STRUCTURE DETERMINATION OF GLYCOPROTEIN DERIVED CARBOHYDRATES

J.F.G. Vliegenthart, P. de Waard, K. Hård and J.P. Kamerling. Bijvoet Center, Dept. of Bio-Organic Chemistry, Utrecht University, Padualaan 8, NL-3508 TB Utrecht, The Netherlands

The structure determination of the carbohydrate chains attached to proteins, by means of high resolution ^{1}H-NMR spectroscopy, still requires the preparation of partial structures. This is due to the occurrence of multiple N- and/or O-glycosylation sites, the microheterogeneity at each glycosylate site and the inherent limitations in the analysis of macromolecules by ^{1}H-NMR. To analyse N,O-glycoproteins, a procedure has been developed wherein the N-linked chains are split off with PNG-ase F. The degree and specificity of the cleavage of the N-linked chains has to be checked carefully. Various methods are available for this purpose, which differ in sensitivity and scope. Subsequently, the residual O-protein is purified and treated with alkaline borohydride to eliminate the O-linked chains. The pools representing N- or O-linked chains are exhaustively fractionated by various HPLC procedures. The identification of purified compounds can often be carried out by ^{1}H-NMR on the basis of our structural reporter group concept. For the characterization of novel structures several 2D-NMR techniques are available to provide addtional evidence. The occurrence of non-carbohydrate substituents like alkyl, acyl, sulfate and phosphate groups gives rise to an increase of the number of structures that occur. The determination of the type of substituent and the precise localization in the carbohydrate chains can often be performed by means of NMR spectroscopy. It should be mentioned that for the analysis of phosphate-containing oligosaccharides we developed a ^{1}H{^{13}P} relayed spin echo difference spectroscopy technique to establish the monosaccharide constituent that is phosphorylated and to characterize the C-atom therein that is substituted. Among the various monosaccharide constituents, the sialic acids occupy a special position, since a large variety of non-carbohydrate substituents can be present in these molecules. NMR can give a significant contribution to the elucidation of the structure of these constituents in intact carbohydrate chains. This is the more relevant in view of our reports on acyl migrations that can take place in sialic acids.

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DEVELOPMENTS IN THE ANALYSIS OF GLYCOLIPIDS AND OLIGOSACCHARIDES; LINKAGE, SEQUENCE AND BRANCHING, (SFC-NCI-MS).

V.N. Reinhold, D.M. Sheeley, J.P. Caesar, M.V. Merritt,

Harvard University School of Public Health.
665 Huntington Ave, Boston, MA 02115.

Linear sequence and branching information can most frequently be obtained by a combination of FAB-MS and/or collision induced decomposition-MS, (CID-MS). Ascertaining inter-residue linkage, however, requires "methylation analysis". These procedures, unfortunately, yield a linkage composition only and are sample costly. An alternative approach to linkage structure has been introduced by periodate oxidation and FAB-MS (1). This chemical modification alters monomer residue molecular weights, dependent on linkage position, which is reflected in the FAB or CID spectrum. We have coupled this strategy with supercritical fluid chromatography (SFC) for sample purification and negative-ion chemical ionization mass spectrometry (NCI-MS) for characterization (SFC-NCI-MS). Consistent with this scheme, high electron affinity groups have been incorporated into these derivatives which brings femtomole detection to high molecular weight ganglioside and N-linked glycans samples.

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The Role of Glycosylation in Glycoprotein Hormone Function

Jacques U. Baenziger, MD, PhD
Washington University School of Medicine, St. Louis, MO 63110

The glycoprotein hormones are a family of closely related proteins, each of which consists of an α- and β-subunit. The α-subunits arise from a single gene and have the identical amino acid sequence for each hormone. The β-subunits are highly homologous in structure but arise from separate genes and confer hormone-specific properties to the dimeric species. hCG (human chorionic gonadotropin) is synthesized by the placental trophoblast whereas LH, TSH, and FSH are synthesized in the anterior pituitary. The oligosaccharides of the glycoprotein hormones have been implicated in a number biologic functions such as activation of adenylcyclase following receptor binding and sorting of LH and FSH to different storage granules within the gonadotroph. The Asn-linked oligosaccharides on LH and TSH are unique, terminating with the sequence SO_4-GalNAcβ1,4GlcNAcβ1,2Manα rather than the common sequence Sialic acidα-Galβ1,4GlcNAcβ1,2Manα found on hCG and FSH. Using a monoclonal antibody specific for the sulfated oligosaccharide we have determined that these sulfated structures are confined to gonadotrophs and thyrotrophs in the bovine pituitary. Thus, the sulfated oligosaccharides display a highly restricted distribution within the pituitary.

Separate GalNAc- and sulfotransferases are required for synthesis of the sulfated oligosaccharides on LH and TSH. We have identified a GalNAc-transferase in pituitary Golgi membranes which will add GalNAc to the synthetic intermediate $GlcNAc_2Man_3GlcNAc_2$. The apparent K_m for transfer of GalNAc to this intermediate is 13 μM when it is on the hormone α-subunit and 2.6 mM when it is present on other glycoproteins such as J-chain. Thus, the GalNAc-transferase recognizes a feature encoded within the peptide of the α-subunit. Oligosaccharide acceptors on hCGβ and LHβ are also specifically recognized by the GalNAc-transferase due to features encoded within their respective peptides. Destruction of the tertiary structure of hCGα and hCGβ does not alter the apparent K_m for GalNAc addition, indicating that recognition is directed primarily at features encoded by the primary sequence. In contrast to hCGβ and LHβ, FSHβ is not recognized by the GalNAc-transferase and, when combined with the α-subunit, masks the α-subunit recognition site. This is the first example of a glycosyltransferase which recognizes features encoded within primary amino acid sequence in order to produce a unique oligosaccharide product. In contrast to the GalNAc-transferase, the sulfotransferase requires only the sequence GalNAcβ1,4GlcNAcβ1,2Manα for transfer. The precise structure of the hormone oligosaccharides modulates their bioactivity, and, in the case of LH and FSH, may play a role in directing these two hormones to separate storage granules within the gonadotroph.

RABBIT LIVER UDP-*N*-ACETYLGLUCOSAMINE:α-3-D-MANNOSIDE β-1,2-*N*-ACETYLGLUCOSAMINYLTRANSFERASE I: CHARACTERIZATION OF A 2.5 KILOBASE cDNA CLONE

M.SARKAR[+], E.HULL[+#], R.J.SIMPSON[&], R.L.MORITZ[&], R.DUNN[$] AND H.SCHACHTER[+#]

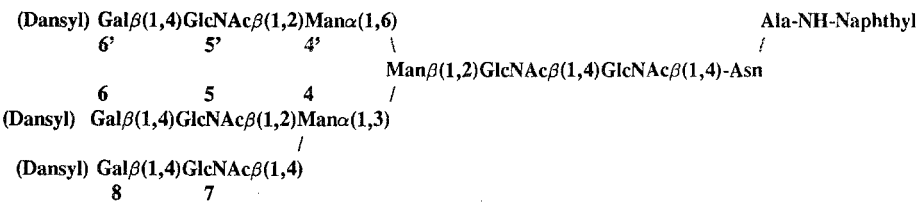
[+]Research Inst., Hosp. for Sick Children, and [#]Dept. of Biochemistry, Univ. of Toronto, Toronto, Ont., Canada, [$]Montreal General Hospital, Montreal, Quebec, Canada, and [&]Joint Protein Structure Laboratory, Ludwig Institute for Cancer Research and The Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia.

UDP-GlcNAc:α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnT I; EC 2.4.1.101) catalyzes an essential first step in the conversion of high mannose to hybrid and complex N-glycans, i.e., UDP-GlcNAc + (Manα1-6[Manα1-3]Manα1-6)(Manα1-3)Manβ1-4R to (Manα1-6[Manα1-3]Manα1-6)(GlcNAcβ1-2Manα1-3)Manβ1-4R + UDP where R is GlcNAcβ1-4(+/-Fucα1-6)GlcNAc-Asn-X. Glycerol, detergent, and salts were removed from highly purified rabbit liver GnT I (Y.Nishikawa *et al.*, J.Biol.Chem. **263**, 8270, 1988) by "inverse-gradient" reversed-phase microbore high performance liquid chromatography (RP-HPLC) (R.J.Simpson *et al.*, Eur.J.Biochem. **165**, 21, 1987). This procedure relies on the ability of proteins to interact strongly with small pore size (60-120 A°) reversed-phase packings at high concentrations of 1-propanol (90-100%). Under these conditions, detergents, glycerol and salts wash through the column. Retained proteins can be recovered from the column by addition of an ion-pairing agent (e.g., trifluoroacetic acid) into the mobile phase and elution with a gradient of decreasing 1-propanol concentration. Peptides were generated from 10 μg of GnT I by digestion with pepsin-trypsin-thermolysin. Eight of these peptides were purified by multi-dimensional RP-HPLC (R.J.Simpson *et al.*, Eur.J.Biochem. **176**, 187, 1988) and 103 amino acid assignments were made. Gene cloning was carried out by mixed oligonucleotide-primed polymerase chain reaction (PCR) amplification of rabbit liver single-stranded cDNA using sense and anti-sense 20-24 bp primers (K.W.Moremen, Proc.Nat.Acad.Sci. USA, **86**, 5276, 1989; C.C.Lee *et al.*, Science **239**, 1288, 1988). Total RNA was prepared from rabbit liver and single-stranded cDNA was synthesized using reverse transcriptase primed by a mixture of three anti-sense synthetic primers based on the peptide sequences. Two primer-dependent DNA fragments (0.50 and 0.45 kb) were obtained. Northern blot analysis of rabbit liver poly(A)[+]RNA with the 0.5 kb probe indicated a single 3 kb mRNA. A 1.6 kb cDNA clone was obtained by screening a rabbit liver cDNA library in λgt10 with the 0.5 kb probe. A 3.0 kb cDNA clone was isolated on re-screening the library with an 80 bp probe from the 5'-end of the 1.6 kb clone. On subcloning the 3.0 kb DNA into pGEM-7z, a 0.5 kb DNA fragment near the 5'-end was deleted. The remaining 2.5 kb cDNA contained a 447-amino acid coding sequence with a single putative trans-membrane (hydrophobic and helical) segment near the 5'-end. All eight peptide sequences were located. None of the 9 Asn residues were in an Asn-X-Ser(Thr) sequence indicating that the protein is not N-glycosylated. RNA was transcribed off the pGEM-7z/2.5 kb insert recombinant DNA and *in vitro* translation using rabbit reticulocyte lysate yielded a 52 kDa protein with GnT I activity. There is no sequence homology to other previously cloned glycosyltransferases but GnT I appears to have a domain structure typical of these enzymes (J.C.Paulson and K.J.Colley, J.Biol.Chem. **264**, 17615, 1989), i.e., an amino-terminal domain, a trans-membrane domain, a proline-rich "neck" region and a large carboxy-terminal catalytic domain. GnT I is the first medial Golgi-localized glycosyltransferase whose gene has been cloned. Sequence comparison with α-mannosidase II, another medial Golgi enzyme, has identified a possible medial Golgi localization consensus sequence. (Supported by the MRC of Canada).

DETERMINING DISTANCE AND FLEXIBILITY WITHIN TRIANTENNARY GLYCOPEPTIDE BY FLUORESCENCE ENERGY TRANSFER

Kevin G. Rice, Pengguang Wu, Ludwig Brand, and Yuan C. Lee
Department of Biology, Johns Hopkins University
Baltimore, Maryland USA

Fluorescence energy transfer has been used as a molecular ruler to measure distances in biopolymers such as proteins, oligonucleotides, and lipids. More advanced applications of this technique include measuring the distribution of distances between two fluorophores separated on short flexible segments. In an effort to understand the relationship of the solution conformation of a triantennary glycopeptide to its binding affinity for the asialoglycoprotein receptor, we have prepared triantennary glycopeptide probes containing two different fluorophores attached at terminal positions in the molecule. Fluorescent glycopeptide probes were prepared from triantennary glycopeptide after oxidation with galactose oxidase. Introduction of dansyl (acceptor) into one of each galactosyl-C6-aldehyde (either the 6', 6, or 8) and naphthyl (donor) into the glycopeptide N-terminus resulted in three, doubly labelled, fluorescent glycopeptide derivatives as well as singly labelled intermediates. With these probes we measured inter-terminal distances and conformational flexibility of the triantennary oligosaccharide by fluorescence energy transfer.



Average distances between the donor and the acceptor pairs were calculated using the Förster equation, measuring the efficiency of energy transfer from the degree of quenching of the donor in the presence and absence of acceptor. The distribution of distances between the donor and acceptor was calculated from the lifetime of the donor in the excited state. The donor alone showed a nearly single lifetime which became multiple lifetimes in the presence of acceptor. These quantitative data were used to generate distance distribution curves between the donor and acceptor pair. These results corroborate previous results obtained from NMR and energy calculations showing that the 6' branch has a greater degree of flexibility than both the 6 or 8 branches of triantennary. This approach should be useful for determining distances and flexibility in a variety of complex oligosaccharide structures.

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Refined Chemical Techniques for the Quantitative Recovery of Intact N- and O- linked Oligosaccharides From Glycoproteins

R B Parekh, C E Warren, A Merry, and J Bruce. Oxford GlycoSystems Limited, Unit 4 Hitching Court, Blacklands Way, Abingdon, Oxon. OX14 1RG

Analysis of the glycosylation of a glycoprotein or glycopeptide generally involves as an initial step, the release, and subsequent recovery free of peptide, of N- and O- linked glycans. Several methods have been proposed to release either N- or O- glycans from glycoproteins, but to date no method has been shown to be generally valid for the simultaneous release in intact form of both. Such a method would be extremely useful in the analysis of the total glycosylation of a glycoprotein. Evidence will be presented of a chemical method involving the use of hydrazine which has been optimised to quantitatively release in intact form both N- and O- linked glycans. Using this optimised method several glycoproteins have been analysed, including bovine fetuin, porcine thyroglobulin, hen ovalbumin, bovine pancreatic ribonuclease B, human α1 - acid glycoprotein, horse-radish peroxidase, human serum IgA and human plasminogen. In these, and several other glycoproteins, the yield of N- and O- linked oligosaccharides was quantitative (>90%) as judged by quantitative monosaccharide composition analysis of the starting glycoprotein and the liberated oligosaccharides. Furthermore, the N- linked type oligosaccharides so released all possessed an intact chitobiose core (>96%) and the O- linked type oligosaccharides an intact Galβ1------->3 GalNAc core (>96%) as judged by 500 MHz - 1-D-H'NMR, P-4 gel filtration and exoglycosidase analysis. By a detailed kinetic analysis of the reactions involved, this chemical procedure has been optimised to ensure high yield of intact N- and O- linked glycans irrespective of oligosaccharide or peptide structure. The method is therefore of general value, and in view of the quantitative aspect of the recovery, relevant in the analysis of small amounts of glycoproteins. Further, the recovered oligosaccharides are in the unreduced form, and so can be used to form a variety of useful derivatives.

