TUMOR ASCITES CELLS. A.P. SHERBLOM, S. Bharathan and C.E. Moody. Department of Biochemistry, University of Maine, Orono, ME 04469.

Cell surface sialomucins have been proposed to "mask" tumor antigens, preventing destruction of certain tumors by the immune system of the host. The NK-(natural killer cell)-resistant 13762 rat mammary tumor ascites cell lines (MAT-B1 and MAT-C1) contain two major cell surface glycoproteins: ASGP-1, a 600-700 kd sialomucin; and ASGP-2, a 120 kd glycoprotein which binds Con A. Previous work has shown that detergent extracts of ascites cell membranes contain a complex of ASGP-1 and ASGP-2 (Sherblom and Carraway, JBC 255, 12051-12059, 1980). The ascites cells can be made susceptible to NK cytolysis by treatments (i.e. trypsin, or long-term culture) which result in the loss of ASGP-1 (Sherblom and Moody, Cancer Res. 46, 4543-4546, 1986). In the current study, we have examined the effect of culturing MAT-B1 and MAT-C1 sublines with tunicamycin, which blocks N-linked (ASGP-2) but not O-linked (ASGP-1) glycosylation. Susceptibility of ascites cells to cytolysis by NK cells increased relative to controls following a 24 h incubation with tunicamycin, and optimal killing (35% in an 18 h assay) was observed at a drug concentration of 5 μg/ml. Under these conditions, tunicamycin was not directly toxic, since viability ranged 88-98% in the treated cells. Synthesis of glycosylated ASGP-1 and ASGP-2 was monitored by ^{14}C-glucosamine labeling and SDS PAGE of labeled cells. The ratio of dpm recovered in ASGP-2/ASGP-1 ranged between 0.4-0.6 for cells cultured in the absence of tunicamycin. Treatment with 5 μg/ml tunicamycin completely inhibited glycosylation of ASGP-2 and resulted in a 60% reduction in labeled ASGP-1 (dpm/μg protein) for both cell lines. Potentially both ASGP-2 and ASGP-1 are required for NK resistance, since glycosylated ASGP-2 may be critical for stabilizing ASGP-1 at the cell surface.

SUBSTRATE .SPECIFICITIES OF GLYCOSYLTRANSFERASES THAT MODIFY PLANT GLYCOPROTEINS. K.D. JOHNSON and M.J. Chrispeels. Department of Biology, University of California, San Diego, La Jolla, CA 92093.

Structural studies on a variety of plant glycoproteins indicate that plants can process high-mannose, N-linked oligosaccharides to complex, biantennary sidechains containing peripheral N-acetylglucosamine, and core fucose and xylose residues. In an effort to understand these carbohydrate processing events in plants, we have characterized enzyme activities corresponding to GlcNAc transferase, a core fucosyltransferase, and a xylosyltransferase from a Golgi-enriched membrane fraction of *Phaseolus vulgaris* cotyledons. Each glycosyltransferase has a pH optimum of 6.3, and each cofractionates with Golgi markers on a linear sucrose gradient. On the basis of acceptor specificities and product analyses, we have determined that the initial steps leading toward complex oligosaccharide formation in beans is similar to the pathway described for mammalian systems. Bean cotyledons have GlcNAc transferase activity, which adds a GlcNAc to $Man_5(GlcNAc)_2Asn$. The product of this reaction can be further processed by the Golgi membranes to $(GlcNAc)_2Man_3$-$(GlcNAc)_2Asn$, implying the presence of α-mannosidase II and GlcNAc transferase II activities. Fucosyltransferase transfers a fucose to the proximal GlcNAc residue of $GlcNAcMan_5$-, $GlcNAcMan_3$-, or $(GlcNAc)_2Man_3(GlcNAc)_2Asn$, while the xylosyltransferase exhibits significant activity toward only the latter two substrates. Thus, the addition of fucose and xylose to plant N-glycans requires the prior action of GlcNAc transferase I and, in the case of xylose, α-mannosidase II.

CHROMATIN GLYCOSYLATION: EVIDENCE FOR O-GlcNAc MODIFICATION OF CHROMOSOMAL PROTEINS

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A novel form of peptide glycosylation was recently described by our lab in which single N-acetylglucosamine residues are linked via an O-linkage (O-GlcNAc) to either serine or threonine residues of proteins (Torres and Hart, J. Biol. Chem., 259:3308). Further investigations on this type of protein modification have revealed that, in contrast to all other previously observed forms of glycosylation, proteins containing O-GlcNAc reside predominantly in either the cytoplasm or nuclei of cells (Holt and Hart, J. Biol. Chem., 261:8049; Holt et al., J. Cell Biol., 104:1157). A membrane-bound, cytoplasmically-oriented glycosyltransferase responsible for this modification has been identified by our lab (see Haltiwanger, et al. abstract). We have further sought to identify what types of proteins contain O-GlcNac in order to try to elucidate the nature of its function. Here we report the existence of O-GlcNAc residues on proteins associated with purified *Drosophila* chromatin. Labelling of polytene chromosomes with either Wheat Germ Agglutinin or ^{3}H-galactose (via galactosyltransferase) results in an *in situ* labelling pattern of the chromosomes, visualized by fluorescence microscopy and emulsion autoradiography, respectively, that is similar to aceto-orcein staining. Galactosyltransferase-labelling of purified *Drosophila* chromatin results in the incorporation of high levels of ^{3}H-galactose into protein. This incorporation is only dissociated by beta-elimination of the protein, and the released carbohydrate migrates as galactose-beta 1,4- GlcNAcitol dissacharide on a P-4 column, suggesting the existence of O-GlcNAc on purified chromatin. We are currently in the process of identifying the chromosomal proteins containing this modification.

BIOSYNTHESIS OF XYLOGLUCAN. GORDON MACLACHLAN. Department of Biology, McGill University, Montreal, Quebec, CANADA H3A 1B1.

Xyloglucan is present in growing regions of all dicot plants at concentrations comparable to cellulose, to which it is tightly adsorbed by hydrogen bonding by virtue of its 1,4-B-glucan backbone. It is hydrolysed at unsubstituted glc units by endo-1,4-B-glucanase to equal amounts of two oligosaccharides. Glc_4Xyl_3 and $Glc_4Xyl_3GalFuc$ appear to alternate in authentic dicot xyloglucan in a very regular sequence. Its biosynthesis from any constituent sugar can be detected unambiguously by identifying these repeating subunits amongst the hydrolysis products, both of which hydrolyse further to products including isoprimeverose (1,6-α-xylosylglucose). Golgi membranes from pea epicotyls contain xylosyl and fucosyl transferases which will decorate endogenous nascent xyloglucan chains or exogenously-supplied xyloglucan, even in the absence of chain elongation. However, the incorporation of glc from UDP- or GDP-glc into xyloglucan precursors continues for only a few glc units unless UDP-xyl is also present, in which event both sugars are incorporated to generate the complete xyloglucan backbone with DP up to 1000.

PROTEOLYTIC PROCESSING OF LYSOSOMAL ENZYMES.
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Virtually all soluble lysosomal enzymes are synthesized as propolypeptides which are subject to a number of post-synthetic modifications, including proteolytic processing; the biological role of proteolytic processing remains somewhat obscure. The proteolytic processing of a number of lysosomal enzymes including N-acetyl-β-hexosaminidase (Hex), α-fucosidase (Fuc), cathepsin D (cath D), and glucocerebrosidase (GLase) was analyzed in normal cultured human fibroblasts by metabolic labeling with [^{35}S]Met in conjunction with subcellular fractionation on Percoll gradients, immunoprecipitation, and SDS-PAGE. In time-course studies, newly synthesized cath D was processed to mature peptide faster than the precursors of Hex α and β polypeptide chains, which in turn were processed more rapidly than Fuc and GLase precursors. Fuc precursor and GLase intermediates were apparent in lysosomal fractions, where they were slowly converted to mature polypeptides. Treatment of fibroblasts with Ep-459 (an irreversible inhibitor of lysosomal cathepsins) resulted in the complete inhibition of Fuc processing, and partial inhibition of Hex and cath D processing; there was no effect on the maturation of GLase, consistent with the previously proposed absence of proteolytic processing during its maturation (which exclusively involves carbohydrate modification). Precursors of Hex α and β polypeptide chains, in addition to Fuc precursor and a Hex β chain intermediate of M_r 38 kDa, were seen predominantly in dense lysosomal fractions of Ep-459-treated cells (along with mature α and β Hex polypeptides). In contrast, both mature and partially processed cath D polypeptides were seen in both non-lysosomal and lysosomal fractions. The results demonstrate that proteolytic processing of lysosomal enzymes is not requisite for delivery to the lysosome, but do not rule out a role for the cleaved propeptide sequence in intracellular translocation. The differential distribution of cath D polypeptides (compared to Fuc, Hex, and GLase), in addition to the occurrence of partially processed cath D in "non-lysosomal" fractions of Ep-459-treated cells, suggests the existence of subsorting mechanisms for "lysosomal" enzymes, and further points to the localization of both processing proteinases and cathepsin D in subcellular compartments distinct from "dense lysosomes". Supported by USPHS Grants DK-38593, NS-23131, and HD-06426.

SIALO-GLYCOPROTEINS FROM TUMOR CELL MEMBRANES ARE DIGESTED BY HUMAN SERUM
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In order to identify new tumor antigens that might permit detection of cancer at earlier stages, we have studied the fate of asn-linked, sialo-glycoproteins from the surface membranes of tumor cells during in vitro and in vivo exposure to serum from cancer patients and from normal controls. Human HT-29 colorectal carcinoma cells, double-labeled with 14C-glucosamine and 3H-mannose, radiolabeled glycoproteins solubilized from the membranes of HT-29 cells with NP-40, or glycopeptides released by pronase from membrane glycoproteins were treated with serum from normal individuals or from cancer patients either before or after surgery and were analyzed by a novel gel filtration method on DuPont GF HPLC columns. Treatment of intact cell surface membrane-associated glycoproteins or of pronase-released glycopeptides with serum samples caused a degradation of larger molecules into low MW fragments. The digestion of high MW glycoproteins and corresponding production of low MW fragments increased with time, reaching a maximum in 12 to 24 hrs, was reproducible and was directly proportional to serum volume. The digestive activity appeared to be enzymatic since it was destroyed by prior heat treatment of serum samples. The possibility that the effect was due to a neuraminidase activity in the serum was ruled out since all but one of the glycoproteins identified was sensitive to V. cholerae neuraminidase. In contrast, several high and intermediate MW glycoproteins and glycopeptides were extremely sensitive to serum digestion while others were resistant for as long as 7 days. The low MW product which resulted from the treatment was composed of a large number of glycopeptide fragments. This serum dependent selective proteolysis of sialo-glycoconjugates bears significance to the potential detection of circulating tumor cell membrane derived antigens by monoclonal antibodies. Our results suggest that glycoprotein antigens which are degraded by contact with serum may not be detectable by antibodies with specificities for intact structures. Furthermore, the low molecular weight oligosaccharide fragments produced by this digestive effect may collect in the circulation of cancer patients and play a role in facilitating the metastasis of tumor cells, in blocking the attachment of cytotoxic immunoeffector cells to tumors, or in disrupting the receptor-mediated endocytosis system in hepatocytes.

IMMUNOGOLD LOCALIZATION OF A PLANT α-GLUCOSIDASE: OCCURRENCE IN THE ENDOCYTIC AND EXOCYTIC ORGANELLES OF SOYBEAN AND MUNGBEAN CELLS.
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An α-glucosidase (100 kd) purified from mungbean hypocotyls has been immunolocalized at the subcellular level using a monospecific antibody and protein A-colloidal gold. The α-glucosidase can be found in abundance in the the Golgi complex, at the plasma membrane, and in the cell wall of mungbean cells. The antibody crossreacts with a protein (100 kd) from soybean suspension culture (line SB1) cells. The subcellular distribution of the protein in SB1 cells is similar to that found in mungbean cells. Protoplasts made from SB1 cells also contain the protein at the plasma membrane and Golgi complex. In addition, the α-glucosidase was found in multivascular bodies and endosomes in soybean protoplasts. The endocytic compartment of the plant protoplasts was marked with the electron-dense probe, cationized ferritin. This observation is consistent with the hypothesis that plant cells can support entry of extracellular or plasma membrane-bound hydrolytic enzymes via an endocytic pathway similar to that found in animal cells.
Supported by NSF (L.R.G.) and NIH (A.D.E.).

GENETIC EVIDENCE THAT ACTION OF cAMP-DEPENDENT PROTEIN KINASE IS AN OBLIGATORY STEP FOR β-ADRENORECEPTOR REGULATION OF PROTEIN N-GLYCOSYLATION.

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The biosynthesis of oligosaccharide and their attachment to protein asparagine residues is a multi-step process. Though the individual enzyme in this pathway has been documented, but there is little information on the regulation of N-linked protein glycosylation. Earlier, we reported that enhanced β-adrenoreceptor regulation of protein N-glycosylation in rat parotid acinar cells is mediated via adenosine 3',5'-cyclic monophosphate (cAMP). The increased oligosaccharide-PP-Dol synthesis and its turnover following such treatment is primarily due to the activation of "key" glycosyltransferases of the dolichol-cascade pathway. Phosphorylation *in vitro* of RER membranes with the cAMP-dependent protein kinase (cAPK) showed enhanced Man-P-Dol synthase activity (~40-80%) to the same degree as observed in the isoproterenol ($1x10^{-5}M$) treated membranes. This change in enzyme activity was not associated with an alteration in apparent K_m for GDP-mannose, but the V_{max} was increased 2-fold. Treatment of *in vitro* phosphorylated membranes with alkaline phosphatase led to a 80-90% inhibition in the enzyme activity. A similar increase in protein N-glycosylation by cAMP-related stimuli has also been observed in capillary endothelial cells, human fibroblasts, C_6-glioma and Chinese hamster ovary (CHO) cells. Several cAPK-deficient mutants of CHO cells however, showed only 7-23% increase in protein glycosylating activity in the presence of 8 Br-cAMP. The majority of these mutants also exhibited altered Man-P-Dol synthase activity. The kinetic data indicated a 3-4 fold higher K_m for GDP-mannose when analyzed in the presence and absence of saturating level of Dol-P. This was especially true for the CHO mutant 10248 which has an altered type I regulatory subunit, binds cAMP poorly, and also has no detectable type II kinase. In addition, the mutant 10248 is defective in phosphorylation of a 50 kDa cellular substrate. These results, therefore, suggested that normal expression of cAPK gene is an obligatory requirement for enhanced Man-P-Dol synthase activity and protein N-glycosylation as well in the presence of cAMP-related stimuli. (BRSG and (NIH R01 HL35011).

INCREASED HYPOXANTHINE LABELLING OF PLASMODIUM FALCIPARUM MALARIA IN HEMPAS ERYTHROCYTES[+]

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The invasion of malaria in normal and HEMPAS blood was studied. Biochemical studies on HEMPAS (hereditary erythroblastic multinuclearity with positive acidified serum test) indicate a truncated Band 3 glycoprotein, the carbohydrate portion of which is reported to be shorter than in normal cells (J. Biol. Chem. 1987, 262, 7195-7206). Band 3, especially the carbohydrate portion, is thought to be crucial for invasion of Plasmodium falciparum (Science, 1985, 228, 75-77).

Infected and normal red blood cells were synchronized by mannitol treatment, followed by schizont isolation by gelatin treatment at 47 hours. The culture was then diluted 1:20 with control or HEMPAS cells to give an infectivity not greater than 1 percent. 0.05 ml of 0.01 mCi/ml ^{3}H-hypoxanthine was added every 4 hours to 0.1 ml of sample in 96 well microtiter plates. The cells were harvested and the incorporated label determined with scintillation counting. The incorporation of ^{3}H-hypoxanthine was nearly twice as much in HEMPAS than in control cells over most of the life cycle of the parasite. The relationship between the levels of incorporation of labelled hypoxanthine and infectivity is under investigation.

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SIALYL DIMERIC-LEWIS-X ANTIGEN EXPRESSED ON MUCIN-LIKE GLYCOPROTEINS IN COLON CANCER METASTASIS Yoshifumi Matsushita, Stuart Hoff, David M. Ota, Karen R. Cleary and Tatsuro Irimura: Departments of Tumor Biology, General Surgery and Pathology, The University of Texas, M. D. Anderson Cancer Center, Houston, TX 77030.

Many oncofetal carbohydrate antigens were previously reported, but their relationship with malignant behavior especially metastatic potential was not known. We have compared immunochemically and histochemically reactivity of monoclonal antibodies (MAbs, kindly provided by Dr. Sen-itiroh Hakomori, Biomembrane Institute) generated against $Lewis^X$ antigens with human primary colorectal carcinoma at different stages and metastases to examine whether the expression was related to metastasis. MAb FH6 specific for sialyl dimeric-Le^X (NeuAc2-3Gal1-4(Fuc1-3)GlcNAc1-3Gal1-4(Fuc1-3)GlcNAc-R) was shown associated with liver metastases. Namely, (a) the binding of MAb FH6 to monolayers of human HT-29 colon carcinoma cell line was inhibited to a stronger extent by extracts from metastatic lesions than by those from primaries, and (b) histochemical examination with MAb FH6 in combination with ABC staining revealed that an equivalent or higher proportions of carcinoma cells were positive in metastases than primaries. We have extracted liver metastasis of colon carcinoma with a buffer containing 4 M guanidine-HCl or with 0.5% Nonidet P40, and attempted to identify molecules reactive with MAb FH6. High molecular weight mucin-like molecules were found reactive with this MAb after separation on Sepharose 2B and DEAE-Sephacel column chromatography followed by dot blot assays. Neuraminidase treatment of the tissue extracts diminished the reactivity confirming the specificity. Electrophoretic separation using 3 or 4% polyacrylamide gels, with or without blotting to nitrocellulose membrane followed by antibody bindings also indicated that MAb FH6 bound to mucin-like high molecular weight glycoproteins which had very slow mobility on polyacrylamide gels and were apparently distinct from sialomucins bound with wheat germ agglutinin or MAb 115D8 (anti-human mammary fat globule membrane mucin). (Supported by USPHS Grant RO1-CA39319)

HUMAN COLONIC SULFOMUCIN AND AN ANTI-SULFOMUCIN MONOCLONAL ANTIBODY: Takao Yamori, Leah Hager, Hitomi Kimura, David M. Ota, Karen R. Cleary and Tatsuro Irimura, Departments of Tumor Biology, General Surgery and Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030.

