Cooperative Interactions of Oligosaccharide and Peptide Moieties of a Glycopeptide Derived from IgE with Galectin-9

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We previously showed that galectin-9 suppresses degranulation of mast cells through protein-glycan interaction with IgE. To elucidate the mechanism of the interaction in detail, we focused on identification and structural analysis of IgE glycans responsible for the galectin-9-induced suppression using mouse monoclonal IgE (TIB-141). TIB-141 in combination with the antigen induced the galectin-9-induced suppression using mouse monoclonal IgE. Sequential digestion of TIB-141 with lysyl endopeptidase and trypsin resulted in the identification of a glycopeptide (H-Lys13-Try3; 48 amino acid residues) with a single N-linked oligosaccharide near the N terminus capable of neutralizing the effect of galectin-9 and another glycopeptide with two N-linked oligosaccharides (H-Lys13-Try1; 16 amino acid residues) having lower activity. Enzymatic elimination of the oligosaccharide chain from H-Lys13-Try3 and H-Lys13-Try1 completely abolished the activity. Removal of the C-terminal 38 amino acid residues of H-Lys13-Try3 with glutamyl endopeptidase, however, also resulted in loss of the activity. We determined the structures of N-linked oligosaccharides of H-Lys13-Try1. The galectin-9-binding fraction of pyridylaminated oligosaccharides contained asialo- and monosialylated bi/tri-antennary complex type oligosaccharides with a core fucose residue. The structures of the oligosaccharides were consistent with the sugar-binding specificity of galectin-9, whereas the nonbinding fraction contained monosialylated and disialylated biantennary complex type oligosaccharides with a core fucose residue. Although the oligosaccharides linked to H-Lys13-Try3 could not be fully characterized, these results indicate the possibility that cooperative binding of oligosaccharide and neighboring polypeptide structures of TIB-141 to galectin-9 affects the overall affinity and specificity of the IgE-galectin interaction.

Human galectin-9 (Gal-9) was originally discovered as a novel tumor antigen of unknown function in patients with Hodgkin disease (1). Although the first biological activity ascribed to Gal-9 was eosinophil chemoattractant activity (2), subsequent studies established that Gal-9 is a multifunctional regulator of the immune response and homeostasis (3). Gal-9 is a member of the galectin family of glycan-binding proteins, which is characterized by affinity for lactosamine type disaccharides and consensus amino acid residues in the carbohydrate recognition domain (CRD). Gal-9 consists of two CRDs joined by a linker peptide and thus belongs to the so-called “tandem repeat type” subclass of the family. The N-terminal CRD (N-CRD) and C-terminal CRD (C-CRD) of tandem repeat type galectins generally have different sugar binding specificities (4). Previous studies have demonstrated that most, if not all, biological activities of galectins depend on the interaction between CRD(s) and the oligosaccharide moieties of glycoconjugate receptors.

Several receptors/binding partners for Gal-9 have been reported to date. Gal-9 down-regulates the Th1 (T helper type I) response through Tim-3 (T-cell immunoglobulin mucin-3) expressed on the cell surface of fully differentiated Th1 cells (5). CD44 (6), Epstein-Barr virus latent membrane protein-1 (7), and GLUT2 (glucose transporter 2) (8) have also been identified as Gal-9 receptors. In addition to these cell surface glycoproteins, Mishra et al. (9) reported that Gal-9 regulates polarization of Madin-Darby canine kidney cells via interaction with Forssman glycosphingolipid. The oligosaccharide moiety of Forssman glycosphingolipid, Forssman pentasaccharide, exhibits exceptionally high affinity for the N-terminal CRD of Gal-9 (4). In contrast to Forssman glycosphingolipid, the structures and glycosylation site(s) of glycoprotein glycans through which Gal-9 exerts its effect are generally difficult to determine unequivocally. Currently, the glycans responsible for the interaction with Gal-9 have not been identified in most glycoprotein receptors except for GLUT2. Vertebrate GLUT2 has a single N-glycosylation site in the first extracellular loop. Although the oligosaccharide structure has not been directly determined, a tetra-antennary complex type glycan bearing little or no sialic acid, which was inferred from plant lectin-binding profiles, is suggested to act as the receptor for Gal-9 (8).