TOPOLOGY OF THE POLY-α-2,8-SIALYLTRANSFERASE IN *E. COLI* K1 AND ENERGETICS OF POLYSIALIC ACID CHAIN TRANSLOCATION ACROSS THE INNER MEMBRANE. **F.A. TROY, T. JANAS, T. JANAS AND R.I. MERKER. DEPT. OF BIOL.CHEM., SCHOOL OF MEDICINE, UNIVERSITY OF CALIFORNIA, DAVIS, CA 95616.**

Introduction - Our studies seek to understand how synthesis, transmembrane translocation and surface expression of the α-2,8-linked polysialic acid (polySia) capsule in neuroinvasive *E. coli* K1 strains are regulated, and to determine how biogenesis of the poly-α-2,8-sialyltransferase (polyST) complex occurs. The genes required for polySia synthesis and export have been cloned and shown to be surprisingly complex, requiring 17 kB of DNA divided into 3 functional regions. The purpose of this study was two-fold: 1) to determine the topological orientation of the polyST; and 2) the energetics of polySia chain synthesis and translocation across the inner membrane (IM).

Topology - Using sealed membrane vesicles of defined orientation, either right-side-out vesicles (ROV) or inside-out vesicles (IOV), we have shown that the functional domain of the polyST complex is located on the cytoplasmic surface of the IM. This conclusion was confirmed by partial trypsinolysis which showed that >90% of the activity was lost when IOV were treated with trypsin. In contrast, there was only a slight decrease in polyST activity when ROV were treated with trypsin before inversion. *In vivo* labeling experiments with ^{14}C-Sia in K1 cells unable to degrade Sia (sialic acid aldolase, *nan*A4, mutation) have shown that polySia chains with degree of polymerization (DP) >85-200 are found inside the cell. This unexpected finding requires that chain polymerization must precede translocation across the IM.

Energetics - The proton electrochemical potential gradient ($\Delta\mu H^+$) was shown earlier to be required for maximal polyST activity (JBC 259, 12769 [1984]). Present studies have shown that the high energy phosphoryl potential of ATP is also required to maintain full activity. To determine if the $\Delta\mu H^+$ is required for translocation of polySia chains across the IM, we developed an *in vivo* system that allows us to study directly the translocation step. The system uses spheroplasts prepared from K1 cells that are unable to degrade sialic acid (*nan*A4 mutation). After pulse-labeling the spheroplasts with ^{14}C-Sia, synthesis and translocation are followed kinetically. PolySia chains that have been translocated across the IM are differentiated from those chains remaining inside by their sensitivity to depolymerization by endo-N-acylneuraminidase (Endo-N). All of the polySia chains synthesized within the first 10 min. were internal. During the next 20 min., ca. 40-60% of the chains were translocated across the IM. When inhibitors that dissipate the pH gradient across the IM (e.g. CCCP) were added after 10 min., there was an ca. 80% inhibition of polySia chain translocation. We conclude that the $\Delta\mu H^+$ is required to move the polySia chains across the IM.

Summary - 1) The functional domain of the polysialyltransferase is localized on the cytoplasmic surface of the inner membrane; 2) polySia chains are polymerized inside the cell before being translocated across the inner membrane; 3) full activity of the polyST complex requires $\Delta\mu H^+$ and ATP; 4) translocation of polySia chains across the inner membrane also requires energy provided by the $\Delta\mu H^+$. (Supported by NIH Grant AI-09352.)

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MICROSOMAL TOPOGRAPHY OF THE GLcNAC-TRANSFERASES WHICH CATALYZE THE BIOSYNTHESIS OF GLcNAc-P-P-DOLICHOL AND $(GLcNAc)_2$-P-P-DOLICHOL

Edward L. Kean, Case Western Reserve University, Cleveland, Ohio 44106

The orientation of the N-acetylglucosaminyl transferases which catalyze the biosynthesis of GlcNAc-P-P-dolichol and $(GlcNAc)_2$ was examined using microsomes isolated from livers of the embryonic chick. Dolichol-phosphate liposomes were used as the exogenous substrate, thus avoiding the use of detergents. The formation of both of these intermediates of the dolichol pathway was almost completely inhibited after trypsinization of the microsomes under conditions in which microsomal integrity was maintained as evaluated by determining the latency of mannose-6-phosphatase. Using dolichol (^{32}P)-phosphate liposomes, it was shown that trypsinization did not interfere with the binding of liposomes to the microsome, suggesting that the effect of trypsinization was on the GlcNAc-transferases. Although dolichol-phosphate liposomes associate with the microsomes during incubation, it was possible by sucrose-density centrifugation to separate a population of liposomes which were not bound in this manner. GlcNAc-lipids isolated from the free liposomes were shown to contain both the mono-GlcNAc and $(GlcNAc)_2$-derivatives in proportions similar to those obtained from the microsomes. These studies strongly suggest a non-luminal, cytoplasmic orientation of the N-acetylglucosaminyltransferases concerned with the biosynthesis of the first two intermediates of the dolichol pathway.

CHARACTERIZATION OF A NOVEL FUCOSYLTRANSFERASE ACTIVITY IN DICTYOSTELIUM.
Beatriz Gonzalez-Yanes & Christopher M. West, Department of Anatomy & Cell Biology, University of Florida College of Medicine, Gainesville, FL 32610-0235.

A mutant which is unable to synthesize GDP-fucose was used to screen for GDP-fucose: fucosyltransferase activities capable of utilizing endogenous acceptors. Vegetative amoebae were gently lysed by passage through a nuclepore filter and sedimented at 170,000 g for 2.5 hrs. Incorporation of DPMs from GDP-^{14}C-fucose into TCA-insoluble material was generally greater for the supernatant (S170) than for the pelleted fraction (P170), whereas greater than 95% of cellular acid phosphatase and alpha-glucosidase II activities were sedimented. Inclusion of protease inhibitors sufficient to block degradation of actin and myosin did not affect the activity in the S170 or its distribution. The S170 activity was specific for the ß-anomer of GDP-fucose and was inhibited by either EDTA or Triton X-100. Though the wild-type S170 fraction exhibited no activity in this assay, this fraction was able to potentiate the activity of the mutant S170 fraction, suggesting that a wild-type S170 activity existed but was acceptor-limited.

A similar analysis was carried out for the distribution of fucosides formed after long-term metabolic labeling of cells with ^{3}H-fucose. SDS-PAGE of fractionated cells disclosed a single predominant protein species (FP23), containing ca. 5% of total cellular ^{3}H-DPMs, in the S170 fraction. This species was not apparent in the P170 fraction by 1-D SDS-PAGE, or in the supernatant fraction recovered by sonication and resedimentation of the P170 fraction.

Analysis of the products of the in vitro fucosylation reactions by SDS-PAGE revealed that FP23 was also the predominant acceptor species for the S170 fucosyltransferase activity. In concordance with the analysis of the in vivo reaction products, FP23 was not detected in the product of the P170 reaction. The data suggest the existence of a cytoplasmic fucoprotein, modified by the action of a cytoplasmic fucosyltransferase, which would utilize a non-Golgi pool of GDP-fucose.

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PORCINE SUBMAXILLARY MUCIN

Allen E. Eckhardt, Yang Wang, Candace S. Timpte, John L. Abernethy, Jr., Yun Zhao and Robert L. Hill. Department of Biochemistry, Duke University Medical Center, Durham, NC, 27710.

Porcine submaxillary mucin (M_r = 2-4 x 10^6) is a long, extended molecule with serine or threonine occuring on an average of one in every three residues. About three-fourths of the hydroxyl groups of the serine and threonine residues are in 0-glycosidic linkage with GalNAc or an oligosaccharide with GalNAc at the carbohydrate-protein linkage region. The sequence of mucin cDNA reveals that the 220 amino acid residues at the COOH-terminus of mucin form a globular domain with very few 0-linked oligosaccharides. The remainder of the molecule is devoid of secondary structures and contains all of the 0-linked oligosaccharides. This region (>2500 residues) contains from 18-25 tandemly repeated sequences that are 81 residues in length and appear to be identical in sequence. The sequence of the molecule from its NH_2-terminus to the tandem repeats is currently being determined by sequence analysis of genomic DNA.

Although the tandemly repeated regions appear to have the same 81 residue amino acid sequence, the cDNA and genomic sequences encoding these regions differ slightly among the repeated sequences without changing the amino acid sequence. Southern blots of genomic DNA from individual pigs are in accord with these sequence variations. This fact, together with the observation that the number of repeated sequences may vary between individuals, suggests that the genes encoding submaxillary mucin are prone to genetic variations.

The amino acid sequence of mucin does not reveal a consensus sequence adjacent the serine and threonine residues that is recognized by the GalNAc transferase that glycosylates mucin. The substrate specificity of the transferase is being examined, however, with pure enzyme and synthetic substrates in order to assess structural features required for glycosylation. The pig submaxillary gland transferase (M_r = 66,000-70,000) has been purified 1300x to a specific activity of 1.65 µmole/min/mg. Although it readily glycosylates a variety of peptides containing serine or threonine, no more than 10-15% of the serine or threonine residues can be glycosylated under saturating conditions. Further aspects of the substrate specificity and the structure of the transferase are under study.

Supported by the National Institutes of General Medical Sciences, NIH, Grant GM25766.

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CLONING STUDIES ON BSM AND CANINE TRACHEAL MUCIN.

E.A. Davidson, Department of Biochemistry and Molecular Biology, Georgetown University School of Medicine, Washington, DC 20007.

Screening of a bovine submaxillary gland cDNA library with polyclonal anti apo-BSM antibodies identified a 2.0 Kb clone. Sequence analysis showed a single open reading frame coding for a 563 residue protein (59 KDa), a figure in agreement with that indicated by translation studies. The amino terminal domain (1-339) is high in thr (25%), ser (15%) and gly (14%) whereas the carboxyl domain is high in cys (13%), asp (7%) and aromatics (Tyr, 6%). RNA blot analysis using DNA probes corresponding to the specific regions indicates that these domains are not artifacts arising from the library. Repeat sequences (11 x 3), a potential cleavage site and N-glycosylation sites have been identified. Sequence homologies to other proteins are evident only for the presumed glycosylation domain. (PSM apomucin for example). Transcripts exhibited tissue specificity as well.

Combined chemical and enzymatic deglycosylation of canine tracheal mucin glycoprotein allowed preparation of antibodies specific for peptide sequences. Screening a canine tracheal epithelium cDNA library with these polyclonal sera resulted in identification of a 2.1 Kb clone. Although not full length, an open reading frame was present coding for over 500 A.A. Neither terminus is present. Similarities to the BSM data above are the presence of a Ser, Thr rich domain characterized by clusters of the hydroxyamino acids; there is no indication in the region thus far sequenced of any significant repeat motif. A non or poorly glycosylated region is also apparently present but is not unusually cysteine rich. Although the anti-canine mucin antibody cross-reacts with deglycosylated human tracheo-bronchial mucin, a human clone has not yet been identified by antibody screening. Further comparisons between the canine and human mucins indicate considerable homology.

HUMAN INTESTINAL MUCIN CORE PROTEINS

Y.S. Kim, J. Gum, J.C. Byrd, N. Toribara, S. Ho, and R. Dahiya. GI Research Lab (151M2), VA Medical Center, 4150 Clement St., San Francisco, CA, 94121, Dept. of Medicine, University of California, San Francisco, CA, 94143.

Mucins are major secretory products of the gastrointestinal tract and are thought to play important roles in cytoprotection and lubrication. Until recently, the structures of apomucin proteins have been poorly understood. We have purified, by gel filtration and CsCl density gradient centrifugation, a threonine-rich sialomucin from nude mouse xenografts of the LS174T human colon cancer cell line and a serine-rich fucomucin from normal human small intestine. To examine the polypeptide backbone structure of these mucins, both xenograft and small intestinal mucins were deglycosylated with HF. Antibodies against the deglycosylated small intestinal mucin (SIB) bound to both SIB and the deglycosylated xenograft mucin (HFB), while anti-HFB bound to HFB much more than to SIB. Neither antibody bound to the heavily glycosylated native mucins. The antibodies were used to screen a human small intestine λgt11 cDNA library. Two groups of partial cDNA clones were obtained and sequenced. One group (MUC-2), was identified by anti-HFB. The MUC-2 cDNAs contained tandem repeats of 69 nucleotides which code for a protein rich in threonine and proline. The second group (MUC-3), identified by anti-SIB, contained tandem repeats of 51 nucleotides which code for a protein rich in both threonine and serine. Both the MUC-2 and the MUC-3 genes are polymorphic. Partial sequencing of MUC-2 genomic DNA suggests that a variable number (35 to 50, depending on allele) of tandem repeats are clustered together in the molecule. The MUC-2 gene maps to chromosome 11 and the MUC-3 gene maps to chromosome 7. Thus, both are distinct from the human mammary mucin gene (MUC-1) which maps to chromosome 1 and consists of 60 nucleotides. Immunohistochemical studies using polyclonal antibodies prepared against synthetic mucin repeat peptides and Northern analysis of cells and tissues indicate that the three known mucin genes are regulated independently of each other.

UTILIZATION OF A UNIQUE NUCLEOTIDE PROBE TO IDENTIFY cDNA ENCODING HUMAN TRACHEOBRONCHIAL MUCIN GLYCOPROTEIN.

Mary C. Rose*, Peter Charles*, Daoud Meerzaman* and Brian M. Martin+. *Dept. of Pediatrics and Dept. of Biochemistry and Molecular Biology, George Washington University and Department of Pulmonary Medicine, Children's National Medical Center, 111 Michigan Ave, N.W. Washington D.C. 20010 and +Clinical Neuroscience Branch, National Institute of Mental Health, ADAMHA, Bethesda, MD 20892.

Human mucin glycoproteins are highly glycosylated macromolecules (80-90% carbohydrate by weight) which contain several hundred oligosaccharide chains in O-glycosidic linkage to threonine and serine residues (1). Human tracheobronchial mucin (TBM), purified by column chromatography and density centrifugation in the presence of reducing and dissociating agents (2,3), contains both glycosylated and non-glycosylated domains. Several peptides from the non-glycosylated domains of TBM have recently been purified and their amino acid sequence determined. A sequence of 22 amino acids was obtained for TR-3A, the major non-glycosylated tryptic peptide of TBM (3).

