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British Society for Matrix Biology Meeting, Manchester, 5–6 April 2004

Grappling with the glycome

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The spring 2004 meeting of the BSMB was held at Hulme Hall, University of Manchester on 5–6 April. The theme of the meeting was recent advances in our understanding of the structure and biological function of saccharides, with a particular emphasis on glycosaminoglycans. The meeting was organized by Dr Malcolm Lyon (University of Manchester) and Dr Robert Lauder (University of Lancaster) and was supported financially by Europa Bioproducts, Sigma-Aldrich, Dionex and Calbiochem, who all exhibited their products. There were 11 invited speakers: one from the USA, three from continental Europe (Sweden, France and the Netherlands) and seven from the UK. The meeting attracted 100 registered delegates evenly split between 50 BSMB members (including 10 students) and 50 nonmembers (including 14 students). A further nine short presentations were selected from the submitted poster abstracts.

Robert Haltiwanger (State University of New York at Stony Brook, New York, USA) opened the meeting by introducing the glycocalyx at the cell surface and its structural complexity. He highlighted the variety of cell-surface glycans that modulate cellular communications, discussing their importance in signal transduction events, before concentrating on the specific role of *O*-fucose modifications in modulating the Notch signalling pathway. The extracellular domain of Notch contains 36-tandem epidermal growth factor (EGF)-like repeats many of which are sites for both *O*-fucose and *O*-glucose type glycosylation. Comparing the phenotypic similarities between knockouts of *O*-fucosyltransferase-1 and Notch in *Drosophila* and mice, he emphasized the importance of *O*-fucose modifications in Notch function. He further developed this theme by considering Fringe, a β1,3-*N*-acetylyglucosaminyltransferase, that is involved in the elongation of the *O*-fucose monosaccharide in some EGF-like repeats and that can further modulate Notch signalling. The sites of Fringe modification may be encoded within the sequences of specific EGF-like repeats. There are suggestions that these enzymatic modifications may influence Notch–ligand interactions. Before concluding, he identified *O*-glucose modification as an additional, highly conserved modification, clustered at the central EGF-like repeats of Notch that may additionally prove to influence Notch biology.

Paul Crocker (University of Dundee, Dundee, UK) gave an overview of the siglec family of transmembrane proteins, whose members include sialoadhesin, CD22 and CD33, defining them as adhesive and signalling molecules of the immune system that recognize sialylated proteins. He emphasized the importance of siglec extracellular domain size in regulating *trans vs. cis* interactions with cell surface ligands, using as an illustration that observed unmasking of siglec binding sites towards external ligands, following a sialidase treatment to destroy *cis* interactions at the cell surface. He then focused on siglec 7, commenting on its unmasked activity when expressed on CHO cells as compared to its masked activity on natural killer (NK) cells, where it is naturally expressed. He introduced the preference of siglec 7 for masked activity on NK cells, where it is naturally expressed. He introduced the preference of siglec 7 for cells as compared to its sialidase treatment to destroy signalling molecules of the immune system that recognize sialylated proteg particular to its specificity, the possible functions of siglec 7 were then considered. The high levels of expression of α2–8-linked disialic acid on NK cells, the masking of siglec-7 in NK cells by *cis* interactions and the apparent rapid evolution of the sialic acid-binding domains within the siglec family suggests that siglec-7 has adapted to engage in *cis* interactions that regulate NK cell activation.

Anne Imberty (CERMAV-CNRS, Grenoble, France) described how PA-IL and PA-IIL, the galactose- and fucose-binding lectins of *Pseudomonas aeruginosa* are associated with the virulence of this bacterium. *P. aeruginosa* colonizes the lungs of patients with cystic fibrosis (CF), where it becomes a serious pathogen. Its specific binding is dependent upon the increased fucosylation and high levels of Lewis a epitopes present in CF patients. The crystal structures of PA-IL and PA-IIL bound to their respective monosaccharide ligands were shown. She drew particular attention to two coordinated calcium ions that appear to be intimately involved in lectin-ligand recognition. Modelling studies were used to look at the potential binding of PA-IIL to more complex oligosaccharide structures and these, together with the results of enzyme-linked lectin binding assays, suggested that the Lewis

a epitope could be the ligand recognized by these lectins in the lungs of CF patients.

