The glycosylation by O-linked N-acetylgalactosamine (O-GlcNAc) is an abundant and dynamic reversible post-translational modification implicated in protein regulation that appears functionally more similar to phosphorylation than classical glycosylation. The monosaccharide N-acetylgalactosamine is attached in a β-linkage to serine or threonine hydroxyl groups by the UDP-GlcNAc-peptide-β-GlcNAc transferase and removed by the N-acetyl-β-D-glucosaminidase.

We have previously identified using a proteomic approach numerous O-GlcNAc proteins in skeletal muscle; these proteins are implied in the glycolytic pathway and energetic metabolism. We have also identified myosin heavy chain, a key component of muscular contraction, to be O-GlcNAc modified. We propose that O-GlcNAc modifications might control muscle metabolism and could be implicated in the interaction between contractile proteins.

Since O-GlcNAc was recently demonstrated to play a role in modulating cellular function in response to nutrition and in stress conditions, we have investigated the implication of the glycosylation/deglycosylation process in the development of atrophy and contractile dysfunction in skeletal muscle considering the animal model of hindlimb unloading (HU), characterized by a decrease in the force of contraction, and known to induce a significant atrophy observed in postural muscles. We showed a decrease of the O-GlcNAc level in the atrophied slow muscle and an opposite increase in fast muscle (which is not altered by HU). These variations of O-GlcNAc are correlated with a variation of the O-GlcNAc process enzymes activities, and could be associated with a differential expression of heat shock proteins. These results strongly suggest that O-GlcNAc variation may prevent fast muscle against atrophy induced by hindlimb unloading.

We developed a carbohydrate sequencing database named “CabosDB.” CabosDB consists of oligosaccharide database, lectin database and glycoprotein database. The oligosaccharide database contains oligosaccharide structures described by carbohydrate sequence markup language (CabosML), their mass spectra and the experimental conditions. Mass spectra were automatically interpreted by comparing to theoretical values of fragment structures. Oligosaccharide database has a web service for rapid identification of oligosaccharide structures of mass spectra. User can determine the structure of analyte using interface software that connects Shimadzu/Kratos AXIMA QIT with our web service. Lectin database contains lectin molecules and lectin map. Lectin molecules include family, CRD, amino acid sequences, function, tertiary structures and binding sites. Lectin map includes affinity constants between lectins and oligosaccharides measured by frontal affinity chromatography. Glycoprotein database contains glycoprotein sequences and glycan binding sites identified by IGOT method. CabosDB has a web interface to access their information including graphical representation of data (glycan binding sites and affinity constants etc.). We also developed the glycan editor which allows users to search these information using glycan structures as query.

Identification and Quantification of N-Linked Glycoproteins Using Immobilized Aminophenylboronic Acid, Multiplex Isobaric Tagging, and Mass Spectrometry

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Quantitative proteome profiling is an emerging technology for the detection of clinical diagnostic or prognostic makers and the discovery of proteins as therapeutic targets. The complete proteome analysis of any species is as yet difficult due to the enormous complexity of proteomes. However, biologically or clinically important information can be obtained by comprehensively analyzing specific, information-rich subsets of the proteome that are selectively isolated. Protein glycosylation has been recognized as a very common post-translational modification. Here we show a method for the selective capturing, identification and quantification of glycoproteins that contain N-linked glycans. It consists of the 1,2- or 1,3-diol conjugation of glycoproteins to aminophenylboronic acid (APBA) immobilized on the magnetic bead, multiplexing isobaric tagging after specific release of N-linked glycopolypeptides using peptide-N-glycosidase (PNGase F). The released peptides are then identified and quantified by MS/MS.
27.4 Glycoproteomic Analysis Based on Multiple Enzymes Printing Technology
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Proteome analysis using two-dimensional gel electrophoresis (2DE) combined with peptide mass fingerprinting (PMF) are widely used because of their relative simplicity and high resolution. Recently, it was proposed that direct PMF analysis on the membrane blotted from 2DE gel coupled with micro-dispense technology using Chemical Inkjet Printer (Shimadzu). The technology enables multiple enzyme reaction against membrane-immobilized single protein spot, such as peptide N-glycosidaseF (PNGaseF) to release the N-linked oligosaccharides and protease digestion for PMF analysis. We also proposed a strategy for rapid identification of the oligosaccharide structure based on MSn analysis using MALDI-QIT-TOF MS. We also proposed a strategy for rapid identification of the oligo-

