Glycosylation Specific for Adhesion Molecules in Epidermis and Its Receptor Revealed by Glycoform-focused Reverse Genomics*

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Glycosylation of proteins greatly affects their structure and function, but traditional genomics and transcriptomics are not able to precisely capture tissue- or species-specific glycosylation patterns. We describe here a novel approach to link different "omics" data based on exhaustive quantitative glycomics of murine dermis and epidermis. We first examined the dermal and epidermal N-glycome of mouse by a recently established glycoblotting technique. We found that the Galα1-3Gal epitope was solely expressed in epidermis tissue and was preferentially attached to adhesion molecules in a glycosylation site-specific manner. Clarified glycomic and proteomic information combined with publicly available microarray data sets allowed us to identify galectin-3 as a receptor of Galα1-3Gal epitope. These findings provide mechanistic insight into the causal connection between the genotype and the phenotype seen in Galα1–3GalT-1-deficient mice and transgenic mice expressing endo-β-galactosidase C. Because humans do not possess the Galα1–3Gal structure on their tissues, we further examined the human dermal and epidermal N-glycome. Comparative glycomics revealed that the GalNAcβ1-4GlcNAc (N,N'-diacetyldiacetamide) epitope, instead of the Galα1-3Gal epitope, was highly expressed in human epidermis. Molecular & Cellular Proteomics 8:232–244, 2009.

Posttranslational protein glycosylation changes the biological and physical properties of glycoconjugates, which include functions as signals or ligands to control their distribution, antigenicity, metabolic fate, stability, and solubility (1). Cells in the epidermis, which forms a major part of the epithelial barrier, undergo desquamation and are continuously being renewed (2), a process that requires changes in adhesion. Because glycoproteins are often involved in adhesion between cells and their extracellular matrices, the glycoproteome of the epidermis may therefore provide new insight into the functional roles of protein glycosylation. Mammalian epidermal glycoconjugates have mostly been studied histochemically using lectins (3) or monoclonal antibodies (4). Although these studies revealed that the cell surfaces of keratinocytes in the epidermis contain numerous glycoconjugates, these approaches cannot provide detailed structural information about the oligosaccharides or their carrier proteins.

We previously clarified the quantitative glycomic profile of murine dermis and epidermis using novel aminoxy-based isotope tags and MALDI-TOF MS analysis (5), which revealed distinct features of the N-glycosylation profile of dermis and epidermis. We found that murine epidermal glycoproteins have a high abundance of high mannose-type oligosaccharides, and the striking roles of lysosomal enzymes in epidermis during lipid remodeling and desquamation were further discussed. This study was the first to demonstrate the usefulness of quantitative gross N-glycan profiling for performing systematic glycoform-focused proteomics. To advance and accelerate this approach, we recently established a novel technology platform for large scale quantitative glycomics based on the glycoblotting technique (6–8). In this method, glycans derived from biological samples are selectively captured onto novel high density hydrazide beads (BlotGlyco H) for highly efficient purification of oligosaccharides from complex biological samples. The captured oligosaccharides are subjected to on-bead methyl esterification to stabilize sialic acid(s) for the simultaneous quantitation of neutral and sialylated oligosaccharides by MALDI-TOF MS and are finally recovered as arbitrary derivatives by imine exchange chemistry.

In the current study, we first used this newly established glycoblotting technique to re-examine both the neutral and sialylated N-glycome of murine dermis and epidermis. We detected 75 oligosaccharides, more than twice the amount detected previously when sialic acids were removed, thus enabling us to perform a more detailed comparison of tissue-specific N-glycosylation profiles. We found that the expression of oligosaccharides containing the Galα1–3Gal epitope was only detected in epidermis, although many non-reducing terminal epitopes tend to vanish in epidermis. Tracing from
the glycome back to the proteome and transcriptome allowed us to identify a group of proteins that carry Galα1–3Gal epitope and a receptor that can recognize Galα1–3Gal epitope. The glycans identified in mice epidermis could account for the phenotypes observed in transgenic mice expressing endo-β-galactosidase C. Finally human epidermal glycomics was evaluated aiming to address the question of whether any alternative glycan structures play a role similar to that of the Galα1–3Gal epitope in murine epidermis.

**Experimental Procedures**

**Skin Samples and Tissue Preparation**—Male hairless mice (Hos/Hr-1) were obtained from Sankyo Labo Service (Tokyo, Japan). They were fed a standard mouse diet and water ad libitum. Full thickness skin samples were taken from the dorsal area of 7–12-week-old animals. Animal experiments were performed according to the respective regulatory standards of Hokkaido University. Normal human skin samples were obtained from patients during resection operations. The medical ethics committee at Hokkaido University approved all of the described studies, and all of the participants gave their written informed consent. After removal of excess subcutaneous fat from the skin samples, the epidermis was peeled from the dermis by heat separation at 60 °C for 30 s. The epidermis and dermis were minced and heated at 90 °C for 10 min in 10 mM ammonium bicarbonate, then defatted as described by Bligh and Dyer (9), and lyophilized.

**N-Glycan Release**—N-Glycans were released from tissues in previously optimized conditions (10) with minor modifications. Each defatted and lyophilized tissue equivalent (3 mg) was suspended in 0.02% 1-propanesulfonic acid, 2-hydroxy-3-lauramido propionic acid in 10 mM ammonium bicarbonate; reduced with DTT; S-carbamoylmethylated; and digested with trypsin (Sigma). Following deglycosylation by treatment with peptide-N-glycosidase F (PNGase F; Hoffmann-La Roche), the samples were digested with Pronase (Calbiochem). The supernatant was evaporated to dryness and redissolved in 120 μl of 10 mM ammonium bicarbonate.