Unique nucleotide probes for TBM were generated from the amino acid sequence of TR-3A by polymerase chain reaction (PCR). Primers (64-fold degenerate) for the DNA coding and non-coding strands of TR-3A were synthesized in the 5' to 3' direction for each strand and first strand cDNA (synthesized from human nasal polyp mRNA) was used as template. A 66 base pair product was amplified, subcloned into m13mp18, and sequenced. The nucleotide sequence of two inserts (PC1 and PC5) differed by four bases, each in the third position of a codon. As the deduced amino acid sequence of the peptides encoded by PC1 and PC5 were identical to the amino acid sequence of TR-3A obtained by Edman degradation analysis (3), PC1 and PC5 appear to be unique nucleotide probes for TBM. The unique nucleotide probes hybridized to transcripts in mucus-rich tissue (trachea, nasal polyps) but not in muscle. Anti-sense PC5 hybridized to transcripts of 7.6, 4.3, and 2.0 kb from human tracheal and nasal polyp RNA on Northern blots. A probe for the tandem repeat of human intestinal mucin (4) also hybridized to transcripts of the same size in nasal polyp and tracheal RNA.

A mixture of nucleotide probes (including PC1 and PC5) was used to screen a human cDNA lung library in lambda gt11 (Clontech). Fourteen positive clones were identified on screening four millon plaques. Inserts from positive clones were amplified by PCR using lambda forward and reverse primers. Amplified products ranging in size from 1000 to 300 bases were obtained from eight of the positive clones and reacted with one or more of the nucleotide probes used for screening. These clones are presently being sequenced and the results of these analyses will be presented.

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This research was funded by NIH grant HL33152.

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REGULATION OF COLON CARCINOMA SIALOMUCIN EXPRESSION

Tatsuro Irimura, Andrew M. McIsaac, Regina C. Sutton, David F. Dohi, Yoshifumi Matsushita, Karen R. Cleary, and Marsha L. Frazier.
The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030.

Studies with pathological specimens and with experimental models of HT-29 variant cells in nude mice indicated that higher levels of sialomucins as determined by WGA-binding to electrophoretically separated high molecular weight glycoproteins and by metabolic incorporation of [^{3}H]glucosamine to high molecular weight components are associated with metastatic human colon carcinoma cells. In HT-29 LMM cells grown in nude mice, differential levels of WGA-reactive sialomucins are apparently due to (1) their clonal origins, and (2) previously unknown microenvironmental factors. We have recently found a soluble protein factor from normal colon connective tissues may be responsible for the increased production of sialomucins as determined by WGA binding. Conditioned media of established human colon fibroblasts contains similar activity but it is not accounted for by TGF-β or IL6. We have partially purified this soluble factor, which we are tentatively calling mucomodulin, by a combination of ion exchange chromatography and gel filtration on Sephacryl S-200. Colon carcinoma cells when treated with an effective dose of mucomodulin did not show changes in morphology or proliferation rates. The effect on WGA-binding was reversible upon the cessation of the treatment. DEAE-cellulose elution profiles of the oligosaccharides produced by HT-29 LMM cells in the presence of benzyl-N-acetylgalactosaminide, untreated or treated by mucomodulin, did not show significant differences, indicating that sialylation of O-linked sugar chains was not affected. Differential binding of RCA1 or PNA after removal of sialic acid from the high molecular weight glycoproteins also indicated that the degree of sialylation did not change after mucomodulin treatment of HT-29 cells. Northern blot analysis using cDNA for the polymorphic epithelial mucin core polypeptide as a probe demonstrated that HT-29 LMM cells expressed this gene and that the level of mRNA became elevated after mucomodulin treatment of the cells. HT-29 sublines selected *in vitro* for high levels of WGA reactive sialomucins were shown to produce a higher degree of sialylation in their O-linked sugar chains than low sialomucin variants. These results indicate that sialomucin expression by human colon carcinoma cells is regulated by their clonal origins as well as by microenvironmental factors, and that sialylation and core polypeptide synthesis may correspond to these mechanisms.

Supported by NCI grants RO1-CA39319 and 50231, THEP grant 1549, and a grant from National Foundation for Cancer Research.

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GANGLIOSIDE-MODULATED PROTEIN PHOSPHORYLATION SYSTEMS

Kai-Foon Jesse Chan
Laboratory of Experimental Neuropathology
National Institute of Neurological Disorders and Stroke
National Institutes of Health, Bethesda, MD 20892, U.S.A.

Gangliosides are sialic acid-containing glycosphingolipids ubiquitous in eukaryotic cells. These glycoconjugates have been implicated to play important roles in a number of biological processes including cellular communication, differentiation, and development. However, their exact functional roles in many of these events are still unclear. Studies in this laboratory revealed that gangliosides, in particular, polysialogangliosides, can exert profound modulatory effects on several protein phosphorylation systems, at least under in vitro conditions.

In synaptosomal membrane and myelin preparations, some of the actions of gangliosides may be mediated through direct regulation of the intrinsic enzymic activities of two novel ganglioside-dependent protein kinases. These include a ganglioside-stimulated protein kinase (PKJ) and a ganglioside-inhibited protein kinase (PKL). It is possible that perturbation of gangliosides in the brain can confer a synchronistic action on the regulation of these two protein phosphotransferase systems. The mechanisms involved in the mobilization of gangliosides for biomodulator function are unknown at present. Prelimary studies indicated that the sialic acid-containing oligosaccharide derived from G_{M1} can enhance the activity of PKJ, but the effect is at least ten times less potent than G_{M1}. By contrast, the oligosaccharide derived from asialo-G_{M1} is completely ineffective.

Gangliosides also can modulate the activities of protein kinase C (PKC) and a Ca^{2+}/calmodulin-dependent protein kinase (CaMK-2), provided their allosteric effectors such as Ca^{2+}/phosphatidylserine and Ca^{2+}/calmodulin, respectively, are present simultaneously. Because the substrate specificities of PKJ, PKL, PKC, and CaMK-2 are distinct from one another, alteration of the phosphorylation of the various target substrates by gangliosides may lead to a synergistic effect in generating appropriate physiological responses.

In skeletal muscle, phosphorylation of glycogen phosphorylase is increased by addition of gangliosides to the soluble extract. This ganglioside-stimulated effect is in part due to an activation of phosphorylase b kinase, a key enzyme in glycogen metabolism.

Phosphorylation of specific proteins also can be regulated by direct interaction of gangliosides with the protein substrates. This substrate-directed effect has been observed in the protein kinase C-catalyzed phosphorylation of myelin basic protein as well as the matrix protein M of vesicular stomatitis virus.

The physiological significance of the various ganglioside-modulated protein phosphorylation systems still remains to be established. Nevertheless, the wide range of putative functional roles of gangliosides suggests that this class of glycoconjugates can serve as multifunctional biomodulators.

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THE ROLE OF GANGLIOSIDE GM1 IN CELL GROWTH AND SIGNAL TRANSDUCTION PATHWAYS.
Sarah Spiegel, Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, DC 20007

Gangliosides have long been implicated in cell growth regulation. A further insight into the physiological role of gangliosides has emerged through the use of the binding or B subunit of cholera toxin. The B subunit, a protein which binds specifically to ganglioside GM1 on the cell surface, has profound effects on cellular proliferation of diverse cell types. The B subunit induced proliferation of resting thymocytes and of quiescent, non-transformed mouse 3T3 fibroblasts. However, in contrast to its effect on resting cells, the B subunit inhibited the growth of *ras*-transformed 3T3 fibroblasts (Ha-, Ki-, and N-*ras*) as well as normal 3T3 cells during rapid growth phase. THe B subunit also diminished the growth of rat glioma C6 cells with elevated levels of GM1 and mouse neuroblastoma cells. In addition, while the B subunit potentiates the effect of EGF, insulin, bombesin, platelet derived growth factor, and even unfractionated fetal calf serum in quiescent cultures of 3T3 fibroblasts, it inhibits DNA synthesis induced by TPA via protein kinase C. The dual effects of the B subunit were also related in this case to different stages of the cell cycle. Recently, we demonstrated that the biphasic effect of the B subunit is determined by the growth stage of the cells and the inherent ganglioside intracellular signaling network and not by the concentration of cell surface GM1. The biphasic response has also been observed in other cells. The discovery that the interaction of the B subunit with endogenous ganglioside GM1 leads to opposing effects on the growth of 3T3 fibroblasts, depending on their state of growth, or depending on the context of other growth factors, led us to suggest that endogenous gangliosides may play a fundamental role as regulators of both positive and negative signals for cell growth.

The use of the B subunit has provided a unique tool to begin an investigation of the molecular mechanisms underlying the action of ganglioside GM1 and the signal transduction pathways utilized by endogenous ganglioside GM1 to modulate cellular proliferation have been extensively investigated. We have found that the binding of the B subunit does not elicit the classical intracellular second messenger systems, such as cAMP, DAG (an endogenous activator of protein kinase C), or IP3 (which mobilizes Ca^{+2} from internal stores) since the B subunit did not activate adenylate cyclase, Na^{+}/H^{+} exchange, phospholipase C, or protein kinase C. However, the B subunit mediated a large increase of intracellular free calcium resulting from a net influx from extracellular sources. This suggests that endogenous ganglioside GM1 might be involved in the regulation of Ca^{2+} fluxes in many other cellular processes. The rise in $[Ca^{+2}]_i$ by itself was not sufficient to explain the effects of the B subunit since Ca^{+2} ionophores in contrast to the B subunit, did not stimulate DNA synthesis in quiescent 3T3 fibroblasts and did not increase the synthesis of numatrin, a nuclear protein whose synthesis is closely correlated to cellular commitment for mitogenesis. Recently, we have shown the involvement of a pertussis toxin-sensitive GTP-binding protein in a late event of DNA synthesis mediated through the interaction of endogenous gangliosides with the B subunit. The underlying mechanism of the involvement of this Gi protein in DNA synthesis is not yet clear, but is clearly not related to any effects of coupling to phospholipase C or adenylate cyclase, or on Ca^{+2} influx. Taken together, the results described above imply that gangliosides may modulate cellular proliferation through an as yet undefined growth signaling pathway. Increased attention is now aimed at the elucidation of new signal transduction pathways and their connection to ganglioside functions.

Acknowledgement: This work was supported by NIH Research Grants 1RGM39718 and 1RO1GM43880.

ROLE OF GANGLIOSIDE AND ITS CATABOLITE IN THE MODULATION OF TRANSMEMBRANE SIGNAL TRANSDUCTION

Sen-itiroh Hakomori. The Biomembrane Institute and University of Washington, 201 Elliott Ave. W., Seattle, Washington 98119, USA

Gangliosides are ubiquitous membrane components of essentially all eukaryotic cells and function as modulators of transmembrane signal transduction, resulting in the regulation of cell proliferation. Taking GM_3 in A431 cells as an example, a systematic study of the effect of GM_3, lyso GM_3, de-N-acetyl GM_3, N,N-dimethylsphingosine on the EGF receptor-associated kinase and protein kinase C have been undertaken (1-4). GM_3 strongly inhibits EGF-dependent receptor phosphorylation, while lyso GM_3 inhibits protein kinase C. N,N-dimethylsphingosine promotes various protein kinases, including EGF-receptor kinase and other kinases associated with cell proliferation.In addition, the use of 1-phenyl-2-decanoylamino-3-morpholino-1-propanol, particularly its D-threo isomer (D-PDMP) which inhibits UDP-Glc: ceramide Glc transferase (5) resulting in the accumulation of ceramide and N,N-dimethylsphingosine and the depletion of GM_3 will be presented. Taking IL-2 dependent proliferation of T-cell line (CTLL) as an example, D-PDMP decreases in GM_3 level and increases N,N-dimethylsphingosine; both of which may cooperate to enhance IL2-dependent tyrosine phosphorylation of several functionally unknown proteins (Mw. 55K, 85K, 100K) leading to inhibition of cell growth possibly through exhaustion of intracellular ATP (6). Thus, a cell growth regulatory mechanism operating via dimethylsphingosine level could be equally important as that operating via GM_3 level and quantities of these two molecules may cooperatively regulate transmembrane signalling.

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MOLECULAR GENETIC BASIS OF THE HISTO-BLOOD GROUP ABO SYSTEM.
Fumi-ichiro Yamamoto, Henrik Clausen, and Sen-itiroh Hakomori. The Biomembrane Institute and University of Washington, 201 Elliott Ave. W., Seattle, WA 98119, USA.

The blood group ABO antigens were the first major human alloantigens recognized, and are responsible for failure of unmatched blood transfusions. While the A and B antigens were shown to be inherited as simple Mendelian dominant characters, and the three-allelic model for the inheritance of the ABO genes was proposed by Bernstein early in this century, the molecular genetic mechanism controlling expression of ABO antigens has remained unknown. The A and B genes are thought to encode the glycosyltransferases to synthesize either A or B antigen, whereas the O gene is thought to be silent or give rise to an enzymatically inactive protein. Recently, the soluble form of α1→3 N-acetylgalactos-aminyltransferase (A transferase) was isolated from human lung tissue (J Biol Chem 265: 1139-1145). Based on partial amino acid sequence, we have cloned and sequenced cDNA encoding this transferase (J Biol Chem 265: 1146-1151).

In the present study, we cloned and sequenced cDNA from cell lines of different ABO status, using the A transferase probe, in order to differentiate the base sequence between ABO genes. A total of four cDNA libraries were constructed in λgt10 with poly(A)$^+$ RNA from human colon adenocarcinoma cell lines SW948 (phenotype O, patient blood type O), SW48 (AB, AB), Colo205 (O, unknown), and SW1417 (B, B), and nucleotide sequences of cDNA clones, which were hybridized with the A transferase probe, were determined and compared. The A and B genes differ in a few single base substitutions, changing four amino acid sequences (176, 235, 266, and 268) which may cause differences in specificity of A and B transferases (α1→3 galactosyltransferase). A critical single base deletion was found in the O gene. This deletion changes the frame of codons and results in an entirely different, inactive protein which is incapable of modifying the H antigen. Nucleotide sequence analysis of cDNA clones identified allele-specific restriction enzyme cleavage sites at three of the four substitutions between A and B allelic cDNAs as well as the single-base deletion found in O allelic cDNAs. We confirmed the structural ABO differences by analysis of genomic DNAs prepared from 14 blood samples with different ABO status, as evidenced by digestibility with these diagnostic restriction enzymes.