27.5 Glycoproteomic Analyses of Hepatitis B Virus-infected Hepatoma Cells
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Hepatitis B virus (HBV) long term infection is a major cause of hepatocellular carcinoma (HCC). Several million people are suffering from HBV infection and following hepatic cancer. It is well known that HBV infect into hepatocyte through unknown receptor, proliferate, and eventually induce HCC. However, the underlying molecular mechanisms are still largely unknown. Our colleague has previously found HBV infection can suppress N-acetylgalactosaminyltransferase III (GnT-III) which is involved in formation of complex type N-glycan, and re-introduction of this enzyme reduce HBV production in HBV-infected hepatoma cell line, HB611. To further gain insight into the association between HBV infection and changes of specific glycosyltransferase, we conducted comprehensive analyses of HB611 and its derivative cell line, named HB611/GnTIII using proteomic and glycomic approaches. HB611/GnTIII secreted much less amount of HBs antigen in ELISA proportionally to GnTIII expression, despite secretion of other proteins has shown few change. Unexpectedly, two dimensional electrophoresis approach revealed little proteins has been changed by introducing GnTIII in its expression pattern. Lectin blot with EPHA which specifically recognizes GnTIII-catalyzed structure of N-glycan, showed increased staining pattern of subset of specific proteins. Simple purification procedure using lectin column chromatography in combination with SDS-PAGE following by MS analysis revealed several cell surface proteins involved in cell-cell contact and cell-matrix binding can be substrate for GnTIII. Structural analysis of N-glycan and function of these glycoproteins have been studied further in this study.

27.6 Fucosylated Haptoglobin Is a Novel Marker for Pancreatic Cancer; a Detailed Analysis on the Oligosaccharide Structure and a Possible Mechanism for Fucosylation
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Changes in oligosaccharide structures have been reported in malignant transformations and, thus could be used for tumor markers in certain types of cancer. In the case of pancreatic cancer cell lines, a variety of fucosylated proteins are secreted into their conditioned media. To identify fucosylated proteins in the serum of patients with pancreatic cancer, we performed western blot analyses using Aleuria Aurantica Lectin (AAL), which is specific for fucosylated structures. An approximately 40 kD protein was found to be highly fucosylated in pancreatic cancer and an N-terminal analysis revealed that it was the β chain of haptoglobin. While levels of fucosylated haptoglobin have been reported in other diseases such as hepatocellular carcinoma, liver cirrhosis, gastric cancer and colon cancer, the incidence was significantly higher in the case of pancreatic cancer. Fucosylated haptoglobin was observed more frequently at the advanced stage of pancreatic cancer and disappeared after an operation. A mass spectrometry analysis of haptoglobin purified from the serum of patients with pancreatic cancer and the medium from a pancreatic cancer cell line, PSN-1 showed that the α1-3/α1-4 /α1-6 fucosylation of haptoglobin was inincreased in pancreatic cancer. When a hepatoma cell line, Hep3B was cultured with the conditioned media of pancreatic cancer cells, the secretion of haptoglobin was dramatically increased. These findings suggest that fucosylated haptoglobin could serve as a novel marker for pancreatic cancer. Two possibilities were considered in terms of the fucosylation of haptoglobin. One is that pancreatic cancer cells, themselves, produce fucosylated haptoglobin; the other is that pancreatic cancer produces a factor, which induces the production of fucosylated haptoglobin in the liver.
27.7 Capillary Electrophoresis; Electrospray Mass Spectrometry for Rapid and Sensitive Analysis of N-Glycans as 9-Fluorenylmethyl Derivatives