Anne Dell (Imperial College, London, UK) gave an overview of mass spectrometric strategies for both high throughput glycomics and glyco-proteomics. She described the application of both MALDI-TOF MS and ES MS/MS not only for identifying carbohydrates from single proteins but also from very complex mixtures such as cell and tissue extracts. She then introduced a number of specific applications in which these approaches had proven valuable and instructive. Protocols for comparing resting and activated lymphocytes showed differences in glycosylation patterns of murine T and B cells upon activation. Changes in the *O*-glycan patterns of activated $CD8^+$ T cells during development were also addressed. In addition, MS provided a rapid glycan screening technique to assess changes in both *O*- and *N*-linked glycosylation in the organs of knockout mice and also for distinguishing core 1 and core 2 antennae *O*-glycans on the CA125 ovarian cancer antigen. MS techniques have also provided valuable data on sperm–egg interactions. Sequencing and site analysis of the *O*-glycans on the murine and human forms of the *zona pellucida* glycoprotein (ZP3), the putative sperm receptor revealed identical glycosylation patterns when expressed on mouse eggs. Interestingly, human ZP3 in transgenic mice interacted with murine but not human sperm. Therefore, it appears that host specific *O*-glycosylation is essential for sperm–egg recognition.

Ten Feizi (Imperial College, London, UK) described the use of carbohydrate microarrays as a potential high throughput technology to assess the specific carbohydrate-binding properties of proteins. Particular emphasis was given to protein–carbohydrate interactions in the immune system. To prepare microarrays, carbohydrates, either synthesized or released from proteins, cells or tissues, can be conjugated to lipids by reductive amination forming novel, lipid-linked oligosaccharides or neoglycolipids (NGLs). Such NGLs can easily be immobilized onto nitrocellulose membranes, via their lipid tails, where they can be probed for interaction with a soluble protein. Repertoires of NGLs, either homogeneous species or heterogeneous mixtures, can be assembled as microarrays to allow the selection of specific ligands by carbohydrate-binding proteins. Subsequent thin layer chromatography and mass spectrometry can be used to characterize positive-binding species. Specific examples used to illustrate the use of such approaches included the interactions of the cytokine INF-α and the chemokine RANTES with sulfated carbohydrate and chondroitin sulfate oligosaccharide microarrays.

Lan Jin (University of Edinburgh, Edinburgh, UK) discussed the novel elucidation of the conformation of heparin oligosaccharides by ion mobility mass spectrometry. In this gas-phase technique, ions are impelled to travel, under the influence of a weak electric field, through a very low density of inert gas. Under these conditions, the time of arrival of ions at the detector depends upon their collision frequency, which is related to their molecular cross sections. A heparin disaccharide and three different tetrasaccharides were prepared and studied. Theoretical gas-phase conformations were generated by molecular modelling and compared to experimental cross sectional areas derived by ion mobility MS. A close correlation was obtained with these small structures, and the method clearly holds promise for probing GAG conformation.

Tim Rudd (University of Liverpool, Liverpool, UK) introduced quartz crystal microbalance-dissipation (QCM-D) and its use in the study of protein/GAG interactions. By analysing the damping of oscillation of the crystal, after adsorption of molecules onto its surface, QCM-D can be used to gain new insights into complex formation. QCM-D measures both the mass of the molecules adsorbed to a surface, in addition to the energy dissipated by the surface. Experimentally, heparin oligosaccharides were immobilized onto a gold-sputtered QCM-D surface and then exposed to various protein ligands, e.g. FGF-1, FGFR1, HGF/SF. The viscoelastic properties of the resulting complexes could be determined from the measured dissipation, which yields information on their shape/conformation. For example, it was determined that complexes of a heparin octasaccharide with FGF-1 were more dissipative than complexes with HGF/SF. It was suggested that the oligosaccharide may have flexible hinge regions and that differential binding of specific growth factors above, or at, the hinge could radically alter the flexibility and thus dissipation of the complex. Interestingly, GAG complexes with FGFR1 were less dissipative (more rigid) than those with FGF-1.