We have previously shown that sulfated high molecular weight mucin-like glycoproteins (sulfomucins) are produced by human colonic mucosa and at lower amounts by primary colon carcinomas in their non-invasive luminal portions. The amount further decreases at the invasive edge of primary carcinomas and distant metastases. A concomitant increase of sialylated mucins is detected in some of the metastatic foci. We have partially purified sulfomucins from normal colonic mucosa, and prepared monoclonal antibodies (MAbs) against this fraction using mouse myeloma cells. One of these antibodies binds to a component electrophoretically co-migrated with metabolically ^{35}S-sulfate labeled sulfomucins. This MAb designated as 91.9H is an IgG1. Histochemical examinations reveals that MAb 91.9H binds to the same cells as those stained with high iron diamine method, but their subcellular localization was slightly different from each other. The differential reactivities of MAb 91.9H to normal mucosa, primary colorectal carcinoma and metastases are consistent with differential amounts of sulfomucins as determined by metabolic ^{35}S-sulfate labeling followed by chromatographic separation on DEAE-cellulose and enzymatic identification. After further purification of sulfomucins, >40% of sulfate labeled materials can be precipitated by MAb 91.9H-coated protein A-agarose. The antibody reactivity appears to be diminished by chemical desulfation with diluted anhydrous hydrochloric acid. Alkaline reduction releases ^{35}S-sulfate labels from the partially purified molecules suggesting that sulfate groups are attached to O-linked carbohydrate chains on sulfomucins. (Ref.: Yamori et al., Cancer Res., 47: 2741, 1987; supported by USPHS Grant RO1-CA39319)

Proteoglycans

STRUCTURAL CHARACTERIZATION OF DERMATAN SULFATE PROTEOGLYCAN FROM HUMAN DERMIS. Hari G. Garg, Elizabeth P. Siebert and David A. Swann, Shriners Burns Institute, Boston, MA 02114.

Proteoglycans (PGs) are implicated in scar formation; in order to understand their role, dermatan sulfate (DS) PGs were isolated and characterized from human scar tissue (Collagen Rel. Res.: in press). To further assess the similarities and differences between these DS.PGs and human skin, DS.PGs were isolated from normal human dermis tissue with 4M Gdm.Cl containing protease inhibitors, using ion exchange chromatography followed by 20, 30, 40, 50 and 75% (v/v) ethanol precipitation. The 20 and 30% ethanol fractions contained dermis DS.PGs; of the total uronic acid present, 94% consisted of iduronic acid. The M_r of dermis DS.PG was 66,000, while alkaline borohydride treatment of dermis DS.PG yielded DS glycosaminoglycan (GAG) chains with a M_r of 17,500. The presence of xylitol in the liberated GAG chains suggests a xylosyl-serine linkage between GAG chains and core protein. The sulfate residues were predominantly attached at C-4 of galactosamine. The protein core peptides released after chondroitinase ABC treatment had M_r values of 21,500 and 14,000. The NH_2-terminus sequence of these peptides was: NH_2-Asp-Glu-Ala-O-Gly-Ileu-Gly-Pro-Glu-Val-Pro-Asp-Asp-Arg-Asp(?)-Phe-Glu-Pro-Ser(?)-Leu. This study suggests that the protein core peptides of DS.PGs from scar and normal human tissue are similar, but that the dermis DS.GAG chains from scar tissue are larger in size. (Supported by research funds from the Shriners Hospitals for Crippled Children.)

CLONING AND LONG TERM CULTURE OF HUMAN CHONDROSARCOMAS PRODUCING KERATAN SULFATE. Joel A. Block, Sven E. Inerot, Steven Gitelis, James H. Kimura, Departments of Biochemistry and Orthopedic Surgery, Rush-Presbyterian-St. Luke's Medical Center, Chicago, IL 60612-3864.

The physical properties of cartilage are due to the organization of proteoglycans (PGs) and collagen in the extra-cellular matrix. Models, such as the Swarm rat chondrosarcoma, provide insight into the biosynthesis and structure of these macromolecules. Non-human systems, however, have limitations; for example, rats produce no keratan sulfate (KS), a glycosaminoglycan (GAG) that comprises about 25% of the GAGs in human large aggregating proteoglycans. We report the first stable long-term culture and cloning of human chondrocyte-like cells that produce a normal pattern of KS and elaborate matrix reminiscent of normal cartilage. Malignant chondrocytes were enzymatically liberated from a fresh human chondrosarcoma and cultured alternately in 1% ultra low temperature gelling agarose and in monolayer. Cells were cloned at 5 months. One clonal cell line (KC2H3) and the parent mass-tumor line (1052202KC) were screened by indirect immunofluorescence with a monoclonal antibody (5-D-4) directed against KS and found to be positive. Biochemical analysis, including GAG content as determined by keratanase and chondroitinase ABC digestions of A1 fractions, revealed that both cell lines produced large aggregating PGs and KS, but in markedly different proportions: 11% KS by 1052202KC vs. a normal 26% KS by KC2H3. This study demonstrates the practicality of long-term maintenance and clonal analysis of human chondrosarcomas. It is the first demonstration of a phenotypically stable clonal cell line that produces normal proportions of KS; and it is the only clonal model of mature human chondrocyte PG metabolism.

DEVELOPMENT OF AN IN VITRO BINDING ASSAY TO CHARACTERIZE THE INTERACTION BETWEEN SMALL PROTEOGLYCANS AND COLLAGEN.
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Electron microscopy has shown that small proteoglycans are associated with collagen fibrils in tendon. This interaction is strong, requiring collagen disruption to extract the proteoglycans. The objective of this study was to develop an in vitro system to examine the interaction between small dermatan sulfate proteoglycans of tendon and collagen. Radiolabeled proteoglycans were generated from cell or explant culture of bovine deep flexor tendon, using $Na_2{}^{35}SO_4$ to label the glycosaminoglycan chain or ^{3}H-leucine for the core protein. The proteoglycans were then purified by ion-exchange and sieve chromatography. During the binding assay proteoglycans were added to a preformed gel of type I collagen from bovine skin. The binding was performed in 5% BSA to minimize nonspecific proteoglycan adhesion to the plates. Maximum binding was achieved under the roughly physiological conditions of 0.1 M NaCl and pH 7.4. Equilibrium was achieved in 24 hrs. at 37°C and 48 hrs. at 4°C. Once bound, the proteoglycan resisted elution, even when rinsed in solutions up to 4 M NaCl for 24 hrs. When binding occured in the presence of up to 1% Triton X-100 or Tween 20, there was no significant change. However, binding was sharply decreased in 1% SDS. Proteoglycans bound equally to native and pepsin treated collagen. However, preliminary data indicated very little binding to gelatin. Binding to native rat tail tendon collagen was the same as to bovine type I collagen. Both free glycosaminoglycan chains and core protein depleted of glycosaminglycan chains by chondroitinase ABC digestion were bound to collagen.

(Supported by NIH AR36110)

WESTERN BLOTTING OF PROTEOGLYCANS FOLLOWING AGAROSE-ACRYLAMIDE GEL ELECTROPHORESIS. R. Heimer. Dept. Biochem., Molec. Biol., Thomas Jefferson University, Philadelphia, PA. 19107

In qualitative as well as quantitative analyses of proteoglycans (PG) in solution it is common practice to label the PG biosynthetically with $^{35}S(SO_4)$ and ^{3}H(glucosamine), and evaluate the distribution of these markers through CsCl density gradient ultracentrifugation and subsequent anion exchange and gel permeation chromatography. This technology can be replaced totally through use of agarose-acrylamide gel electrophoresis, electrotransblotting to nitrocellulose, and reacting membranes with monoclonal antibodies to epitopes characteristic of PG. Maximum sensitivity of detection, approx. at the 1 ng PG level, can be obtained through employing ^{125}I-labeled second antibody and autoradiography. Autoradiographs scanned by laserbeam densitometry provide quantitative information when appropriate standards are included in the assays. The technique is particularly useful when analysis of multiple samples is required. Another advantage is that electrophoretically transblotted PG remain in the membrane which can be subjected to a variety of probes without concern for half life.

By giving details of each step in the procedure, it will be shown that complex mixtures containing approx. 1,000 parts protein to 1 part PG can be resolved by electrophoresis without preliminary purification. For instance, meaningful results are obtained with as little as 1 µl synovial fluid, for which we employed commercially available monoclonal antibodies to chondroitin sulfate, keratan sulfate and the hyaluronate binding region on the core proteins. Analyses with these antibodies were done sequentially on one blot, removing each antibody prior to the application of the next with 3M NaSCN. Quantitative analyses allowed to establish PG profiles for distinct categories of rheumatic joint disease.

CHLORATE-A MODULATOR OF HEPARAN SULFATE BIOSYNTHESIS. J.M.Keller and K.M.Keller. Univ. Health Sci./Chicago Medical School, North Chicago, IL 60064.

Swiss mouse 3T3 cells, when grown in the presence of increasing concentrations of chlorate, an inhibitor of PAPS biosynthesis, produce heparan sulfate glycosaminoglycan chains containing increasingly less sulfate. As determined from ^{35}S-sulfate incorporation into chains and their behavior on ion-exchange chromatography, the chains from cells grown in the presence of 5 mM chlorate contain only about 6 % of the sulfate present in the heparan sulfate from non-treated cells. These undersulfated chains are sensitive to nitrous acid at pH 4.5, which indicates that many glucosaminyl groups have unsubstituted amino groups. There is no significant alteration in the length of the chains. Cells producing the undersulfated heparan sulfate chains do not demonstrate any alterations in their growth control. However, the spreading behavior of the cells is altered, with the cells showing a flat rounded morphology rather than the more typical fibroblastic appearance of the untreated cells. Alterations in the cytoskeleton as reflected in changes in the distribution of vinculin and microfilaments are apparent. The sulfation of chondroitin chains is also inhibited, but at a lower chlorate concentration, which does not alter either growth control or the spreading ability of the cells. These data indicate that (a) 3T3 cell surface heparan sulfate proteoglycan is involved in cell spreading, but not growth control, (b) the use of chlorate should be a valuable method for the study of the biosynthesis and structure/function relationships of heparan sulfates and other sulfated glycosaminoglycans and (c) the temporal sequence of the heparan sulfate chain modification reactions predicted from results of studies with cell free extracts also operates in the cell. (Supported in part by NIH grant CA 02136.)

CELLULAR LOCATION OF PROTEOGLYCANS AND GLYCOSAMINOGLYCAN FREE CHAINS

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We have studied the location of proteoglycans and "free" GAG chains in cultures of BALB/c 3T3 cells by use of detergent extraction, trypsin treatment and degradation by Flavobacterial heparinase.

Cells were labeled with [^{35}S]-sulfate and [^{3}H]-glucosamine and total GAGs isolated by chromatography on Sepharose. Specific GAGs were then identified by treatment with chondroitinases, hyaluronidase, heparitinase and nitrous acid. Chondroitin sulfates, dermatan sulfate and heparan sulfate were found to be present in the proteoglycan and the free chain species in about a 1:2 ratio in the cell associated fraction. No free chains appeared to be present in the medium. Localization of the GAG polymers was carried out by treatment of the cell layer with trypsin or crude Flavobacterial heparinase under conditions preventing enzyme internalization. Trypsin treatment removed about 2/3 of the total proteoglycans and only a minor and variable portion of free chains. A very significant finding was that crude heparinase, on the other hand, removed over 90% of total cellular GAGs. This shows that most of the proteoglycans as well as free chains including trypsin-resistant material are present on the cell surface. This is contrary to most literature reports relying on trypsin treatment only. It also indicates that free chains are not merely intermediates in the degradation pathway, as usually implied, but may serve a significant function in cell membranes. Pulse chase studies indicated that most of the free chains are not directly derived from membrane proteoglycans.

EFFECT OF UV-LIGHT AND VITAMIN E INTAKE ON ALBINIC RAT SKIN GLYCOSAMINOGLYCANS. Maria D. Longas[1], Durga K. Bhuyan[2], Kailash C. Bhuyan[2] and Christine M. Gutsch[1]. Purdue University Calumet, Department of Chemistry and Physics[1], Hammond, IN 46323 and The Mount Sinai School of Medicine, Department of Ophthalmology[2], New York NY 10029.

The increment of glycosaminoglycan (GAG) concentration, effected by ultraviolet (UV) light irradiation of rat skin, was reversed by dietary vitamin E. Four weeks old Sprage Dawley rats, maintained on a regular rodent diet, were separated into three groups of eight subjects each; dl-α-tocopherylacetate (2,000 IU/Kg of food) was added daily to the diet of group III. After one week, groups II and III were shaved on the back and irradiated with 2.3 milliwatts/cm^2, using UV light of 350-400 nm with maximal intensity at 365 nm. Light exposure was six hours daily for two weeks, while the diet remained unchanged. The skin of the sacrificed animals was exhaustively digested with proteolytic enzymes, and GAG precipitated sequentially with 18%, 35% and 45% (v/v) ethanol. Infrared spectra of the products demonstrated that three dermatan sulfates with different degrees of sulfation were precipitated with 18% and 45% ethanol. Chondroitin-4-sulfate (Ch-4-S) precipitated with 35 % ethanol. In the controls (set I), dermatan sulfate (DS) and Ch-4-S represented, respectively, 0.098% and 0.007% (w/w) of wet surgically-delipidated skin. Their concentration increased, respectively by 2.34 and 3.88-folds, upon irradiation (set II). The GAG content was not significantly altered in irradiated skin of rats kept on a vitamin E-rich diet (set III). Infrared spectra of DS isolated from the latter group with 18% ethanol (DS_{18}) showed intensification of bands at 840 cm^{-1} and 785-805 cm^{-1}.

The data show that three chemically different dermatan sulfates and Ch-4-S are the major GAG of seven-weeks-old, albinic, rat skin. Their concentration increased upon exposure to UVA-light, but was not significantly affected when vitamin E was included in the diet. The content of equatorial sulfates with bands at 840 cm^{-1} and 785-805 cm^{-1} was elevated in DS_{18} of irradiated skin of vitamin E-treated rats (Set III).

BOVINE AORTIC ENDOTHELIAL CELL PROTOEGLYCANS. P. V. TRESCONY, T. R. Oegema, R. Eisenstein. Department of Orthopaedic Surgery, University of Minnesota, Minneapolis, MN 55455

Bovine aortic endothelial cells in culture were labeled with $^{35}SO_4$ for 24 hours and proteoglycans were isolated from the medium, a CHAPS extract of the cell layer, and a 4M guanidine·HCl extract of the residual matrix by ion-exchange chromatography. CHAPS extracted a unique proteoglycan pool not found in the residual matrix which contained 50% of the proteoglycan label. CHAPS-extractable proteoglycans were resolved into two distinct bands, HS-A and HS-B, by large pore composite agarose-polyacrylamide gel electrophoresis which were isolated by electroblotting to DEAE-cellulose paper and found to be single chains containing little or no attached core protein. The size of HS-A and HS-B were determined by Sepharose CL-6B gel filtration chromatography to be Mr = 6.5 x 10^4 and Mr = 3.0 x 10^4, respectively. Trypsin accessiblity experiments indicated that HS-B was an intracellular species and that HS-A was located in part at the cell surface after 24 hours of labeling. Continuous labeling in the presence of heparan sulfate degrading enzymes over 4 hours indicated that both HS-A and HS-B or their precursors cycled to the cell surface. Label-chase experiments showed that a species with an electrophoretic mobility similar to HS-A was present within 7 minutes of labeling and that a species with a mobility similar to HS-B appeared within 60 minutes. The maximum amount of intracellular HS-A label occured within 1 hour after a 30 minute label and preceded the HS-B maximum by about 1.5 hour. After a 24 hour label the ratio of HS-A to HS-B remained constant over 24 hours and both turned over with a half-life of roughly 24 hours. A model is proposed where a proteoglycan precursor to HS-A and HS-B was synthesized and resided at the cell surface for about 1 hour. HS-A chains were generated by proteolysis and internalized and HS-B chains were then derived by limited endoglycosidic cleavage of a portion of HS-A chains. Both species subsequently resided in a stable intracellular pool and turned over with a $t_{1/2}$~20 hours.

Glycosyltransferases

A PLANT FUCOSYLTRANSFERASE WITH "LEWIS" SPECIFICITY.

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A Triton X-100 extract of mung bean seedlings, prepared essentially as described by Szumilo *et al.* (Biochemistry *26* (1987) 5498) was found to transfer L-fucose from GDP-fucose to βDGal(1→3)βDGlcNAc-OR (**1**) (R= $(CH_2)_8COOMe$) when assayed using a recently reported SepPak assay (Glycoconjugate J. *5* (1988) 49). The product of this fucosyltransferase activity on **1** was isolated and shown by ^{1}H-NMR to be the trisaccharide βDGal(1→3)[αLFuc(1→4)]βDGlcNAc-OR, the human Lewis-a blood-group determinant. No transfer to βDGal(1→4)βDGlcNAc-OR (**2**) could be detected, nor was βDGal-OR a substrate.

The H-Type 1 trisaccharide αLFuc(1→2)βDGal(1→3)βDGlcNAc-OR was also a substrate for the plant fucosyltranserase, presumably forming the Lewis-b determinant αLFuc(1→2)βDGal(1→3)[αLFuc(1→4)]βDGlcNAc-OR as does the human Lewis enzyme.The absolute specificity of this enzyme for Type 1 sequence (**1**) appears to be unique since other known fucosyltransferases act on both sequences **1** and **2**. The enzyme activity does not require manganese and inhibition by GDP was negligible. The K_m's for GDP-fucose and **1** are approximately 0.1 and 1 mM, respectively.

AFFINITY PURIFICATION OF GDP-L-FUC:*N*-ACETYL-β-D-GLUCOSAMINIDE α1→6FUCOSYLTRANSFERASE (α1→6FUCT) FROM HUMAN FIBROBLASTS.

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α1→6FucT has been purified 37,000-fold from cultured human skin fibroblasts using detergent extraction, chromatofocusing and affinity chromatography. The key step in the purification procedure was the affinity support prepared with asialo-agalactotransferrin glycopeptide (GN-GN), the substrate for the enzyme (*Anal. Biochem. 168, 367, 1988*). Human serotransferrin was desialylated by mild acid, then Pronase digested followed by removal of the terminal Gal residues with jack bean β-galactosidase. Further purification was by filtration on Biogel P-2 and P-10. This glycopeptide was conjugated to activated CH-Sepharose 4B (GN-GN-Sepharose) at 1.2 μmol/ml of gel. α1→6FucT was solubilized from the fibroblasts by a two-step extraction with detergents and was assayed as described (see ref.). The enzyme was partially purified by chromatofocusing and the fractions (pH 7.1 to 7.4) were combined and applied to GN-GN-Sepharose in the presence of 30 μM GMP. After washing with 25 mM Tris, pH 7.5, 5 mM $MgCl_2$, 10% glycerol and 30 μM GMP, the column was eluted with the buffer without GMP and in the buffer containing 1 M NaCl and no GMP. The enzyme activity was recovered in the high salt eluate. Affinity purified α1→6FucT was free of α1→3FucT and the exoglycosidases which were examined. Attempts to further purify α1→6FucT continue to be negative, suggesting the advantage of affinity chromatography utilizing a known substrate for the enzyme rather than a related compound.