We previously reported that Gal-9 specifically binds to human and murine IgE with relatively high affinity (10). The...
interaction inhibited IgE-antigen complex formation, whereby degranulation of RBL-2H3 cells was suppressed. The effect of Gal-9 was completely abolished in the presence of 20 mM lactose. In addition, degranulation became insensitive to Gal-9 when the experiment was performed with a periodate-treated IgE preparation (i.e., IgE with an oxidized oligosaccharide moiety). These results indicate that the interaction between Gal-9 and IgE glycan(s) is indispensable for the suppressive effect of Gal-9. In the present study, we aimed at identification of glycosylation site(s) and structural analysis of IgE glycans responsible for the Gal-9-induced suppression of RBL-2H3 cell degranulation. Because most studies, including our previous ones, concerning the in vivo function and therapeutic potential of Gal-9 were performed with a mouse model (11–17), mouse monoclonal IgE was used as a target molecule for both human and mouse Gal-9.

Experimental Procedures

Cloning and Sequence Analysis of TIB-141 cDNA—An anti-2,4,6-trinitrophenyl (TNP) mouse monoclonal IgE-producing hybridoma cell line (IGELb4) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). The term TIB-141 will be used as the name of the monoclonal antibody in this paper, although it is the ATCC catalogue number for the hybridoma cell line. The cDNA sequences of both the L and H chains of TIB-141 were determined to confirm previous reports by other investigators. Total RNA was extracted from IGELb4 hybridoma cells by the method of Chomczynski and Sacchi (18). About 2 μg of total RNA was reverse transcribed using the GeneAmp RNA PCR kit with oligo(dT) primers (PerkinElmer Life Sciences). cDNAs for the light chain (L chain) and heavy chain (H chain) of TIB-141 were amplified using gene-specific primer pairs of mIGLK-1/-2 and mIGH-1/-2, respectively

GeneAmp RNA PCR kit with oligo(dT) primers (PerkinElmer Biosystems). The sequences (terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI Prism 3130 genetic analyzer (Applied Biosystems). The sequences (supplemental Fig. S1) were deposited in the DDBJ database under the accession numbers LC031494 (H chain) and LC031495 (L chain). The sequence of the L chain completely coincided with those reported previously, whereas there were differences in the sequence of the H chain between the present data and the data reported by Liu et al. (20). In the DNA database, however, we found DNA sequences identical to that of the H chain shown in supplemental Fig. S1. Currently, it is not clear whether the differences are due to sequencing error, PCR artifacts, or genetic variation of the hybridoma cell line. The sequences determined in the present study were used to interpret the results described below.

Expression and Purification of Recombinant Gal-9—Expression of tag-free recombinant proteins, human stable form Gal-9 (hG9Null), and mouse stable form Gal-9 (mG9Null), in Escherichia coli BL21(DE3) was carried out as described previously (22). Recombinant proteins were purified by affinity chromatography on a lactose-agarose column (Seikagaku Corp., Tokyo, Japan). The purified proteins were dialyzed against PBS and then sterilized by filtration. All of the preparations were found to be >95% pure, as judged on SDS-PAGE. The protein concentrations were determined using BCA protein assay reagent (Pierce) and BSA as a standard. The concentrations of peptides purified by HPLC were determined from the UV absorbance at 215 and 225 nm using the formula, concentration (mg/ml) = (A215 nm − A225 nm) × 0.144 (23).

Cell Culture—The rat basophilic leukemia cell line (RBL-2H3) was provided by the RIKEN BioResource Center (Ibaraki, Japan). RBL-2H3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 milliunits/ml penicillin and 100 μg/ml streptomycin at 37 °C under a 5% CO2, 95% atmosphere. Mouse hybridoma cell line IGELb4 was maintained under the same conditions as those described above. To prepare serum-free conditioned medium, a nearly confluent culture of hybridoma cells was diluted 1:4 in E-RDF medium supplemented with RD-1 (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) and then cultured for 4 days. This procedure was repeated again, resulting in a 1:16 dilution of the original serum-containing medium. At the end of the culture, cells were collected by centrifugation and then resuspended in fresh serum-free medium (E-RDF + RD-1) at a concentration of 5 × 106 cells/ml. Cells were subcultured every 5 days. IGELb4 grew equally as well in serum-free medium as in serum-containing medium. Serum-free conditioned medium obtained by centrifugation of the culture was supplemented with 20 mM Tris-HCl (pH 7.5) and then stored at 4 °C until use. The serum-free medium contained only transferrin and insulin as proteinaceous components.