CLONING, SEQUENCE, AND EXPRESSION OF HAMSTER UDP-GlcNAc:DOLICHOL PHOSPHATE GlcNAc-1-P TRANSFERASE Mark A. Lehrman and Xiaying Zhu. Department of Pharmacology, UT-Southwestern, 5323 Harry Hines Blvd., Dallas TX. 75235.

UDP-GlcNAc:dolichol phosphate N-acetylglucosamine-1-phosphate transferase (GPT) catalyzes the initial reaction required for synthesis of dolichol-P-P-oligosaccharides. Our laboratory has now sequenced and expressed a full-length cDNA clone encoding hamster GPT. The cDNA predicts a protein of 408 amino acid residues including 10 hydrophobic segments. Several portions of the hamster GPT sequence constituting one-third of the protein have 60% or greater identity with yeast GPT, and half of the conserved sequence falls within the hydrophobic segments.

Hamster GPT has two copies of a putative dolichol recognition sequence recently identified in four yeast proteins (ALG1, ALG7, DPM1, Sec59) that interact with dolichol. The refined consensus based on six peptides is:

Phe-Ile/Val-X-Phe/Tyr-X-X-Ile-Pro-Phe-X-Phe/Tyr

with invariant residues underlined.

By transient expression in COS-1 cells or stable expression in CHO cells, the cDNA causes a five to ten-fold increase of GPT activity in membrane fractions. The primary product in COS-1 membranes was shown to be GlcNAc-pyrophosphoryldolichol, and the activity was completely inhibitable by tunicamycin.

This cDNA represents the first enzyme of the dolichol-oligosaccharide biosynthetic pathway to be cloned from a vertebrate source and demonstrates structural homology between the enzymes of the yeast and mammalian pathways. Future efforts will be directed at mapping functional domains of the enzyme.

CLONING AND CHARACTERIZATION OF GlcNAc-1-P-TRANSFERASE cDNA FROM MOUSE MAMMARY GLANDS. B.Rajput, J.Ma and I.K.Vijay. Dept. of Animal Sciences, U. of Maryland, College Park, Md 20742.

The enzyme GlcNAc-1-P-transferase (GPT), catalyzes the first step in the assembly of the dolichol-linked oligosaccharides required for asparagine linked (N-linked) glycosylation in eukaryotes, and thus is a potential target for the regulation of biosynthesis of N-linked glycoproteins. An earlier study from our laboratory showed that three key enzymes including GPT underwent differentiation-related activation during ontogeny of the mouse mammary gland and that prolactin appeared to play a role in this regulation (Eur.J.Biochem.154,57-62(1986)). In order to study the developmental and hormonal regulation of GPT at the molecular level, it was deemed necessary to isolate the cDNA coding for GPT from mouse mammary glands. A cDNA fragment encoding the putative GPT in rat liver (kindly provided by Dr. Lehrman) was used as a probe to screen a λgt11 library prepared from mRNA isolated from mouse mammary glands. Upon screening $1.3x10^6$ phage, 7 positive plaques were isolated. The clones were characterized by restriction enzyme, PCR, Southern, Northern and DNA sequence analyses. These data showed that the clones shared DNA sequences in common with the rat liver GPT cDNA. Probes generated from the 5' and 3' ends of the cDNAs isolated thus far were used for a second round of screening in order to obtain full length cDNA sequence. Five more positive plaques were found and they are currently being characterized.

SITE DIRECTED MUTAGENESIS OF *ESCHERICHIA COLI* CMP-N-ACETYLNEURAMINIC ACID SYNTHETASE. Gerardo Zapata, Jane Makovitch, and Willie F. Vann. Laboratory of Bacterial Polysaccharides, CBER, FDA, Bethesda, Md. 20892.

CMP-N-acetylneuraminic acid (CMP-neuAc) synthetase catalyzes the formation of the substrate for sialyltransferases. The role of amino acid residues in the function of *Escherichia coli* CMP-neuAc synthetase has been investigated by several site directed mutagenesis methods. The amino acid sequence of CMP-neuAc synthetase only has 2 cysteine residues. Enzymatic activity and thermal stability are not affected by iodoacetamide or iodoacetic acid. Each of these residues was separately mutated to glycine by the polymerase chain reaction (PCR) method of Higuchi *et. al.*. Significantly less CMP-neuAc synthetase activity was detected in strains harboring these mutations. The effects of mutating cys129 and cys329 do not appear to be equivalent. Thus, either important cysteines are not accessable to alkylation or cys129 and cys329 are involved in *in vivo* folding.

CMP-neuAc synthetase has sequence homology with CMP-ketodeoxyoctulosonic acid (CMP-KDO) synthetase. Random mutations were generated in defined regions in both the amino and carboxyl termini of CMP-neuAc synthetase by using a novel PCR approach. Mutation of isoleucine-5 in the amino terminal sequence of CMP-neuAc resulted in low levels of CMP-neuAc synthetase activity. Isoleucine-5 occurs in a region of homology with CMP-KDO synthetase. These studies suggest that isoleucine-5 may be important for activity.

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Modifications of Biological Activities Of Interleukin-2 By Chemical Glycosylation.

Subramaniam Sabesan And T. Juhani Linna
E. I. du Pont de Nemours & Co., Central Research & Development Department, Wilmington, DE 19880-0328, USA.

Interleukin-2 (IL-2) is a glycoprotein of 15,000 m. wt. and is secreted by antigen activated T-lymphocytes. Known as T cell growth factor, IL-2 causes the activation and proliferation of T cells. In addition, it augments the activities of cytolytic natural killer (NK) cells and induces lymphokine activated killer (LAK) cells. Adoptive immunotherapy using LAK cells and interleukin-2 has been shown clinically to be useful in the treatment of some cancers and may be of value in infectious diseases.

IL-2 made through recombinant DNA technology contains insoluble inclusion bodies in bacterial cells and is therefore deficient in desired physical properties, such as solubility and thermal stability. In addition, IL-2 has very short serum half life. To improve these physical and pharmacological properties, we have chemically glycosylated the rIL-2 with synthetic carbohydrates. Oligosaccharides having a tether have been made through chemical and combined chemical and enzymatic procedures and have been linked to lysine residues of rIL-2 through the acyl azide chemistry. Sialylated IL-2 has been prepared enzymatically by using glycosylated IL-2s containing the lactosamine core. The glycosylated rIL-2 (grIL-2) has been shown to be readily soluble in biological buffers. We have proved through circular dichroism measurements that the glycosylation results in very little change in the conformational properties of the molecule. In addition, the glycosylation dramatically increases its thermal stability up to 90°C. The biological activities appear to be differentially retained and depend on the degree of glycosylation. Whereas most of the T cell growth factor (TCGF) activity has been lost upon glycosylation, the NK augmenting and LAK generating properties have been relatively well maintained, when only few of the lysines are glycosylated. Such differential activity seen for the first time, may be clinically useful to reduce the toxicity possibly associated with T cell activation. These results agree with and can be supported by the published crystal structure of IL-2 and the knowledge of the receptors on cells that mediate the TCGF and NK - LAK activities.

MODULATION OF LACTOSIDE-BINDING LECTINS DURING DIFFERENTIATION OF EMBRYONAL CARCINOMA CELLS

Reuben Lotan, Dafna Lotan, Dunia Carralero, and Brad Amos

Department of Tumor Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Tx. 77030

Cell surface carbohydrates have been implicated in intercellular and cell-extracellular matrix interactions during embryogenesis, development, and in cancer. It has been suggested that some of the carbohydrate-mediated interactions involve recognition of specific carbohydrates by carbohydrate-binding lectin-like molecules on the cell surface. We use murine embryonal carcinoma cells as a model for studying the involvement of endogenous lactoside-binding proteins and cell surface glycoproteins in such interactions before and after induction of differentiation by retinoids. Murine F-9 embryonal carcinoma cells were found to contain a low level of two lectins with M_r 14.5 and 34 kDa. Treatment of these cells with either β-all trans retinoic acid (RA) or Ch55, a chalcone carboxylic acid with retinoid-like properties, resulted in the formation of cells with the properties of primitive endoderm. This differentiation was accompanied by a rapid decrease in the expression of a 34 kDa lactose-binding lectin. This decrease occurred before any overt alterations in the morphology or growth of these cells in response to RA. After 48 h of treatment the 34 kDa lectin was undetectable by immunoblotting. We propose that this decrease is associated with suppression of the growth and the expression of the transformed phenotype of the cells. Subsequent to the disappearance of the 34 kDa lectin there was an increase in the amount of a 14.5 kDa lectin. The level of this molecule increased several fold after 48 to 72 h of treatment with RA and paralleled the increased expression of the B1 chain of laminin. RA-induced differentiation of the F-9 cells resulted in the increased cell surface expression of the 14.5 kDa lectin as determined by indirect immunofluorescence. In an attempt to identify endogenous glycoconjugates in the F-9 cells that bear oligosaccharides complementary to the lectins' carbohydrate-binding site, we analyzed the glycoproteins synthesized by undifferentiated and differentiated F-9 cells using metabolic labeling with several radioactive monosaccharide precursors followed by SDS:PAGE and fluorography. The result demonstrated changes in the glycosylation of specific glycoproteins of Mr 400, 250, and 175 kDa and the synthesis of laminin B1 chain. RA also increased the level of two lysosomal associated membrane glycoproteins (LAMP-1 and LAMP-2), and their ability to bind ^{125}I-labeled L-phytohemagglutinin (L-PHA). LAMP-1 and L-PHA-reactive glycoproteins were localized to intracellular vesicles, presumably lysosomes, and to the cell surface. The intracellular and the cell surface expression of these glycoproteins was found to increase after RA-induced differentiation. Both laminin and LAMP glycoproteins have been reported to contain polylactosamine oligosaccharide chains, which are bound effectively by lactoside-specific lectins. Indeed, the lectins present in an aqueous extract of F-9 cell were adsorbed on immobilized EHS sarcoma laminin and eluted specifically with lactose but not with 0.5 M NaCl. Double-labeling with anti-lectin (14.5 kDa) and anti-laminin antibodies revealed that the lectin was co-localized with the extracellular matrix glycoprotein, laminin. These results indicate that the 14.5 kDa lectin may serve as a laminin binding protein in this system. Studies are underway to determine whether the lectins bind also LAMP glycoproteins.

THE MOLECULAR NATURE OF A NULL ALLELE OF THE H α(1,2)FUCOSYLTRANSFERASE GENE IN A BOMBAY (O_h) INDIVIDUAL. Lowe, J. B., Kelly, R. J., Larsen, R. D., and Ernst, L. K. The Howard Hughes Medical Institute and the Department of Pathology, Univ. of Michigan Medical School, Ann Arbor, MI 48109-0650. The H blood group antigen serves as the precursor for the action of glycosyltransferases determined by the ABO locus. It consists of Fucα(1,2)Gal linkages on erythrocyte glycoconjugates, and is constructed by an α(1,2)fucosyltransferase encoded by the H blood group locus. Rare individuals have been identified (of the Bombay [O_h] or para-Bombay phenotypes) that do not express red cell H antigens, nor a corresponding α(1,2)fucosyltransferase. The molecular basis for the null phenotype in these individuals has not been defined. We have recently isolated genomic DNA sequences corresponding to the H blood group locus. DNA sequence analysis of 8174 base pairs of the wild type α(1,2)fucosyltransferase gene, and of the corresponding sequence of an allele isolated from a Bombay individual, identified 6 nucleotide sequence differences between these alleles. One of these differences is a point mutation that creates a stop codon within the coding sequence, and is predicted to truncate the α(1,2)fucosyltransferase polypeptide 50 amino acids from it usual COOH-terminus. To confirm that this sequence change was responsible for the apparent inactivation of this Bombay allele, this mutation, and each of the others, were separately introduced into the wild type sequence background, in a mammalian expression vector. These vectors were each transfected into COS-1 cells, and extracts prepared from the transfected COS-1 cells were tested for α(1,2)fucosyltransferase activity. These analyses confirmed that the point mutation in the coding sequence is the molecular lesion that inactivates this allele, and that each of the other sequence differences is functionally neutral in the transfection system. Molecular analysis of other null alleles is in progress.

MOLECULAR BIOLOGY OF MAMMALIAN PROCESSING MANNOSIDASES

K.W. Moremen, J.S.Schutzbach*, W.T.Forsee*, P.Neame**, J. Bischoff†, H.F.Lodish†, and P.W.Robbins Center for Cancer Research, M.I.T., Cambridge, MA 02139, *Department of Microbiology University of Alabama, Birmingham, AL 35294, **Shriners Hospital, Tampa, FL 33612, and †The Whitehead Institute, Cambridge,MA 02139.

The processing of mammalian Asn-linked oligosaccharides is initiated soon after the co-translational transfer of the lipid-linked oligosaccharide to the nascent polypeptide chain. Three glucosyl residues are removed in the endoplasmic reticulum (ER), and six of the nine mannosyl residues are removed in the ER and Golgi complex. At least three enzymes are involved in the mannose trimming reactions; a dMM-resistant α1,2-mannosidase in the ER, a dMM-sensitive α1,2-mannosidase in the ER or Golgi, and an α1,3/α1,6-mannosidase (mannosidase II) in the Golgi complex. Each of these three enzymes has been cloned from mammalian cDNA libraries by a method employing the mixed oligonucleotide-primed amplification of cDNA.

The polypeptide sequence of the rat dMM-resistant ER α1,2-mannosidase revealed no membrane spanning domain or signal sequence but the sequence does bear a striking homology to the vacuolar α-mannosidase from *Saccharomyces cerevisiae*. Northern blots of rat tissue poly $(A)^+$ RNA revealed a single message of ~3.5 kb and transient expression of the cDNA in COS cells also resulted in a 400-fold increase in α-mannosidase activity.