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SIALYLTRANSFERASE ACTIVITY IN MELANOMA AND OTHER CULTURED HUMAN CANCER CELLS. I. J. THAMPOE, K. Furukawa, E. Vellvé, and K. O. Lloyd. Memorial Sloan-Kettering Cancer Center, New York, NY

Quantitative changes in ganglioside expression have been reported to accompany malignant transformation, for example GD3 expression is enhanced in human melanoma. An analysis of mechanisms that control ganglioside synthesis and expression merits study. Here we present data on the enzyme CMP-NeuAc:GM3 sialyltransferase (GD3 synthase). Enzyme activity on membrane preparations was measured in an *in vitro* assay that quantitated ^{14}C-NeuAc incorporation into GM3. Assays were carried out in 0.1M cacodylate-HCl pH 6.0 containing 0.1% Triton CF-54 and 10mM Mg^{2+} and 25µM GM3.

Using these conditions, GD3 synthase activity was assayed on a panel of 47 human cancer cell lines; the products were also analyzed by TLC and fluorography. The presence of cell surface gangliosides in these cell lines was determined using a panel of monoclonal antibodies detecting GM3, GM2, GD3, and GD2. The relationship between *in vitro* reaction products and ganglioside content was further examined in 7 representative cell lines by TLC-resorcinol and -immunostaining methods.

In the panel studied, melanoma cell lines exhibited the highest levels of enzyme activity; GD3 was found to be the major reaction product and the most prominent ganglioside *in vivo*. In contrast, very low levels of GD3 synthase were detected in cultured normal melanocytes. Neuroblastoma and some astrocytomas also contained significant levels of enzyme activity and GD3 was the major reaction product. *In vivo*, however, GD2 and not GD3 was the major ganglioside in these cell types. Although epithelial cancer cell lines, in general, contained low levels of GD3 synthase, high levels of ^{14}C-NeuAc incorporation was observed in some breast, renal, and lung cancer cell lines; the reaction products did not, however, correspond to GD3. In summary, GD3 synthase levels and GD3 surface expression were particularly high in melanoma cell lines.

CHARACTERIZATION OF THE EXPRESSION OF β-GALACTOSIDE α2,6 SIALYLTRANSFERASE IN CHINESE HAMSTER OVARY AND COS-1 CELLS. J. C. Paulson, K. Colley, E. U. Lee, and J. Roth.* Department of Biological Chemistry and Molecular Biology Institute, UCLA School of Medicine, Los Angeles, CA 90024 and *Biocenter, University of Basle, CH-4056 Basle, Switzerland.

Cloning of a full length cDNA of the rat liver Galβ1,4GlcNAc α2,6 sialyltransferase [J. Biol. Chem. (1987) **262**,17735] has allowed the construction of vectors suitable for expression of sialyltransferase in Chinese hamster ovary (CHO) cells and Cos-1 cells to analyze the basis for the subcellular localization of the enzyme and the consequences of expressing glycosyltransferases not normally produced by a cell. CHO cells normally contain N-linked oligosaccharides which terminate with the SAα2,3Gal sequence. In contrast, stable transformants expressing the α2,6 sialyltransferase also produce carbohydrates containing the SAα2,6Gal sequence, which allow the expressing cells to be detected using the SAα2,6Gal specific *Sambucus nigra* agglutinin (SNA) [J. Biol. Chem. (1987) **262**,1596]. Thus, as might be expected, expression of this enzyme alters the structures of the cell surface carbohydrate groups of CHO cells. Although the expressed sialyltransferase has not been detected by immunocytochemistry at the electron microscope level, the enzymatic product of the enzyme is readily detected using the SNA lectin. Although the sialyltransferse is normally thought to be localized in the trans-Golgi, the SNA staining is found throughout the Golgi apparatus stack, in addition to all post-Golgi membrane structures. The significance of finding the product of the enzyme in the *cis*-, *medial*-, and *trans*- Golgi cisternae is not yet clear. Immunofluorescence localization of the enzyme expressed in Cos-1 cells under the late adenovirus promotor shows the enzyme to be localized in the perinuclear region, consistent with its expected localization in the Golgi apparatus. Pulse chase studies in both CHO and Cos-1 cells show that the enzyme remains intracellular. Site-directed mutagenesis experiments to produce 'mis-localized' mutants of the enzyme are in progress. Supported by NIH Grants GM-27904 and GM-11557.

EVOLUTION OF α1-3 GALACTOSYLTRANSFERASE IN MAMMALS. U. Galili, S.B. Shohet,* and B.A. Macher,† Cancer Research Institute, University of California, San Francisco, CA*, and Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, CA†.

The enzyme α1→3 galactosyltransferase catalyzes the following reaction:

Galβ1-4GlcNAc-R + UDP Gal → Galα1-3Galβ1-4GlcNAc-R + UDP

Studies on the expression of the glycosylation product Galα1→3Galβ1→4GlcNAc-R on nucleated cells revealed the presence of such epitopes in amounts ranging between 10^6 to 30×10^6 per cell on fibroblasts, epithelial cells, endothelial cells, and smooth muscle cells from almost all non-primate mammalian species studied. Among primates, Galα1→3Galβ1→4GlcNAc epitopes are abundant on nucleated cells of prosimians and New World monkeys, but are not found on nucleated cells of Old World monkeys, apes, and humans. The latter group, however, constantly synthesizes large amounts of IgG antibodies (1% of circulating IgG) against this epitope. The absence of Galα1→3Galβ1→4GlcNAc epitopes in humans was found to result from a diminished activity of the enzyme α1→3 galactosyltransferase, rather than from the lack of appropriate acceptor for the enzyme. Thus, bovine α1→3 galactosyltransferase was found to be capable of generating Galα1→3Galβ1→4GlcNAc residues on lactosamines of human thyroglobulin. Our studies suggest that α1→3 galactosyltransferase activity was suppressed in ancestral Old World primates, some 20 to 30 million years ago, before divergence of ancestral apes and Old World monkeys. There are indirect indications that the gene for α1→3 galactosyltransferase is present within the human genome, but is largely suppressed. These include the finding of cryptic Galα1→3Gal epitopes on human red cells, which are unmasked in aged and certain pathologic red cells, and observations on binding of anti-Gal to human thyroid tissue and to various human cell lines. Hypothetically, anomalous activity of α1→3 galactosyltransferase in man would result in the *de novo* synthesis of Galα1→3Galβ1→4GlcNAc epitopes. Since 1% of circulating IgG antibodies in man recognize this epitope (the natural anti-Gal antibody), such an anomalous activity of α1→3 galactosyltransferase may result in the initiation of autoimmune processes in man.

MURINE β-1,4-GALACTOSYLTRANSFERASE: EVIDENCE FOR TWO FORMS OF THE PROTEIN BASED ON CLONING STUDIES. N.L. Shaper, G.F. Hollis† and J.H. Shaper*. The Oncology Center and *Department of Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, Baltimore, MD 21205 and †Monsanto Co., St. Louis, MO 63198.

We have isolated overlapping cDNA clones representing the full-length transcript for murine β-1,4-galactosyltransferase. The full length cDNA is approximately 4.1 kb and contains an unusually long 3'-untranslated region (2582 bp), a coding region of about 1200 bp and a heterogeneous 5'-untranslated region. Inspection of the coding region indicates three distinct structural features: 1) a large, potentially glycosylated, COOH-terminal domain (355 amino acids), which is positioned within the Golgi lumen and contains both the catalytic site and the α-lactalbumin-binding site, 2) a single transmembrane domain of 20 amino acids, and 3) a short NH_2-terminal domain.

Primer extension analysis, S1 protection analysis and RNA blotting demonstrate the presence of two sets of mRNA transcripts which differ in length by about 200 bp. The 5' boundary of the long transcripts maps upstream of two in-frame ATGs. The 5' boundary of the short transcripts maps between these two ATGs.

These results demonstrate that two related forms of β-1,4-galactosyltransferase of 399 and 386 amino acids are synthesized as a consequence of alternative translation initiation. Both forms of the enzyme are identical in primary structure with the exception that the long form (399 amino acids) has a NH_2-terminal extension of 13 amino acids which, in part, potentially encodes a cleavable signal sequence. The structural implications, topological distribution and potential biological significance of the two forms of the enzyme will be discussed.

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DEFECT IN GALACTOSYLTRANSFERASE (GT) FOUND IN A VARIANT OF CONGENITAL DYSERYTHROPOIETIC ANEMIA TYPE II (HEMPAS) PATIENT. M.N. Fukuda*, K. Masri*, and A.Dell†, * La Jolla Cancer Research Foundation, CA. and †Imperial College of Technology, London.

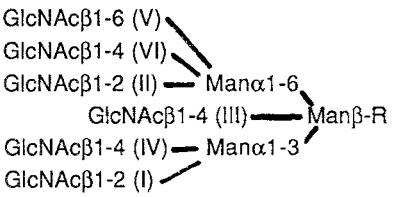
Congenital dyserythropoietic anemia type II or HEMPAS is a genetic disease inherited by an autosomally recessive mode. The primary defect of HEMPAS is lowered activity of N-acetylglucosaminyltransferase II (GnT II) (J. Biol. Chem. (1987) 262, 7195). In addition to typical HEMPAS cases, many variants have been recognized. One such variant, G.K.'s erythrocytes exhibit relatively low Ij antigen levels. Cell surface labeling using galactose oxidase/NaB[^{3}H]$_4$ incorporated almost no radioactivity into G.K.'s erythrocytes. The fast atom bombardment-mass spectrum of N-glycans isolated from G.K. erythrocyte membranes contains a key signal, m/z 1682, for the truncated trimannosyl hybrid oligosaccharide, the structure which specifically result from GnT II deficiency. The spectrum also contains signals for high mannose oligosaccharides, which were not observed in other HEMPAS cases, suggesting a further anomaly in the variant. GnT II and GT activities in G.K.'s microsomal membranes was 43% and 24% of normal levels, respectively. Western blotting using anti-GT antibodies showed reduced levels of the 65kd band which corresponds to the membrane bound form of GT. GT in G.K.'s serum was, however, higher (28.0 nmole/hr/ml) than normal levels (16.7 ± 3.2 nmole/hr/ml). These results strongly suggest that the variant G.K. is defective both in GnT II and in the membrane bound form of GT. High levels of soluble GT but reduced amounts of membrane bound GT in G.K. suggest that the genetic mutation results in increased proteolytic cleavage of membrane bound form GT.

SPONTANEOUS OR MUTAGEN-INDUCED ACTIVATION AND DE-ACTIVATION OF A β1,4-N-ACETYLGLUCOSAMINYLTRANSFERASE III ACTIVITY IN CHO CELLS. SANDRA SALLUSTIO AND PAMELA STANLEY. DEPT. OF CELL BIOLOGY, ALBERT EINSTEIN COLLEGE OF MEDICINE, BRONX N.Y., 10461.

LEC10 is a dominant glycosylation mutant of Chinese hamster ovary (CHO) cells which expresses a developmentally regulated β1,4-N-acetyl-glucosaminyltransferase III (GlcNAc-TIII) activity not detectable in parental CHO cells. In order to investigate the genetic events that give rise to GlcNAc-TIII expression, the frequency of mutation to the LEC10 phenotype was determined after treatment of Pro$^-$5 parental cells with the mutagens ethylmethanesulfonate (EMS), N-methyl-N-nitrosoguanidine (MNNG), ICR-191, ethylnitrosourea (ENU) and 5-azacytidine. Three of the mutagens (EMS, MNNG, and ICR-191) increased the frequency of the LEC10 phenotype 6-10-fold over the spontaneous frequency ($\leq 1X10^{-7}$). However, ENU and 5-azacytidine did not significantly alter the frequency of LEC10 phenotypes in the population. Variable degrees of GlcNAc-TIII expression were observed among independent isolates with activities ranging from 3 to 21 nmol/hr/mg protein. The low forward mutation frequency was not matched by the frequency of reversion of LEC10 mutants to the parental phenotype. Revertants selected for resistance to erythro-phytohaemagglutinin (E-PHA) were obtained at spontaneous frequencies of $\sim 1.5X10^{-4}$ and arose at a rate of $\sim 3X10^{-5}$/cell/generation. None of the revertants possessed GlcNAc-TIII activity. Several independent revertants were subjected to selection for re-reversion (i.e. back to the LEC10 phenotype) and two frequencies of re-reversion were observed. Three revertant lines gave rise to LEC10 mutants at high frequency ($\sim 10^{-4}$) while seven others gave rise to LEC10 mutants at the low frequency typical of parental cells. Those with a high re-reversion frequency did not appear to have acquired a general mutator phenotype. The combined data indicate that the high reversion frequency of LEC10 mutants reflects unusual genomic changes rather than a multitude of genes whose products affect GlcNAc-TIII expression. The various cell lines isolated should be useful in investigating the regulation of GlcNAc-TIII expression once the gene encoding the GlcNAc-TIII enzyme is cloned.

SYNTHESIS OF BRANCHED N- AND O-GLYCANS. Inka Brockhausen and Harry Schachter. Research Institute, Hospital for Sick Children, Toronto, Ont., Canada.

Cells interact with their environment at least in part by means of highly branched glycans on their surfaces. There are six GlcNAc-transferases (GnT I to VI, see scheme below) which initiate the addition of antennae to the common $Man_3GlcNAc_2Asn$ core of N-glycans. There are also several GnT involved in the synthesis of the branched O-glycan cores (core types 2 and 4, see below) and in the synthesis of the branched I determinant [GlcNAcβ1-6(GlcNAcβ1-3)Gal-] attached to some of these cores. Correlations have been reported between the levels of some of these GnT and differentiation, cancer or oncogenic transformation, metastatic potential and anemia. To understand the basis for these correlations, it is essential to determine the synthetic controls for branching. We have used HPLC assays for these GnT to determine sequential paths for the synthesis of both the N- and O-glycans. For example, GnT I must act before GnT II, III and IV, and both GnT I and II must act before GnT V and VI. GnT V is the only one among the six N-glycan branching GnT which can be assayed in the absence of Mn^{2+}. GnT I, II, IV and V act poorly, if at all, on bisected oligosaccharides but GnT VI acts equally well on bisected and non-bisected substrates. GnT VI is also unusual in that it has a rather high Mn^{2+} concentration optimum (about 100 mM) with the synthetic substrates GlcNAcβ1-6(GlcNAcβ1-2)ManαR (R = methyl or -1-6Manβ-$(CH_2)_8COO-CH_3$). GnT VI has been found in some avian, but not in mammalian tissues. (Support by Canadian MRC and CF Found.).



O-glycan cores:
(1) Galβ1-3GalNAc-
(2) GlcNAcβ1-6(Galβ1-3)GalNAc-
(3) GlcNAcβ1-3GalNAc-
(4) GlcNAcβ1-6(GlcNAcβ1-3)GalNAc-

UDP-GlcNAc: POLYPEPTIDE GlcNAcTRANSFERASE
A membrane-bound, cytoplasmically oriented enzyme

R. S. Haltiwanger, G.D. Holt, and G.W. Hart.
Johns Hopkins Medical School
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The last four years has seen the discovery of a new form of protein-saccharide modification in which single residues of N-acetylglucosamine (GlcNAc) are attached in O-linkage to protein (Torres and Hart, *J. Biol. Chem.* **259**:3308). Since this report, a variety of researcher have shown that, in contrast to all other known forms of glycosylation, O-GlcNAc is found predominantly on proteins within the cytoplasmic and nucleoplasmic compartments of the cell (Holt and Hart, *J. Biol. Chem.* **261**:8049, and Holt et al., *J. Cell Biol.*, **104**:1157). This unexpected orientation of proteins bearing O-GlcNAc has provoked great interest in identifying proteins which undergo this form of modification, with particular regard to how carbohydrate addition effect the proteins' functions. Here we describe the identification of a UDP-GlcNAc: polypeptide N-acetylglucosaminyl-transferase. Using substrate generated from a 65 kDa cytosolic O-GlcNAc-bearing glycoprotein of human erythrocytes (Holt et al., *J. Biol. Chem.* **262**:14847), we have developed an assay for the enzyme catalyzing the transfer of GlcNAc to hydroxy amino acids at specific sites along the polypeptide backbone. The transferase is inhibited by UDP, but not by GlcNAc or tunicamycin, and has been determined to have an absolute requirement for manganese cation; no other cation reconstitutes activity after treatment with EDTA. Interestingly, the transferase has also been determined to be membrane bound (activity sediments at 160,000 x g), and oriented towards the cytoplasm since it is detectable under conditions in which GlcNAc transferase I activity is latent. Current work is underway to further characterize the peptide:GlcNAc transferase. This work is supported by HD13563 and RSH by an Arthritis Foundation Post-Doctoral Fellowship.

GLYCOSYLATION SITE-SPECIFIC OLIGOSACCHARIDE PROCESSING: THE INFLUENCE OF PROTEIN STRUCTURE. S. C. Hubbard. M.I.T. Cambridge, MA 02139

The Sindbis virus glycoproteins, E1 and E2, were used to evaluate the effects of local protein structure on the processing of N-linked oligosaccharides by Golgi enzymes. The conversion of oligomannose to N-acetyllactosamine (complex) oligosaccharides is hindered to different extents at the four glycosylation sites, so that the complex/oligomannose ratio decreases in the order E1-Asn139 > E2-Asn196 > E1-Asn245 > E2-Asn318. The processing steps most susceptible to interference were deduced from the oligosaccharide compositions at hindered sites in virus from baby hamster kidney cells (BHK), chick embryo fibroblasts, and normal and hamster sarcoma virus-transformed hamster fibroblasts. Persistence of $Man_{6-9}GlcNAc_2$ was taken to indicate interference with α-mannosidase I; $Man_5GlcNAc_2$, with GlcNAc transferase I; and unbisected hybrid glycans, with α-mannosidase II. Taken together, the results indicate that all four sites acquire a precursor oligosaccharide ($Glc_3Man_9GlcNAc_2$) with equally high efficiency, but α-mannosidase I, GlcNAc transferase I and α-mannosidase II are all impeded at E2-Asn318 and, to a lesser extent, at E1-Asn245. Since previous work has indicated that these two sites are less sterically accessible than E2-Asn196 or E1-Asn139, the simplest explanation for the results is that these three enzymes are susceptible to steric hindrance. E1-Asn245 consistently carried an unusually high proportion (1/5 to 1/2 of chains, depending on the host and cellular growth status) of unbisected hybrid glycans, probably because of protein structural hindrance of α-mannosidase II at this site. In contrast to the first three Golgi processing steps, sialic acid and galactose transfer to hybrid glycans (in BHK cells) is virtually quantitative even at E2-Asn318. E2-Asn318 carried no complex oligosaccharides, but the structures of those at E1-Asn245 indicate almost complete GlcNAc transfer by GlcNAc transferase II, sialylation, and galactosylation. Thus, these later steps may be less susceptible to steric hindrance than α-mannosidase I, GlcNAc transferase I and α-mannosidase II. Other factors must also be involved, since the complex glycans at E2-Asn196 are smaller and contain less sialic acid than those at E1-Asn245, although the latter site is less sterically accessible.