Purification of Mouse IgE—The serum-free conditioned medium of IGELb4 hybridoma cells was used as the source of TIB-141. About 200 ml of the conditioned medium was applied to a ceramic hydroxyapatite column (Bio-Scale Mini CHT Type I cartridge, 5 ml; Bio-Rad) equilibrated with 10 mM sodium phosphate buffer (pH 7.5) and then stored at 4 °C until use. The soluble protein fraction was collected and subjected to second hydroxyapatite chromatography. The dialysate was applied to a Bio-Scale CHT2-I column (Bio-Rad) equilibrated with 10 mM sodium phosphate buffer (pH 7.5). After washing the column with 10 ml of equilibration buffer, the adsorbed material was eluted with 0.2 M sodium phosphate buffer (pH 7.5). The eluate was dialyzed against PBS and then subjected to second hydroxyapatite chromatography. The dialysate was applied to a Resource Q column (1 ml; GE Healthcare) equilibrated with 20 mM Tris-HCl (pH 8.0). After washing the column with 10 ml of equilibration buffer, the adsorbed material was eluted with a linear gradient of 10–500 mM sodium phosphate buffer (pH 7.5) over 40 ml. Fractions containing TIB-141 were pooled and then dialyzed against 20 mM Tris-HCl (pH 8.0). The dialysate was applied to a Resource Q column (1 ml; GE Healthcare) equilibrated with 20 mM Tris-HCl (pH 8.0). After washing the column with 10 ml of equilibration buffer, the adsorbed material was eluted with a linear gradient of 0–0.5 M NaCl in the buffer over 30 ml. The peak fractions containing TIB-141 were pooled, dialyzed against PBS, and then sterilized by filtration. The sterilized preparation was used as purified TIB-141 (Fig. 1). The final yield was ~10 mg of purified TIB-141/liter of conditioned medium. To determine the N-terminal amino acid sequences of H and L chains, purified TIB-141 was subjected to
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SDS-PAGE under reducing conditions, and the resolved chains were transferred to a PVDF membrane (Millipore Corp., Billerica, MA). The blotted proteins were stained with CBB R-250 and cut out, and then the N-terminal amino acid sequences were determined with a gas phase sequencer. The N-terminal amino acid sequences of the H and L chains of purified TIB-141 were determined to be EVQLQQSGAE and DVLMTQTPLS, respectively.

Lysyl Endopeptidase Digestion of IgE and Isolation of Glycopeptides—Purified TIB-141 was reduced and pyridylethylated by the method of Fullmer (24). About 12 mg of pyridylethylated TIB-141 was dissolved in 6 ml of 0.1 M Tris-HCl (pH 9.5), 10 mM CaCl₂, and then digested with 0.2 AU of lysyl endopeptidase (Achromobacter proteinase I; Wako Pure Chemical Industries, Osaka, Japan) for 6 h at 37 °C. The reaction mixture was acidified with TFA and then applied to a μBondasphere C18 column (150 × 3.9 mm; Waters, Milford, MA). After washing with 0.1% TFA, the column was developed with a linear gradient of 0–80% (v/v) acetonitrile in 0.1% (v/v) TFA. Alternatively, the enzyme digest was subjected to affinity purification before reversed-phase HPLC (RP-HPLC). After adjusting the pH between 7.8 and 8.0 with HCl, the digest (1 ml) was applied to an hG9Null-immobilized column (about 3 mg of hG9Null immobilized on a 1-ml HiTrap NHS-activated HP; GE Healthcare). The column was washed with 5 ml of 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl (TBS). Gal-9-interacting peptides were eluted with TBS, 0.2 M lactose.

Trypsin Digestion of P23 and Endoproteinase Glu-C Digestion of a Tryptic Fragment of P23—About 150 μg of the lyophilized P23 preparation (see “Results”) was dissolved in 200 μl of 50 mM Tris-HCl (pH 8.5), 1 mM CaCl₂. After the addition of 8 μg of trypsin (Roche Diagnostics, Mannheim, Germany), the reaction mixture was incubated for 6 h at 37 °C. The proteolytic fragments were isolated by RP-HPLC as described above. About 100 μg of lyophilized P23-T11, a tryptic fragment of P23 (see “Results”), was dissolved in 200 μl of 25 mM NH₄HCO₃ (pH 7.8). After the addition of 5 μg of endoproteinase Glu-C (Roche Diagnostics), the reaction mixture was incubated for 6 h at 25 °C and then subjected to RP-HPLC.

The molecular weights of the peptides derived from TIB-141 were determined by MALDI-TOF MS performed on an ultralife-Xtreme instrument (Bruker Daltonics, Bremen, Germany).