Partial clones for the dMM-sensitive α1,2-mannosidase have been isolated from rabbit, rat, and mouse cDNA libraries based on protein sequence data derived from the purified rabbit liver enzyme. Although the 5' end of the open reading frame remains to be isolated, several features are clear from the partial sequence data. The lack of a membrane spanning domain within the COOH-terminal 53 kDa of the open reading frame suggests that the membrane spanning domain will reside in the remaining ~5-10 kDa of NH_2-terminal sequence. The catalytic domain of the enzyme also contains a helix-loop-helix (E-F hand) calcium binding consensus sequence as predicted by the tight Ca^{2+} requirement for the enzyme activity. Unlike the dMM-resistant α1,2-mannosidase, the mRNA encoding the dMM-sensitive enzyme is heterogeneous in size, with three messages in rabbit liver (~2.2, 2.5, and 4.3 kb) and two in rat and mouse tissues (2.8 and 4.7 kb). DNA sequence analysis has revealed that these messages differ in their 3'-untranslated regions by employing alternate use of polyadenylation signals. The ratio of the multiple bands remained constant among the several rat tissue poly $(A)^+$ RNAs examined, but the total level of expression was quite variable, with highest expression levels in adrenal and liver tissues. Although no similarities were found with other entries in protein or DNA databases, the polypeptide sequence was found to be homologous to the recently cloned ER Man_9-specific processing α-mannosidase from *Saccharomyces cerevisiae* (A.Camirand, A.Heysen, and A.Herscovics, abstract this meeting).

Mannosidase II has been cloned from a mouse cDNA library. The protein sequence and topology data all agree with a model of a type II transmembrane glycoprotein containing an uncleaved signal/anchor domain. The enzyme contains a six amino acid NH_2-terminal cytoplasmic domain, a single transmembrane domain, a stem region followed by a potential protease cleavage site, and a large COOH-terminal catalytic domain. The mannosidase II message is ~7.5 kb, almost 2.5 times larger than the open reading frame, with most of the untranslated region residing on the 3'-end of the message. These features of mannosidase II topology, domain structure, and message organization, in combination with the above results for the dMM-sensitive α1,2-mannosidase, suggest that the similarities among the Golgi transferases reported previously [J.C.Paulson and K.J.Colley (1989) J.Biol.Chem. 17615-17618] may also extend to the Golgi hydrolases. (Supported by NIH grants CA26712, CA14051, CA16777, and GM38643 and a fellowship from the Juvenile Diabetes Foundation)

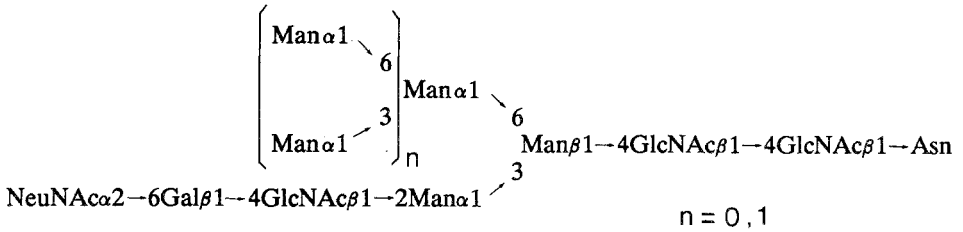
ABSTRACT 26 WITHDRAWN

HEMPAS, GENETIC DEFECT IN GLYCOPROTEIN GLYCOSYLATION.

Michiko N. Fukuda, La Jolla Cancer Research Foundation, La Jolla, CA 92037.

HEMPAS (hereditary erythroblastic multinuclearity with positive acidified serum lysis test) is a rare genetic anemia in humans inherited by autosomally recessive mode. In HEMPAS erythrocyte membranes, Band 3 and Band 4.5 glycoproteins have short Asn-linked oligosaccharides, while in normal erythrocytes these glycoproteins have polylactosaminoglycans. Polylactosamines are accumulated in HEMPAS erythrocytes as polyglycosylceramides. Thus it appears that a genetic factor of HEMPAS blocks the glycosylation of glycoproteins by polylactosamines and shifts these carbohydrates to the lipid acceptors (1).

Structural analysis of HEMPAS Band 3 carbohydrates showed the presence of truncated oligosaccharides as follows:



The lowered activity of N-acetylglucosaminyltransferase II (GnT II) in the same HEMPAS patient's cells suggested a deficiency of GnT II (2).

Recently, we have analyzed new HEMPAS case, G.C., and found that carbohydrates from G.C. HEMPAS erythrocytes contain high mannose-type oligosaccharides as well as hybrid and complex-type oligosaccharides. Enzyme activity assay showed normal level of GnT II, but significantly reduced α-mannosidase II (α-MII) activity in G.C. cells. Northern blot of total RNA and poly(A)$^+$ mRNA from G.C. cells showed significantly reduced amount (less than 10% of normal) of α-MII message at 7.6 Kb. Since no abnormal α-MII mRNA species has been detected, the most likely gene defect in G.C. lies in the promoter region of α-MII gene, resulting in an inefficient transcription initiation. This is the first evidence showing that HEMPAS is caused primarily by a gene defect encoding the enzyme involved in the synthesis of Asn-linked oligosaccharide (3).

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ROLE OF SULFATED GLYCOCONJUGATES IN TUMOR CELL INTERACTIONS WITH THROMBOSPONDIN AND LAMININ. David D. Roberts, Vivian Zabrenetzky, Giulia Taraboletti, Elise Kohn, Henry Krutzsch, C. N. Rao, and Lance Liotta. Laboratory of Pathology, NCI, NIH, Bethesda, MD 20892.

The adhesive glycoproteins laminin and thrombospondin modulate adhesion, migration and growth of tumor cells. These activities are mediated by binding of laminin and thrombospondin to several types of receptors on the cell surface and in the extracellular matrix. For example, thrombospondin binds to protein receptors on melanoma cells via its carboxyl-terminal domain and stimulates attachment and haptotactic migration. Thrombospondin also binds to heparan sulfate proteoglycans and sulfated glycolipids via the amino-terminal domain and binding to the latter stimulates melanoma cell spreading and chemotaxis. The drug suramin is a sulfonated molecule that has antitumor activity *in vivo*. It bound to both laminin and thrombospondin and inhibited their binding to sulfatide. Suramin was therefore used to further examine the role of sulfatide-binding sites on thrombospondin and laminin in promoting tumor cell adhesion and migration. Suramin inhibits melanoma cell spreading and chemotaxis to thrombospondin and haptotaxis, chemotaxis and adhesion of melanoma cells to laminin. These effects of suramin are specific, as the drug was not cytotoxic and did not inhibit cellular responses which do not require binding to sulfated glycoconjugates, including haptotaxis induced by thrombospondin or adhesion or migration of melanoma cells induced by fibronectin. These results confirm that the sulfatide-binding domain of thrombospondin mediates spreading and chemotaxis and suggest that the sulfatide-binding activity of laminin participates in adhesion and migration of melanoma cells to laminin. Following proteolysis, a 50 kDa sulfatide-binding fragment of laminin was isolated by gel filtration and heparin affinity chromatography. The purified fragment bound specifically and with high affinity to sulfatides and to melanoma and breast carcinoma cells. The fragment partially inhibited binding of labelled intact laminin to both cell lines. Thus, the sulfatide-binding site of laminin contributes significantly to binding of laminin to these tumor cells.

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ALTERED GLYCOSYLATION OF SURFACE GLYCOPROTEINS IN TUMOR CELLS AND ITS CLINICAL APPLICATION

Akira Kobata, Department of Biochemistry, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan

Appearance of large *N*-linked sugar chains in the surface glycoproteins is one of the most widely observed phenomena in malignant cells, and is known as the Warren-Glick phenomenon. By comparative structural study of the *N*-linked sugar chains of the surface glycoproteins of cultured cells and their malignant transformants, it was revealed that the enlargement of the sugar chains is induced by the increase of the GlcNAcβ1→6Manα1→6 group as well as the elongated outer chains.

The enzymatic basis of this interesting phenomenon was elucidated by comparative study of *N*-acetylglucosaminyltransferase (GnT) activities of BHK and Py-BHK cells. No GnT III activity was detected in the homogenate of both cells. Same levels of GnTs I, II, IV and VI were detected in both cells. On the contrary, GnT V activity that forms the GlcNAcβ1→6Manα1→6 group was significantly higher in Py-BHK cells than BHK cells. This evidence, together with the report by Van den Eijenden that GnT VI, responsible for the outer chain elongation, works most favorably on the sugar chains with the Galβ1→4 GlcNAcβ1→6Manα1→6 group, indicated that enhancement of GnT V activity is the key to lead to the Warren-Glick phenomenon.

We have recently extended this line of study by using another viral transformation system. 3Y1 cells, a cell line established from Fischer rat embryo can be effectively transformed by adenovirus 12. The advantage of this transformation system is that the cells can be equally transformed by the adenovirus DNA. This opened a way to investigate the mechanism of the genetic regulation, which induces the altered glycosylation in oncoviral transformants. By the comparative study of the *N*-linked sugar chains of surface glycoproteins of 3Y1 cells transfected with adenovirus 12 gene and its fragments, the increase of 2,6-branched triantennary and tetraantennary oligosaccharides was found to be positively correlated to the tumorigenic potential of the transformed cells. The data also indicated that glycosylation of cellular glycoproteins is differently affected by the expression of specific regions of the adenovirus genome, and the combined action of E1 and E4 gene products is important for the enhanced expression of GnT V.

Comparative study of the *N*-linked sugar chains of normal human esophageal epithelium and esophageal squamous carcinoma revealed that the carcinoma have increased amount of sugar chains with the GlcNAcβ1→6Manα 1→6 as well as the Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc group. These data indicated that the Warren-Glick phenomenon occurs widely in human esophageal carcinoma.

MOLECULAR PATHWAYS THAT DICTATE GAL(β1-4)GLCNAC α2-6 SIALYLTRANSFERASE GENE EXPRESSION. Joseph T.Y. Lau, Terrance P. O'Hanlon, and XueCheng Wang, Dept. of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, NY 14263.

The coding information for the β-galactoside α2,6-sialyltransferase is dispersed among greater than 40 kb of rat genomic DNA. Alternate usage of exon sequences results in a family of related mRNAs that are generated in a tissue specific manner. At least some of these mRNAs encode polypeptide products that are significantly divergent from the hepatic β-galactoside α2,6-sialyltransferase. Hepatic transcription of the sialyltransferase is regulated at least in part by glucocorticoids. Putative binding elements for the liver specific transcriptional activators, LF-B_1 (HNF-1) and DBP, are located 49 bp and 210 bp, respectively, upstream of the cap site, suggesting tissue specificity in the utilization of this promoter region. A physically distinct promoter region is responsible for the transcription of the major kidney mRNAs from this sialyltransferase gene. The functionality of these promoter regions is confirmed by their ability to express the bacterial CAT gene in sialyltransferase-CAT constructs. In addition, there is heterogeneity among the sialyltransferase transcripts expressed from the kidney promoter. This heterogeneity is presumably the result of alternate splicing events. Implications of these findings to sialyltransferase regulation will be discussed. (Supported by NIH GM38193 and the Buffalo Foundation)

EXPRESSION-CLONING OF cDNAS THAT RESCUE GENETIC DEFECTS IN CHINESE HAMSTER OVARY CELL GLYCOSYLATION MUTANTS. Michael Heffernan and James W. Dennis. Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, 600 University Avenue, Toronto, Ontario, M5G 1X5 and the Department of Medical Genetics, University of Toronto.

Chinese hamster ovary cells (CHO) have been used extensively for the isolation and characterization of somatic cell mutants, particularly glycosylation mutants. Although the biochemical defects in many of the CHO glycosylation mutants have been identified, the genes involved have not been cloned. Since altered glycosylation can be detected at the cell surface with plant lectins and anti-carbohydrate antibodies, it should be possible to introduce and express a cDNA library in mutant CHO cell lines and pan or select transfected cells, which bear cDNA that corrects recessive mutations or creates a selectable dominant phenotype. An expression cloning system has not been developed for use in CHO cells and since numerous glycosylation mutants have been isolated from these cells, we sought to devise a system similar to that used in COS cells. The CDM8 shuttle vector recently used to clone α1-3Fuc-T using COS-1 cells (Lowe et al.) was constructed with both polyoma and SV40 viral origins of replication (ori) to allow amplification of the vector in mouse (eg. WOP) or simian cell lines (eg COS-1), which express the polyoma and SV40 virus large T antigens, respectively. For use in CHO cells we constructed a modified CDM8 vector, pMH, where the polyoma and SV40 origins of replication were replaced with the 400bp noncoding region of the hamster papovavirus, presumed to be the origin of replication. CHO cells were stably transfected with plasmids for expression of the early genes of polyoma virus and hamster papovavirus. CHO transfectants, expressing hamster large T antigen, supported the efficient replication of the pMH vector while CHO cells, expressing polyoma large T antigen, supported replication of the vector CDM8. These results establish that CDM8 or pMH can be used as an expression vector for cloning of cDNA in CHO cells.

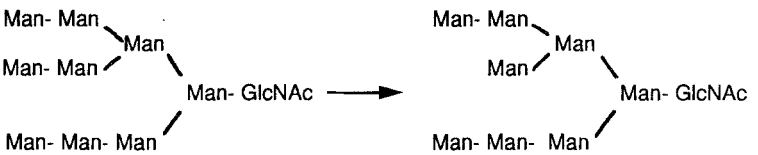
Wild type CHO and lec4 cells, a glycosylation mutant deficient in GlcNAc-TV activity, were transfected with a vector for expression of viral large T antigen (designated CHOP and CHOP4, respectively). Hen oviduct mRNA was used to construct a cDNA library in CDM8 and was transiently expressed in CHOP4 cells. After 48 h of expression, the cells were panned on L-PHA coated plates and plasmids from the attached cells were isolated and amplified in E. coli. After 3 cycles of transfection, panning, and plasmid amplification, a small pool of plasmids remained, which, when transfected into CHOP4 cells, enhanced GlcNAc-TV activity. Isolation and characterization of individual cDNA clones is in progress.

In an effort to clone a cDNA encoding sialic acid hydroxylase, mRNA from D33W25, a dominant WGA-resistant mutation of MDAY-D2 tumour cells, was used to construct a cDNA library in CDM8. D33W25 cells express sialic acid hydroxylase activity, while the gene appears to be inactive in MDAY-D2 and CHO cells. The cDNA library was transiently transfected into CHOP cells and after 48 h the cells were placed in WGA-containing selective medium. Three weeks later, WGA-resistant colonies were selected and five independent isolates were shown to contain identical sized inserts which appear to confer a WGA-resistant phenotype. Characterization of the inserts is in progress.

Using this system, we are attempting to clone cDNAs encoding other glycosyltransferases and sugar-nucleotide transporters deficient in CHO and murine mutant cells.

ISOLATION OF A SPECIFIC PROCESSING α-MANNOSIDASE GENE FROM SACCHAROMYCES CEREVISIAE. A. Camirand, A. Heysen and A. Herscovics. McGill Cancer Centre, McGill University, Montreal, QC, Canada, H3G 1Y6.