ASPARAGINE-LINKED OLIGOSACCHARIDE BIOSYNTHESIS IN SACCHAROMYCES: THE ALG2 MUTATION. B.J. Jackson and P.W. Robbins. MIT Center for Cancer Research, Cambridge, MA 02139

Some intricacies of asparagine-linked glycosylation become clearer through study of yeast alg mutants, isolated by Huffaker and Robbins (1983. PNAS USA 80:7466). Alg2 mutants accumulate $Man_2GlcNAc_2$ and $Man_1GlcNAc_2$ as lipid-linked intermediates at the non-permissive temperature, and transfer these oligosaccharides to protein under conditions where cells are viable (Jackson et. al., submitted). Thus these small oligosaccharides can orient into the lumen of the endoplasmic reticulum in vivo.

Co-reversion of the phenotypes of temperature-sensitivity and defective biosynthesis of lipid-linked oligosaccharides suggests a single defect is responsible for both phenotypes. Three plasmids have been isolated that can correct both defects. Integrational analysis confirmed that the cloned gene is ALG2, the gene that, when defective, produces the phenotypes described. Upon disruption of the coding sequence of ALG2, lethality ensues. This result, in concert with data on phenotypes of mannoprotein mutants (Ballou, 1982. Mol. Biol. of Yeast Saccharomyces, CSH Lab), suggests the metabolic block in synthesis of $Man_3GlcNAc_2$ is the 1,6 mannosyltransferase (rather than the 1,3). DNA sequencing of ALG2 provides some support for ALG2 encoding a mannosyltransferase, and reveals homology between ALG2 and DPM1, the gene encoding dolichylphosphomannose synthase, in the region hypothesized to participate in dolichol binding (Orlean et. al., submitted). Biochemically, plasmids correct the oligosaccharide defect in isolated membranes prepared from plasmid-bearing cells. These data are consistent with the ALG2 gene coding for a 1,6 mannosyltransferase or a positive regulator of that enzyme.

CHROMATOGRAPHIC ANALYSIS OF SUGAR NUCLEOTIDES.

Joseph D. Olechno and Steven R. Carter, Dionex Corp., Sunnyvale, CA 94086

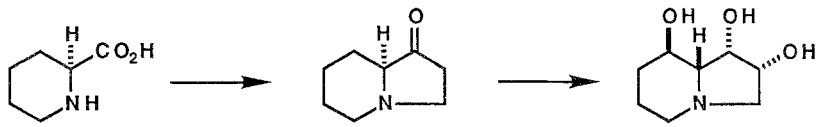
Nucleotide diphosphate sugars (sugar nucleotides) such as uridine diphosphate glucose (UDPG) are important substrates for many glycosyltransferases and are important intermediate metabolites in their own right. Two examples of the latter are the biosynthesis of sialic acid beginning with UDP-N-acetylglucosamine and the epimerization of galactose to glucose through the UDP intermediate. While there has been some success in separating the various nucleotide diphosphate sugars by HPLC, both speed and resolution have been lacking. Since the separations of carbohydrates by pellicular anion exchange resins has been so successful, we have applied this technique to these carbohydrate containing molecules and will show examples of their chromatography under various pH conditions.

While pulsed amperometry has proven very useful for the detection of carbohydrates, the conditions required for detection are such that alkaline hydrolysis of the nucleotide diphosphate sugars may take place during chromatography. Comparisons of UV and PAD detection for sensitivity and linearity will be shown as well as effects of pH on nucleotide diphosphate sugars.

Glycosidase Inhibitors

BIOSYNTHESIS OF SWAINSONINE AND RELATED INDOLIZIDINE ALKALOIDS. Thomas M. Harris, Department of Chemistry and Center in Molecular Toxicology, Vanderbilt University, Nashville, TN 37235.

Swainsonine, a potent inhibitors of α-mannosidases, is a metabolite of both fungi and higher plants. Studies of swainsonine biosynthesis in the fungus *Rhizoctonia leguminicola* have shown that it is derived from pipecolic acid. Condensation with malonate gives 1-oxoindolizidine which undergoes reduction to the alcohol and then hydroxylation at C-2. The resulting diol undergoes further hydroxylation at C-8 and epimerization at C-8a to give swainsonine. The epimerization involves replacement of the 8a proton. No intermediates have been detected in these final steps, but it is postulated that iminium ions are involved. *R. leguminicola* produces a second major indolizidine metabolite slaframine; it also is formed from pipecolate via 1-oxoindolizidine. The routes to swainsonine and slaframine diverge in the reduction of 1-oxoindolizidine, hydride being delivered from the β-face in the first case and the α-face in the second. The biosynthesis of swainsonine in the plant *Astragalus oxyphysus* has also been investigated and found to be qualitatively identical to that in the fungus. Neither slaframine nor indolizidine precursors to it were detected among the metabolites in the plant. All available evidence points to biosynthesis of swainsonine in *A. oxyphysus* involving plant enzymes rather than fungal contamination. [Supported in part by USPHS grants]



L-Pipecolic acid 1-Oxo-indolizidine Swainsonine

SWAINSONINE AUGMENTS HUMAN LYMPHOKINE-ACTIVATED KILLER (LAK) CELL GENERATION Terry L. Bowlin, Mohinder S. Kang, and Prasad S. Sunkara. Merrell Dow Research Institute, Cincinnati, OH 45215

Activation and expansion of functionally mature lymphocyte populations is endogenously regulated by secreted and membrane bound glycoproteins. Interleukin 2 (IL 2) is a secreted glycoprotein which acts as a lymphocyte activation and/or proliferative signal for appropriate target lymphocytes expressing membrane bound glycoprotein IL 2 receptors (IL 2R). T cells, B cells and natural killer cells (lymphokine activated killer, LAK) have been shown to be responsive to IL 2. In this regard, we have recently established that swainsonine (SW), an inhibitor of mannosidase II that is involved in glycoprotein processing, augmented T cell IL 2 production, IL 2R expression, and IL 2 induced proliferation following mitogen stimulation. We now report that SW augments IL 2 induced LAK generation in vitro. Human mononuclear leukocytes (MNL) were incubated with various concentrations of SW (0.01-100 μM) and/or IL 2 (1-100 U/ml) for up to 72 hrs. SW treated MNL exhibited a substantial increase in high-mannose type oligosaccharides based upon [2-^{3}H] mannose labeling, susceptibility to α-mannosidase, and subsequent chromatography on sizing and binding to con-A sepharose columns. Cytolytic activity was assessed against LAK sensitive human myelogenous leukemia cells (K562). At non-saturating concentrations of IL 2 (10 U/ml) SW augmented LAK induction, in a dose-dependent manner, up to two-fold. Cytolytic activity generated in the presence of swainsonine at a low concentration of IL 2 (e.g. 10 U/ml) was similar to that observed with higher concentrations of IL 2 (e.g. 100 U/ml) alone. Furthermore, surface phenotypic analysis, utilizing fluorescent monoclonal antibodies, revealed that pretreatment with SW increased the frequency of IL 2R (CD25) positive lymphocytes, in a dose-dependent manner, greater than two-fold. In contrast, SW treatment did not alter the number of CD2, CD3, CD4, or CD8 positive lymphocytes. Furthermore, MNL pretreated with SW and subsequently restimulated with recombinant IL 2 exhibited a two-fold increase in [^{3}H]-TdR incorporation. These data indicate that SW can augment the frequency and cytolytic activity of human LAK in vitro. Therefore, SW may be useful in lowering the concentrations of IL 2 required for LAK generation in vitro and in vivo.

ELEVATED DOL-PP-OLIGOSACCHARIDES IN NCL FIBROBLASTS TREATED WITH PROCESSING INHIBITORS. P.F.Daniel[1], D.Sauls[1], R-M.Boustany[1], R.Cacan[2] and A.Verbert[2]. [1]E.K.Shriver Center, Waltham, MA 02254; [2]Lab. de Chimie Biologique, USTL, Villeneuve D'Ascq 59655, France.

The Neuronal Ceroid Lipofuscinoses (NCL) are a group of autosomal recessive, neuro-degenerative disorders of unknown etiology, which differ in their age of onset and histopathology of the storage bodies. Patients with juvenile or late infantile NCL (JNCL or LINCL) have been reported to have greatly increased levels of Dol-PP-Oligosaccharides (Dol-PP-OS) in their cerebral gray matter. It is unclear whether this is due to a primary gene defect affecting Dol-PP-OS synthesis or turnover, or whether this is a secondary phenomenon due to an impairment of membrane function. In an attempt to investigate this further we have examined the effects of two inhibitors of glycoprotein processing on the level of Dol-PP-OS in cultured skin fibroblasts from controls and from 2 JNCL and 2 LINCL patients. Cells were cultured for 7 days in the presence of either N-methyl-deoxynojirimycin (MdNM) or deoxymannojirimycin (dMM) or both inhibitors together at a concentration of 1 mM. Dol-PP-OS were extracted with C/M/W 10:10:3, cleaved with mild acid, digested with endo H, reduced, perbenzoylated and the component oligosaccharides analyzed by HPLC. In the absence of inhibitors or in the presence of dMM alone, <0.1 nmol OS/mg protein was detected in control, JNCL or LINCL cells. In the presence of MdNM alone or MdNM + dMM, 2.5, 4.1 and 3.7 nmol total OS/mg were accumulated in control, JNCL and LINCL fibroblasts, respectively. Peaks comigrating with M_4G and M_5G were the major species present, with smaller amounts of M_6G and M_7G.

Short term labeling experiments with [2-^{3}H]mannose indicated a greater incorporation into Dol-PP-OS in both JNCL and LINCL fibroblasts in comparison with controls. It was postulated that this might be caused by a reduced turnover of Dol-PP-OS due to a genetic deficiency of a dolichol-specific phosphodiesterase. However, preliminary experiments have indicated a greater release of OS and OS-phosphate in NCL cells than in the controls. Hence, the cause of the elevated Dol-PP-OS in NCL cells remains obscure.
Supported by grant NS24279 from NINCDS and by CNRS.

SYNTHESIS OF POLYHYDROXYLATED AMINO COMPOUNDS AS GLYCOSIDASE INHIBITORS, George W. J. Fleet, Dyson Perrins Laboratory, Oxford University, South Parks Road, Oxford OX1 3QY, UK

Problems and solutions in the synthesis of hydroxylated piperidines (such as deoxymannojirimycin, fucodeoxynojirimycin and the corresponding lactams), pyrrolidines, octahydroindolizines (6-epicastanospermine and related compounds) and the recently isolated alexines (a novel class of pyrrolizidine) will be discussed.

Some brief comments will be made on structural relationships in regard to the inhibition of glycosidases, including the unpredictable effect of N-alkylation on selectivity of glycosidase inhibition.

EFFECTS OF A LYSOSOMAL β-GALACTOSIDASE INHIBITOR ON POLYLACTOSAMINE CATABOLISM IN CULTURED HUMAN FIBROBLASTS. Larry W. Hancock[1], Probal Banerjee[1], Glyn Dawson[1], and J.N. BeMiller[2].[1]Depts. of Peds. & Biochem., U. of Chicago, IL 60637 and[2]Whistler Ctr. Carb. Res., Purdue U., West Lafayette, IN 47907.

As previously described by others, β-D-galactopyranosylmethyl-p-nitrophenyl triazene (βGalMNT) completely inhibited lysosomal β-galactosidase (β-Gal) activity without effect on other lysosomal enzyme activities. After a single addition of βGalMNT (20 μM) to the culture medium, treated cells recovered 50% of their initial β-Gal activity within 120 h, indicative of the *de novo* synthesis of enzyme; in labeling experiments, βGalMNT was added to the culture medium at 24 h intervals to maintain supression of enzyme activity. To assess the effects of βGalMNT on glycoprotein catabolism, treated and control cells were labeled with [^{3}H]GlcN and the 100,000 X g supernatant fractions were analyzed for the accumulation of labeled storage oligosaccharides on Bio-Gel P-4; oligosaccharides corresponding to storage products derived from typical N-linked structures were seen in treated cells. To analyze βGalMNT effects on glycoprotein-associated polylactosamine chains, the 100,000 X g pellets of [^{3}H]GlcN-labeled cells were digested with Pronase and the resultant glycopeptide (GP) preparations were analyzed on Sephadex G-50. GP's from treated cells showed an elution profile similar to that observed for GP's from control cells, with an enhanced accumulation of labeled GP's in the void volume. When pooled GP's were treated with endo-β-galactosidase (E-β-Gal) and rerun on Sephadex G-50, there was negligible effect on the elution profile of GP's from control cells. In contrast, E-β-Gal digestion of GP's from βGalMNT-treated cells resulted in the generation of significant amounts of low molecular weight oligosaccharides with a concomitant loss of radioactivity from the void volume. These findings could be explained by i) inhibition of glycoprotein-associated polylactosamine catabolism or processing secondary to βGalMNT inhibition of lysosomal β-Gal activity (suggesting a processive mechanism of catabolism or processing for these structures), or by ii) direct inhibition of mammalian E-β-Gal activity by βGalMNT. Further studies, utilizing cell lines which express high proportions of polylactosamine structures or which are deficient in lysosomal β-Gal activity, will distinguish between the possibilities. Supported by USPHS Grants DK-38593 & HD-06426, and the BRF.

A New α-Glucosidase Inhibitor from Astragalus lentiginosus. Irena Pastuszak, Russell J. Molyneux* and Alan D. Elbein. University of Texas Health Science Center, San Antonio, Texas and USDA Labs, Albany, CA.

Methanolic extracts from the leaves of Astragalus lentiginosus contain the indolizidine alkaloid, swainsonine, which is a potent inhibitor of lysosomal α-mannosidase and also of the glycoprotein processing mannosidase II. The isolation of swainsonine from these extracts involves an ion-exchange chromatography on a column of Dowex-50-Nh_4+ and gradient elution with increasing concentrations of NH_4OH. During this procedure, we detected a peak of α-glucosidase-inhibitory activity that was clearly distinct from the swainsonine peak. On silica gel plates run in $CHCl_3$:CH_3OH:NH_4OH:H_2O (175:65:5:6), this inhibitor of α-glucosidase ran considerably faster than swainsonine. This faster-moving band was eluted from the plates and further purified by radial chromatography to where only a single Ehrlich staining spot was observed by TLC. This material was subjected to GLC (as the TMS derivative) and gave two major peaks eluting at 8.65 minutes (19.7% of total) and 9.07 minutes (78.9% of total). Swainsonine elutes from this GLC column at 14.18 minutes and castanospermine at 17 minutes. Mass spectrometry of the material indicated a molecular ion of 157 while NMR analysis suggested that these compounds were indolizidine alkaloids with two hydroxyl groups, probably on the 5-membered ring. Thus, at this stage this material is apparantly a mixture of 2 dihydroxy-indolizidines, differing in their stereochemistry. This mixture was tested against a variety of exoglycosidases to determine its activity. The mixture inhibited amyloglucosidase (an α1,4,α1,4-glucosidase) with a Ki of about $5x10^{-5}$M, whereas castanospermine inhibits with a Ki of about $5x10^{-7}$M. The inhibition of this enzyme was of a competitive nature. On the other hand, the dihydroxyindolizidines did not inhibit β-glucosidase, α or β-mannosidase or α- or β-galactosidase, even at very high concentrations (100-200μg/ml).

Australine, A Pyrrolizidine Alkaloid That Inhibits Amyloglucosidase and Glucosidase I. Joseph E. Tropea, Russell J. Molyneux*, Y.T. Pan, G.P. Kaushal and Alan D. Elbein. University of Texas Health Science Center, San Antonio and *USDA, Albany, CA.

During the isolation of castanospermine from the seeds of Castanospermum australe, we observed the presence of other alkaloids when methanolic extracts were subjected to thin-layer chromatography. By a combination of ion-exchange chromatography and preparative centrifugal thin-layer chromatography, a new alkaloid was purified to homogeniety, and subsequently crystallized from ethanol. Mass spectrometry and NMR indicated that this compound, given the trivial name australine, was a pyrrolizidine alkaloid containing three hydroxyl groups and a hydroxymethyl group. The absolute configuration of this new alkaloid was established as (1R,2R,3R,7S,7aR)-3-hydroxymethyl-1,2,7-trihydroxypyrrolizidine by X-ray diffraction analysis. Due to its polyhydroxylated nature, this alkaloid was tested against a variety of exoglycosidases to determine whether it had any biological activity. Australine proved to be a potent competitive inhibitor of amyloglycosidase (an α1→4 and α1→6 glucosidase) but had no activity against β-glucosidase, α- or β-galactosidase on α- and β-mannosidase. Australine also inhibited the glycoprotein processing enzyme, glucosidase I, but was inactive against glucosidase II. In cell culture, australine blocked glycoprotein processing and caused the accumulation of glycoproteins having a $Glc_3Man_{7-9}(GlcNAc)_2$ oligosaccharide structure. This observation is consistant with an inhibition of glucosidase I. Thus, australine represents the first glycosidase inhibitor with a pyrrolizidine ring structure, and which shows selective inhibition of glucosidase I.

POLYHYDROXY BICYCLIC ALKALOID GLYCOSIDASE INHIBITORS FROM LEGUMINOSAE. RUSSELL J. MOLYNEUX, Alan D. Elbein and Joseph E. Tropea. Western Regional Research Center, Agricultural Research Service, 800 Buchanan St., Albany CA 94710, and University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284.

A number of polyhydroxy-indolizidine, -pyrrolidine, and -piperidine alkaloids have now been isolated from members of the plant family Leguminosae. These alkaloids are potent inhibitors of glycosidases and profoundly influence glycoprotein processing. The alteration of this fundamental biochemical pathway produces a variety of significant effects in animals, plants and insects. Of particular interest are the indolizidine alkaloids swainsonine, an alpha-mannosidase inhibitor which occurs in the locoweeds (Astragalus and Oxytropis spp.), and castanospermine, an alpha- and beta-glucosidase inhibitor which is found in the Moreton Bay chestnut (Castanospermum australe).