**N-Glycan Purification and Derivatization**—N-Glycans in the de-N-glycosylated sample were purified and labeled by using a previously described method (7). Briefly 20-μl aliquots of epidermis and dermis samples were applied to a polymer displaying hydrazide functionality at high density (BlotGlyco H), 180 μl of 2.5% dihydroxybenzoic acid (DHBA; 10 mg/ml in 30% acetonitrile; Bruker Daltonics, Bremen, Germany), and an aliquot (1 μl) was deposited on the stainless steel target plate. The aoWR derivatized sample was analyzed to elucidate the relative quantities of the different oligosaccharides present in each tissue. MALDI-TOF data were obtained using an Ultraflex time-of-flight mass spectrometer (Bruker Daltonics) equipped with a LIFT-TOF/TOF unit controlled by the FlexControl 2.2 software package. All of the spectra were obtained using the reflectron mode with an acceleration voltage of 25 kV, a reflector voltage of 26.3 kV, and a pulsed ion extraction of 160 ns in the positive ion mode. The results were obtained by accumulating the signals of 1,000 laser shots. The signal intensity of each mass was automatically calculated by FlexAnalysis 2.0. Estimations of N-linked type oligosaccharide structures were obtained by entering the peak masses into the GlycoMod Tool (Swiss Institute of Bioinformatics) and GlycoSuite (Proteome Systems).

**Neutral N-Glycan Profiling by MALDI-TOF**—Each purified aoWR-derivatized sample was dried and diluted with 10 μl of 2.5 dihydroxybenzoic acid (DHBA; 10 mg/ml in 30% acetonitrile; Bruker Daltonics, Bremen, Germany), and an aliquot (1 μl) was deposited on the stainless steel target plate. The aoWR derivatized sample was analyzed to elucidate the relative quantities of the different oligosaccharides present in each tissue. MALDI-TOF data were obtained using an Ultraflex time-of-flight mass spectrometer (Bruker Daltonics) equipped with a LIFT-TOF/TOF unit controlled by the FlexControl 2.2 software package. All of the spectra were obtained using the reflectron mode with an acceleration voltage of 25 kV, a reflector voltage of 26.3 kV, and a pulsed ion extraction of 160 ns in the positive ion mode. The results were obtained by accumulating the signals of 1,000 laser shots. The signal intensity of each mass was automatically calculated by FlexAnalysis 2.0. Estimations of N-linked type oligosaccharide structures were obtained by entering the peak masses into the GlycoMod Tool (Swiss Institute of Bioinformatics) and GlycoSuite (Proteome Systems).

    1 The abbreviations used are: PNGase F, peptide-N-glycosidase F; ConA, concanavalin A; aoWR, N-α-aminoxy-acetyl-L-tryptophanylarginine methyl ester; LactoNAc, N,N’-diacetyllactosaminide (GalNACβ1–4GalNAC); PA, 2-aminopyridine; ODS, octadecylsilyl silica; MDSF, matrix-dependent selective fragmentation; Ah, arthritaniol hydrazine; SPR, surface plasmon resonance; Fuc, fucose; M2, Man1,GlcNAc2; M3, Man2,GlcNAc2; M3F, Man2,GlcNAc2,Fuc; M4, Man3,GlcNAc2; NeuAc, N-acetyllactosaminide; NeuGc, N-glycolylneuraminic acid; MHC, major histocompatibility complex; Susd 2, sushi domain-containing protein-2; α3GalT, α1,3-galactosyltransferase; GU, glucose unit.

The beads were washed with dioxane, H2O, methanol, and H2O, and then 20 μl of 20 mM N-α-aminoxy-acetyl-L-tryptophanylarginine methyl ester (aoWR) and 180 μl of 2% acetic acid in acetonitrile were added. The solutions were heated at 80 °C for 45 min to transfer the glycans captured on the beads to aoWR, and the glycans were eluted with H2O. To remove the excess aoWR, an aliquot of each sample was applied to a MassPREP hydrophilic interaction chromatography EMulsion Plate (Waters, Milford, MA) according to the manufacturer’s instructions with minor modifications. Following washing with 1% acetic acid, the wells were equilibrated with 1% acetic acid in 95% acetonitrile. After the addition of each sample, the wells were washed with equilibration solution and eluted with 1% acetic acid in 5% acetonitrile.

**N-Glycan Profiling by MALDI-TOF**—Each purified aoWR-derivatized sample was dried and diluted with 10 μl of 2.5 dihydroxybenzoic acid (DHBA; 10 mg/ml in 30% acetonitrile; Bruker Daltonics, Bremen, Germany), and an aliquot (1 μl) was deposited on the stainless steel target plate. The aoWR derivatized sample was analyzed to elucidate the relative quantities of the different oligosaccharides present in each tissue. MALDI-TOF data were obtained using an Ultraflex time-of-flight mass spectrometer (Bruker Daltonics) equipped with a LIFT-TOF/TOF unit controlled by the FlexControl 2.2 software package. All of the spectra were obtained using the reflectron mode with an acceleration voltage of 25 kV, a reflector voltage of 26.3 kV, and a pulsed ion extraction of 160 ns in the positive ion mode. The results were obtained by accumulating the signals of 1,000 laser shots. The signal intensity of each mass was automatically calculated by FlexAnalysis 2.0. Estimations of N-linked type oligosaccharide structures were obtained by entering the peak masses into the GlycoMod Tool (Swiss Institute of Bioinformatics) and GlycoSuite (Proteome Systems).

**Neutral N-Glycan Profiling by the Two-dimensional Mapping Technique**—Each sample was also analyzed by derivatization with 2-aminopyridine (PA) followed by the two-dimensional mapping technique as described previously (11, 12). Briefly N-glycans in the de-N-glycosylated sample were purified by gel filtration and derivatized with PA and sodium cyanoborohydride. After removal of unreacted PA by Sephadex G-15 (GE Healthcare), the PA-oligosaccharides were further purified by collecting the elution from amide-80 (4.6 × 250 mm, Tosoh, Tokyo, Japan) using HPLC. The mixture of PA-oligosaccharides was applied to an octadecylsilyl silica (ODS; 6 × 15 mm, Shimadzu, Kyoto, Japan) HPLC column, and the elution times of the individual peaks were normalized with reference to the PA-derivatized isomalto-oligosaccharides of polymerization degree 4–20 and represented by GU (ODS). Then individual fractions separated on the ODS column were applied to the amide-80 column. Similarly the retention time of the individual peaks on the amide-80 column were represented by GU (amide). Thus, a given compound from these two columns provided a set of GU (ODS) and GU (amide) values, which corresponded to coordinates of the two-dimensional sugar map. By comparison with the coordinates of reference PA-oligosaccharides, the N-glycans from skin were identified. Identification was confirmed by co-chromatography with a candidate reference on the columns and sequential exoglycosidase digestion. Molar ratios of N-glycans were calculated from the individual peak areas.