We have purified a proteolytically-released soluble form of a yeast α-mannosidase (60 kDa) which removes a single specific mannose residue from $Man_9GlcNAc$ (Jelinek-Kelly and Herscovics (1988) J. Biol. Chem. 263: 14757).



The purified enzyme migrated as two bands of about 44 and 22 kDa on SDS-PAGE under reducing conditions. The amino acid sequences of N-terminal and tryptic fragments from the 44 kDa band were used to design degenerate oligonucleotides which served as primers for PCR reactions on a template of yeast genomic DNA. A YEp24 yeast genomic library was screened with the labelled PCR products and seven related positive clones were purified. A common 4.6 kb Xba I- BamHI fragment was subcloned into pBluescript and sequenced on both strands. A 1.6 kb ORF situated within the Xba I- BamHI fragment contained nucleotide sequences encoding peptides identical to the N-terminal and tryptic amino acid sequences of the 44 kDa band. Furthermore, a sequence encoding the N-terminal amino acid sequence of the 22 kDa band was found within the same ORF, downstream of the region encoding the 44 kDa peptides. The protein encoded by the ORF has a MW of about 63 kDa and possesses a putative non-cleavable signal sequence near the N-terminal region, and a calcium-binding consensus sequence. The 3' non-coding region of the gene has putative polyadenylation signals situated 780 and 600 nucleotides downstream from the TAG codon . Subcloning of a 3.3 kb fragment containing the ORF into the shuttle vector YEp352 and transformation of the vacuolar mannosidase-disrupted yeast strain MK1-11B resulted in an 8- to 10-fold overexpression of the specific α-mannosidase activity. (Supported by NIH grant GM-31265 and by an FRSQ fellowship to A. Camirand).

THE GOLGI APPARATUS LOCALIZATION/RETENTION SIGNAL IS LOCATED IN THE STEM REGION OF THE β-GALACTOSIDE α2,6-SIALYLTRANSFERASE

Karen J. Colley and James C. Paulson Department of Biological Chemistry, UCLA School of Medicine, Los Angeles CA 90024-1737.

To investigate the basis for the localization of the β-galactoside α2,6-sialyltransferase (ST) to the the Golgi apparatus of cells, we have expressed altered ST molecules in CHO and Cos-1 cells and determined their localization by immunofluorescence and biochemical analysis. The ST is a class II membrane protein which has been localized to the *trans* cisternae of the Golgi and the *trans* Golgi network. It possesses a 9 amino acid cytoplasmic tail, a 17 amino acid signal-anchor domain and an extended, 35 amino acid stem region which tethers the large COOH-terminal catalytic domain to the rest of the molecule. Previous results have suggested that the stem region of the ST contains sequences which are responsible for its localization in the Golgi apparatus. Replacement of the first 57 NH_2-terminal amino acids of the ST with a cleavable signal peptide and expression of this construct in CHO cells, resulted in the rapid secretion of the active ST catalytic domain. These data suggested that the ST Golgi apparatus localization signals must reside within the cytoplamic tail, signal anchor and/or stem regions of the enzyme (Colley, K. J., Lee, E. U., Browne, J. K., Adler, B. and Paulson, J. C. (1989) J. Biol. Chem. 264, 17619-17622). Deletion of the core amino acids of the cytoplasmic tail resulted in a Δtail-ST molecule which behaved like wild type ST and was localized to the Golgi apparatus of Cos-1 cells. Remarkably, when ST sequences were altered by oligonucleotide-directed mutagenesis to include signal cleavage sites at the COOH-terminal end of the signal anchor domain, the resulting signal cleavage-ST (sc-ST) molecules, which contained only the stem region and catalytic domain of the ST, were largely retained within the Cos-1 cells and predominantly localized to the Golgi apparatus. These results suggested that the stem region of the ST contains sequences required for localization to the Golgi apparatus. Based on these results we have initiated an in depth study of the stem region of the ST to further define the amino acid residues involved in the Golgi apparatus localization. The results of analyses of the stem region by detailed oligonucleotide-directed mutagenesis and the construction of fusion proteins will also be presented.(Supported by NIH grants GM 27904 and GM 11557 and an American Cancer Society, California Division Senior Fellowship S-28-90).

SUBCELLULAR LOCALIZATION OF β1,4-GALACTOSYLTRANSFERASE (GT).

Daisuke Aoki, Craig Dubois, and Michiko N. Fukuda, La Jolla Cancer Research Foundation, La Jolla, CA 92037.

Subcellular localization of GT in *trans* Golgi cisternae has been well documented. In order to investigate the retention of GT in the Golgi apparatus, we have transfected Cos-1 cells with the eukaryotic expression vector pcDNAI containing human GT cDNA[1)2)] and examined intracellular distribution of the expressed protein by immunofluorescence microscopy. GT is a type II membrane-anchored protein composed of a 23 amino acid residue NH_2-terminal cytoplasmic tail, a 20 amino acid membrane-anchoring domain, and a presumptive "stem" region of 34 amino acids followed by large catalytic domain including the COOH-terminus. Since GT is universally present in mammalian cells, the expression vector was designed to encode a chimeric protein, which is composed of the entire GT molecule fused to a 59 amino acid residue COOH-terminal region of human chorionic gonadotropin α-subunit (hCGα) as a reporter. The localization of the expressed fusion protein was then immunocytochemically analyzed using rabbit anti-hCG antibodies followed by FITC conjugated anti-rabbit IgG. The immunostaining of the transfected Cos-1 cells clearly demonstrated that this chimeric protein expression was restricted to a region of Golgi membranes, as expected. This expression system allowed us to determine which domain is responsible for the Golgi retention of this enzyme, by analyzing the intracellular location of chimeras which had been altered by site-directed mutagenesis. Two deletion mutants, which are lacking 19 amino acids in the NH_2-terminal cytoplasmic tail or 10 amino acids immediately after the membrane anchoring domain, were found to be localized to the Golgi in the same manner as the wild-type GT-hCGα. However, in the case of another mutant which is lacking 6 amino acids (37-42) from the membrane anchoring domain, the intensity of staining of the Golgi was much weaker than that of the wild-type or the other two deletion mutants. These preliminary results suggest that the membrane anchoring domain may contain the amino acid sequence necessary for the retention of GT within the Golgi membrane.

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Synthetic Oligosaccharides and the Antibody Binding Site for the *Salmonella* Serogroup B Polysaccharide: A Comparision of Ligand Mapping Studies with a High Resolution Crystal Structure

David R. Bundle

Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, K1A 0R6.

A family of oligosaccharide epitopes based on a linear trisaccharide with isomeric 3,6–dideoxyhexose branches define the serology of the majority of *Salmonella* that cause typhoid and enteric fevers. Diagnostic and protective antibodies are characterized by the immunodominant role of 3,6–dideoxyhexose residues in the recognition of the bacterial cell surface antigen.

$$[\rightarrow 2)\ \alpha\text{–D–Man}\ (1\rightarrow 4)\ \alpha\text{–L–Rha}\ (1\rightarrow 3)\ \alpha\text{–D–Gal}\ (1\text{–}]$$
$$\uparrow$$
$$X$$

Serogroup B	X = 3,6–dideoxy–D–galactose	(abequose)
Serogroup A	X = 3,6–dideoxy–D–glucose	(paratose)
Serogroup D	X = 3,6–dideoxy–D–mannose	(tyvelose)

Purified bacterial lipopolysaccharide and synthetic glycoconjugates were used to select murine hybridoma antibodies that bound bacterial O–antigens from *Salmonella* serogroup B. One antibody, an IgG1λ, selectively recognized epitopes involving a branched trisaccharide containing 3,6–dideoxygalactose (abequose). Antibody Fab was crystallized in the presence of a dodecasaccharide composed of three chemical repeating units of the O–antigen. The crystals diffracted to 2.3Å resolution and the structure of the complex was solved by molecular replacement. The oligosaccharide binds on the surface of the Fab fragment with the abequose residue buried in a small cleft formed at the VL/VH interface. The antigen chain lies nearly perpendicular to this interface. Aromatic amino acid side chains line the sides of the site (3 His, 3 Trp and 1 Phe) and the electron density for the trisaccharide, Abe[Gal]Man, uniquely positions each monosaccharide residue. The conformation of the trisaccharide in the bound state is close to that inferred from solution NMR measurements and predicted by simple potential energy calculations, HSEA. The stereochemical requirements of the 3,6–dideoxy–D–galactose residue dominate the binding site and both hydroxyl groups are hydrogen bonded to the protein, as predicted by studies with synthetic ligands.

The antibody site has been mapped by panels of synthetic oligosaccharides, modified by single site deoxygenation to identify crucial polar contacts between the carbohydrate epitope and the protein binding site. Microcalorimetry showed that both enthalpy ($\Delta H = -4.9$kcal/mole) and entropy ($-T\Delta S = -2.4$kcal/mole) favour binding of the natural epitope ($\Delta G = -7.3$kcal/mole) and all but one of the monodeoxy trisaccharides. The enthalpy/entropy compensation often observed for oligosaccharide lectin interactions was only observed for a 4–deoxymannose trisaccharide epitope. The inferences drawn from immunochemical mapping experiments can now be compared with the crystal structure of the complex. These observations form the basis for planning mutations of synthetic genes that code for the Fab, which has been expressed in *E. coli*.

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ENZYMES IN CARBOHYDRATE SYNTHESIS. *C.-H. Wong*, Department of Chemistry, Research Institute of Scripps Clinic, La Jolla, CA 92037

Enzymes hold great potential for the synthesis of both natural and unnatural carbohydrates. Many monosaccharides, oligosaccharides, and related substances of biomedical importance can be prepared effectively based on a combined chemical and enzymatic approach. Enzymatic reactions are stereospecific and are often carried out under mild conditions without protection of functional groups. This presentation describes our recent work on the use of aldolases for the synthesis of monosaccharides, and of glycosyl transferases with cofactor regeneration for the synthesis of oligosaccharides. It is expected that the increasing availability of these two types of enzymes based on cloning techniques will have a significant impact on synthetic carbohydrate chemistry.

SYNTHETIC CHEMISTRY AND GLYCOBIOLOGY OF L-FUCOSE

Khushi L. Matta

Department of Gynecologic Oncology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA

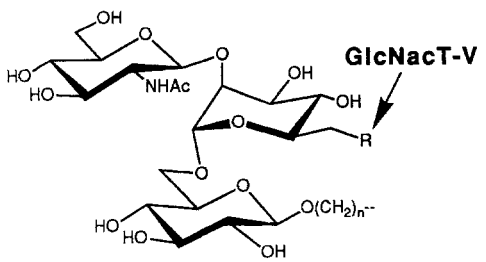
The chemistry of L-fucose has been a topic of major interest to us due to the variety and significance of biochemical applications. A new synthetic strategy based upon the use of methyl 3,4-O-isopropylidene-2-O-(4-methoxybenzyl)-1-thio-ß-L-fucopyranoside as an α-L-fucosylating reagent has been developed in our laboratory and a series of nitrophenyl oligosaccharides containing α-L-fucose as a terminal unit have been accomplished. Current availability of compounds such as Fucα1→2Galß→1 O-$C_6H_4NO_2$(p), Fucα1→3GlcNAcß1→O$C_6H_4NO_2$(p), Fucα1→6GlcNAcß1→O-$C_6H_4NO_2$(p), and Fucα1→6Galß1→O$C_6H_4NO_2$(p) enables us to develop rapid assay procedures for highly specific α-L-fucosidases (which do not act on aryl α-L-fucopyranosides). It also allows us to gain information on the specificity of these enzymes from different sources. Some specific data on α-L-fucosidases obtained from leeches, earthworms and human tissues along with the accomplished purification of α-L-fucosidase from earthworms will be discussed. With the aid of synthetically modified analogs such as MeO-2Galß1→3GlcNAcß1→OBn and OSO_3-3Galß1→4GlcNAc selective assay methods have been developed for specific α-L-fucosyltransferases. Applications of these methods will be discussed. Chemical syntheses of synthetic antigens such as Fucα1→3GlcNAcß1→6Galß1→O-$C_6H_4NO_2$(p), Fucα1→3GlcNAcß1→6GalNAcα1→O$C_6H_4NO_2$(o) and Fucα1→3GlcNAcß1→6Manα1→O-$C_6H_4NO_2$(p) have been accomplished. Immunological studies are currently in progress in our laboratory.

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

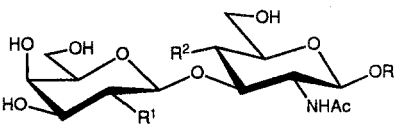
APPLICATIONS OF SYNTHETIC CARBOHYDRATE CHEMISTRY TO THE STUDY OF GLYCOSYLTRANSFERASES. Ole Hindsgaul[1], Michael Pierce[2], and Monica M. Palcic[3]
[1]Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada, [2]Department of Cell Biology and Anatomy, University of Miami Medical School, Miami, Florida 33101 USA, [3]Department of Food Science, University of Alberta, Edmonton, Alberta, T6G 2P5 Canada.

Synthetic analogs of naturally occuring oligosaccharides find broad application in many areas of glycobiology. Such structures are frequently used as ligands in the study of carbohydrate-protein interactions with antibodies, lectins and bacterial or viral receptors. In addition to these more classical uses, oligosaccharide analogs are becoming increasingly useful in the study of glycosyltransferases where they have primarily been used as specific acceptors in enzyme assays. We have recently reported (J. Biol. Chem. 265, 1990, 6759) that removal of the active hydroxyl group on the synthetic GlcNAcT-V acceptor 1 resulted in the production of an inhibitor (**2**, Ki=70μM) which was specific for that enzyme. The BSA-conjugate of inhibitor **2** has since been immobilized on agarose and the resulting affinity-support has been used in the purification of GlcNAcT-V. Glycosyltransferase inhibitors such as **2** also have the potential to alter the glycosylation patterns of cell surface glycoproteins and glycolipids and may therefore become powerful tools in the study of the function of glycosylation.

The preparation of additional deoxygenated oligosaccharides is in progress and these are also being evaluated as potential glycosyltransferase inhibitors. To date, the 2'-deoxy disaccharide **3** has been found to be an inhibitor for the human α(1→2) fucosyltransferase and the 4-deoxy-trisaccharide **4** was an inhibitor for the Lewis α(1→4) fucosyltransferase from mung bean but not from human milk. A new approach for the preparation of deoxy-oligosaccharides involves the chemical preparation of deoxy-sugar nucleotides and their transfer to synthetic oligosaccharide acceptors using glycosyltransferases. To date, 3, 4 and 6-deoxy-GlcNAc and 3-deoxy-Fuc have been found to act as donor substrates for GlcNAcT's I and II and the human Lewis fucosyltransferase.