Additional inhibitors have now been isolated from C. australe, including 6-epicastanospermine, fagomine, and a novel alkaloid, isomeric with castanospermine, which has been named australine. The latter is a potent and specific inhibitor of amyloglucosidase. This alkaloid possesses a pyrrolizidine ring structure rather than the indolizidine ring system characteristic of castanospermine and other bicyclic alkaloid glycosidase inhibitors. Australine therefore expands the group of known glycosidase inhibitors to a more general class of alkaloids. This information should prove of predictive value in evaluating new naturally-occurring or synthetic alkaloids for useful biochemical and biological properties.

GLYCOSIDASE INHIBITORS AS ANTIVIRALS AGAINST HUMAN IMMUNODIFICIENCY VIRUS (HIV). P.S. Sunkara, P.S. Liu, M.S. Kang, A.S. Tyms*, T.L. Bowlin and A. Sjoerdsma. Merrell Dow Research Institute, 2110 E. Galbraith Road, Cincinnati, OH 45215 and *St. Mary's Hospital and Medical School, London W21PG, U.K.

The objective of the present investigation was to develop potent inhibitors of glycosidases that are involved in glycoprotein processing by mammalian cells, as potential antiretroviral agents. Analogs of the known inhibitor, castanospermine, were synthesized and evaluated for their effect on the glycosidases based on the ability of compounds to accumulate glycoproteins with $G_3M_9(GlcNac)_2$ oligosaccharide in cell cultures. The compounds were further evaluated for their antiretroviral activity against Moloney murine leukemia virus (MoLV) in the XC plaque assay. There was a good correlation between the ability of these compounds to accumulate oligosaccharide precursors and inhibition of MoLV replication. Several 6-O-acyl derivatives of of castanospermine were 10 times as potent against MoLV than castanospermine. These inhibitors were further evaluated for their ability to inhibit HIV induced syncytia formation in CD4 positive HeLa T_4 cells and a T cell line (JM cells). The data indicate that these compounds were at least two to ten fold as active as castanospermine in inhibiting HIV replication. These above results suggest the potential usefulness of these novel glycosidase inhibitors as chemopreventive and therapeutic agents in the treatemnt of Acquired Immune Deficiency Syndrome (AIDS).

Mutants in Glycoconjugate Research

EXPRESSION OF A PHASEOLIN GLYCOSYLATION MUTANT IN TRANSGENIC TOBACCO SEED. L.Griffing, M.Bustos, R.Klassy, T.Hall. Department of Biology, Texas A&M University, College Station, TX 77843.

Site-directed mutagenesis of a gene coding for the β-subunit of phaseolin, the storage protein of the common bean, Phaseolus vulgaris, has been used to assess the role of glycosylation on protein targeting and stability in transgenic tobacco seeds. A two-base pair substitution on the codon for the Asn_{252} residue resulted in the loss of one of two N-linked glycans present in normal phaseolin also expressed in tobacco seeds. During seed maturation, the mutant protein ("dglyl") is less stable to proteolytic degradation than its normal counterpart. The presence of the remaining glycan (Asn_{341}) appears to stabilize a smaller glycopeptide cleavage product. Dglyl phaseolin is targeted to protein bodies and can be localized using immunogold EM in 14-25 DPA tobacco embryos and endosperm. Supported by grants from NSF to L.R.G. and T.C.H.

α-L-FUCOSIDASE IN FUCOSIDOSIS. R.A. DiCioccio[1], J.K. Darby[2], J.S. O'Brien[3], and P.J. Willems[4]. [1]Dept. of Gynecologic Oncology, Roswell Park Memorial Institute, Buffalo, NY, [2]Dept. of Genetics, Stanford University, Stanford, CA, [3]Dept. of Neurosciences, UCSD, LaJolla, CA, [4]Dept. of Medical Genetics, University of Antwerp, Belgium.

Fucosidosis is an autosomal recessive lysosomal storage disease with a deficiency of fucosidase activity in tissues and body fluids. Exponentially growing lymphoid cell cultures from two fucosidosis patients had 2.7-fold and 8.7-fold less extracellular fucosidase protein and 28.8-fold less intracellular fucosidase protein with negligible catalytic activity as compared to the mean of 19 controls. The ratios of extracellular to intracellular fucosidase protein in fucosidosis cultures were 11.6-fold and 3.6-fold more than controls. During a 1.5 h pulse with ^{35}S-methionine, fucosidase was synthesized by fucosidosis and control cells as an intracellular form (Mr = 58,000). During a subsequent 21 h chase with unlabeled methionine, mutant enzyme was almost entirely processed to an extracellular form (Mr = 62,000). In contrast, only 25-30% of control enzyme was processed to an extracellular form (Mr = 62,000) with the remainder retained intracellularly (Mr = 60,000). Intracellular and extracellular enzyme forms apparently were glycoproteins containing polypeptide chains of Mr = 52,000. Newly made intracellular enzyme contained high mannose carbohydrate chains and processed intracellular and extracellular enzyme contained complex carbohydrate chains. The mutation(s) causing the two fucosidosis cases affected the catalytic activity, quantity, and secretion of fucosidase in lymphoid cells. (Supported by DK32161, NSO8682, and HD18983).

EVIDENCE OF GENE AMPLIFICATION IN TUNICAMYCIN-RESISTANT CHINESE HAMSTER OVARY CELLS. J. R. Scocca and S. S. Krag, Department of Biochemistry, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, Md. 21205.

Chinese hamster ovary cells stably resistant to 27 ug/ml of tunicamycin (TM) were selected by culturing wild-type cells (WTE6) in progressively higher increments of tunicamycin. Membranes prepared from the TM-resistant cells have elevated levels of the target enzyme, dolichol-phosphate:UDP-N-acetylglucosamine 1-phosphate transferase activity (Waldman, Oliver, and Krag, J. Cell. Phys. 131, 302-317, 1987). When HindIII restriction digests of genomic DNA from a clonal derivative (3E11) of the population of TM-resistant cells were probed with a 1.8 kb yeast tranferase DNA probe (ALG7), two cross-hybridizing DNA bands were found. Only traces of cross-hybridizing DNA were detected using this probe in genomic DNA prepared from WTE6 cells. Three other restriction enzyme digests of genomic DNA from 3E11 cells gave different patterns of cross-hybridizing DNA but again the level of cross-hybridizing DNA was elevated three to four-fold compared to WTE6. The level of cross hybridizing DNA appeared further elevated over wild-type in 3E11 cells cultured continuously in the presence of TM prior to isolation of the genomic DNA. The amount of DNA on each lane of the nitrocellulose filters was normalized using a probe prepared from the CHO gene for hypoxanthine phosphoribosyl transferase. This data suggests that moderate amplification of the glucosaminyl-1-phosphate transferase gene has occurred in 3E11 cells, consistent with the previously observed chromosomal translocations and a 15-fold increase in enzymatic activity. (Work supported by NIH GM36570).

Membrane Anchors

DEMONSTRATION OF A GLYCOSYL-PHOSPHATIDYLINOSITOL ANCHOR FOR THE I-ANTIGENIC GLYCOPROTEIN OF THYROID CELL SURFACE

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The blood group I-active glycoprotein of calf thyroid plasma membrane (GP-3, M_r=20,000, approximately 80% carbohydrate) contains two sulfated N-linked carbohydrate units with branched, I-antigenic polylactosamine chains capped by α-1,3-linked galactose and α-2,6-linked sialic acid as well as eight O-linked saccharide units ranging in size from mono- to hexasaccharide (JBC 260, 15332-15338, 1985). Deglycosylation of the glycoprotein with trifluoromethanesulfonic acid, which resulted in the removal of 92% of the carbohydrate, yielded a peptide (M_r=4,400) of about 40 amino acids. This would indicate that the ten saccharide chains are distributed along a protein core of limited size, leaving little peptide available for interaction with a lipid bilayer.

Despite its dense glycosylation, GP-3 was found to extract into the detergent phase upon Triton X-114 partitioning consistent with its membrane localization. Digestion with phosphatidylinositol specific phospholipase C (PI-PLC) converted the glycoprotein into an aqueous-soluble form with a markedly slower migration upon SDS-polyacrylamide gel electrophoresis. Nitrous acid treatment specifically cleaved GP-3 at a GlcN residue which was recovered as anhydromannitol after $NaBT_4$ reduction and acid hydrolysis; moreover, this treatment, like the PI-PLC digestion, delipidated the glycoprotein. Surprisingly, N-acetylation of GlcN, which abolished its sensitivity to nitrous acid, also prevented cleavage of the lipid from the glycoprotein by PI-PLC; in contrast reductive N-methylation had no effect on enzyme susceptibility, suggesting the importance of a positive charge on GlcN. The PI-PLC treated glycoprotein was unreactive with an antibody (anti-CRD) against the glycan component of trypanosome variant surface glycoprotein (VSG). The GP-3 glycan, after radiolabeling by nitrous acid-$NaBT_4$ treatment, could be liberated from the protein by hydrazinolysis; it displayed an apparent M_r=1,600 on Bio-Gel P-4 and did not bind to Bandeiraea simplicifolia I-agarose indicating that it lacked α-linked galactose. The data suggest that the thyroid I-active glycoprotein is anchored to the cell surface via a glycosyl-PI containing a non-N-acetylated glucosamine residue which is required for PI-PLC cleavage; the glycan appears to have a distinct structure from that of VSG.

BIOSYNTHESIS OF GLYCOSYL-PHOSPHATIDYL-INOSITOL MEMBRANE ANCHORS

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In recent years, many membrane glycoproteins have been found to be anchored by glycosyl-phosphatidylinositol lipids at their C-termini (***Ann. Rev. Biochem. 57, 285-320***). The abundance and ease of isolation of the variant surface glycoproteins (VSGs) of African trypanosomes has made them the best models for studying the biochemistry of these lipid anchors. The apparent addition of the lipid anchor within 1-minute of VSG synthesis (***PNAS 82, 3207-3211***) led us to postulate the existence of a pre-assembled anchor precursor that is transferred *en bloc* to nascent VSG. Such a glycosyl-phosphatidylinositol anchor biosynthetic precursor was subsequently identified and characterized (***J. Biol. Chem. 261, 12147-12153***).

Using pulse-chase radiolabelling with [^{3}H]ethanolamine and [^{3}H]mannose, as well as degradation by HNO_2 and PI-PLCs, we have identified a putative biosynthetic precursor for the membrane anchor of the murine lymphocyte THY 1.2 antigen. In other studies, trypanosomes have been used to develop a cell-free system to study the pathway of anchor biosynthesis. This system efficiently incorporates both sugar precursors and lipid precursors into VSG's anchor, the previously identified anchor precursor, and numerous other intermediates. We are also purifying the galactosyltransferase that adds α-linked galactosyl residues to VSG's anchor. The VSG system is ideally suited for studying the biosynthesis and attachment of these important molecules. (Supported by NIH AI21334 and the MacArthur Fdn.)

THE GLYCOSYL-PHOSPHATIDYLINOSITOL ANCHOR OF CELL SURFACE PROTEINS.
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A diverse group of cell surface proteins are anchored to the membrane by covalent linkage to a single phosphatidylinositol molecule located in the lipid bilayer. The phosphatidylinositol and the protein are connected by a novel glycan which is amide-linked to the alpha-carboxyl of the C-terminal amino acid through a phosphoethanolamine residue. The reducing end of this glycan is a glucosamine which is glycosidically linked to the inositol ring of the phosphatidylinositol moiety. The detailed structures of the glycans are known for relatively few proteins but biosynthetic labelling and chemical and enzymic degradation studies suggest that many features of the glycosyl-phosphatidylinositol (GPI) anchors have been conserved during evolution.

The full physiological role played by the GPI anchors is uncertain but it is clear that they are crucial for membrane attachment of the protein since degradation of the GPI-anchor by bacterial phosphatidylinositol-specific phospholipases C (PI-PLC) will release the proteins from the membrane. GPI-anchor degrading enzymes have also been identified in eukaryotic organisms. One of these enzymes is a phospholipase C (GPI-PLC) and is membrane associated whereas a phospholipase D (GPI-PLD) has been found in the plasma of several mammalian species. In contrast to the bacterial PI-PLC these eukaryotic anchor-degrading enzymes are unable to hydrolyse other phospholipids including phosphatidylinositol. However the GPI-PLC and GPI-PLD are able to act on a wide variety of GPI-anchored proteins. This suggests that structural features of the glycan may play an important role in determining the ability of the GPI-PLC and GPI-PLD to hydrolyse the anchors. The restricted substrate specificity of these phospholipases also suggests that they may be involved in the release of GPI-anchored proteins from the cell surface in response to physiological stimuli.

A UBIQUITOUS FAMILY OF CELL MEMBRANE INTERCALATED HEPARAN SULFATE PROTEOGLYCANS
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It is widely believed that there is a family of heparan sulfate proteoglycans (HSPG) characterized by a core protein containing a highly hydrophobic domain inserted into the cell membrane. However, evidence for intercalation has to date been circumstantial, i.e. detergent extraction, hydrophobicity, and micellar insertion. We have isolated an HSPG from a wide variety of cells which share many characteristic features: a core protein of ~80 kD substituted with 3 heparan sulfate chains (~30 kD), 2-3 N-linked oligosaccharides, and 10-20 O-linked oligosaccharides. This HSPG is highly hydrophobic (as assessed by elution from octyl-Sepharose) and is tightly associated with the cell membrane (requiring high detergent concentrations for efficient extraction). To determine whether this class of HSPG is truly intercalated into the cell membrane we isotopically labeled cultures of osteoblast-like cells (UMR 106-01), parathyroid cells, and ovarian granulosa cells with ^{35}S-sulfate and ^{3}H-leucine precursors. Membrane preparations were isolated from these cultures which were then incubated with a highly hydrophobic iodinated photoactivatable carbene precursor ([^{125}I]TID) under conditions which have previously been shown to label the amino acids in proteins which span the plasma membrane. Membranes were extracted with 8 M urea containing 2% triton X-100 and the proteoglycan fraction isolated by anion exchange chromatography. Following further purification, an HSPG pool was prepared which was subjected to both gel filtration chromatography and SDS-PAGE with and without prior treatment with heparitinase. The data clearly argue that the HSPG has been labeled with the [^{125}I]TID and that the iodine label co-migrates with the ^{3}H-label in the core protein.

STRUCTURE OF THE GLYCOINOSITOL PHOSPHOLIPID ANCHOR ATTACHED TO HUMAN ERYTHROYCTE ACETYLCHOLINESTERASE. W. L. Roberts, S. Santikarn, V. N. Reinhold, J. J. Myher, A. Kuksis, and T. L. Rosenberry. Case Western Reserve University, Cleveland, OH 44106, Harvard University, Boston MA 02115 and University of Toronto, Toronto, Ontario M5G 1L6.

Acetylcholinesterase (AChE) is anchored on the extracellular face of human erythroyctes (E^{hu}) exclusively by a covalently linked glycoinositol phospholipid. Fragments of the anchor were obtained after Pronase digestion and base hydrolysis or after nitrous acid deamination. Fast atom bombardment mass spectrometry of these fragments revealed a structure that shares several features with the anchor of variant surface glycoproteins from trypanosomes. Both anchors contain a similar backbone of three linear mannose residues with a phosphodiester linkage at their nonreducing terminus to an ethanolamine in amide linkage to the C-terminal amino acid. A glucosamine with a free amino group at their reducing terminus is linked to an inositol phospholipid. Unique features of the E^{hu} AChE anchor include an additional phosphoethanolamine branching from the mannose distal to the C-terminus and the composition of its inositol phospholipid. This phospholipid includes an alkylacylglycerol, 83% of which contains an 18:0 or 18:1 1-alkyl group and a 22:4, 22:5 or 22::6 2-acyl group, and an unusual direct acylation of an inositol group with palmitic acid (16:0). Many proteins with glycoinositol phospholipid anchors are selectively released from cell membranes by phosphatidylinositol-specific phospholipase C (PIPLC). However, in some cells, particularly E^{hu}, this release is only partial. For example, PIPLC released only about 5% of the AChE from E^{hu}. Selective hydrolysis of the E^{hu} AChE inositol phospholipid revealed that PIPLC resistance arose entirely from the palmitoyl group on the inositol. Removal of this fatty acid with NH_3-saturated methanol generated an inositol phospholipid which was cleaved with PIPLC to produce an alkylacylglycerol product. In contrast, the inositol palmitoylation did not prevent anchor-specific phospholipase D in mammalian sera from cleaving the E^{hu} AChE anchor.

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THE VARIANT SURFACE GLYCOPROTEIN OF TRYPANOSOMA BRUCEI: INTERMEDIATES IN THE BIOSYNTHESIS OF THE GLYCOLIPID ANCHOR

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A novel class of membrane proteins, characterized by covalently-attached glycosyl-phosphatidylinositol anchors, has been identified in recent years. The anchors are probably synthesized *in vivo* as precursor glycolipid molecules, to which newly synthesized proteins are transferred. The biosynthetic pathway of these events is yet to be elucidated.

The variant surface glycoprotein (VSG) of *Trypanosoma brucei* is the best characterized example of this protein class. We have developed a cell-free system to investigate the biosynthesis of its glycolipid anchor (W.J. Masterson *et al*, manuscript submitted). This system works effectively to incorporate radioactive sugars and lipids into several glycolipid species, including the putative anchor precursor, as well as into the VSG.

We are currently employing this system to study anchor biosynthesis by generating radioactively labeled intermediate species. These moieties have been isolated and characterized with respect to their glycosidase, lipase, and nitrous acid susceptibility and their behavior on gel filtration and anion exchange (Dionex) chromatography. Through these and kinetic studies, we hope to establish an overview of the glycolipid anchor biosynthetic pathway. (Supported by NIH AI21334 and the MacArthur Foundation.)

Carbohydrate-binding Proteins

CARBOHYDRATE-MEDIATED ADHERENCE OF *FUSOBACTERIUM NUCLEATUM*. A. PRAKOBPHOL, B. GILLECE-CASTRO, CUI TIAN-YI, H. LEFFLER, A. BURLINGAME and S. FISHER (University of California, San Francisco, CA 94143).