Removal of N-Glycans with Peptide-N-Glycosidase F—Digestion of glycopeptides with peptide-N-glycosidase F (PNGase F; Takara Bio, Shiga, Japan) was carried out according to the manufacturer's instructions under native conditions.

β-Hexosaminidase (β-HEX) Release Assay—The β-HEX release assay was performed as described previously (10) with modifications. RBL-2H3 cells were inoculated onto 96-well plates (2 × 10⁴ cells/well). After 24-h culture, the cells were washed once with assay buffer (Hanks' balanced salt solution containing 20 mM HEPES-NaOH, pH 7.5, and 1 mg/ml BSA) and then stimulated by the addition of 0.3 μg/ml TIB-141 and 0.048 μg/ml TNP-conjugated BSA (TNP-BSA; Biosearch Technologies, Novato, CA). Galectins were added 10 min before the addition of TIB-141 unless otherwise stated. The culture medium was collected 60 min after the addition of TNP-BSA. After washing with assay buffer, cells were lysed with 0.1% Triton X-100. β-HEX activity in the culture medium and cell lysate was determined as described previously (10). To determine the neutralizing activity of glycopeptides derived from TIB-141 toward Gal-9-induced suppression of degranulation, Gal-9 was mixed with the glyccopeptides before the addition to the reaction mixture.

Western Blot Analysis—RBL-2H3 cells were lysed with reducing SDS-PAGE loading buffer. Samples were run on 12.6% gels. The separated proteins were transferred to PVDF membranes, followed by immunodetection with antibodies against phospho-p44/42 MAPK (Thr202/Tyr204), p44/42 MAPK, and actin (Cell Signaling Technologies, Danvers, MA) and peroxidase-labeled goat anti-rabbit IgG antibodies (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). ECL Western blotting detection reagents (GE Healthcare) were used.

Purification and Structural Analysis of N-Glycans—Preparation and purification of pyridylaminated (PA-)N-glycans from glycopeptides derived from TIB-141 were carried out as described previously (25, 26), except that the PA-glycans were first subjected to affinity chromatography on an hG9Null-immobilized column as described above. PA-glycans dissolved in TBS were applied to the column. After washing the column with TBS, adsorbed glycans were eluted with TBS, 0.2 M lactose. The flow-through fraction and eluate were used as unadsorbed and adsorbed fractions, respectively. The unadsorbed and adsorbed fractions obtained on affinity chromatography were separately fractionated by anion exchange chromatography on a Mono Q column (0.5 × 5 cm; GE Healthcare). The neutral glycan fraction (flow-through fraction) designated as “N”, and the adsorbed anionic fractions designated as A1–A4 according to the elution order (supplemental Fig. S2) were further fractionated by size fractionation chromatography on a TSKgel Amide 80 column (0.46 × 7.5 cm; Tosoh). The molecular size of each PA-glycan is given in terms of glucose units based on the elution times of PA-isomalto-oligosaccharides. The size-fractionated glycans were finally purified by RP-HPLC on a Cosmosil 5C18-S column (0.2 × 25 cm; Nacalai Tesque, Kyoto, Japan).
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when the cells were stimulated with TIB-141 and TNP-BSA. hG9Null and mG9Null inhibited the phosphorylation in accordance with their effects on degranulation (Fig. 2B).

**Lysyl Endopeptidase Digestion of TIB-141 and Isolation of Galectin-9-interacting Peptides**—Protease digestion of S-pyridylethylated TIB-141 (PE-TIB-141) and subsequent analysis of proteolytic peptides were performed to identify N-glycan(s) with high affinity for Gal-9 and thus responsible for Gal-9-induced inhibition of degranulation. As the first step, PE-TIB-141 was digested with lysyl endopeptidase and then subjected to affinity purification on an hG9Null-immobilized column. The predicted peptide fragments resulting from the proteolysis of the H chain and L chain of PE-TIB-141 are summarized in supplemental Tables S1 and S2, respectively. The unfraccionated digest, the affinity-purified fraction, and the unbound fraction (flow-through fraction) were individually analyzed by RP-HPLC (Fig. 3). About 40 peaks, including minor ones, were observed in the chromatogram of the unfraccionated digest. Among the major peaks of the chromatogram (designated as P1–P23 in Fig. 3A), six peaks (P13, P18, P19, P21, P22, and P23) were almost exclusively recovered in the affinity-purified fraction, and three peaks (P4, P16, and P17) were detectable for both the affinity-purified and unbound fractions (Fig. 3, B and C). Essentially the same results were obtained on using an mG9Null-immobilized column for affinity purification (data not shown). The nine major peaks detected in the chromatogram of the affinity-purified fraction were isolated and examined for their ability to neutralize the effect of G9Null on degranulation. Only the P23 preparation reversed the effect of hG9Null (Fig. 4A). The fractions exhibited no effect toward degranulation in the absence of Gal-9 (Fig. 4A, hatched bars). In addition to P23, P22 showed weak activity as to mG9Null-induced suppression of degranulation (Fig. 4B). When the major peaks except for P23 were reexamined at a higher concentration, P22 but not the other peaks of the affinity-purified fraction clearly antagonized the effects of both hG9Null and mG9Null (Fig. 4C). Other major peaks (P1–P3, P5–P15, and P20) and minor peaks (indicated by asterisks in Fig. 3A) were ineffective under the assay conditions used (data not shown).