Joyce Taylor-Papadimitriou (Guy's Hospital, London, UK) introduced MUC1, the major epithelial mucin expressed by breast carcinomas. Its extracellular domain has a tandem repeat structure bristling with *O*-glycans. Joyce has shown convincingly that MUC1 exhibits changes in *O*-glycoyslation in breast cancer. Increased levels of ST3 Gal 1 enzyme in breast cancer induces a switch from the addition of mainly core 2-based *O*-glycans to Sialyl T *O*-glycans. Although Sialyl T *O*-glycans can exist in normal cells outside the breast, in the breast they are cancer associated, and their presence increases mammary cancer growth and can be immunosuppressive. In up to 30% of breast cancers, *O*-glycosylation is terminated early, yielding GalNAc (Tn) or Sialyl Tn *O*-glycans on MUC1. Sialyl Tn glycans, coinciding with increases in the ST6GalNac I enzyme, are highly tumour-specific. Joyce concluded with a very interesting discussion of how this could be exploited for immunotherapeutic intervention in breast cancer. An immunization protocol using MUC1-bearing Sialyl Tn *O*-glycans as

immunogen has been used in mice with some success to provide protection against tumour growth. This has encouraged a presently running clinical trial of a similar strategy in breast cancer patients.

Tony Day (University of Oxford, Oxford, UK) gave a comprehensive introduction to the massive GAG, hyaluronan (HA). Though it is the simplest GAG in sequence, it nevertheless supports a wide range of diverse biological activities. Tony suggested that a key to the behaviour of HA is that it can adopt a variety of different conformational states in solution with potentially fast interchanges between them. He then summarized the many HA-binding proteins (hyaladherins) and suggested that the hyaladherin may determine the particular HA conformation stabilized. He went on to discuss results indicating that changes in the architecture of HA-protein complexes may contribute to the variety of biological functions that are attributed to HA. Particular attention was given to TSG-6 and CD44. Molecular modelling studies indicated significant differences in their HA-binding domains and suggested that much larger perturbations in HA structure ensue following binding to CD44 than to TSG-6. Also the binding of TSG-6 to HA enhances the ability of HA to then bind CD44, suggesting TSG-6 induces favourable alterations in HA organization. Lastly, Tony discussed the role of TSG-6 in the covalent modification of HA by attachment of 'heavy chains' derived from the interα-inhibitor. This cross-linking can provide an alternative way of rearranging HA architecture and appears to be important in cumulus cell expansion in ovulation and fertilization.

Mark Ritchie (Waters MS Technology Centre, Manchester, UK) gave a comprehensive overview of the practise of mass spectrometry, before concentrating on its specific application to the analysis of *N*-glycosylation in the large, gel-forming, salivary mucin MUC5B. Though *O*-glycosylation dominates in such mucins, there are abundant potential sites for *N*-glycosylation, though little is known about its structure. He then described how, experimentally, the mucin can be trypsinized and resulting *N*-glycosylated peptides can be identified by capillary LC Q-TOF MS. Analysis of candidate ions by MS/MS revealed that there is *N*-linked occupancy of numerous sites as well as microheterogeneity of structure.

Ulf Lindahl (Uppsala University, Uppsala, Sweden) opened the session on complex glycosaminoglycans with a comprehensive overview of heparan sulfate (HS) structure and function. Pulling together the major contribution of his own lab, with the work of other groups, on HS biosynthesis, HS-binding specificities of various proteins and the results of biosynthetic enzyme knockouts, he challenged the audience with the question: how regulated does HS structure need to be? HS clearly is highly regulated in its synthesis and final structure, with much structural diversity existing between HS from different organs. Recent evidence also demonstrates the existence of an extracellular 6-*O*-sulfatase that can also modulate HS structure postsynthetically. It has been assumed that tightly regulated structure imparts specific protein-binding properties, though antithrombin III still remains the only case in which a precise structure–function relationship between a defined HS sequence and a protein has been elucidated. The essential role of HS in development has clearly been shown by the failure of gastrulation in mouse embryos lacking HS-polymerizing enzymes. However, knockouts of individual enzymes (e.g. NDST-1, 2-OST-1 or GlcA C5-epimerase) responsible for the postpolymeric modification, and thereby the distinctive regulated structure of HS show lethality only at late stages of embryo development. By then, much, apparently normal, organ development has occurred, even though this must have involved the appropriate co-ordinated activity of known HS-binding morphogens/growth factors. Similarly, transgenic overexpression of the heparanase enzyme, an endo-β-glucuronidase which partially fragments HS chains, gives rise to apparently normal and fertile animals despite their much reduced HS chain length.