A proline-rich glycoprotein (PRG) is the major constituent of human parotid saliva. In previous studies [Anal. Biochem. (1987) 164, 5-11] we showed that this glycoprotein can serve as a receptor for the gram-negative pathogen *Fusobacterium nucleatum*. Recently, we demonstrated that this receptor activity was a function of the N-linked oligosaccharides carried by this glycoprotein; removal of these oligosaccharides by PNGase digestion abolished receptor activity. Experiments using an overlay technique to screen a standard glycolipid library showed that the highest affinity receptor sites for *F. nucleatum* were sugar termini containing Galß1->4 GlcNAc and Galß1->3 GalNAc. Unexpectedly, the addition of fucose to these termini abolished receptor activity in all cases. To better understand the structural requirements for adherence of this bacterium to a potential biological receptor, we investigated the carbohydrate structures carried by the major oligosaccharides of PRG. The sugar composition of the pooled oligosaccharide fraction was determined by gas-liquid chromatography. Based on three mannose residues, the molar ratios of fucose, galactose, N-acetylglucosamine and N-acetyl neuraminic acid were 5.2, 2.6, 3.5 and 0.4, respectively. The reducing termini of these oligosaccharides were derivatized using the UV-absorbing moiety, octyl *p*-amino benzoate and the products were separated by HPLC. Using liquid secondary ion mass spectrometry, we determined a partial sequence from the non-reducing terminus of the oligosaccharides comprising each of the resulting fractions. Both biantennary and triantennary structures were present, many of which had fucose termini. These results suggest that *F. nucleatum* adheres to galactose-containing receptors and that this activity is inhibited by the presence of fucose. Since both fucose and galactose are major components of the PRG from human parotid saliva, the mechanisms identified in the screening assays appear to be of potential biological significance. (This study was supported by USPHS Grants RR-01614 and DE-07244)

SIALIC ACID-DEPENDENT ADHESION OF MYCOPLASMA PNEUMONIAE TO PURIFIED GLYCOPROTEINS. David D. Roberts, Lynn Olson*, Michael F. Barile*, Victor Ginsburg, and Howard C. Krivan. Laboratory of Structural Biology, NIDDK, NIH; *Mycoplasma Laboratory, Center for Drugs and Biologicals, FDA, Bethesda, MD 20892.

Several purified glycoproteins including laminin, fetuin, and human chorionic gonadotropin promote dose-dependent and saturable adhesion of Mycoplasma pneumoniae when adsorbed on plastic. Adhesion to the proteins is energy dependent as no attachment occurs in media without glucose. Adhesion to all of the proteins requires sialic acid, and only those proteins with α2-3-linked sialic acid are active. The α-subunit of human chorionic gonadotropin also promotes attachment, suggesting that a simple biantennary asparagine-linked oligosaccharide is sufficient for binding. Soluble laminin and 3'-sialyllactose but not 6'-sialyllactose inhibit attachment of M. pneumoniae to laminin. M. pneumoniae also adhere to sulfatides adsorbed on plastic. Dextran sulfate, which inhibits M. pneumoniae attachment on sulfatide, does not inhibit attachment on laminin and 3'-sialyllactose does not inhibit attachment on sulfatide, suggesting that two distinct receptor specificities mediate binding to these two carbohydrate receptors. Both 3'-sialyllactose and dextran sulfate partially inhibit M. pneumoniae adhesion to a human colon adenocarcinoma cell line (WiDr) at concentrations which completely inhibit binding to laminin or sulfatide, and in combination they inhibit binding of M. pneumoniae to these cells by 90%. Thus, both receptor specificities contribute to M. pneumoniae adhesion to cultured human cells.

BACTERIAL ADHESION: IDENTIFICATION OF GLYCOLIPID RECEPTORS FOR MANY PULMONARY PATHOGENS. Howard C. Krivan Laboratory of Structural Biology, NIDDK, NIH, Bethesda, MD 20893

Bacteria adhere with often exquisite specificity to epithelial cell surfaces, which is an important initial step in the pathogenesis of infectious disease. Previous data have shown that Pseudomonas bacteria isolated from patients with cystic fibrosis bind specifically to asialo GM1 and asialo GM2 by recognizing at least the GalNAcβ1-4Gal sequence in these glycolipids (1). To further examine the possible role of carbohydrates as adhesion receptors, a variety of gram-positive and gram-negative pulmonary pathogens were tested for binding to glycolipids. The classic triad of infectious bacteria found in cystic fibrosis, Pseudomonas aeruginosa, Haemophilus influenzae, and Staphylococcus aureus, along with other bacteria commonly implicated in typical pneumonia, such as Streptococcus pneumoniae and certain Escherichia coli, bind specifically to asialo Cad (GalNAcβ1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer), fucosylasialo GM1 (Fucα1-2Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer), asialo GM1 (Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer), and asialo GM2 (GalNAcβ1-4Galβ1-4Glcβ1-1Cer) (2). They do not bind to a large number of reference glycolipids tested, including the gangliosides GM1, GM2, GM3, GD1a, GD1b, GT1b, and Cad. These data suggest that these bacteria require at least terminal or internal GalNAcβ1-4Gal sequences unsubstituted with sialyl residues for binding. Another lung pathogen, Mycoplasma pneumoniae, does not bind to glycolipids containing GalNAcβ1-4Gal sequences, but instead recognizes sulfated glycolipids (3). The specific binding of M. pneumoniae to sulfatides is both energy and temperature dependent, and is markedly inhibited by dextran sulfate. Asialo GM1 and sulfatide were found in human lung and trachea and are possible adhesion receptors for infection.

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CELL-SURFACE ANAPLASMA MARGINALE PROTEINS AND INTERACTION WITH BOVINE ERYTHROCYTES

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The rickettsia Anaplasma marginale, is the causative agent of an enzootic hemoparasitic disease that affects cattle in tropical and subtropical regions of the world. Little is known about the molecular events associated with the specific interaction of A. marginale and its target cell, the bovine erythrocyte. Preliminary studies on the specific A. marginale proteins involved in the invasion of red cells revealed five to seven Anaplasma proteins able to bind either to erythrocytes or their ghosts. These polypeptides include those with estimated molecular weights of 105, 91, 61, 38 and 29 kD. Among them, the 91 kD polypeptide, a major protein in the parasite, showed a host-specific ability to bind to bovine but not human erythrocytes. Oligosaccharide fractions isolated from bovine red blood cell-surface glycoproteins blocked the binding of the Anaplasma proteins to their target cells. The effect was observed at concentrations as low as 10 nM, suggesting that the parasite proteins have a marked affinity for red cell surface carbohydrates. The erythrocyte-binding proteins may represent the A. marginale receptors involved in early steps that take place during invasion of red cells. A specific group of carbohydrates may be acting as Anaplasma target ligands at the erythrocyte surface.

A METHOD FOR EFFICIENT ATTACHMENT OF A GLYCOPEPTIDE TO PROTEINS.

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A neoglycoprotein that carries an oligosaccharide of known, defined structure is a useful tool in the studies of carbohydrate-binding systems. We prepared a linking compound which can attach a glycopeptide (or other amino-containing ligand) efficiently to proteins. The compound, which has a masked aldehydo group at one end and a hydrazide group at the other end, can be prepared in two steps as shown below.

$$(MeO)_2CHCH_2NH_2 + HOOC(CH_2)_4COOMe \xrightarrow{EDAC} (MeO)_2CHCH_2NHCO(CH_2)_4COOMe$$

$$\xrightarrow{NH_2NH_2} (MeO)_2CHCH_2NHCO(CH_2)_4CONHNH_2$$

The linking compound was converted efficiently to azide using N_2O_4 under anhydrous conditions, and then reacted with a glycopeptide at pH 8.5. The derivative is purified by gel filtration, and then converted to aldehydo form by mild acid hydrolysis in 50% trifluoroacetic acid at room temperature. After removal of trifluoroacetic acid, the product was coupled to protein by reductive alkylation at neutral pH, using pyridine borane as reducing agent.

When the protein amino group was in large excess (> 6 fold) over the aldehydo-glycopeptide, the glycopeptide was incorporated into bovine serum albumin with efficiency higher than 83%.

ABUNDANT SOLUBLE LACTOSE BINDING LECTINS OF RAT AND MOUSE INTESTINE.

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The intestinal mucosa is rich in glycoconjugates. The chick intestinal mucosa contains an abundant lactose binding lectin which has been shown to interact with glycoconjugates in that tissue (1). Here we report studies of several lactose binding lectins in rat and mouse intestinal mucosa that could also interact with endogenous glycoconjugates.

The lectins were isolated by extraction with lactose followed by affinity chromatography on lactosyl-Sepharose and anion and cation exchange chromatography. Together they constitute at least 1% of the total soluble protein. We have studied their chemical properties, amino acid sequence (including use of tandem mass spectrometry), carbohydrate specificity, immunological cross reactivity with other lactose binding lectins and, for some, localisation by immunohistochemistry.

Lectins of subunit molecular weight of about 14 kD from both rat and mouse intestine had properties like those of similar molecular weight from other rat tissues, such as lung, and were found primarily in the non-epithelial part of the intestine. The other intestinal lectins had subunit molecular weights of 17-20 kD and pIs of between 7 and more than 9 and were different from all other known lactose binding lectins. Some 17 kD lectins were related immunologically, by carbohydrate specificity (2) and/or by amino acid sequence to the rat lung lectin RL29 (3) and the mouse lectin CBP35 (4). Immunohistochemistry using anti-RL29 showed that these are abundant in the epithelial cells of small and large intestine.

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BIOCHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION OF K-1735P MELANOMA GALACTOSIDE-BINDING LECTINS AND THEIR MODULATION BY DIFFERENTIATION INDUCERS*

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Endogenous carbohydrate-binding proteins (lectins) have been found in a variety of normal cells and tissues and their expression was found to be developmentally regulated. We have demonstrated that many different tumor cells contain galactoside-specific lectins and that the expression of the lectins is correlated with the transformed and the metastatic phenotype. In the present study we have purified lectins from melanoma cells, compared them to those from normal cells and studied their modulation after differentiation. Lectins purified by affinity chromatography on immobilized asialofetuin from extracts of the UV-induced mouse K-1735P melanoma cells appeared as two polypeptides [L-14.5 (14.5 kDa) and L-34 (34 kDa)] in one-dimensional polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulfate (SDS). However, in two-dimensional electrophoresis (isoelectric focusing followed by SDS:PAGE) the L-14.5 polypeptide was resolved into three acidic forms of pI 4.6, 4.9, and 5.8; whereas the L-34 was resolved into two polypeptides of pI 4.9, and 5.3. Antibodies directed against galactoside-binding lectins from rat and bovine lungs, mouse 3T3 fibroblasts, and mouse UV-2237 fibrosarcoma cells reacted with the K-1735P lectins in immunoblots and normal mouse lung extracts were found to contain cross-reactive proteins that co-migrated with the two melanoma lectins. Indirect immunofluorescence staining using the above antibodies demonstrated that both L-14.5 and L-34 were expressed on the surface of viable K-1735P cells. Treatment of these cells with 1 μM β-all trans retinoic acid or 1 mM dibutyryl cAMP for 5 days induced morphological differentiation, inhibition of anchorage-dependent and anchorage-independent growths, and a selective decrease in the L-34 lectin level. Growth inhibition by starvation for serum factors, which did not induce differentiation, had no effect on the level of L-34. Retinoic acid treatment of other murine melanoma cells such as B16-F1 and B16-F10, which suppresses their transformed phenotype and enhances morphological differentiation also decreased selectively the expression of L-34 without altering the level of L-14.5 lectin. These results demonstrate that the melanoma lectins are immunologically related to normal cell lectins and that the two polypeptide species are expressed on the cell surface. Further, they demonstrate that the L-34 lectin level can be modulated by agents that enhance differentiation.

ANTI-3-FUCOSYLLACTOSAMINE (3-FL) ANTIBODIES: STRUCTURE-FUNCTION RELATIONSHIPS. Donald M. Marcus and Hirohisa Kimura. Departments of Medicine, Microbiology and Immunology, Baylor College of Medicine, Houston, TX 77030

We are studying the structure and regulation of murine and human antibodies (Abs) against the 3-FL antigenic determinant (also known as Le^x or X). On the basis of Southern filter hybridization and partial sequences of 3 monoclonal antibodies (mAbs) against 3-FL, we suggested previously that there was restricted gene usage by these Abs. We have recently obtained complete sequences of the heavy and light chains of five more anti-3-FL Abs and extensive data on three other Abs. The heavy chains of all eight Abs are encoded by V_H441 of the X24 V_H family, 7 of the mAbs use the J_H4 segment and 1 uses J_H3. All of the light chains are encoded by V_K24B and J_K1. The restricted use of gene segments by these Abs is in contrast to the use of multiple V_H and V_L segments by murine Abs against (α1-6) dextran and group A streptococcal carbohydrate. The V_H441 gene segment is also used to encode Abs against levan and galactan, and the amino acid sequences of the V_H regions of these three families of Abs may be virtually identical. As illustrated in the Table below, however, the CDR3 regions of the Abs are quite different and apparently play a major role in determining the specificity and affinity of the Abs. We are currently undertaking site-directed mutagenesis to test this hypothesis.

	CDR3								
	95	96	97	98	99	100	100A	101	102
Anti-3-FL									
PM81	Q	L	G	E	N	A	M	D	Y
Anti-Galactan									
X44	L	H	Y	Y	G	Y	A	A	-
Anti-Levan									
UPC 10	N	W	D	V	G		F	-	-

ROLE OF SULFATION AND STRUCTURE IN INTERACTIONS OF FUCOIDAN WITH THROMBOSPONDIN. Lijuan Zhang, Victor Ginsburg, and David Roberts, Laboratory of Structural Biology, NIDDK, NIH, Bethesda, MD 20892.

Fucoidan, a sulfated homofucan [extracted from F. vesiculocus] containing 35% sulfate by weight, strongly inhibits the binding of thrombospondin to sulfatide with 50% inhibition at 0.2 µg/ml. It is much more inhibitory than other sulfated polysaccharides with similar sulfate contents. By ion exchange chromatography on DEAE-sephacel eluted with a linear gradient of sodium chloride (0-3 M) in 0.1 M HEPES, fucoiden was fractionated into 9 components. The fractions had different contents of sulfate ranging from 20% to 45%. Their ability to inhibit thrombospondin binding to sulfatide varied directly with sulfate content with 0.1 µg/ml to 0.1 mg/ml required for 50% inhibition by the fractions with highest and lowest sulfate contents, respectively. The molecular weights of each fraction as estimated by gel filtration on Sephacryl S-400 were the same as unfractionated fucoidan. After desulfation in dimethyl sulfoxide, fucoidan no longer inhibited. Resulfation using pyridine .SO_3 complex in dimethyl sulfoxide restored some inhibitory activity but at comparable sulfate contents the resulfated fucoidan was less active than native fucoidan. Partial acid hydrolysis did not yield any fragments of fucoidan with inhibitory activity, but sulfate was partially lost under the conditions used. Synthetic L-fucose sulfate, which contains 50% sulfate (1.7 equivalent/mole), inhibited thrombospondin binding by 50% at 24 µg/ml, and is 50 times more inhibitory than other hexose sulfates tested. Thus, the activity of fucoidan depends strongly on its sulfate content and the thrombospondin binding site probably interacts with more than one sulfate ester. The activity of fucose sulfates as inhibitors, however, suggests that specific interactions with fucose may contribute to the strong inhibitory activity of fucoidan.

INTERACTIONS OF CONCANAVALIN A WITH ASPARAGINE-LINKED GLYCOPEPTIDES. FORMATION OF HOMOGENEOUS CROSS-LINKED LATTICES IN MIXED PRECIPITATION SYSTEMS. C.F. Brewer, L. Bhattacharyya and M.I. Khan. Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461.

We have previously shown that certain oligomannose and bisected hybrid type glycopeptides are bivalent for binding to the D-mannose/D-glucose specific lectin concanavalin A (Con A) (Bhattacharyya, L., Ceccarini, C., Lorenzoni, P., and Brewer, C.F. (1987) J. Biol. Chem. 262, 1288-1293). Each glycopeptide gives a quantitative precipitation profile with the protein which consist of a single peak that corresponds to the binding stoichiometry of glycopeptide to protein monomer (1:2). We have shown that the affinities of the primary and secondary sites of the glycopeptides influence their extent of precipitation with the lectin (Bhattacharyya and Brewer, submitted). In the present study, we demonstrate that equimolar mixtures of any two of the glycopeptides results in a quantitative precipitation profile which shows two protein peaks. Using radiolabeled glycopeptides, the precipitation profiles of the individual glycopeptides were determined. The results show that each glycopeptide forms its own precipitation profile with the protein which is independent of the profile of the other glycopeptide. The relative precipitation maxima of the glycopeptides is determined by mass-action equilibria involving competitive binding of the two carbohydrates to the protein. These equilibria, in turn, are sensitive to the relative amounts and affinities of the carbohydrates at both their primary and secondary sites. These findings indicate that each glycopeptide forms a unique homogeneous cross-linked lattice with the lectin which excludes the lattice of another glycopeptide in a mixture. These results have important implications for the structure-function properties of asparagine-linked carbohydrates and lectins, as well as other multivalent binding systems.

FLOW CYTOMETRIC ANALYSIS OF HUMAN ERYTHROCYTES PROBED WITH FITC-LABELLED LECTINS AND IMMUNOLOGLOBULINS. Karol A. Gutowski, Diane E. Brede, Jerry L. Hudson, and David Aminoff, Institute of Gerontology and the Department of Pathology, The University of Michigan, Ann Arbor, Michigan 48109 U.S.A.

Glycophorin-like sialoglycoproteins are present on the RBC of many mammalian species. In humans, glycophorin is responsible for the blood groups M and N specificities. Data from our laboratories, and that of others implicate glycophorin, as the responsible molecule that determines the life span of RBC in circulation. Flow cytometric procedures (FC) have been used extensively in immunology and oncology to follow changes in cell surface markers and in functional parameters of many normal and malignant cells, but so far have had but limited applications to RBC. We believe that FC would represent a valuable tool to explore the dynamics of physiological and pathological changes in both the structural and functional properties of RBC, and hereby report on some of our initial findings using fluorescently labelled lectins and immunoglobulins. Fluorescein isothiocyante labelled wheat germ, elderberry and peanut agglutinins as well as goat-anti-human IgG and IgM were used for these studies: FITC-WGA, -EBA, -PNA, -GAH-IgG, -GAH-IgM, respectively. The following tentative conclusions can be drawn from our preliminary results: 1. The time-dependent change in size and shape of RBC can be monitored, and serve as a time scale to compare structural and functional changes in RBC with their age in vivo, 2. There is a gradual decrease in sialic acid content of RBC with decreasing size (increasing age), and a very rapid decrease at the "twilight zone" just prior to the sequestration of RBC from circulation. This is more effectively demonstrated with EBA than with WGA. PNA does not react with RBC, 3. There is a greater susceptibility of the smaller RBC to sialidase treatment, 4. There is a low level reactivity of RBC of all ages with both autoimmune IgG and IgM, with a greatly enhanced reactivity with the small cells in the "twilight zone," 5. Both the bound IgG and IgM autoimmune antibodies of the smallest RBC are effectively eluted with beta-methyl, but not alpha-methyl-galatoside.

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STUDIES ON THE EFFECT OF KETOCONAZOLE ON FUSION OF RAT L_6-MYOBLASTS. S.Wayne, J.C.Jamieson, M.A. Spearman and J.A.Wright, Department of Chemistry and Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Canada, R3T 2N2.