**MALDI-TOF/TOF MS of PA-derivatized N-Glycans Using the Matrix-dependent Selective Fragmentation (MDSF) Method**—Some of the PA-derivatized N-glycans also were analyzed by MALDI-LIFT-TOF/TOF MS using MDSF according to the procedure described previously (13, 14). α-Cyano-4-hydroxycinnamic acid (Bruker Daltonics) was prepared as a saturated solution in 3:1 (v/v) acetonitrile/water. The desalted PA-derivatized N-glycan samples were dissolved in water, applied on the target spot of the stainless steel target plate, mixed with 1 μl of matrix solution (either 2,5 dihydroxybenzoic acid or...
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α-cyano-4-hydroxycinnamic acid), and dried at room temperature. All measurements were performed using an Ultraflex TOF/TOF mass spectrometer equipped with a reflector and controlled by the FlexControl 2.2 software package (Bruker Daltonics). In MALDI-TOF MS reflector mode, ions generated by a pulsed UV laser beam (nitrogen laser, λ = 337 nm, 5 Hz) were accelerated to a kinetic energy of 23.5 kV. Metastable ions generated by laser-induced decomposition of the selected precursor ions were analyzed without any additional collision gas. In MALDI-LIFT-TOF/TOF mode, precursor ions were accelerated to 8 kV and selected in a timed ion gate. The fragments were further accelerated by 19 kV in the LIFT cell, and their masses were analyzed after the ion reflector passage. Masses were automatically annotated by using the FlexAnalysis 2.2 software package.

**Preparation of Glycopeptides**—Defatted and lyophilized murine epidermis (30–50 mg) was dissolved in a solution of 7 M guanidine hydrochloride, 0.5 M Tris-HCl, pH 8.5, and 10 mM EDTA; reduced with DTT, and 5-carboxymethylated. The alkylated proteins were dialyzed against 10 mM ammonium bicarbonate and digested with trypsin. The digested proteins were applied to a concanavalin A (ConA)-agarose (Seikagaku Co., Tokyo, Japan) column equilibrated with a solution of 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM Tris-HCl buffer, pH 7.5. After the column was washed with equilibrated buffer, the glycopeptides carrying biantennary complex-type oligosaccharides were eluted with buffer containing 10 mM methyl α-D-glucopyranoside. The eluted glycopeptides were then separated on an ODS column using HPLC with a linear gradient of acetonitrile (0–32%) in 0.1% formic acid. Chromatography was carried out at a flow rate of 1 ml/min at room temperature and was monitored at 214 nm. The glycopeptide mixture was separated into 100 fractions and dried with a centrifugal vacuum concentrator. The fractionated glycopeptides were dissolved in 10 μl of 30% acetonitrile. A portion (1 μl) of each fraction was deglycosylated by PNGase F and dissolved in the matrix solution.

**Glycopeptide Identification by MALDI-TOF/TOF**—Each fraction with and without PNGase F treatment was mixed with 2,5-dihydroxybenzoic acid (10 mg/ml in 30% acetonitrile) and then applied to the MALDI target plate. MALDI-TOF and MALDI-TOF/TOF data were obtained using an Ultraflex time-of-flight mass spectrometer as above. For fragmentation ion analysis in the TOF/TOF mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell, and their masses were analyzed after passing the ion reflector. Masses were automatically annotated by using FlexAnalysis 2.0. External calibration of MALDI-TOF MS was carried out using singly charged monoisotopic peaks and fragments of a mixture of human angiotensin II (m/z 1046.542; Bruker Daltonics), bombesin (m/z 1619.823; Bruker Daltonics), and adrenocorticotropic hormone (18–39) (m/z 2465.199; Bruker Daltonics).

**Protein Identification by Database Search**—Peak lists were generated from the MS/MS spectra using Bruker FlexAnalysis (Version 2.0) and were processed by the Mascot™ (Version 2.1, Matrix Science, London, UK) search algorithm to assign peptides based on the Mass Spectrometry Protein Sequence Database (MSDB database updated February 27, 2005, 1,942,918 sequences), a database containing 75,031 mouse genome sequences. The database was searched for tryptic peptides with up to one misscleavage and a mass tolerance for the precursor ions of 1.2 Da and for the fragment ions of 2.0 Da. All cysteine residues were treated as being carboxamidomethylated. Deamidation of asparagines caused by deglycosylation was considered. We first screened the candidate peptides with probability-based MOWSE (molecular weight search) scores that exceeded their thresholds (p < 0.05) and with MS/MS signals for y- or b-ions > 5. If the peptide did not contain the consensus tripeptide sequence for N-linked glycosylation (NX(S/T)) the data were eliminated regardless of the matching score. In total, 14 sets of MS/MS data were obtained.

**Determination of the Relative Quantities of the Microheterogeneous Glycoforms Present at Each N-Glycan Binding Site**—Following the fractionation of ConA-bound fractions (eluted with buffer containing 10 mM methyl α-D-glucopyranoside or 100 mM methyl α-mannopyranoside), each fraction was further analyzed by reversed-phase chromatography as described previously (5). The relative quantitation of the microheterogeneity of different glycoforms present at a particular N-glycan binding site was determined by comparing the signal intensities upon mixing the same volume from each successive fraction that contained the same peptide backbone.

**Purification of Oligosaccharide Having Galα1–3Gal Epitope and Labeling with Biotin**—Purification of oligosaccharide having the Galα1–3Gal epitope and its labeling with biotin were performed as described previously (7). Briefly alkylated and dialyzed murine epidermal proteins were digested with trypsin/PNGase F and were subjected to glycoblotting as described above. The blotted oligosaccharides were recovered as anthraniloyl hydrazine (Ah) derivatives, which are fluorogenic and suitable for chromatographic separation. Ah-derivatized oligosaccharides were subjected to ConA immobilized affinity chromatography and normal-phase HPLC according to the procedure described previously (15). Purified oligosaccharides (100 pmol) were dissolved in 2% acetic acid in 98% acetonitrile and were incubated with aminooxy biotin (Biotium, Inc.) (2 nmol) to promote the conversion of Ah derivatives to biotin derivatives. To remove the excess aminooxy biotin, an aliquot of samples was applied to an amide-80 column.