**Identification of Galectin-9-interacting Peptides**—To identify the substances present in the peak fractions, especially P22 and P23, the P4–P23 preparations with and without treatment with PNGase F were subjected to SDS-PAGE, N-terminal amino acid sequence analysis, and MALDI-TOF MS analysis. The P23 preparation contained a major component with an apparent molecular weight of 20,000–23,000 (P23H, PG1) and 12 kDa (P23C, PG2) were determined to be DFTCHVT, FTCHVT, FTCHVT, and FTCHVT, respectively. The band disappeared upon treatment with PNGase F, with the concomitant appearance of high-molecular-weight substances present in the peak fractions, especially P22 and P23. MALDI-TOF MS analysis of the P23 preparation contained a major component with an apparent molecular weight of 20,000–23,000 (P23H, PG1) and 12 kDa (P23C, PG2) were determined to be DFTCHVT, FTCHVT, FTCHVT, and FTCHVT, respectively. The band disappeared upon treatment with PNGase F, with the concomitant appearance of high-molecular-weight substances present in the peak fractions, especially P22 and P23. The N-terminal amino acid sequence analysis of P23H, PG1, PG2, and PG3 was performed. The N-terminal amino acid sequence of P23H was determined to be DFTCHVT, FTCHVT, FTCHVT, and FTCHVT, respectively. The band disappeared upon treatment with PNGase F, with the concomitant appearance of high-molecular-weight substances present in the peak fractions, especially P22 and P23. The N-terminal amino acid sequence analysis of P23H, PG1, PG2, and PG3 was performed. The N-terminal amino acid sequence of P23H was determined to be DFTCHVT, FTCHVT, FTCHVT, and FTCHVT, respectively. The band disappeared upon treatment with PNGase F, with the concomitant appearance of high-molecular-weight substances present in the peak fractions, especially P22 and P23. The N-terminal amino acid sequence analysis of P23H, PG1, PG2, and PG3 was performed. The N-terminal amino acid sequence of P23H was determined to be DFTCHVT, FTCHVT, FTCHVT, and FTCHVT, respectively. The band disappeared upon treatment with PNGase F, with the concomitant appearance of high-molecular-weight substances present in the peak fractions, especially P22 and P23. The N-terminal amino acid sequence analysis of P23H, PG1, PG2, and PG3 was performed. The N-terminal amino acid sequence of P23H was determined to be DFTCHVT, FTCHVT, FTCHVT, and FTCHVT, respectively. The band disappeared upon treatment with PNGase F, with the concomitant appearance of high-molecular-weight substances present in the peak fractions, especially P22 and P23.
S-pyridylethylated H-Lys13 (supplemental Table S1), a mass peak at $m/z = 9684.3$, which is closely similar to the mass ([M + H]$^+$) of S-pyridylethylated H-Lys13 deglycosylated at three potential glycosylation sites (monoisotopic mass = 9683.837), was detected on MALDI-TOF MS analysis (Fig. 5C). The removal of N-glycan with PNGase F results in the conversion of an amino acid residue (Asn to Asp) at the N-glycosylation sites with a concomitant increase of 1 mass unit/site. It is most probable that P23 represents partly deglycosylated H-Lys13.