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Most proteins show characteristic biological roles after modification with glycans. Comprehensive analyses of glycans are important for understanding the protein function. In contrast to proteomics, high-throughput glycomics analysis is still in developing stage. In this study, glycosyl amine forms of reducing end of glycans released from glycoprotein with peptide: N-glycanase F (PNGase F) were directly derivatized with 9-fluorenylmethyl chloroformate (Fmoc-Cl), and analyzed by capillary electrophoresis-electrospray ionization mass spectrometry (CE-ESI MS). Procedure: Glycoprotein samples (10 μg) were digested with PNGase F (1 unit) in phosphate buffer (pH 8.5). The mixture was incubated at 37 °C for 2 h. Fmoc-Cl in acetone (200 μL, 50 mg/mL) was added to the mixture, and the mixture was incubated at 37 °C for 1 h. After removing the excess reagent and protein by extraction of the mixture with chloroform, a portion of the aqueous layer was analyzed by CE-MS. CE-ESI and CE-ESI/MS using Fmoc derivatives are a powerful technique for the structure analysis of glycans derived from high-mannose, siaiso- and asialo- complex and hybrid-type oligosaccharides. We applied the method to the analysis of glycans derived from some antibody pharmaceuticals, and found that the pharmaceutical products from different cells showed characteristic patterns of carbohydrates. We will also show that Fmoc-CE-MS technique is useful for identification of oligosaccharides released from the band observed on 2-DE gel after in-gel digestion. The method requires only 4 hours to perform the whole procedures including the release of glycans, derivatization with Fmoc and analysis by CE-ESI MS.

27.8 Titanium Dioxide, a Promising Approach for GPI-anchored Peptide Enrichment

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GPI-anchored proteins (GPI-APs) compose a subset of post-translationally modified proteins that are localized to the cell surface via a lipid anchor. The synthesized proproteins are targeted to the endoplasmic reticulum (ER) where after proteolytic cleavage of the C-terminal peptide, the already assembled GPI anchor is attached to the “new” C-terminus, w-site, of the protein. Identification of GPI-APs and assignment of the w-site involves time-consuming and laborious techniques. Bioinformatic tools have been developed to assist identification of GPI-APs. However, more and better experimental data on verification of GPI-anchors are necessary to improve these predictions tools. Therefore, new and improved methods for selective purification of GPI-APs are required. Here, we present a selective enrichment procedure for GPI-anchored peptides based on affinity purification using TiO2, which in combination with tandem mass spectrometry allowed characterisation of standard GPI-APs as well as identification of GPI-APs from S. cerevisiae. After purification of plasma membranes and cell walls from S. cerevisiae, the proteins were dephosphorylated with alkaline phosphatase immobilized on paramagnetic beads and subsequently treated with PI-PLC or β-glucanase to release the GPI-APs from membrane and cell-wall. Released proteins were proteolytically digested and peptides carrying the remaining GPI-anchor were purified by TiO2 microcolumns and analysed by mass spectrometry. The assignment of diagnostic ions commonly observed in the fragment ion spectra after collision induced dissociation of the GPI-anchor assisted in verification of GPI-anchored peptides derived from GPI-APs.

27.9 ASC_GLY_FLUO; a New Reagent for the Selective Characterization of Glycoproteins

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A key aspect to successful proteomics studies is the ability to precisely analyse post-translational modifications of proteins. Glycosylation is one of the most common and complex of these modifications. It results in thousands of unique, bioactive glycoproteins that can be circulating, membrane-bound or confined in the cytoplasm. Glycoprotein detection is currently performed using modified Periodic Acid Schiff (PAS) methods. Following SDS-PAGE proteins are fixed in the gel and revealed by oxidation and fluorescent Schiff base formation. These classical methods for in-gel carbohydrate detection are very selective, but lack sensitivity (detection limit of 25–100 ng of carbohydrate). In order to improve sensitivity, a fluorescent dye can be used. Although this staining procedure is quite selective for glycoproteins, some non-specific protein staining occurs in a gel formulation-dependant manner. We have developed a new fluorescent reagent for the selective detection of glycoproteins in polyacrylamide gels. Our fluorescent probe contains a boronic acid, whose affinity for diols is very well known and a highly fluorescent ruthenium complex. This allows for the specific and sensitive direct detection of glycoproteins in gels. Our new reagent was first tested on SDS PAGE with standard glycoprotein samples and/or molecular weight markers. This new fluorescent probe performed well on these proteins as the observed detection limit was 5–10 times lower than those observed with the Periodic Acid–Schiff base reagent. The next step which is currently under investigation in the laboratory is to apply this new fluorescent detection to more complex proteic samples from HCT 116 cells separated on 2D gel electrophoresis.
**Approaches to the Mucin-type O-Glycoproteome of Drosophila melanogaster S2 Cells**