**Surface Plasmon Resonance Analysis**—The aminooxy-labeled oligosaccharide (10 pmol) was introduced onto a streptavidin preimmobilized sensor surface (sensor chip SA-5 (Biacore AB, Uppsala, Sweden)). Recombinant murine galectin-3 and -7 (R&D Systems, Inc.) were purified with Superdex 200 10/300 GL (GE Healthcare) to remove excess carrier protein. Interactions were monitored by surface plasmon resonance (SPR) using a Biacore 1000 system (Biacore AB) as described previously (16). The reference flow cell sensorgram (determined by injection over a blank surface) was subtracted from the corresponding sensorgrams to abolish baseline drift, bulk, and nonspecific interaction contributions.

**RESULTS AND DISCUSSION**

**Gross N-Glycan Profiling of Murine Dermis and Epidermis**—We analyzed the murine dermal and epidermal N-glycomes using a recently established glycoblotting technique (7) that utilizes a polymer displaying high density hydrazide functionality (BiotGlyco H) coupled with solid-phase methyl esterification of sialic acids and subsequent tag conversion by aowR, a labeling reagent (MS probe) for highly sensitive MALDI-TOF MS. As shown in Fig. 1, the MALDI-TOF MS spectra obtained from each sample differed substantially; the epidermal glycome tended to be of smaller molecular size than dermal glycome. We detected 75 oligosaccharides in either dermis or epidermis, more than twice the number detected previously when sialic acids were removed (5). This great increase is attributable not only to the addition of a variety of sialylated species but also to improved detection sensitivity from the drastically improved signal-to-noise ratio obtained with the glycoblotting technique because of its extremely high purification power. The structure and relative abundance of each detected oligosaccharide are summarized.
We quantified the relative abundance of each type of structure in the observed oligosaccharides (Fig. 2). High mannose-type oligosaccharides, including Man2GlcNAc2 (M2), Man3GlcNAc2 (M3), Man3GlcNAc2Fuc (M3F), and Man4-GlcNAc2 (M4) were more highly expressed in epidermis than in dermis (Fig. 2a). The relative abundance of M2, M3, M3F, and M4, which are considered to be degraded products according to the well characterized N-glycan biosynthesis (17), was much higher in epidermis than in dermis (Fig. 2b). Sialylated species were scarcely observed in epidermis, whereas 40% of dermal N-glycans were found to be sialylated (Fig. 2a). In mice, the sialylated species include both N-acetylneuraminic acid (NeuAc) and N-glycolylneuraminic acid (NeuGc), but NeuGc was the major sialic acid form (Fig. 2c). The relative abundance of oligosaccharides having one or more fucose residues was also substantially lower in epidermis than in dermis; fewer than 30% of epidermal oligosaccharides were modified with fucose, whereas 50–60% of dermal oligosaccharides were fucosylated (Fig. 2d). The relatively smaller molecular size observed for the epidermal N-glycome may in part be attributable to decreased sialylation as well as fucosylation. This finding, in conjunction with the marked increase of M2, M3, M4, and their fucosylated analogues in epidermis, suggests that glycoforms of epidermal glycoproteins are often trimmed by several glycosidases. These observations most likely reflect the unique epidermal environment, which is rich in degradation enzymes, including various glycosidases, because of the desquamation of living cells (18). Thus although many non-reducing terminal epitopes vanished, interestingly the expression of oligosaccharides containing the Galα1–3Gal epitope was only detected in epidermis (Fig. 2e). The presence of the Galα1–3Gal epitope in mice epidermis was predicted from the histochemical studies using Griffonia simplicifolia-I (GS-I), a lectin known to bind to the Galα1–3Gal epitope (19), although the histochemical approach does not clarify whether the glycoconjugate of interest is a glycolipid or an N-glycosylated or O-glycosylated glycoprotein.

Reverse Proteomics/Genomics of the Galα1–3Gal Epitope—The unique expression profile of Galα1–3Gal in mouse epidermis prompted us to identify proteins that carry the Galα1–3Gal epitope in murine epidermis. Because all the observed glycans having the Galα1–3Gal epitope were biantennary oligosaccharides, we used ConA as an affinity reagent for the enrichment of the glycopeptides of interest. Immobilized
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TABLE I

Observed signals of oligosaccharides released from murine skin glycoproteins

Oligosaccharides indicated with an asterisk were detected also by a two-dimensional (2D) mapping technique. Structures were determined by a two-dimensional mapping technique combined with sequential exoglycosidase digestions. Green square, GlcNAc; light blue square, GalNac; yellow circle, Man; dark blue circle, Gal; red inverted triangle, Hex, hexose; HexNac, N-acetylhexosamine; dHex, deoxyhexose.