John Gallagher (University of Manchester, Manchester, UK) spoke about the diversity in sulfation patterns within heparan sulfate. HS has regions that are highly N/O-sulfated, termed S-domains, interspersed with low/nonsulfated regions, enriched in *N*-acetylated disaccharide units, termed NA-domains. Recent studies have looked at the specificity of action upon HS of the K5 lyase enzyme isolated from the K5A bacteriophage. K5 lyase breaks down the glycocalyx of *Escherichia coli* K5 polysaccharide $(GlcNAc\ 1–4\ GlcA)_n$, which has an identical structure to polymeric unmodified heparan, the precursor of HS. This enzyme could partially fragment mature HS, and it has a preference for cleaving within HS sequences containing four or more *N*-acetylated units. Interestingly, *N*-sulfation inhibited the enzyme. Therefore, the K5 lyase was able to protect defined and unique regions of alternating *N*-acetylated and *N*-sulfated disaccharides, which are known to be common in the HS chain, but have not been easily accessible for analysis before. These regions would appear to occur as transition zones between the NA-domains and S-domains of HS. Subsequently, he described a fast gel filtration approach for analysing the stability and stoichiometries of complex formation between heparin oligosaccharides of defined length and proteins. For example, as well as being able to investigate binary heparin–FGF1 interactions, it was possible to also investigate the formation of ternary complexes between heparin-FGF1 and the receptor FGFR2A.

Tarja Kinnunen (University of Liverpool, Liverpool, UK) switched emphasis to the *Caenorhabditis elegans* developmental model, and what it can tell us about the role of HS in

neuronal migration. Expression of the syndecan-1 heparan sulfate proteoglycans (*HSPG*) gene (*sdn-1*) in neuronal cells, and the HS 2-O-sulfotransferase gene (*hst-2*) in various (neuronal, hypodermis and gonad) cells, coincide with the start of morphogenesis in mid-embryonic development. A *sdn-1* deletion mutant lacking the HS attachment site had specific neurone and axon path-finding defects. Similarly, *Hst-2* mutants are also defective in neuronal migration but with a more diverse phenotype. In both *sdn-1* and *hst-2* mutants, specific serotonergic neurones (HSNs) fail to migrate to their positions in the vulva, giving rise to a defective egg-laying phenotype. However, in contrast to *sdn-1* mutants, the *hst-2* mutants have normal migration of canal-associated neurones (CANs), suggesting that 2-*O*-sulfation of HS is essential for HSN, but not CAN, migration.

Malcolm Lyon (University of Manchester, Manchester, UK) discussed new approaches for mapping protein–GAG interactions, using HGF/SF as a model protein. GAG oligosaccharides can be fluorescently tagged at their reducing termini using 2-aminoacridone. After incubation with a protein, the mixture can be run in a gel mobility shift assay on a native PAGE gel to assess protein binding. When oligosaccharides bind to the protein, the free oligosaccharide band becomes depleted and a new, slower-migrating band of oligosaccharide-protein complex appears. HGF/SF was shown to bind to a minimal size HS tetrasaccharide, but a hexasaccharide in the case of DS. Two highly truncated variants of HGF/SF, called NK1 and NK2, had identical binding properties to the full-length HGF/SF, which points to the GAG-binding site being located solely in the N-terminal region encompassed by the smaller NK1 protein. The concept was also discussed of how zero-length cross-linking of fluorescently tagged minimal oligosaccharides to proteins, followed by proteolytic digestion, can potentially liberate fluorescent neo-glycopeptides. These could be recovered, using their anionic character, and potentially analysed to identify the site of GAG attachment and thus binding.