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A survey of the fruitfly genome identifies numerous orthologs of O-glycoproteins known from higher and lower eukaryotes. Nevertheless, only few Drosophila mucin-type glycoproteins have been characterized to date, and the lack of a defined consensus sequence makes the prediction of O-glycosylation difficult based on amino acid sequence alone. Since mucin-type O-glycosylation of Drosophila melanogaster was expected to be restricted to the Tn-antigen and T-antigen structure, we applied techniques based on lectin affinity chromatography to identify the O-glycoproteome of the non-differentiated S2 cell line. GC/MS analyses of methylated O-glycans released from glycoproteins by b-elimination confirmed the expected structures GalNAc-oMe and Gal-GalNAc-oMe. We therefore used immobilized lectins from Artocarpus integrifolia (Jacalin) and Arachis hypogaea (PNA) with T-antigen specificity, and Helix pomatia agglutinin (HPA) with Tn-antigen specificity for consecutive lectin affinity chromatography of S2 cell extracts. Proteins in ultrafiltrated lectin eluates were precipitated prior to 2D-electrophoresis. 2-Dimensional western blot analyses with monoclonal antibodies directed against the Tn-antigen (5F4, IE3) and the T-antigen (A78-G/A7, HH8) detected independent patterns of O-glycoproteins in the lectin eluates. A multiplexed fluorescence detection strategy using the glycoprotein specific dye Pro-Q Emerald 488 in combination with Sypro Ruby total protein stain was applied in preparative 2-D experiments to exclude contamination with non-glycosylated proteins. Double positive protein spots were processed by automated spot picking, in-gel digestion, and peptide mass fingerprinting by MALDI-TOF mass spectrometry, followed by database searches using the Mascot search engine. Using this strategy, we have successfully identified several members of the Drosophila O-glycoproteome expressed in the untreated wild type Schneider S2 cell line. Individual analyses of the identified proteins and their potential functions will be discussed.

**Development of Pattern Recognition Methods Using Lectin Map for Identification of Oligosaccharide Structures**

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Recent progress in high-throughput, frontal affinity chromatography makes it possible to construct “Lectin Map,” which provides comprehensive, quantitative data on affinities between 100 lectins and 100 glycans. The completion of the Lectin Map is expected to contribute greatly as an essential and fundamental resource for glycoproteomics. In this context, cluster analysis of a currently developing Lectin Map has been performed preliminarily, and suggested that affinity patterning could work satisfactorily for the structural analysis of oligosaccharides. Here we present two novel computational methods for extracting useful lectins from the Lectin Map to identify oligosaccharide structures. One is a discriminating method, which determines appropriate lectins to discriminate each one of the denoted structural features of interest by means of Support Vector Machine, a powerful binary classifier. The other is a combination method to search for an optimized set of lectins, which can distinguish each member of the oligosaccharide library by comparing the affinity scores in a comprehensive manner. To validate the utility of the above methods, the affinities between 45 lectins and 50 N-glycans were examined. The discriminating method determined some lectins as identifiers for certain structural categories of N-glycan (e.g. high-mannose-type, complex-type, bisecting-GlcNAc, fucosylated, galactosylated) successfully. Furthermore, combining these lectins could also separate more complicated structures. On the other hands, combination of a relatively small number of lectins (e.g. <6) was found to be effective for discrimination of the 50 N-glycan structures. Taken together, our methods using the developing Lectin Map have proved to be highly promising for glycan profiling of even more diverse oligosaccharides.
Role of Branching N-Glycans and the Functional Glycomics

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Over 50% of proteins undergo glycosylation and functional glycomics is one of the most important strategies for proteomics in order to explore the protein functions.