Previous studies have shown that fusion of rat L_6 myoblasts is dependent on the presence of $GlcNAc_2Man_{8-9}$ oligosaccharides on cell surface fusogenic glycoproteins (Spearman, Jamieson and Wright, (1987) Exptl Cell Res. *168*, 116-126). Since high mannose containing oligosaccharides utilize dolichol phosphate mannose as mannose donor, inhibition of synthesis of dolichol phosphate would be expected to affect lipid-linked intermediates of glycoprotein biosynthesis. Ketoconazole acts as a lanosterol demethylase and cytochrome P450 inhibitor; P450 is needed for the synthesis of oxysterol intermediates which control HMGCoA reductase activities (Gupta,*et al.* J.Biol.Chem. *261*, 8348-8356). Because of its inhibitory effect on HMGCoA reductase, ketoconazole would be expected to act as an inhibitor of synthesis of dolichol phosphate and glycoprotein biosynthesis. We have tested this compound on glycoprotein biosynthesis in myoblasts, particularly as it affects the fusion reaction. Fusion of myoblasts was very sensitive to ketoconazole with concentrations as low as 2μM inhibiting fusion by 90%. Binding of concanavalin A and wheat germ agglutinin was significantly reduced in cells treated with ketoconazole indicating a reduction in cell surface N-linked glycoproteins. Inhibition of fusion was accompanied by a reduction in the creatine phosphokinase activity of the cells showing that biochemical differentiation is also affected. Incorporation of labelled mannose from GDP-mannose into lipid-sugar and lipid-oligosaccharide was also reduced in presence of ketoconazole. Reversal experiments showed that cells would resume to fuse when ketoconazole was removed; in addition, concanavalin A and wheat germ agglutinin binding, creatine phosphokinase activities and incorporation of mannose from GDP mannose returned to normal showing that inhibition of fusion was not the result of cell damage. Experiments in which medium was supplemented with cholesterol, dolichol phosphate and mevalonate were also carried out. The results suggest that ketoconazole has a significant inhibitory effect on myoblast fusion because of its effect on cell surface glycoprotein biosynthesis most likely by interfering with the synthesis of dolichol phosphate.

Supported by M.R.C. Canada.

EVIDENCE FOR THE ROLE OF GLYCOPRHORIN IN AGING AND SEQUESTRATION OF MAMMALIAN ERYTHROCYTES. **David Aminoff: Institute of Gerontology and Department of Biological Chemistry, The University of Michigan, Ann Arbor, MI, 48109.**

Red blood cells (RBC) have a definite life span in circulation. Data emanating from our laboratory and those of others ~~to~~ implicate a significant role to the physiological desialation of glycophorin. The evidence in support of this hypothesis is outlined below and will be discussed in detail at the meetings.
1) Glycophorin-like molecules have been demonstrated on RBC surface of most mammalian species, 2) Careful chemical analysis of the carbohydrate moiety of glycophorin has demonstrated the preponderance of O-glycosyl tetrasaccharides with small amounts of the two isoforms of the trisaccharide; there is little or no evidence for the presence of dis--accharide or monosaccharide chains, 3) We have demonstrated the presence of more galactose oxidase and sialyl transferase (GOST) reactive sites on senescent, as compared to young, RBC, 4) The structures of the oligosaccharide chains in glycophorin, suggested a specific procedure to isolate the GOST sites as senescence factor glycopeptides SFG(s) from senescent RBC, 5) These SFG(s) are recognized by spleen monocytes, and this recognition is destroyed by treatment with beta-galactosidase, 6) SFG(s) are very potent inhibitors of autologous erythrophagocytosis, inhibiting at nanomolar concentrations, 7) Treatment of rat RBC with various glycosidases has demonstrated the potential of: (a) "Artificial Aging" of RBC by treatment with sialidase, (b) "Rejuvenation" of the artificially aged RBC by the appropriate enzymatic removal of either the terminal beta-Gal, or the Gal-beta(1-3)GalNAc residues, 8) Flow cytometric analysis of RBC with fluorescently tagged lectins and goat anti-human IgG and IgM have indicated (a) a gradual decrease in sialic acid content of RBC with decreasing size (increasing age) of cells and a very rapid decrease at the "twilight zone" just prior to the sequestration of RBC from circulation, (b) greater susceptibility of the smaller RBC to sialidase treatment, (c) low level reactivity of RBC of all ages with both autoimmune IgG and IgM, with a greatly enhanced reactivity of the small cells in the "twilight zone," and (d) both the IgG and IgM autoimmune reactivity of the smallest RBC are effectively eluted with beta-methyl, but not with alpha-methyl galactoside.

Supported by a grant HL AM 17881 from the National Institutes of Health.

ISOLATION AND CHARACTERIZATION OF A SECOND LECTIN FROM ELDERBERRY (SAMBUCUS NIGRA L.) BARK. Hanae Kaku, Irwin J. Goldstein and Willy Peumans. Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109, and Lab. voor Plantenbiochemie, W. de Croylaan 42, B-3030 Leuven, Belgium

A second lectin has been isolated from Elderberry (S. nigra) bark by affinity chromatography on immobilized asialoglycophorin. This lectin is a blood group non-specific glycoprotein (containing 7.8% carbohydrate consisting of glucosamine mannose and small amounts of fucose and galactose) with M_r = 30,000. It is a Gal/GalNAc-specific lectin which is precipitated by glycoproteins containing GalNAc-terminated oligosaccharide chains (e.g. asialo-ovine submaxillary and hog gastric mucins), and by glycoproteins and polysaccharides having multiple terminal nonreducing D-galactosyl groups as occur in asialoglycophorin, asialolaminin and type 14 pneumococcal polysaccharide.

The carbohydrate binding specificity of the S. nigra lectin II was studied by sugar hapten inhibition of the asialoglycophorin precipitation reaction. The lectin's binding site appears to be most complementary to GalNAcα1→2,3 or 6Gal disaccharide units which are approximately 100 times more potent than melibiose, 60 times more potent than N-acetyllactosamine, and 30 times more potent than lactose.

Interestingly, the blood group A-active trisaccharide containing an L-fucosyl group linked α1,2 to galactose was 10-fold poorer than the parent oligosaccharide (GalNAcα1,3Gal) suggesting steric hindrance to binding by the α-fucosyl group; this explains the failure of the lectin to be blood group A-specific.

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PURIFICATION OF TOMATO LECTIN BY ERYTHROGLYCAN-SEPHAROSE AFFINITY CHROMATOGRAPHY

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Tomato lectin is specific for oligomers of polylactosamines containing 3 repeating Gal(β1-4)GlcNAc(β1-3)-disacchrides. As such it is highly useful for purifying oligosacchrides or glycopeptides with polylactosamine character. We have found the lectin very useful as an affinity reagent for isolating glycoproteins or glycoprotein domains having polylactosamine glycosylation. Conventional preparation of tomato lectin by ovomucoid-Sepharose affinity chromatography was found to be unsatisfactory due to instability of column and bleeding of ovomucoid into eluents requiring the necessity for additional purification steps following affinity chromatography. We prepared a column of human erythrocyte band 3 carbohydrate glycopeptide (erythroglycan) attached to Sepharose as an affinity matrix. The purification of tomato lectin to homogeneity in one step on this column matrix is described in this report.

Oligosaccharide Signalling

CELL-WALL-DERIVED OLIGOSACCHARIDES SIGNAL THE ACCUMULATION OF ANTIMICROBIAL COMPOUNDS IN PLANTS. M. G. HAHN, F. Cervone, J.-J. Cheong, A. G. Darvill, P. Albersheim. Department of Botany and Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602

The accumulation of antimicrobial compounds (phytoalexins) is an important defense response of plants to microbial infection. Phytoalexin accumulation is induced by molecules (elicitors) originating from both fungal and plant cell walls. The best characterized of these elicitors are oligosaccharides that are released from cell wall polysaccharides. The smallest active fungal cell-wall-derived elicitor is a branched hepta-β-glucoside. A number of structurally related oligo-β-glucosides ranging in size from hexamer to decamer have been chemically synthesized. Comparisons of the abilities of these oligo-β-glucosides to induce phytoalexin accumulation demonstrated that there are precise structural requirements for elicitor activity, which supports our previous conclusion that plant cells have a specific receptor for this elicitor. Current work is focussed on demonstrating the presence in plant cells of a specific binding site for the hepta-β-glucoside elicitor. The plant cell-wall-derived elicitors are linear α-(1→4)-linked oligogalacturonides with degrees of polymerization between 10 and 13. In addition to being separately active as inducers of phytoalexin accumulation, the plant and fungal oligosaccharide elicitors act synergistically when applied in combination to plant tissues.

The elicitor-active oligogalacturonides can be released from plant cell walls by fungal endopolygalacturonases. However, *in vitro*, fungal endopolygalacturonases rapidly depolymerize elicitor-active oligogalacturonides to inactive fragments. We have now demonstrated that a constitutive plant polygalacturonase-inhibiting protein (PGIP) enhances the production of elicitor-active oligogalacturonides by altering the rate at which oligogalacturonides released from higher MW polymers are degraded. In the presence of excess PGIP, the release/degradation cycle takes about 48 hours, as compared with less than 15 min in the absence of PGIP. PGIP is a specific, reversible, saturable, high-affinity receptor for endopolygalacturonase. Formation of the PGIP-endopolygalacturonase complex results in increased concentrations of oligogalacturonides that activate plant defense responses by regulating gene expression. The interaction of the plant-derived PGIP with fungal endopolygalacturonases appears to be a mechanism by which plants convert endopolygalacturonase, a factor important for the virulence of pathogens, into a factor that elicits plant defense responses. (Supported by NSF grants DMB-8704022 and DMB-8518488, and DOE grant DE-FG09-85ER13425)

Oligosaccharide Signalling in Plants

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Plants from several families can respond to attacks of insects and microorganisms by activating genes that code for the synthesis of defense proteins, such as proteinase inhibitors, or for enzyme systems that produce complex defense chemicals, such as phytoalexins. Both classes of genes can be activated in tissues of specific plants by oligo- or polysaccharides that can be fragmented from either plant or fungal cell walls by hydrolytic enzymes. The oligosaccharides that induce (or elicit) defensive responses includes β-glucans, chitin and chitosan oligomers from fungal cell walls, and oligogalacturonans from plant cell walls. This suggests that all of these fragments may be involved in a universal early recognition system to activate plant defense genes. Genes coding for two non-homologous proteinase inhibitors, called Inhibitor I and Inhibitor II, have been isolated from potato and tomato genomic libraries and characterized to further understand the mechanism(s) of wound-regulation by oligosaccharides. The structure and expression of genes coding for proteinase Inhibitors I and II from potato and tomato plants will be discussed with respect to their roles in further understanding wound induction. (Supported by Grants from the USDA and NSF.)

OLIGOSACCHARIDE ELICITORS OF COMPONENTS OF THE HYPERSENSITIVE RESPONSE IN HIGHER PLANTS. C. A. WEST, Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90024-1569.

Many higher plants respond to invasions by incompatible races of pathogenic microorganisms by mounting a hypersensitive resistance response (HR) that helps to contain the invading pathogen. The HR involves the death of one or more plant cells and the biosynthesis in the immediate surroundings of a number of components, including antimicrobial antibiotics (phytoalexins), lignin, callose (a β-1,3-glucan), hydroxyproline-rich glycoprotein, and intercellular hydrolases (chitinases, β-1,3-glucanases and endoproteinases) that are thought to have a role in disease resistance. A number of biotic elicitors of the synthesis of HR components in higher plant tissues have been identified as oligosaccharides arising from the degradation of cell walls of either an invading fungal pathogen or the host plant. It is likely that hydrolytic enzymes elaborated by either the fungus or the plant during their interaction are responsible for the release of these elicitors. The best characterized fungal cell wall oligosaccharide elicitors are derived from branched β-1,3-glucans, chitosan (β-1,4-D-glucosaminoglycan) and chitin (β-1,4-N-acetyl-D-glucosaminoglycan). The best characterized elicitors from the plant cell wall are fragments derived from homogalacturonan (α-1,4-D-galacturonide) regions of the pectic substances. The properties of some of these oligosaccharide elicitors and their role in elicitation will be discussed.

INTRACELLULAR TARGETING OF LYSOSOMAL ENZYMES AND MEMBRANE COMPONENTS. Larry W. Hancock. Depts.of Peds. and Biochem., U. of Chicago, IL 60637.

The subcellular distribution of a number of soluble lysosomal enzymes and lysosomal membrane components were analyzed in [^{35}S]Met-labeled cultured human fibroblasts by immunoprecipitation and SDS-PAGE after fractionation on Percoll gradients. Mature polypeptides of the soluble glycosidases N-acetyl-β-hexosaminidase (Hex) and α-fucosidase (Fuc), as well as the membrane-associated glycosidase glucocerebrosidase (GLⱻase) were preferentially associated with dense (>1.07) lysosomal fractions, while the lysosomal membrane glycoprotein Lamp A [Viitala, et al (1988) PNAS 85:3743] was associated with both dense and buoyant fractions, consistent with its previously reported distribution in both lysosomal and plasma membranes. Surprisingly, the proteinase cathepsin D (cath D) was widely distributed among subcellular fractions, in spite of its acquisition of the mannose 6-phosphate recognition marker (as assessed by binding to immobilized mannose 6-phosphate receptor). Hex and Fuc polypeptides also bound quantitatively to this affinity column, while there was no evidence of GLⱻase or Lamp A binding. Treatment of fibroblasts with 1-deoxymannojirimycin (DMM) or Ep-459 (an inhibitor of cathepsins) had no dramatic effect on the subcellular distribution of lysosomal enzymes or membrane components, although there were clear effects on the proteolytic processing (Hex, Fuc, cath D) and carbohydrate processing (GLⱻase, Lamp A) of newly synthesized polypeptides in cells treated with Ep-459 and DMM, respectively. Surprisingly, Hex α-polypeptide chain synthesized in DMM-treated cells was subject to incomplete proteolytic processing, similar to that observed secondary to lysosomal N-acetylneuraminic acid (NeuAc) accumulation in infantile NeuAc storage disease [Hancock, et al (1988) BBRC 152:83]. These results suggest the presence of multiple targeting mechanisms for lysosomal enzymes and membrane components (in addition to the mannose 6-phosphate-mediated system) in cultured fibroblasts, and further imply the existence of sub-populations of hydrolytic organelles (as differentiated by density and complement of hydrolytic enzymes). They further implicate complex N-linked carbohydrate chains in the maintenance of a functional intralysosomal environment, as evidenced by the impaired proteolytic processing of Hex α-chain in DMM-treated fibroblasts. Supported by USPHS Grant DK-38593 and the MOD.

Glycoconjugates in Development

MONOCLONAL ANTIBODIES THAT RECOGNIZE NOVEL CHONDROITIN SULFATE STRUCTURES ARE SPECIFICALLY EXPRESSED DURING DEVELOPMENT. Caterson, B., Mahmoodian, F., Sorrell, J.M. Depts. of Biochemistry & Anatomy, West Virginia University

Chondroitin sulfate (CS) proteoglycans are major components of cartilage and lesser constituents of many other connective tissues. In this paper we present evidence supporting a new function for CS glycosaminoglycans synthesized by connective tissues during development. These studies indicate that novel structures (sulfation patterns) occur within native CS chains synthesized on proteoglycans during normal development. We postulate that these structures bind metabolic growth factors and thus regulate growth and development. Monoclonal antibodies (MAb) were raised using embryonic chick bone marrow proteoglycans as immunogens. MAb specificity was determined in immunoassay analyses against a wide variety of proteoglycan antigen preparations from different animal species. To determine if carbohydrate structures were epitopes for these, MAbs antigen preparations were pretreated with various glycosidases. "Dot Blot" and ELISA immunoassay analyses indicated that 5 or 8 resultant MAbs recognized epitopes present in native CS glycosaminoglycans, i.e. pretreatment of antigens with chondroitinase abolished MAb binding. These new MAb epitopes were present in CS chains of proteoglycans isolated from human, cow, dog, pig, rabbit, chicken and shark cartilages but were not present in Swarm rat chondrosarcoma proteoglycan. Strongest expression of MAb epitopes occurred in young developing tissues and were not significantly expressed in proteoglycans from older mature tissues. Immunohistochemical studies using these five new MAbs to study the occurrence of CS proteoglycans in embryonic chicken hemopoietic tissues indicated that there was a differential expression of these novel CS epitopes in matrix proteoglycans during development and differentiation of the hemopoietic organs. This expression of novel CS structures closely paralleled the cellular events controlled by interactions with regulatory growth factors. These data suggesting that differences in the metabolism of CS proteoglycan are directly related to normal tissue growth and development.

AGE-ASSOCIATED CHANGES IN POLYISOPRENOID METABOLISM IN THE KIDNEYS AND LIVER OF MICE. Dean C. Crick, Jack W. Rip, and Kenneth K. Carroll. Dept. of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1.