| No. | Observed (nM) | composition | epidermis Relative abundance (%) mean ± S.D. (n=3) | 2D structures | dermis Relative abundance (%) mean ± S.D. (n=3) | 2D structures |
|-----|---------------|-------------|------------------------------------------------|---------------|------------------------------------------------|---------------|
| 1   | 1016.4424     | (Hex)1     | 0.3 ± 0.1                                      |               | 0.2 ± 0.2                                      |               |
| 2   | 1178.4952     | (Hex)2     | 6.6 ± 1.5                                      |               | 1.1 ± 0.6                                      |               |
| 3   | 1324.5531     | (Hex)2     | 2.7 ± 0.6                                      |               | 1.4 ± 0.6                                      |               |
| 4   | 1340.5458     | (Hex)3     | 11.2 ± 0.8                                     |               | 1.3 ± 0.7                                      |               |
| 5   | 1381.5749     | (Hex)3     | 0.3 ± 0.1                                      |               | 0.0 ± 0.0                                      |               |
| 6   | 1498.6009     | (Hex)2     | 5.5 ± 0.2                                      | **            | 0.6 ± 0.2                                      |               |
| 7   | 1502.6009     | (Hex)4     | 4.0 ± 0.8                                      | **            | 0.9 ± 0.2                                      | **            |
| 8   | 1543.6274     | (Hex)1     | 1.3 ± 0.1                                      | **            | 0.4 ± 0.1                                      |               |
| 9   | 1548.0567     | (Hex)4     | 0.3 ± 0.1                                      |               | 0.0 ± 0.0                                      |               |
| 10  | 1684.6593     | (Hex)2     | 17.0 ± 1.6                                     | **            | 9.0 ± 1.6                                      | **            |
| 11  | 1698.6575     | (Hex)1     | 0.5 ± 0.1                                      | **            | 0.8 ± 0.2                                      |               |
| 12  | 1705.6822     | (Hex)1     | 1.9 ± 0.6                                      | **            | 0.7 ± 0.1                                      |               |
| 13  | 1749.7099     | (Hex)2     | 1.1 ± 0.1                                      |               | 0.3 ± 0.1                                      |               |
| 14  | 1810.7115     | (Hex)2     | 0.1 ± 0.1                                      |               | 0.0 ± 0.0                                      |               |
| 15  | 1828.7064     | (Hex)5     | 9.5 ± 1.1                                      | **            | 5.0 ± 0.4                                      | **            |
| 16  | 1648.7381     | (Hex)1     | 0.2 ± 0.2                                      |               | 0.1 ± 0.2                                      |               |
| 17  | 1751.7381     | (Hex)1     | 1.7 ± 0.3                                      | **            | 0.9 ± 0.3                                      | **            |
| 18  | 1897.7322     | (Hex)1     | 0.7 ± 0.1                                      |               | 1.1 ± 0.2                                      | **            |
| 19  | 1992.7647     | (Hex)2     | 0.5 ± 0.0                                      | **            | 2.0 ± 0.6                                      | **            |
| 20  | 2008.7588     | (Hex)2     | 1.6 ± 0.1                                      | **            | 0.5 ± 0.1                                      | **            |
| 21  | 2049.7682     | (Hex)2     | 0.3 ± 0.0                                      |               | 0.0 ± 0.0                                      |               |
| 22  | 1989.7592     | (Hex)4     | 6.1 ± 0.6                                      | **            | 2.2 ± 0.1                                      | **            |
| 23  | 1964.7903     | (Hex)1     | 0.1 ± 0.1                                      |               | 0.0 ± 0.0                                      |               |
| 24  | 2010.7912     | (Hex)1     | 0.1 ± 0.2                                      |               | 0.3 ± 0.0                                      |               |
| 25  | 2113.7920     | (Hex)2     | 0.6 ± 0.2                                      |               | 0.5 ± 0.1                                      |               |
| 26  | 2208.7921     | (Hex)1     | 0.3 ± 0.1                                      |               | 0.5 ± 0.1                                      |               |
| 27  | 2029.7868     | (Hex)1     | 0.4 ± 0.2                                      | **            | 0.9 ± 0.0                                      | **            |
| 28  | 2024.6155     | (Hex)1     | 0.5 ± 0.1                                      | **            | 1.7 ± 0.4                                      | **            |
| 29  | 2070.8124     | (Hex)2     | 1.9 ± 0.5                                      | **            | 5.7 ± 0.5                                      | **            |
| 30  | 2095.8441     | (Hex)3     | 0.1 ± 0.1                                      |               | 0.6 ± 0.1                                      | **            |
| 31  | 2111.8379     | (Hex)3     | 0.5 ± 0.1                                      |               | 0.2 ± 0.2                                      |               |
| 32  | 2190.812      | (Hex)2     | 6.3 ± 0.3                                      | **            | 2.4 ± 0.6                                      | **            |
| 33  | 2152.8660     | (Hex)4     | 0.0 ± 0.0                                      |               | 0.0 ± 0.0                                      |               |
| 34  | 2158.8491     | (Hex)1     | 0.0 ± 0.0                                      |               | 0.0 ± 0.0                                      |               |
| 35  | 2172.8442     | (Hex)1     | 0.2 ± 0.1                                      |               | 0.6 ± 0.1                                      |               |
| 36  | 2175.8437     | (Hex)3     | 0.2 ± 0.0                                      |               | 0.0 ± 0.0                                      |               |
| 37  | 2188.8442     | (Hex)2     | 0.1 ± 0.1                                      |               | 0.8 ± 0.1                                      |               |
| 38  | 2191.8560     | (Hex)4     | 0.3 ± 0.1                                      |               | 0.0 ± 0.0                                      |               |
| 39  | 2216.6703     | (Hex)2     | 2.6 ± 0.3                                      | **            | 1.4 ± 1.5                                      | **            |
| 40  | 2252.6552     | (Hex)2     | 0.4 ± 0.1                                      |               | 0.0 ± 0.0                                      |               |
| 41  | 2257.5969     | (Hex)1     | 0.5 ± 0.1                                      |               | 0.7 ± 0.1                                      | **            |
| 42  | 2287.8511     | (Hex)3     | 0.3 ± 0.1                                      |               | 0.0 ± 0.0                                      |               |
| 43  | 2298.9225     | (Hex)2     | 0.5 ± 0.0                                      |               | 0.0 ± 0.0                                      |               |
| 44  | 2312.8648     | (Hex)2     | 4.1 ± 0.7                                      | **            | 1.7 ± 0.8                                      | **            |
| 45  | 2334.8069     | (Hex)3     | 0.1 ± 0.2                                      |               | 0.4 ± 0.0                                      |               |
| 46  | 2350.8068     | (Hex)3     | 0.0 ± 0.0                                      |               | 0.5 ± 0.0                                      |               |
| 47  | 2359.9265     | (Hex)1     | 0.0 ± 0.0                                      |               | 0.3 ± 0.2                                      |               |
ConA columns bind weakly to biantennary complex-type N-glycans, which can be eluted with 10 mM α-methylglucoside (20). Peptides were identified by off-line LC-MALDI-TOF/TOF analysis by analyzing the glycopeptide or by analyzing the peptide following PNGase F digestion.