1: R=OH
2: R=H



3: R^1= H, R^2 = OH
4: R^1= OH, R^2 = H

BIOSYNTHESIS OF GLYCOPHOSPHATIDYL INOSITOL MEMBRANE ANCHORS. *Gerald W. Hart, Tamara L. Doering, Jayne Raper, Laurence Buxbaum, Takeuchi Makoto, and Paul T. Englund.* Department of Biological Chemistry, Johns Hopkins Biological Chemistry, Johns Hopkins Medical School, Baltimore, Maryland 21205.

Many membrane proteins in all eukaryotes are anchored to the plasma membrane at their C-terminus by recently discovered glycophosphatidyl inositol (GPI) structures. The variant surface glycoprotein (VSG) of African trypanosomes, a glycoprotein which allows this parasite to defeat the host immune defenses, is anchored to the membrane by a GPI-anchor that exclusively contains myristic acid as the fatty acid component. Unlike the GPI on VSG, most mammalian GPIs contain a variety of different fatty acids.

The abundance of VSG (~10^7/cell) makes the trypanosome ideal for the study of GPI-anchor biosynthesis. A trypanosome cell-free system (for review see, ***J. Biol. Chem.* 265, 611-614**) has allowed the elucidation of the major steps in the pathway of trypanosome GPI-anchor biosynthesis. GPI is pre-assembled on a subset of endogenous phosphatidylinositols (PIs) that lack myristate. First, GlcNAc is donated from UDP-GlcNAc to the inositol moiety. The GlcNAc-PI is rapidly de-acetylated to form GlcN-PI. GlcN-PI is elongated sequentially by the addition of 3 mannosyl residues in reactions involving intermediates similar to mannosyl phosphoryldolichol. Subsequently, ethanolamine-phosphate is attached at the 6-OH of the terminal mannosyl residue (donor unknown, but probably not CDP-ethanolamine). Finally, a unique series of acyl-exchange reactions (termed "fatty acid remodeling"), that involve GPI-specific phospholipases and acyl transferases, convert the anchor-precursor to a di-myristyl GPI, which is then attached *en bloc* to nascent VSG. Recently, we have also identified putative GPI biosynthetic intermediates in mammalian cell-free systems. Supported by NIH AI21334 and by the MacArthur Foundation.

GLUCOSYLATION OF **GLYCOPROTEINS** IN THE ENDOPLASMIC RETICULUM

Armando J. Parodi , Instituto de Investigaciones Bioquimicas Fundacion Campomar, A. Machado 151 , (1405) Buenos Aires, Argentina

N-linked, high mannose-type oligosaccharides lacking glucose residues (either by the action of glucosidases I and II or in the case of trypanosomatid protozoa because unglucosylated oligosaccharides are transferred to proteins) are transiently glucosylated in mammalian, plant, fungal and protozoan cells. The products formed have been identified as N-linked $Glc_1Man_{5-9}GlcNAc_2$. The glucosylating activity (UDP-Glc:glycoprotein glucosyltransferase) was detected in microsomal membranes of the above mentioned cells using UDP-Glc as sugar donor and denatured thyroglobulin as acceptor. The native form of the glycoprotein was ineffective. No dolichol derivatives were involved in the transfer reaction. The structure of thyroglobulin-linked $Glc_1Man_9GlcNAc_2$ appeared to be identical with that of $Glc_1Man_9GlcNAc_2$-P-P-dolichol, thus suggesting that glucosidase II is also responsible for the in vivo deglucosylation of the newly glucosylated compounds. The glucosyltransferase was found to be loosely attached to the luminal surface of the rough and smooth endoplasmic reticula. It required bivalent cations for activity (Ca^{++} being more effective than Mg^{++} or Mn^{++}) and has been purified several hundred fold from rat liver by standard procedures. In order to evaluate the molar proportion of N-linked oligosaccharides that are glucosylated in normal cells, the trypanosomatid protozoan Trypanosoma cruzi (a parasite transferring $Man_9GlcNAc_2$ in protein N-glycosylation) was grown in the presence of ^{14}C-glucose and concentrations of the glucosidase II inhibitors deoxynojirimycin and/or castanospermine that were up to 1500 fold higher than their I_{50} values for the T. cruzi enzyme. The inhibitors did not have deleterious effects on cellular metabolism. About 50-55 % of total N-linked oligosaccharides contained glucose residues and were identified as $Glc_1Man_{7-9}GlcNAc_2$. The same proportion was obtained when cells were pulse-chased with ^{14}C-glucose in the presence of deoxynojirimycin for 60 min. No evidence for the presence of an endomannosidase yielding GlcMan from the glucosylated compounds was obtained. As on the average the number of N-linked oligosaccharides per molecule in glycoproteins is higher than one, this result suggests that more than 50-55 % of total glycoproteins are glucosylated in the endoplasmic reticulum and that transient glucosylation is a major event in the normal processing of glycoproteins.

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CONTROL OF GLYCOPROTEIN PROCESSING AND TERMINAL GLYCOSYLATION

Richard D. Cummings, University of Georgia, Department of Biochemistry, Athens, Georgia 30602

The molecular regulation of protein glycosylation is poorly understood. Although newly synthesized glycoproteins are generally exposed to a similar battery of glycosidases and glycosyltransferases within the Golgi apparatus, different glycoproteins from a single cell type exhibit substantial differences in the structures of their oligosaccharides and within a glycoprotein there is both site-specific glycosylation and controlled microheterogeneity of oligosaccharide structures. In trying to understand more about the control of these phenomena, we are studying the biosynthesis of N-linked oligosaccharides in the mouse teratocarcinoma cell line F9. Many of the N-linked oligosaccharides in glycoproteins synthesized by these cells contain the repeating disaccharide -3Galß1,4GlcNAcß1- or poly-N-acetyllactosamine (PL) sequence and the terminal sequence Galα1,3Galß1,4GlcNAc-R in addition to more commonly found sialylated structures. In addition we have found that the PL sequences are not present on many surface glycoproteins and appear to be restricted primarily to laminin and the lysosomal associated membrane proteins designated LAMP's. Upon cellular differentiation induced by retinoic acid, there is an increase in the activity of the α1,3 galactosyltransferase and an increase in terminal α-galactosyl sequences.

To address the issue of whether terminal glycosylation is determined by competition between certain glycosyltransferases, we and our collaborators have cloned the gene for the murine α1,3 galactosyltransferase and expressed the gene in Chinese hamster ovary (CHO) cells which lack this activity. Our results demonstrate that in the CHO transfectants the α1,3 galactosyl residues are added to poly-N-acetyllactosamine (PL) sequences on complex-type Asn-linked oligosaccharides, and there is a proportional decrease in the sialylation of these chains; interestingly, there are no differences in the lengths of the PL chains between the parental CHO and the CHO transfectants. Based on these and other results, it is hypothesized that (a) elongation of the PL chains occurs largely within the trans Golgi apparatus; (b) enzymes which synthesize the PL chains recognize specific protein substrates; and (c) that the addition of the terminal sugars α1,3 galactosyl and α2,3 sialyl residues occurs in a more distal compartment of the Golgi apparatus.

Evidence will be presented that these findings may have important biological implications. We have found that PL chains are bound with high affinity by a wide variety of vertebrate ß-galactoside-binding proteins or S-type lectins. The restricted distribution of the PL chains on laminin and LAMP's may indicate that these lectins are involved in cellular interactions with basement membrane and/or organization and deposition of the basement membrane components.

Supported by NIH Grant CA37626 to R.D.C.

BIOSYNTHESIS AND TARGETING OF POLYLACTOSAMINOGLYCAN CONTAINING GLYCOPROTEINS.

Minoru Fukuda, Wei-Chun Wang, Ni Lee, *Sven Carlsson and Mark A. Williams, La Jolla Cancer Research Foundation, La Jolla, CA 92037 and *Dept. of Med. Biochem. & Biophysics, University of Umea, Umea, Sweden.

We have shown that the major glycoproteins containing polylactosaminoglycan in nucleated cells are lysosomal membrane glycoproteins with Mr~120,000. These lysosomal membrane glycoproteins, designated as lamp-1 and lamp-2 in human cells, constitute a large fraction of lysosomal membrane proteins. Our studies revealed the following features of these glycoproteins:

(a) The polypeptide portion of these molecules are ~40K and the majority of the molecules reside in the lumenal side of lysosomes (1-3).
(b) The intralumenal part is extensively decorated by N-glycans and divided into two homologous domains, that are separated by a hinge-like structure (1-3).
(c) The molecule contains 4 loops connected by disulfide bridges, and each domain contains 2 loops. The loop structure distinguishes lamps from the immunoglobulin superfamily (4).
(d) The structures of each lamp-1 and lamp-2 are conserved throughout evolution, and human lamp-1 and lamp-2 are encoded by chromosomes 13 and X, respectively (5).
(e) A significant portion of the N-glycans has very complex poly-N-acetyllactosaminyl structure, and the amount of this structure increases when HL-60 cells differentiate into granulocytic cells. This increase in poly-N-acetyllactosamine is caused by an increased number of N-glycosylation sites that are modified by poly-N-acetyllactosamine (6,7).
(f) The increased amount of poly-N-acetyllactosamine is associated with increased half-lives of lamps, and may result in more stable lysosomes in cells with more phagocytic functions (6).
(g) In contrast to points (e) and (f), the amount of poly-N-acetyllactosaminyl structure decreases when CaCo-2 colonic cells acquire more epithelial property. Thus, the conversion of tumorigenic to less-tumorigenic cells is associated with the decrease of poly-N-acetyllactosamine (7).
(h) The targeting signal of lamps to lysosomes was localized in the short cytoplasmic segment, and the tyrosine residue in the segment was found to be essential for lamps trafficking to lysosomes (8).

We are currently working on the biosynthesis of polylactosaminoglycan in lamps. We will present the most recent developments in these studies. (Supported by CA48737).

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TWO NEW INHIBITORS OF THE GLYCOPROTEIN PROCESSING MANNOSIDASES, MANNOSIDASE I AND MANNOSIDASE II. A.D. Elbein, G.P. Kaushal, I. Pastuszak and J.L. Tropea, Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78284.

We have recently identified two new inhibitors of the glycoprotein processing mannosidases, mannosidase I and mannosidase II. These two compounds represent new and unusual structures that are quite different from any processing inhibitors known thus far. One of these inhibitors, called mannostatin, is produced by the microorganism, Streptoverticillium verticillus, and was identified as 4-amino-5-methylthio-1,2,3-cyclopentanetriol. Mannostatin was as effective as swainsonine as an inhibitor of the aryl-α-mannosidase (IC_{50} = 70 nM), and also as an inhibitor of mannosidase II (IC_{50} = 10 nM with p-nitrophenyl-α-mannoside as substrate and 90 nM with GlcNAc-Man_5-GlcNAc as substrate). However, mannostatin had no activity against mannosidase I. Mannostatin inhibited the processing of the influenza viral hemagglutinin in MDCK cells. The major N-linked oligosaccharide found on the protein produced in the presence of inhibitor was a hybrid type of structure with one or two complex chains on the 3-linked mannose and 2 mannoses on the 6-linked mannose. These structures were characterized by lectin affinity chromatography and also by treatment with various enzymes coupled to gel filtration. Mannostatin is the first glycoprotein progressing inhibitor that has the nitrogen present as an exocyclic amine rather than in the ring.

The second inhibitor is a compound called kifunensine that comes from the actinomycete, Kitasatosporia kifunensine. Kifunensine can be considered to be a cyclic oxamide derivative of 1-aminodeoxymannojirimycin. Kifunensine was found to be a potent inhibitor of the purified plant glycoprotein processing mannosidase I (IC_{50} = 2 x 10^{-8}M), but was inactive against the purified mannosidase II. It also appeared to be inactive towards the endoplasmic reticulum α-mannosidase, although it did inhibit mannosidase I in rat liver Golgi fractions. In cell culture studies, kifunensine inhibited glycoprotein processing of the influenza viral hemagglutinin and caused the accumulation of glycoproteins that contained mostly $Man_9(GlcNAc)_2$ structures. In these experiments, kifunensine was at least 50 to 100 fold more effective than deoxymannojirimycin, making it one of the best glycoprotein processing inhibitors known thus far. These new inhibitors should add important new structures to the growing list of glycosidase and processing inhibitors. Once enough of these structures are known, computer modeling should indicate similarities and differences in these compounds and facilitate chemical synthesis of active inhibitors. [These studies were supported by grants from the Robert A. Welch Foundation and The National Institutes of Health (DK 21800)].

ENGINEERED GLYCANS TO IDENTIFY A VACUOLAR SORTING DETERMINANT IN PHYTOHEMAGGLUTININ-L. Craig D. Dickinson and Maarten J. Chrispeels. Dept. of Biology C-016, University of California San Diego, La Jolla CA 92093.

We are using a novel approach to identify the vacuolar sorting domain in the plant protein phytohemagglutinin (PHA). PHA is a tetrameric vacuolar glycoprotein that has a high mannose glycan on Asn_{12} and a complex (xylose-containing) glycan at Asn_{60}. By site-directed mutagenesis, N-linked glycosylation sites were introduced at various locations in the protein. It is predicted that a glycan added at or near a vacuolar targeting determinant will obstruct interaction with a targeting receptor in the Golgi and thus lead to secretion of the modified protein. Exposed sites for the new glycans were chosen with the help of crystal structures of three related lectins. The effect of glycan additions on secretion of PHA by plant cells will be presented. It will also be interesting to test the effect of these modifications on stability and lectin activity. Five glycan sites have been engineered at surface locations. All five sites are utilized by the plant glycosylation machinery and none result in an unstable protein. Additional surface site are presently being engineered along with sites which, if used, would be expected to interfere with oligomerization or proper folding.

EFFECT OF INHIBITORS OF N-LINKED OLIGOSACCHARIDE PROCESSING ON OPSIN GLYCOSYLATION AND ROD OUTER SEGMENT DISC MEMBRANE MORPHOGENESIS.
Steven J. Fliesler, Hirohumi Arakawa, Lynne Mann, and Lisa Cruz, Bethesda Eye Institute and E.A. Doisy Dept. of Biochemistry and Molecular Biology, St. Louis Univ. Sch. Med., St. Louis, MO 63110.