The amounts of cholesterol, dolichol, and dolichyl phosphate (Dol-P) in kidneys and liver from 1- and 24-mo. old mice were measured after saponification of the tissues. The cholesterol content of kidneys (per g of tissue) decreased by about 4-fold in older animals while the amount of cholesterol/g liver remained constant. The dolichol content of both tissues increased about 2-fold. The amount of Dol-P in kidneys increased 7-fold over 23 mo., while the concentration in liver decreased from 4.5 ug/g to undetectable levels. Metabolic labelling experiments using tritiated water demonstrated that the rate of synthesis of total dolichol (dolichol + Dol-P) in the kidneys did not change as a function of age. However, the kidneys of young mice incorporated 3 times more radioactivity into dolichol than into Dol-P, whereas this was reversed in old mice. In liver, the rate of dolichol synthesis decreased 2-fold over 23 mo. It was not possible to compare the rates of Dol-P biosynthesis between ages as Dol-P was undetectable in livers of old mice even though the [1-^{14}C]Dol-P tracer was recovered in good yield. The relative abundance of the 19-isoprene unit homologs of both dolichol and Dol-P decreased approximately 5% while the 17-isoprene unit homologs increased by a similar amount in both tissues of the old mice. The data indicate that the metabolism of dolichol and Dol-P is independently regulated between tissues. The major age-associated change in dolichol metabolism may be an alteration of the balance between dolichol kinase and dolichyl phosphate phosphatase activities. The extent to which these age-associated changes affect the capacity for N-linked protein glycosylation is yet to be determined. (Supported by the Medical Research Council of Canada)

ENHANCED GLYCOSYLATION OF SPECIFIC GLYCOPROTEINS IN MURINE EMBRYONAL CARCINOMA CELLS (F-9) INDUCED TO DIFFERENTIATE INTO ENDODERMAL CELLS BY RETINOIC ACID Brad Amos and Reuben Lotan Department. of Tumor Biology, M.D. Anderson Cancer Center Houston, TX

Previous studies have demonstrated that increased glycosylation of cell membrane glycoproteins accompanies retinoic acid (RA)-induced in vitro growth inhibition and differentiation in a number of tumor cell lines of both human and murine origin. The present studies were initiated to identify and characterize RA-modulated glycoproteins in the murine embryonal carcinoma cell line, F-9. Other laboratories have shown that F-9 cells are induced to differentiate into endodermal cells in response to RA and that this differentiation is accompanied by alterations in glycoprotein synthesis as measured by a decrease in the size of fucosylated glycopeptides and in the ability of various plant lectins to bind to the cells. We have identified alterations induced by RA in the glycosylation of specific cell surface and cellular glycoproteins. RA treatment of F-9 cells resulted within 3 days in a dose dependent inhibition (IC50=0.2mM) of cell proliferation in monolayer culture. Colony formation in 0.5% agarose was also inhibited at very low concentrations of RA (IC50=0.02nM). Metabolic labeling of the cells with [^{3}H]fucose or [^{3}H]glucosamine (10 μCi/ml, 48 hr) followed by glycoprotein analysis after cell solubilization, polyacrylamide gel electrophoresis, and fluorography revealed that RA enhanced the incorporation of both monosaccharides into glycoproteins of *M*rs 400, 250, and 175 KD. The increased incorporation of fucose into the highest *Mr* glycoprotein was detected as early as 24 h after the addition of RA while the alterations in the other two glycoproteins occurred after 36 h. The 175 KD glycoprotein was detected on the surface of the RA-treated cells by methods that specifically label exposed sialic acid and galactose residues. Another compound with retinoid-like properties, chalcone carboxylic acid (Ch55), also induced the differentiation of the cells and caused an increase in the glycosylation of the same glycoproteins as RA. Treatment of the cells with RA and dibutryl cAMP resulted in differentiation to neuronal-like cells which did not exhibit the same alterations in fucosylation that were seen when the cells were induced to differentiate into endodermal cells by RA alone or Ch55. The increases in the fucosylation and sialylation of the glycoproteins were most probably the result of RA enhanced activities of sialyltransferase and fucosyltransferase. Because the changes in glycosylation of some cell surface glycoproteins precede the alterations in cell growth and differentiation they may be related causally to RA's mechanism of action.

Molecular Biology of Glycoconjugates

MOLECULAR APPROACHES FOR GLYCOPROTEIN CLONING. K.W.Moremen and P.W.Robbins, M.I.T.., 77 Massachusets Ave., Cambridge, MA 02139

Several distinct methodologies are available for the cloning of genes for eucaryotic glycoproteins. A common feature of most of these approaches is the primary screening of a genomic or cDNA libraries inserted into appropriate vectors and amplified in procaryotic or eucaryotic cells. In yeast, the ease of genetic manipulations have allowed the detection of positive clones by complementation of conditionally lethal mutations, increased resistance to drugs, *in vitro* detection of enzyme activities in a mutant backgrounds, and the over-expression of enzyme activities in wild type cells. In higher eucaryotes, the cloning of structural genes generally rely upon the use of immunological or oligonucleotide probes to distinguish positive clones from the general library background. Since primary clones obtained by immunological or oligonucleotide probing methods frequently do not span the entire coding region of the gene of interest, secondary screening is often required to extend the clones beyond the ends of the coding region.

An alternative approach to the primary cloning of eucaryotic genes is the use of protein sequence data to directly amplify a segment of the gene of interest from a cDNA preparation using specific degenerate primers and a modification of the Polymerase Chain Reaction(PCR). This methodology has several advantages over standard cloning techniques since: 1) no primary screening is required, 2) sequencing the fragment in regions adjacent to the primer region yields direct, unambiguous confirmation that the amplified fragment represents a segment of the target coding region, 3) the fragment can be subcloned or used directly as a probe for secondary screening of cDNA libraries.

Initial studies using this approach employed exact match primers to amplify a gene of known DNA sequence, the α2-6 sialyltransferase gene from rat liver. Successful amplification of the 1.2 kb sialyltransferase coding region was demonstrated from several cDNA preparations. The method was then tested on a gene for which there were several regions of known protein sequence, but no previously isolated DNA sequence (mannosidase II). Primers were designed using several of the strategies common in oligonucleotide probe design and used to amplify a segment of the rat liver mannosidase II gene corresponding to 1/3 of the coding region. The authenticity of this fragment has been verified by DNA sequencing and is presently being used as a probe to isolate the full length cDNA clones. Further work on extending the usefulness of the cloning procedure is being pursued by examining the limits of primer design including the limits of inosine substitution, degeneracy of the primers, and basepair mismatch between the primer and the target DNA. This approach should find general use in molecular biology for the primary cloning of genes for which some basic protein sequence data has been obtained. The simplicity, speed, and sensitivity of the PCR cloning procedure should also appeal to investigators who are attempting to clone a gene that has a low abundance in the original mRNA population.

A STUDY OF THE CATALYTIC AND REGULATORY FUNCTIONS OF A SUGAR NUCLEOTIDE SYNTHETASE USING IN VITRO and IN VIVO MUTAGENESIS TECHNIQUES. J. Preiss, A. Kumar and P. Ghosh. Department of Biochemistry, Michigan State University, East Lansing, MI 48824.

The glucosyl donor for synthesis of bacterial glycogen and plant starch is the sugar nucleotide, ADPglucose. The enzyme catalyzing its synthesis, ADPglucose synthetase is inhibited by either ADP, AMP or P_i and activated by glycolytic intermediates. The enzyme has been purified to homogeneity from several bacterial sources and from spinach leaf. The structural gene for the enzyme, glgC, has been isolated from Escherichia coli and Salmonella typhimurium DNA and expressed. Chemical modification studies have indicated that Lys^{39} is involved in binding of the activator, fructose 1,6-P_2, Tyr^{114} is involved in the binding of both the substrates ATP and ADPglucose and the inhibitor, 5'AMP; Lys^{196} is involved in the binding of the substrates. Nucleotide directed mutagenesis of Tyr^{114} to Phe not only decreases the apparent affinity of the enzyme but also alters the allosteric properties of the enzyme. Moreover, the deduced amino acid of an allosteric mutant of the enzyme indicates that substitution of Lys^{296} to Glu and Gly^{336} to Asp increases the apparent affinity of the enzyme for the activator and decreases the apparent affinity for the inhibitor. The mutant enzyme is also less dependent on activator for activity. Thus chemical modification studies in conjunction with recombinant DNA and classical mutagenesis techniques provides much information on the structure-function relationships of a sugar nucleotide synthetase.

THE YEAST GENE FOR DOLICHOL PHOSPHATE MANNOSE SYNTHASE: CLONING, SEQUENCE ANALYSIS AND IN VITRO MUTAGENESIS. P. Orlean, C. Albright and P.W.Robbins, E17-235, M.I.T., 77 Massachusetts Avenue, Cambridge, MA 02139.

The yeast gene for dolichol phosphate mannose (Dol-P-Man) synthase (DPM1) was isolated by screening a yeast genomic DNA library for plasmids that conferred overexpression of in vitro Dol-P-Man synthase activity on individual yeast colonies. The cloned gene is the structural gene for Dol-P-Man synthase since E. coli cells harboring the DPM1 gene express Dol-P-Man synthase activity in vitro, and since the M_r of 30 000 predicted for the protein after DNA sequencing matches that of purified yeast Dol-P-Man synthase (Haselbeck, A. and Tanner, W. (1982) PNAS 79, 1520-1524).

The topography of Dol-P-Man synthesis is an interesting problem, as synthesis and subsequent utilization of this glycolipid apparently occur on opposite sides of the ER membrane. Dol-P-Man synthase is predicted to be a hydrophilic protein with a hydrophobic, membrane spanning region at its C-terminus. Analysis of the deduced amino acid sequence suggests the bulk of the protein lies in the lumen of the ER. This orientation is consistent with Dol-P-Man being involved in lumenal reactions, but it is unclear how the enzyme interacts with GDP-Man on the cytoplasmic side of the membrane. Models for the enzyme's transmembrane topography are being tested.

The C-terminal hydrophobic region of Dol-P-Man synthase includes a stretch of 14 amino acids, many of which have bulky side chains. Such a region has also been identified in proteins involved in early steps in N-glycosylation, and could be involved in binding dolichol.

Dol-P-Man synthase is essential for viability in yeast since disruption of the DPM1 gene is lethal. Dol-P-Man synthase may not be essential because of its role in N-glycosylation, since mutations in other genes that affect late steps in lipid-linked oligosaccharide synthesis do not affect growth. Instead, Dol-P-Man may be essential in yeast because it is required for O-mannosylation, or for some unidentified process. To define the functions of Dol-P-Man synthase in vivo, we are studying conditionally lethal mutants in DPM1. Mutants were isolated following in vitro mutagenesis of the cloned DPM1 gene. Such strains are temperature-sensitive for growth as well as defective in Dol-P-Man synthase activity in vitro. (NIH GM-31318)

MOLECULAR CLONING AND SEQUENCE ANALYSIS OF A cDNA ENCODING RAT LIVER α-L-FUCOSIDASE. K.J. Fisher and N.N. Aronson, Jr. Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, PA 16802.

Catabolism of Asn-linked glycoproteins has been proposed to occur by way of an ordered, bidirectional process in rat liver lysosomes[J.Biol. Chem. 261 5803-5809 (1986)]. In an effort to better understand this mechanism, our laboratory is studying the enzymes involved in glycoprotein degradation at both the biochemical and genetic levels. Recently a 900-bp cDNA clone(FC2) has been isolated from a λgt11 rat liver cDNA library after screening $2x10^6$ plaques with rabbit polyclonal antibodies raised against affinity purified rat liver α-L-fucosidase. Preliminary nucleotide sequence analysis of clone FC2 has revealed it contains both coding and non-coding information. Comparing the 95 nucleotides identified in the coding region of FC2 to a partial human α-L-fucosidase cDNA(AF3) reported by Fukishima *et al.* [PNAS 82 1262-1265 (1985)], there exists a strong correspondence(87%) between the two(asterisks indicate non-homologous nucleotides):

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          1          10          20          30          40
FC2(rat)  GG AAC TTC ACG GAG ATT CTT GCT TGG CTC TAC AAT GAA AGC CCG GTC
                     * * * *     * *                 *       *

AF3       GG AAC TTC ACA AAT TTT CTT TCA TGG CTC TAC AAT GAC AGC CCT GTC
(human)      500         510         520         530         540

           50          60          70          80          90
          AAG GAT CAG GTG GTA GTG AAT GAC CGG TGG GGT CAG AAC TGC TCC TGT
                *           *           *                       *

          AAG GAT GAG GTG GTA GTA AAT  GAC CGA TGG GGT CAG AAC TGT TCC TGT
                550         560         570         580         590
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The non-coding region of FC2 contains a polyadenylation signal(AATAAA) 9 nucleotides upstream from a poly-A tail. A lysogen of clone FC2 established in E. coli strain Y1089 yielded a β-D-galactosidase fusion protein of approx. M_r= 138 KDa. These results suggest that the coding portion of FC2 encodes a peptide with an approx. M_r= 24 KDa, which would be derived from a 600-bp polynucleotide segment. This further suggests that the non-coding region of FC2 contains an approx. 300-bp 3'-untranslated region. Efforts are now underway to completely sequence FC2, as well as to obtain a full length clone of rat liver α-L-fucosidase. Amino acid sequence analysis of rat liver α-L-fucosidase is also being pursued.

MOLECULAR CLONING, EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF A CHITINASE FROM VIBRIO PARAHEMOLYTICUS

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A Sau3A partial digest of V. parahemolyticus DNA was used to make a library in pUC18. One clone in 2000 produced a clear zone of hydrolysis on particulate chitin-agar plates. This construct, designated pC139, was used to transform E. coli DH5 alpha. All of the transformants digested particulate chitin. A 5.4 kilobase pair DNA insert, produced from pC139, was subcloned into Bluescript plasmid, designated pBS139. The molecular weight of the mature protein is 95 kDa for both the recombinant and native Vibrio chitinases. The 95 kDa protein was purified from both the periplasmic and extracellular compartments. Using exonuclease III and mung bean nuclease, nested deletion variants were produced from pBS139 for localization of the structural gene. Deletion variant 2036, consisting of a 2.9 kbp insert, produced 1.8 IU of total activity using host E. coli JM101. The same insert was cloned in the opposite orientation. The resulting construct, R361, produced 19 IU of activity. Therefore, a change in orientation of the insert, relative to the plasmid origin of replication, resulted in an 11-fold increase in the level of chitinase expression. The cellular localization of chitinase also was influenced by the orientation of the insert. Ninty-four percent of the chitinase activity was extracellular for R361; whereas, only 11% was found in the growth medium for 2036. The minimum insert length necessary for chitinase activity and secretion is 1.9 kbp. The corresponding truncated protein has a molecular weight of 67 kDa and is secreted into the periplasm. In both cases, the N-terminal amino acid was determined to be alanine. The prechitinase possesses a signal sequence with a +1 charge and 21 amino acids, 17 of which are hydrophobic. The mature enzyme exhibits a broad pH range of activity and a temperature optimum of 45°C using [^{3}H]-chitin as the substrate. The enzyme retains 100% and 25% activity in the presence of 0.5 M and 4 M NaCl, respectively.

IMMUNOCHEMICAL STUDIES ON LYSOSOMAL β-MANNOSIDASE,

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β-Mannosidosis, an autosomal recessive disorder first diagnosed in goats and more recently in humans, is due to a deficiency of lysosomal β-mannosidase (β-man) activity. Storage material in humans is a Manβ4GlcNAc disaccharide. Guinea-pig liver lysosomal β-man was purified (more than 95% pure by $NaDodSO_4$ polyacrylamide gel electrophoresis and silver staining) and antiserum was prepared in a rabbit. This polyclonal antibody inhibited the hydrolysis of 4-methylumbelliferyl β-mannoside by guinea-pig tissue extracts. There was little cross-reactivity against other lysosomal hydrolases such as α-mannosidase and N-acetyl-β-glucosaminidase. The polyclonal antibody cross-reacted with human fibroblast β-man at a lower dilution of antibody. Western blotting of the purified guinea pig enzyme gave a single broad band at approximately 95-kDa. The antiserum also precipitated a 95KDA protein from radioactively labelled guinea pig fibroblasts, and a 100-kDa protein from [^{35}S]/[^{3}H] labeled human fibroblasts. Evidence was obtained for a 114-kDa precursor. In contrast, a human patient with combined β-man-sulfamidase deficiency contained no β-man cross-reacting material. The antibody-enzyme complex from guinea pig tissue extracts had substantially more (up to 3-fold) β-man activity than expected from assay of total tissue homogenate. Our data suggests the elimination (by immunoprecipitation) of a heat stable inhibitor present in the total extract. Inhibition was also destroyed by freeze-thawing or dialysis, but not by EDTA. This is the first report of the characterization of an antibody to β-man. We have used it to screen a λgt11 guinea pig library and isolate an antibody cross-reacting fusion protein containing a 15-kDa fragment of β-man. The 0.5kb cDNA has a unique seqence with a single glycosylation site, and hybridizes (on Southern blot analysis) preferentially to guinea pig DNA over human DNA. Experiments are underway to verify this cDNA as authentic β-man DNA, isolate larger pieces of the gene and use it to study human β-man deficient mutants. (Supported by USPHS Grants HD-06426, HD-09402 and HD-04583).

DNA-MEDIATED TRANSFORMATION OF N-ACETYLGLUCOSAMINYL-TRANSFERASE I ACTIVITY. James Ripka, Michael Pierce, and Nevis Fregien. Department of Anatomy and Cell Biology, University of Miami Medical School,1600 N.W. 10th Ave, Miami, Fl 33101.

The enzyme, $\alpha(1,3)$ mannoside $\beta(1,2)$ N-acetylglucosaminyltransferase I (GlcNAc-TI), catalyzes the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to N-linked Man_5GlcNA_2 oligosaccharides. This addition represents the first GlcNAc residue transferred during the processing of high-mannose structures into complex oligosaccharides. The mutant Chinese hamster ovary (CHO) cell line, Lec1 exhibits no detectable GlcNAc-TI activity and, therefore, expresses the truncated Man_5GlcNA_2 oligosaccharide on cell surface glycoproteins. Consequently, Lec1 cells are highly sensitive to the mannose-binding lectin Concanavalin A (Con A). In order to clone the GlcNAc-TI gene, Lec1 cells were transformed with human A431 cell DNA by $CaPO_4$/DNA-mediated transformation and plated in the presence of Con A. Transformant colonies that exhibited the parental CHO cell sensitivity to Con A arose at a frequency of 10^{-6}, whereas spontaneous revertants of Lec1 have not been observed (frequency $< 2 \times 10^{-8}$). Lec1 DNA or mock transformations did not result in parental CHO phenotype transformants. DNA from primary transformants was purified and used to transform Lec1, resulting in secondary transformants that expressed the parental CHO phenotype. Both primary and secondary transformants expressed active in vitro GlcNAc-TI activity. While human alu repeat DNA sequences are present in the primary transformants, these sequences were not detected in the secondary transformants. These studies were supported by NIH CA 35377 and AR 38873.

CHARACTERIZATION AND DIFFERENTIAL EXPRESSION OF TWO LECTIN GENES OF *DOLICHOS BIFLORUS*. M.E. Etzler, D.J. Schnell, J.C. Maxwell and J.J. Harada. University of California, Davis, CA 95616.

The *Dolichos biflorus* plant contains a family of lectins, all of which bind to *N*-acetyl-D-galactosamine but which have some differences in their carbohydrate binding properties. Two of these lectins, the seed lectin and DB58, are under different regulatory controls that result in the expression of the seed lectin during late seed maturation and the expression of DB58 during rapid growth of the stems and leaves. The lectins are also targetted to different sites in the cell; the seed lectin is localized in the protein bodies of the cotyledons whereas DB58 is localized in the cell walls of the stems and leaves.

The genes for both of these lectins have been identified and isolated from a genomic DNA library prepared from *Dolichos biflorus* DNA. These genes are located within 3 kilobases of one another. Comparisons of the amino acid sequences deduced from the genomic DNA and cDNAs show that the lectins are 84% homologous in amino acid sequence but have different consensus glycosylation sites which have been confirmed to be the sites of glycosylation in the intact proteins. Each of the lectins is an oligomeric glycoprotein composed of equal amounts of structurally similar subunits. Biosynthetic and structural studies show that this subunit heterogeneity arises by posttranslational COOH-terminal proteolytic processing. (Supported by NIH Grant GM21882 and NSF Grant DMB-86 14246.)