We identified six glycoproteins with seven N-glycosylation sites that carry the Gal\(^{1-3}\)Gal epitope (supplemental Fig. 1; summarized in Table II). Although we only identified a limited number of glycoproteins, they are likely to represent the major Gal\(^{1-3}\)Gal proteins because we identified those glycopeptides with the strongest signal intensities. Among the six glycoproteins identified as carrying Gal\(^{1-3}\)Gal, both desmoglein 1 and desmocollin 1 are components of intercellular desmosome junctions and are involved in the interaction of plaque proteins and intermediate filaments mediating cell-cell interactions (21). Integrin \(\beta4\) is a glycoprotein that associates with the \(\alpha6\) integrin to form the \(\alpha6\beta4\) complex, which functions as a receptor for laminin. Integrin \(\beta4\) also plays a critical structural role in the hemidesmosome of epithelial cells (22). H2-K1 and H2-D1 are components of MHC class I (MHC-I),

![Table I—continued](image-url)
which noncovalently associates with β2–microglobulin (23). MHC-I molecules display peptides from the intracellular pool at the cell surface for recognition by T lymphocytes bearing αβ T cell receptor and mediate cell-cell adhesion by directly binding to CD8 (24–26). The functional role of sushi domain-containing protein-2 (Susd 2) is not known, but it contains the AMOP (adhesion associated domain in MUC4 and other proteins) domain, an ~100-residue extracellular domain that occurs in putative cell adhesion molecules and in some splice variants of MUC4; because this domain is found in extracellular domains involved in cell adhesion (27), it could be indicative of an adhesive role for this protein. All six glycoproteins commonly localize in extracellular space and are all bona fide or predicted type I transmembrane proteins.

We further determined the relative abundance of different microheterogeneous glycoforms present at a particular N-glycan binding site according to a method described previously (5) (Table II). Most glycoproteins identified as Galα1–3Gal epitope carriers were almost exclusively modified with oligosaccharides having the Galα1–3Gal epitope when the fraction weakly bound to an immobilized ConA column was used for the analysis. An exception was a glycopeptide of desmocollin 1 in which oligosaccharides with the Galα1–3Gal epitope were not the major glycoforms. We previously identified the same peptide to be glycosylated with high mannose-type oligosaccharides when a fraction strongly bound to an immobilized ConA column was used for the analysis (5). To clarify the relative quantities of the micro-

**TABLE II**

Summary of glycoproteins identified as Galα1-3Gal epitope carriers

| Glycosylation sites are underlined. ND, not detected. |
|-------------------------------------------------------|
| Localization and protein name (gene name) | SwissProt/TrEMBL Accession No. | Sequence | MASCOT Score | MASCOT Expect | No. 29 | No. 40 | No. 52 | No. 59 | No. 59 | No. 83 |
|-------------------------------------------------------|
| Desmocollin (cytosine or for 1) | Q16490 or Q71881 or Q71882 | QIADTPLEVNLNNWAPK | 63 | 0.0096 | ND | ND | ND | ND | 33.09 ± 17.75 | 66.31 ± 17.75 |
| Desmocollin 1 (desf) | P38449 | NMPPQFQPVATQTGFR | 78 | 3.206-06 | 67.40 ± 4.72 | 19.86 ± 3.01 | 12.63 ± 1.73 | ND | ND | ND |
| Hemidesmosomes | A2A023 | TCCGTTSTLGEPQDR | 53 | 6.0995 | ND | ND | ND | ND | 15.28 ± 11.24 | 55.72 ± 11.24 |
| | | NDCPLTATDDNSNQGFR | 100 | 1.720-06 | ND | ND | ND | ND | ND | 100.0 ± 0.0 |
| MHC molecule | P39091 | YYSGSSGRKIH | 97 | 8.998-05 | ND | ND | ND | ND | 13.76 ± 2.90 | 10.95 ± 2.97 | 79.56 ± 5.38 |
| NOD1 protein (NOD2) | Q83926 | TFLQYGYSQAGKTHY | 37 | 0.053 | ND | ND | ND | ND | ND | 100.0 ± 0.0 |
| Unknown | Q06853 | FCIDVMSSTSSVQTNRE | 45 | 0.0082 | 34.50 ± 2.66 | 16.62 ± 0.88 | 9.69 ± 1.77 | 12.61 ± 1.53 | 5.13 ± 1.10 | 21.99 ± 2.76 |
heterogeneous glycoforms present on the glycopeptides, fractions both weakly and strongly bound to the immobilized ConA column were collected in gross, and the relative quantities of the microheterogeneous glycoforms present at each N-glycan binding site were determined. In addition to seven glycopeptides identified as modified with the Gal$_{1}$-H$_{9251}$1–3Gal epitope, we analyzed two glycopeptides determined previously to be modified with high mannose-type oligosaccharides (Fig. 3). This analysis revealed that the glycopeptide of interest from desmocollin 1 is dominantly modified with high mannose-type oligosaccharides, and the relative proportion of Gal$_{1}$-H$_{9251}$1–3Gal was nominal. The same tendency was observed also for the peptides of the H2-K1 and H2-D1 proteins. The remaining glycopeptides were dominantly modified with oligosaccharides having the Gal$_{1}$-H$_{9251}$1–3Gal epitope. This analysis also revealed that the microheterogeneity of the two different glycosylation sites (underlined) on desmoglein 1 are completely different; LNATDADEPNLNSMIAFK is dominantly modified with oligosaccharides having the Gal$_{1}$-3Gal epitope, whereas TGEINITIVDR is dominantly modified with high mannose-type oligosaccharides. These observations indicate that the Gal$_{1}$-3Gal epitope is preferentially attached to adhesion molecules, and the spatial arrangement of this epitope on the protein is highly regulated.