Tunicamycin prevents glycosylation of opsin and results in aberrant morphogenesis of retinal rod outer segment (ROS) disc membranes in *Xenopus* retina organ cultures (Fliesler *et al.*, ***JCB*** 100: 574, 1985). In contrast, incubation of *Xenopus* retinas with castanospermine prevents post-translational processing of opsin's N-linked oligosaccharides, but does not disrupt normal ROS membrane assembly (Fliesler *et al.*, ***PNAS*** 83: 6435, 1986). To further evaluate the oligosaccharide structural requirements for ROS disc membrane morphogenesis, we incubated *Xenopus* retinas with 1-deoxymannojirimycin (dMM, an inhibitor of Golgi α-mannosidase I) and [^{3}H]Man or [^{35}S]Met/Cys, and examined the effects on opsin glycosylation and disc assembly. SDS-PAGE analysis of retinas and isolated ROS membranes revealed that newly synthesized opsin from dMM-treated retinas had a slightly larger M_r compared with controls. ROS N-linked oligosaccharides (released by N-glycanase, reduced with $NaBH_4$) were analyzed by Bio-Gel P-4 column chromatography before and after digestion with selective glycosidases, and by acetolysis and Glyco-Pak N HPLC. Oligosaccharides from dMM-treated retinas eluted from Bio-Gel P-4 in the range of 12.5-13.5 Glc units, whereas the control samples eluted coincident with 11.5 Glc units. Further analyses indicated that the major opsin glycoform from controls has the structure $GlcNAc(Man)_5GlcNAc_2$, whereas opsin oligosaccharides from dMM-treated retinas have the structure $(Man)_{8-9}GlcNAc_2$. For comparison, the primary oligosaccharide structure of bovine opsin is $GlcNAc(Man)_3GlcNAc_2$ (ca. 60%), whereas $GlcNAc(Man)_5GlcNAc_2$ represents a minor (ca. 30%) isoform (Liang *et al.*, ***JBC*** 254: 6414, 1979; Fukuda *et al.*, ***JBC*** 254: 8201, 1979). Light microscopic autoradiography and electron microscopy revealed that newly synthesized glycoproteins were assembled into morphologically normal ROS discs in both control and dMM-treated retinas. Preliminary studies with swainsonine (a Golgi α-mannosidase II inhibitor) also indicate no effect on disc morphogenesis, as predicted from the determined opsin oligosaccharide structure. These results represent the first analysis of amphibian opsin oligosaccharide structure, and confirm the flexibility of structural requirements for N-linked oligosaccharides involved in ROS membrane assembly. [Supported by NEI/NIH grant EY06045 and RPB, Inc.]

THE ADHESIVE PHENOTYPE OF TRANSFECTED CELLS INDICATES THAT CD44 MAY BE A HYALURONIC ACID RECEPTOR. Tom St. John, Georgia Rees, W. Michael Gallatin, Division of Basic Sciences, Fred Hutchinson Cancer Research Center 1124 Columbia Street, Seattle, WA 98104.

Normal function of the immune system requires that adhesive contacts be formed between many disparate cell types. For example, lymphocytes interact with specialized endothelium in lymphoid tissues as part of a process which targets functionally distinct subsets to appropriate microenvironments. One cell surface glycoprotein, CD44, has been linked to this process.

The broad tissue distribution of CD44 on cells of nonhemopoietic origin would argue for its involvement as an adhesion molecule in other tissue systems as well. Accordingly, we have found that this glycoprotein can promote cell-cell adhesion in another cellular context, namely aggregation of fibroblasts. The function of the CD44 glycoprotein as an adhesion molecule was directly tested by transformation of a primate CD44 cDNA into mouse L cell fibroblasts. We have previously shown that several independent transfectants exhibited a new self-adhesive phenotype, forming large aggregates in when placed in suspension. Variants were derived from a transfectant clone by flow cytometric methods. Aggregation experiments with these variants, expressing greatly differing levels of the transfected CD44 protein, showed that aggregation competence correlated with expression of the transfected gene. This CD44-mediated adhesion was blocked specifically by monoclonal antibodies binding one immunologically defined region of CD44. Nontransfected L cells did not self-aggregate but were capable of adhering to the transfectants indicating that at least one ligand for this adhesion molecule is expressed by mouse fibroblasts.

Paradoxically, untransfected L cells express the murine CD44, but do not show an adhesive phenotype. In order to address the mechanism by which transfected but not endogeneous CD44 molecules confer an adhesive phenotype, we have constructed chimeric CD44 molecules. These molecules consist of the external domain of the primate molecule attached to the cytoplasmic domain of the murine, and the external domain of the murine attached to the cytoplasmic domain of the primate. Independent transfectants of these two constructs indicate that the adhesive phenotype depends upon the cytoplasmic domain of the molecule. This behavior may depend upon the disruption of the normal cytoskeletal contacts of the CD44 molecule, a molecule normally tightly associated with cytoskeletal elements.

The apparent molecular similarity of CD44 to a cell surface hyaluronic acid receptor described by Underhill, the sequence similarity of CD44 to the hyaluronic acid binding link proteins and the implication that CD44 is a receptor for ECM components (W. Carter) prompted us to examine the behavior of these transfected L cells in the presence of hyaluronic acid. Hyaluronic acid induces a "super-aggregation" of these transfectants, but has little if any effect on nontransfected L cells. This aggregation is also strongly blocked by the preincubation of the cells with an anti-CD44 monoclonal antibody.

In order to determine whether CD44 is identical to this hyaluronic acid receptor, the CD44 gene from BHK cells was isolated and transfected into mouse L cells. These transfected cells, but not untransfected L cells, stain with the K3 mAB, originally described by Tarone et al. and shown to recognize this hyaluronic acid receptor. These data suggest that CD44 is either a hyaluronic acid receptor or is very closely and functionally associated with a hyaluronic acid receptor on the cell surface.

BIGLYCAN (BGN) AND DECORIN (DCN): SEQUENCE AND TISSUE DISTRIBUTION

Larry W. Fisher, Bone Research Branch, NIDR, NIH, Bethesda, MD.

Two closely related small proteoglycans, biglycan (PG I) and decorin (PG II or PG-40), have been described in a variety of species. Human biglycan is translated as a putative 42,500 dalton prepro-protein that is then processed into a 38 kDa secreted protein containing a mix of N- and O-linked oligosaccharides and two chondroitin or dermatan sulfate chain whose composition and lengths vary with tissue source. Indirect evidence suggests that occasionally a BGN containing only one glycosaminoglycan chain may be secreted. The human biglycan gene is about 6 kbp and contains 8 exons. BGN has been localized to the end of the long arm of the human X chromosome. Although BGN shares considerable homology to DCN and fibromodulin, both of the latter being collagen-binding proteins, BGN appears to be associated with cell membranes (developing keratinocytes and skeletal myofibers) or territorial matrices (bone and cartilage). BGN can also be found on specific surfaces of polarized cells. One example is the cortical collecting tubules of developing human kidney in which only the lumenal surfaces of the epithelial cells are positive for the epitope.

Decorin is highly homologus to biglycan, containing pre- and pro-regions amino-terminal to a structure dominated by two disulfide clusters separated by a region made of 10-12 repeats of a nominal 24 amino acid consensus sequence. The repeat structure is based on a pattern of hydrophobic amino acids that appears to have been used throughout evolution for protein-protein, protein-cell and/or cell-cell interactions. Decorin, as its name implies, "decorates" the outer surface of collagen fibrils in a periodic fashion. In adult human skin we have observed approximately 5% of the cross-reactive core protein lacking any glycosaminoglycan chain. Due to the close proximity of the epitope(s) to the glycosaminoglycan attachment site, we consider it unlikely that the free core protein is the result of proteolytic degradation. DCN is on the long arm (q) of human chromosome 12 and appears to be a tandem gene. We are currently determining whether or not the second gene is a pseudogene. Immunolocalization (using a monospecific anti-peptide antiserum) and in situ localization (using antisense RNA) showed DCN to be produced by cells associated with developing connective tissues. Decorin was universally seen associated with collagen bundles

PROTEOGLYCANS IN CELL REGULATION. Erkki Ruoslahti, M.D., La Jolla Cancer Research Foundation, La Jolla, CA 92037

The versatility of proteoglycans and their capacities for multiple interactions with other molecules gives them the ability to function as a multipurpose "glue" in cellular interactions. They can bind together extracellular matrix components, mediate the binding of cells to the matrix, and capture soluble molecules such as growth factors into the matrix and onto cell surfaces. The proteoglycan-mediated insolubilization of growth factors provides a means of concentrating growth factor activities and directing them into a geometry appropriate for the architecture of the tissue. During the past several years we have been studying proteoglycan structure and functions by cDNA cloning and gene transfer. The latest proteoglycan we have completed the sequence of, versican, has a very large (2389 amino acids) core protein. Its structure is very interesting in that it has homologies with two different types of lymphocyte homing receptors. Similar to Hermes or CD44, the NH_2-terminal domain contains a hyaluronic acid-binding motif, and the COOH-terminus consists of epidermal growth factor-like repeats, a lectin-like sequence and a sequence found as a repeat in various complement regulatory proteins. These same domains make up the extracellular portion of a family of cell surface receptors some of which are also lymphocyte homing receptors. Judging from its structure, versican may be a matrix equivalent of such receptors and may function in cell recognition; current experiments are designed to test this hypothesis. Gene transfer experiments with decorin, a proteoglycan we had cloned earlier, led to a very interesting observation. It turned out that Chinese hamster ovary cells in which human decorin was expressed at high levels from a cDNA construct changed dramatically, assuming the appearance and growth rate of normal cells. Since the original cells are tumorigenic, this observation suggests that decorin has the potential of reversing the malignant behavior of cells. Its growth regulatory effects are at least in part due to the ability of decorin to bind transforming growth factor β and neutralize the activity of this growth factor. Other cell regulatory growth factors also bind decorin. Current work aims at characterization of such factors.

THE SULFATE-ACTIVATION PATHWAY IN RAT CHONDROSARCOMA

Stephen Lyle, John Westley and Nancy B. Schwartz. Dept. of Biochemistry and Molecular Biology, Univ. of Chicago, Chicago, IL.

PAPS (3'-phosphoadenosine, 5'-phosphosulfate) is the activated sulfate donor used by bacteria, yeast, fungi, plants and all animals to sulfate various macromolecules such as glycosaminoglycans. PAPS is produced by a two-enzyme pathway. ATP sulfurylase (EC 2.7.7.4) catalyzes the formation of APS (adenosine 5'-phosphosulfate) and pyrophosphate from ATP and free SO_4^{-2}. APS kinase utilizes ATP to phosphorylate the APS intermediate and produce PAPS and ADP. A new assay has been developed to measure the activity of both enzymes in the physiologic direction. Incorporation of $(^{35}S)SO_4^{-2}$ into both APS and PAPS are measured simultaneously. The use of this assay has demonstrated a channeling effect between the sulfurylase and kinase of rat chondrosarcoma, since PAPS appears without any detectable lag and without an accumulation of the APS intermediate. The ratio PAPS/APS produced corresponds to a 96% channeling efficiency. Moreover, the velocity of APS kinase in the overall system, utilizing endogenous APS was 8-fold greater than that of the kinase alone, using exogenously added (^{35}S)APS. These results further suggest that APS is channeled between the active sites of sulfurylase and kinase. In addition, ATP sulfurylase and APS kinase have co-purified through gel filtration, ion exchange chromatography, and affinity chromatography, without any diminution of the channeling effect. All of these results demonstrate a close kinetic association between these two activities and suggest that they may reside on a bifunctional protein or in a tightly coordinated complex. Experiments are in progress to distinguish these possibilities.

DEVELOPMENTALLY-REGULATED CHONDROITIN SULFATE PROTEOGLYCANS OF BRAIN CHARACTERIZED WITH MONOCLONAL ANTIBODIES *U. Rauch, P. Gao, A. Janetzko, R.K. Margolis and R.U. Margolis.*
New York University Medical Center, New York, NY 10016, and State University of New York Health Science Center, Brooklyn, NY 11203.

Monoclonal antibodies to five chondroitin sulfate proteoglycans of rat brain and to a probable hyaluronic acid binding protein were used for studies on their localization during cerebellar development, and for the isolation and biochemical characterization of two of the proteoglycans. Immunocytochemical studies demonstrated that all of these antigens have similar localizations and developmental changes, with the exception that only two of the proteoglycans were detected in Purkinje cells. These results indicate that many of the multiple species of brain chondroitin sulfate proteoglycans which we have previously identified are produced by a single type of neuron or glial cell.

One of the chondroitin sulfate proteoglycans (designated 1D1) consists of a major component with an average molecular size of 300 kDa in 7-day brain, containing a 245 kDa core protein and an average of three 22 kDa chondroitin sulfate chains. A 1D1 species with a 150 kDa core protein is also present at 7 days, and during development the molecular size of the proteoglycan decreases progressively to ≈175 kDa at one month (150 kDa core protein), with the appearance in adult brain of a major 80-110 kDa proteoglycan (core protein size = 45 kDa) containing one or two 32 kDa chondroitin sulfate chains. The concentration of 1D1 decreases during development, from 20% of the total proteoglycan protein (0.1 mg/g brain) at 7 days postnatal to 6% in adult brain. In both young and adult animals the proteoglycan contains ≈40% carbohydrate by weight, comprising approximately equal proportions of chondroitin sulfate and glycoprotein oligosaccharides (some of which contain the 3-sulfated HNK-1 carbohydrate epitope).

A large chondroitin/keratan sulfate proteoglycan (designated 3H1) with a size of >400 kDa was also isolated and characterized using antibodies to the keratan sulfate chains. The 3H1 proteoglycan has a core glycoprotein which decreases in size from 360 kDa at 7 days to 280 kDa in adult brain. The proteoglycan contains two to four 25 kDa chondroitin sulfate chains and two to eight keratan sulfate chains of 8.4 to 10 kDa in one week and adult brain, respectively. In contrast to the 1D1 proteoglycan, the concentration of 3H1 increases during development from 3% of the total proteoglycans at 7 days to 11% in adult brain. This proteoglycan also contains a significant proportion of the novel *O*-glycosidic mannose-linked oligosaccharides which we have previously characterized in the chondroitin sulfate proteoglycans of brain (and which are absent from the 1D1 proteoglycan). The two fractions of 3H1 proteoglycan eluted from the immunoaffinity column with 0.5 M NaCl and pH 11 buffer contain 50-60% carbohydrate. The concentration of chondroitin sulfate (12-23% by weight) and glycoprotein oligosaccharides (20-31%) decreases during brain development, while the concentration of keratan sulfate increases from 5-11% at 7 days to ≈18% in the proteoglycan from adult brain. There is also a significant developmental decrease in the branching and/or sulfation of the keratan sulfate chains.