Romain Vivès (Institut de Biologie Structurale, Grenoble, France) followed with a demonstration of how a similar approach has been successfully used to map heparin-binding sites on proteins. Proteins can be immobilized onto heparin-linked beads, using the zero length cross-linking method. Subsequent exhaustive proteolysis of the beads with thermolysin, followed by vigorous washing, leaves specific peptides, derived from the GAG-binding site of the protein, remaining cross-linked to the beads. These peptides can then be identified by direct N-terminal Edman sequencing from the beads. This technique, for the identification of heparin-binding regions within a protein, potentially provides an alternative to more complex and time-consuming approaches, such as site-directed mutagenesis, for mapping critical residues. Two examples used to validate the approach were the chemokine RANTES and the gC envelope protein from the pseudorabies virus.

Toin van Kuppevelt (NCLMS University Medical Centre, Nijmegen, The Netherlands) discussed the potential for using phage-display technology to generate antibodies capable of distinguishing between different HS structures. He began by emphasizing the structural complexity of HS and the challenge this presents. The major technical barrier to acquiring a panel of sequence-specific, anti-HS antibodies is the poor immunogenicity of HS. So, he has proposed the use of semisynthetic antibody phage-display systems, expressing single chain Fvs (scFvs), combined with 'biopanning' techniques, as a potential solution. An additional advantage is that the cDNA encoding a particularly useful scFv can be identified. He described antibodies successfully generated through this procedure that possess differing reactivities towards HS from various tissues. This included antibodies with selectivity towards tumour-associated epitopes, or reactivities with normal, but not rejected, renal tissue. An interesting application was the transfection of tumour cells with cDNA encoding for a specific anti-HS antibody that resulted in a block of HS expression and a reduction in tumour size when the cells were implanted into mice. Finally, the requirement for well-defined oligosaccharide libraries or microarrays to aid the identification of the epitope specificity of such antibodies was underlined.

Claire Johnson (University of Manchester, Manchester, UK) discussed the changes in the levels and sulfation patterns of HS, and specific HSPGs, during the differentiation of embryonic stem (ES) cells into neuroectodermal precursors *in vitro*. She began by introducing the process of directed differentiation *in vitro* of ES cells into neural precursors and also a neural differentiation assay monitored by flow cytometry. A comparison of ES cells and day 6-differentiated cells by FACS analysis, using an HS-specific antibody, revealed an increase in HS expression during differentiation. Parallel structural studies on the extracted HS indicated specific increases in *N*- and 6-*O*-sulfation associated with differentiation. She also revealed a characteristic plaque-like patterning of HS upon ES cells, as visualized by immunocytochemistry, which was lost upon differentiation. RT-PCR data highlighted syndecan-4 as being the only HSPG significantly increased upon differentiation. Although there were also increases in NDST-4 and 3-*O*-ST expression, there was a surprising lack of dramatic alterations in expression of HS biosynthetic enzymes, considering the significant HS structural changes seen.

Briedgeen Kerr (University of Cardiff, Cardiff, UK) reported on the development of a new antibody (BKS-1) specific to a keratanase-generated keratan sulfate (KS) neo-epitope, and its potential applications to the structural analysis of skeletal and corneal KS. The reactivity of BKS-1, which requires keratanase

I treatment of KS but is not generated by keratanase II, was contrasted with that of the previously existing anti-KS antibody, 5D4, which recognizes internal linear sequences of disulfated *N*-acetyl lactosamine disaccharides. Data suggests that BKS-1 recognizes nonreducing *N*-acetylglucosamine 6-sulfate adjacent to a nonsulfated lactosamine disaccharide. Applications of BKS-1 presented, included the analysis of KS substitution of the CS attachment region of cartilage aggrecan during ageing.