Mice deficient in α3GalT-1 develop cortical cataracts within 4–6 weeks of birth (28). Although no other notable pathological abnormality has been detected, these α3GalT-1-deficient animals retain a residual level of the αGal epitope possibly because of compensation by α3GalT-2 (29, 30). Very recently, transgenic mice that systemically express endo-β-galactosidase C, which removes the terminal Gal$_{1}$-3Gal disaccharide, were produced. Flow cytometry and histochemical analyses showed that the Gal$_{1}$-3Gal epitope was completely removed from at least restricted organs including skin, heart, and lung. Of a total of six founder mice obtained, three were unable to survive after birth because they had severe phenotypes, including edema and dry skin that resulted in loss of moisture from the skin surface and finally to cracking of the skin in the neonatal stage (31). Despite the marked pathologic lesions in the epidermis, the dermis was normal in structure (32). These observations agree well with the distribution of the Gal$_{1}$-3Gal epitope in the murine dermis and epidermis shown in the current study. Proteins identified as Gal$_{1}$-3Gal carriers in this study may contribute to the severe phenotypes seen upon failure of proper α-galactosylation.

To further validate these findings, we compared our proteome data with the tissue distributions derived from the enormous amount of expression data present in the SymAtlas
of the Genomics Institute of the Novartis Research Foundation (33). Among the six proteins identified, desmoglein 1, desmocollin 1, and integrin β4 are highly selectively expressed in epidermis, digits, and tongue. H2-K1 and H2-D1 are most highly expressed in B cells and T cells but are also expressed in various other tissues and organs including lymph node, trachea, adipose tissue, epithelium, lung, spleen, and small intestines. Susd 2 is ubiquitously expressed, but it is expressed most highly in umbilical cord and dorsal root ganglia followed by epidermis, trigeminal, and kidney. The transcriptional profiling data of α3GalT-1 shows that it is ubiquitously expressed but is most highly expressed in the female reproductive system (e.g. in fertilized egg, oocyte, blastocytes, and placenta) (supplemental Fig. 2) consistent with previous findings that the terminal Galα1–3Gal epitope is crucial for murine sperm-egg binding (34) and recognition during initial gamete binding (35). The expression levels of Ggta1 in epidermis and eye are either slightly higher or equal to the median, thus making it very difficult to predict the phenotypes detected in α3GalT-1-deficient mice and transgenic mice expressing endo-β-galactosidase C by transcriptomics data alone. We could discover that the Galα1–3Gal epitope specifically modifies adhesion molecules, which can possibly explain the phenotypes seen in transgenic mice, by using the approach to link the glycome to the proteome and transcriptome. The transcriptomics data indicate that desmoglein 1 and integrin β4 are also moderately expressed in the eye; therefore, these molecules may be involved in the incidence of cortical cataracts seen in α3GalT-1-deficient mice.

Identification of Galα1–3Gal Epitope-recognizing Molecule in Murine Epidermis—N-Glycosylation has been shown to modulate the adhesion properties of proteins, such as the association of the α5 and β1 subunits of integrin, and receptor functioning (36, 37). In this regard, the adhesion molecule-specific glycosylation by the Galα1–3Gal epitope in murine epidermis may also be involved in the modulation of the adhesion properties of proteins. As a trial to explore this possibility, genes of which epidermal expression is more than 10-fold over median expression levels were searched on SyMatlas. Among the list, six genes are Gene Ontology-annotated as “sugar binding.” They are Aim1, Lgals3, Lgals7, Mgl1, Mgl2, and Mrc1, each encoding absent in melanoma 1, galectin-3, galectin-7, macrophage galactose N-acetylgalactosamine-specific lectin 1, macrophage galactose N-acetylgalactosamine-specific lectin 2, and macrophage mannose receptor 1, respectively.

Galectins are a group of relatively small lectins whose ability to bind to β-galactosides is evolutionarily conserved among extensive organisms. Galectin-3 is expressed and secreted by various types of cells, especially monocytes, macrophages, mast cells, and epithelial cells including corneal epithelium (38). It is a mitogen capable of stimulating fibroblast cell proliferation in a paracrine fashion through interaction with cell surface glycoconjugates (39). This protein can also exert an antiapoptotic activity underscoring its strong effect on cell growth (40). Unlike galectin-3, expression of galectin-7 is restricted to epithelia that are stratified or are destined to become stratified (41). Galectin-7 is thought to play a role in apoptosis (42). The carbohydrate binding specificities of galectins have been elucidated by many researchers. Notably Hirabayashi et al. (43) extensively analyzed the interaction of galectins, including galectin-3 and -7, by frontal affinity chromatography using 41 glycans (including 12 N-glycans). To our knowledge, however, the interaction of galectin-3 and -7 with N-glycans having the Galα1–3Gal epitope(s) has not been elucidated. Therefore, we examined the interaction of galectin-3 and -7 with various naturally derived N-glycans including the biantennary oligosaccharide having two Galα1–3Gal epitopes at the non-reducing termini (number 63 in Table I) by SPR. Following the biotinylation of each oligosaccharide, they were immobilized onto the streptavidin preimmobilized surface. The molar amount of each immobilized oligosaccharide is presumed to be nearly constant because the amount of streptavidin was constant, and an excess of purified biotinylated oligosaccharide was introduced (16). The validity of the immobilization of each N-glycan was quantitatively confirmed by measuring interactions with ConA and RCA120, whose sugar binding specificities are well characterized (supplemental Fig. 3).

The murine galectin-3 and -7 were passed over the sensor surface at concentrations of 0.7 and 1.3 μM, respectively, and binding was monitored as changes in the SPR signal. As shown in Fig. 4a, the bindings of galectin-3 differed markedly depending on the structure of the immobilized N-glycans. N-Glycans having two Galα1–3Gal epitopes gave the highest increase in the resonance signal followed by an N-glycan with one Galα1–3Gal epitope. N-Glycans terminated with Galβ1–4GlcNAc also interacted with galectin-3, but the response was much lower. These results indicate that galectin-3 has higher affinity to Galα1–3Galβ1–4GlcNAc epitope than to Galβ1–4GlcNAc. Interestingly we observed that the presence or absence of core fucose on the N-glycan with two Galα1–3Gal epitopes affects the interaction with galectin-3; the presence of core fucose further increased the binding of galectin-3. This may be attributable to the conformational change of N-glycan. The presence of core fucose has been reported to have a dramatic effect on the conformation of the Manα1–6 antenna resulting in the reduced flexibility of this antenna (44). Note that N-glycosylation sites of desmoglein-1 and integrin β4 identified to have Galα1–3Gal epitopes are almost solely occupied by the N-glycan having two Galα1–3Gal epitopes and core fucose. On the contrary, the increase in resonance signal upon injection of galectin-7 was marginal (Fig. 4b).