Our group has been interested in the role of glycosyltransferases involved in the biosynthesis of N-glycan branching such as GnT-III, V, and Fut 8 and found the several target molecules for the glycosyltransferase and their genes which play pivotal roles in cancer metastasis, cancer suppression and antibody dependent cellular cytotoxicity activity which is a key role in the antibody therapy against cancer. We developed null mice of Fut 8 gene and the null mice lack core fucose of the glycoproteins as judged by Mass spectrometry and develop the emphymatous changes of the lung. This phenotypic change was found to be due to the constitutive activation of Matrix metalloproteinase (MMP) gene(s) due to aberrant glycosylation of a growth factor receptor which may otherwise suppress the MMP gene expression.

The null mice were rescued by the treatment of the growth factor indicating that the development of lung emphysema in the null mice was actually due to dys-regulation of the growth factor signaling as judged by the functional glycomics approach. These data indicate that functional glycomics may open a new insight into the role of glycoproteins in vivo and in vitro.

Characterization of Glycoproteins and N-Glycans of Recombinant Human Erythropoietin by Liquid Chromatography and Mass Spectrometry

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Glycoproteins are biomolecular macromolecules in which carbohydrates are attached covalently to asparagine (N-glycans) or serine/threonine (O-glycans) residues of the protein amino acid backbone. Glycoprotein focuses on; structural characterizations of the protein amino acid sequence and profiling of the attached carbohydrates.

This approach is based on dual digestion, firstly by a protease that will cleave amino acid sequence at specific sites, followed by a digestion with glycosidase that will cleave the N-glycans from the peptides produced by the first digestion. After this dual digestion, peptide fragments with released N-glycans, glycopeptides (O-glycans) and all other peptides can be separated by reversed phase chromatography and analyzed by mass spectrometry. With this approach, we have fully structurally characterized the heavily glycosylated macromolecule of recombinant human Erythropoietin at the picomolar level in solution by mass spectrometry. This study shows that only after deglycosylation it was possible to determine disulphide bridges position and the location of N-glycan occupation sites. Furthermore, conformation of the correct protein sequence with very high sequence coverage was achieved. To this extent, both MALDI-TOF with parallel PSD and nanoscale LC-ESI-MS/MS were utilized. The analysis of the N-glycans was made by a noval hybrid quadrupole time-of-flight mass spectrometer in with accurate mass assignments after purification of the carbohydrates on a carbon column. Sugars of high purity were analyzed in negative ion mode using nanoelectrospray analysis. NanoLockSpray with internal calibration and accurate mass measurement provides unambiguous N-glycans and protein amino acid sequence analysis.
Characterization of Site-specific Glycans of Large Glycoproteins, Fibronectin, and Apolipoprotein B-100, Using a Method of Hydrophilic Affinity Isolation of Glycopeptides and MALDI Mass Spectrometry

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Isolation of glycopeptides utilizing hydrogen bonds between glycopeptide glycans and carbohydrate-gel matrix in organic phase facilitates characterization of site-specific glycan structures of glycoproteins (Wada, Y., Tajiri, M., Yoshida, S., Anal. Chem. 76:6560–6565, 2004). The recovery of glycopeptides was improved in the presence of manganese divalent cation in the binding solution. The method followed by MALDI linear TOF and MALDI QIT multiple-stage tandem MS was applied to the analysis of glycan heterogeneities at seven N-glycosylation sites in each of the plasma and cellular fibronectins. Beside the global sialylation of plasma isoform, a remarkable site-specific difference was found in fucosylation; cellular fibronectin was largely fucosylated except for site-6, while only sites-5 and 7 were fucosylated in the plasma isoform. In addition, a new O-glycosylation site was located at the connecting segment between the fibrin- and heparin-binding domain and the collagen-binding domain. Considering that another site of O-glycosylation was within the connecting strand 3 (III CS), O-glycosylation of fibronectin was suggested to play a significant role in segregating the neighboring domains and thus keeping the steric structure of fibronectin and domain functions. In a similar way, the complete glycan structures, including a so far undetermined site, of apolipoprotein B-100 of over 4,500 amino acid residues were characterized.

The results indicated that the method of glycopeptide enrichment will serve smart resources to the analysis of site-specific glycans in glycoproteomics.