James Fawcett (University of Cambridge, Cambridge, UK) presented the Sigma-Aldrich lecture in which he considered the roles of proteoglycans (PGs) in the regeneration and plasticity of the central nervous system. He focused upon the chondroitin sulfate proteoglycans (CSPGs), which are up-regulated following injury and appear to have predominantly inhibitory roles in regeneration and plasticity. He illustrated this with results from animal studies in which the injection of chondroitinase ABC at the site of neural injury enabled partial recovery of function and commented that the relatively rapid timescale of the observed improvements pointed towards an increased plasticity rather than regeneration as the likely cause. After defining plasticity, he developed this theme by introducing the concept of perineuronal nets, emphasizing the number of CSPGs found within these structures. Referring to examples of ocular dominance plasticity, he suggested that the developmental appearance of perineuronal nets coincided with loss of plasticity. Considering the roles of specific CS structures, he defined chondroitin 6-sulfate (C6S) as being more inhibitory than chondroitin 4-sulfate (C4S) and suggested that oversulfation may promote axon growth. He went on to discuss the up-regulation of CS 6-*O*-sulfotransferase and HS 2-*O*-sulfotransferase mRNA expression and concomitant increases in C6S and HS staining, at sites of injury and described how the treatment of cells in culture with tumour growth factor α (TGF-α) or TGF-β, cytokines known to be released following injury, resulted in similar increases. Finally, he discussed the PGs identified within perineuronal nets, emphasizing PG differences between nets from different brain regions.

Gavin Brown (University of Lancaster, Lancaster, UK) concluded the meeting with an ultrastructural investigation of the corneas of dermatopontin-knockout mice. He described how the collagen fibrils of the cornea are organized into lamellar layers, highlighting the importance of the uniformity of both fibril diameter and interfibrillar spacing, with the near hexagonal lattice arrangement of fibrils, providing corneal transparency. By reference to macular corneal dystrophies, as well as the lumican PG-knockout mouse, which both result in corneal opacity, he introduced the additional major contribution of KSPGs to the maintenance of corneal transparency. Commenting on the observed KS-mediated interaction between lumican KSPG and dermatopontin, he described the phenotype of the dermatopontin-knockout mouse, emphasizing the loss of lamellar structure and reduction in stromal thickness at the posterior region and consequent reduced corneal thickness. Though there is a 40% reduction in collagen content within the posterior region of the stroma and an increase in the interfibrillar space, there is no change in fibril diameter. The conclusion is that the loss of dermatopontin results in disruption of the long-range order of collagen fibrils.

Speaker's Abstracts

Regulation of signal transduction by glycosylation

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The incredible diversity found in cell-surface glycoconjugate structures led researchers over 30 years ago to propose that complexity in carbohydrates must play a role in cellular communication. Recent studies from a number of laboratories have confirmed this hypothesis, demonstrating that cell-surface glycoconjugates play significant roles in signal transduction events. One striking example is the effect of *O*-fucose modifications on the Notch-signalling pathway. Notch is a cell-surface receptor that is essential for proper development. The extracellular domain of Notch contains up to 36-tandem epidermal growth factor-like (EGF) repeats, many of which are predicted to be modified at putative consensus sequences with *O*-fucose and *O*-glucose saccharides. Genetic alterations (by knockout or RNAi methodologies) in the enzyme responsible for the addition of *O*-fucose to Notch, protein *O*-fucosyltransferase-1, result in severe, embryonic lethal phenotypes resembling Notch mutants. Thus, *O*-fucosylation appears to be essential for proper Notch function. Elongation of the *O*-fucose monosaccharide by the β1,3-*N*-acetylglucosaminyltransferase, Fringe, modulates Notch function, either increasing or decreasing response from ligands depending on context. Although it is now clear that *O*-fucose modifications affect Notch signalling, the molecular mechanism by which this occurs is not known. As an initial step in understanding how *O*-fucose glycans affect Notch function, we are mapping *O*-fucose modifications to specific sites on Notch. Already, we have demonstrated that *O*-fucose modifies one of the EGF repeats involved in ligand binding, suggesting that the sugars may play a role in Notch–ligand interactions. Experiments to test the role of *O*-fucose modifications at specific sites are in progress. We have also found that