Although similar gene expression patterns do not necessarily mean that these two gene pairs are related, our analysis clearly demonstrated that galectin-3 preferentially binds to N-glycan with the Galα1–3Gal epitope than those without this epitope and thus strongly suggested that the extracellular
portion of cell surface adhesion proteins (e.g., desmoglein and integrin) would associate with galectin-3 via Galα1–3Gal epitopes on their N-glycans at epidermis. The biological significance of the interaction of galectin-3 with cell surface adhesion proteins needs to be further elucidated. Galectin-3 may mediate cell-cell/cell-extracellular matrix interactions or act as deadhesion molecule as shown in the interaction of thymocytes and thymic microenvironmental cells (45).

**Glycomics of Human Dermis and Epidermis**—The Galα1–3Gal structure is abundantly expressed in glycoproteins and glycolipids of nonprimate mammals and New World monkeys but is not present in Old World monkeys, apes, and humans (46–48). Therefore, we turned our focus to human epidermal glycomics and glycoproteomics. To our knowledge, the glycomics of the human dermis and epidermis have not been studied extensively.

We quantitatively analyzed human dermal and epidermal glycome using the same procedure described for the murine glycomics; the MALDI-TOF spectra are shown in supplemental Fig. 4. We estimated the structure and relative abundance of each signal (supplemental Table 1), and we analyzed the relative abundance of each type of structure in the oligosaccharides observed in each sample (Fig. 5). The glycomic profiles of both the epidermis and dermis were fairly similar between mouse and human. The high mannose-type oligosaccharides (including M2, M3, and M4 and their fucosylated analogues) were relatively abundant (Fig. 5, a and b), and the sialylated and fucosylated species were of relatively low abundance in human epidermis (Fig. 5, c and d) as they were for mice. The interspecies differences in dermal glycomic profiles were mostly limited to the extent and form of sialylation: human contains only NeuAc, whereas mouse contains both NeuAc and NeuGc. As a result, the signals observed in the higher molecular mass region of the dermal glycome spectra often shifted by 16 or 32 Da increments between human and mouse.

As expected, no glycans carrying the Galα1–3Gal epitope were observed in human dermis or epidermis. Instead we found that the expression of oligosaccharides with the GalNAcβ1–4GlcNAc (N,N′-diacetyllactosediame; LacdiNAc) epitope was exceptionally high in the human epidermis (Fig. 5e). The presence of the LacdiNAc epitope was confirmed based on two-dimensional LC mapping as well as the MDSF method in MALDI-TOF/TOF mass spectrometry as exemplified in supplemental Fig. 5. The LacdiNAc epitope is common on the glycoproteins of invertebrates, such as helminth parasites, but is also found on some mammalian glycoproteins (49). In vertebrates, the group of glycoproteins that carries the LacdiNAc epitope is diverse and includes membrane as well as secreted glycoproteins, enzymes, hormones, transport proteins, and protective glycoproteins (50, 51).

It is not clear at this point whether the LacdiNAc epitope plays an important role in human epidermis as the Galα1–3Gal epitope does in murine epidermis. However, it is noteworthy that it was reported that a remarkably strict co-localization of galectin-3-reactive binding sites with desmoglein was found by immunohistochemical analysis of human corneal and conjunctival epithelia (52). Although no detailed mechanism underlying the interaction of galectin-3 and desmosomal proteins has been elucidated to date, the LacdiNAc epitope may mediate the interaction of these molecules. It may be interesting to note that the conjunctival epithelium from the patient with Stevens-Johnson syndrome, a serious and potentially life-threatening cutaneous drug reaction, expressed no galectin-3-reactive binding sites (52). Future glycomics and glycoproteomics analyses of various skin disorders would enable the significance of the presence of the LacdiNAc epitope in epidermis to be elucidated.
Conclusion—In this study, we evaluated the murine skin glycomics and glycoproteomics in detail, including sialylated species, based on a recently established glycoblotting technique and MALDI-TOF/TOF MS analysis. One of the key findings was that the Gal\(^{1–3}\)Gal epitope is highly selectively attached to adhesion molecules of murine epidermis in a glycosylation site-specific manner. These findings provide mechanistic insight into the causal connection between the genotype and the phenotype seen in Gal\(^{1–3}\)GalT-1-deficient mice and transgenic mice expressing endo\(-\)galactosidase C. Because protein glycosylation is very complicated, it is not possible with the current methodology to fully characterize the N-glycosylation of proteins on a proteome-wide scale in terms of the glycosylation site, oligosaccharide structure, and quantitative microheterogeneity. Very complex forms of transcriptional and post-translational modifications can, in principle, be analyzed with state-of-the-art top-down MS methods if sufficient amounts of a purified protein are available (53); however, major technological breakthroughs are needed to achieve the purification of the protein(s) of interest and high throughput protein analysis.

The correlation function of the searchable SymAtlas database was then used to refine genes with an expression similar to proteins identified to carry Gal\(^{1–3}\)Gal epitope. Molecular interaction analysis using naturally derived N-glycans with or without the Gal\(^{1–3}\)Gal epitope experimentally demonstrated that N-glycan with not only Gal\(^{1–3}\)Gal epitope but also the core fucose is a preferred glycan ligand for galectin-3. Finally comparative glycomics of murine and human epidermis indicated that the LacdiNAc epitope, rather than the Gal\(^{1–3}\)Gal epitope, was highly expressed in human epidermis, although its functions remain to be elucidated.

Glycoform-focused reverse genomics is a novel approach that links data from different omics by first obtaining exhaustive quantitative glycomics data and then tracing from the glycome back to the proteome and transcriptome. This reverse approach allows novel classification of proteins and genes with regard to the significance of glycosylation in a way that is not possible with the information that can currently be obtained from genomic, transcriptomic, and traditional proteomic information alone. This concept would find wide application for the identification of diagnostic biomarkers and therapeutic targets.

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