The major band detected for the P22 preparation had an apparent molecular weight of 32,000–35,000 (P22-C), and a major 18-kDa band (P22-PG) appeared after digestion of the preparation with PNGase F (Fig. 5, A and B). The N-terminal amino acid sequences of P22-C and P22-PG were determined to be XFTCHVT and DFTCHVT, respectively. Redigestion of the P22 preparation with lysyl endopeptidase resulted in the formation of a component with a molecular weight indistinguishable from that of P23 (data not shown). Furthermore, mass peaks consistent with H-Lys14, -Lys15, and -Lys16 peptides were detected for the tryptic digest of P22 by MALDI-TOF MS analysis (data not shown). These results suggest that the P22 preparation contained H-Lys13 with an elongated C-terminal end (i.e., a proteolytic fragment of TIB-141 was formed as a consequence of incomplete digestion with lysyl endopeptidase). P22 was not further characterized in the present study.
A theoretical digest of the H chain with lysyl endopeptidase contains seven peptides with potential N-glycosylation site(s) (H-Lys10, -Lys13, -Lys16, -Lys19, -Lys22, -Lys28, and -Lys33), and H-Lys13 was identified in the P23 (and P22) fraction as described above. Among other peptides, H-Lys10, -Lys16, -Lys19, and -Lys28 were identified in P17, P16, P4, and P18/19, respectively, on N-terminal amino acid sequence analysis and MALDI-TOF MS analysis of PNGase F-treated preparations (data not shown). H-Lys22 and -Lys33 were not detected in the affinity-purified fraction. No glycopeptide was detected in the P13 and P21 fractions, but instead the P13 fraction contained L-Lys10 and H-Lys18, and the P21 fraction contained L-Lys7 and H-Lys26 as major components. It is possible that the peptides (non-glycopeptides) have affinity for a glycopeptide(s) and are recovered in the affinity-purified fraction.

Trypsin Digestion of P23 and Characterization of the Resulting Peptides—The H-Lys13 peptide, the major component of the P23 fraction, has three potential N-glycosylation sites (Fig. 6A). It is most probable that the peptide is glycosylated at all three sites, as judged by MALDI-TOF MS analysis of the P23 fraction treated with PNGase F as described under “Identification of Gal-9-interacting Peptides.” To further define the glycosylation site(s) to which an oligosaccharide(s) with high affinity for G9Null is attached, the P23 preparation was digested with trypsin and then analyzed by RP-HPLC (Fig. 6B). The identity of three major peaks was established by N-terminal amino acid analysis and MALDI-TOF MS analysis (data not shown) after treatment with PNGase F; P23-T1, P23-T7, and P23-T11 correspond to H-Lys13-Try1, H-Lys13-Try4, and H-Lys13-Try3, respectively (Fig. 6A). P23-T12 seems not to be a peptide peak based on the analyses. H-Lys13-Try2 (a pentapeptide) was not detected in the chromatogram. The results also show that H-Lys13 was cleaved at all possible sites, although the second site (Arg-Pro) is known to be resistant (but not completely resistant) to trypsin digestion (29). The three major peak materials were isolated and then examined for their ability to antagonize the effect of hG9Null on degranulation. The results are shown in Fig. 7 in comparison with the activity of P23. The P23-T11 preparation nearly completely reversed the effect of hG9Null at 10 μM. The dose-response curves show that the specific activity of P23-T11 is about one-third that of P23. Activity of P23-T1 was detectable but was 10 times lower than that of P23-T11. P23-T7 was inactive in the assay up to 20 μM (data not shown). To examine the effect of the peptide moiety of P23-T11 (H-Lys13-Try3) on Gal-9-induced inhibition of degranulation, P23-T11 was deglycosylated with PNGase F and then purified by RP-HPLC. The deglycosylated P23-T11 preparation did not antagonize the effect of hG9Null at 20 μM (Fig. 7, filled triangle).

Endoproteinase Glu-C Digestion of P23-T11 and Characterization of the Resulting Peptides—P23-T11 (H-Lys13-Try3) is a relatively large peptide consisting of 48 amino acid residues with an N-glycan near the N terminus (Fig. 6C). In order to determine the effect of the peptide moiety of P23-T11 on its ability to neutralize the effect of G9Null on degranulation, P23-T11 was further digested with endoproteinase Glu-C and then analyzed by RP-HPLC. P23-T11-G1 and P23-T11-G2 were identified as H-Lys13-Try3-Glu1 and H-Lys13-Try3-Glu2 (Fig. 6C and D), respectively, by N-terminal amino acid sequence analysis and MALDI-TOF MS analysis (data not shown). The neutralizing activity of P23-T11-G1, a C-terminally truncated...
form of H-Lys13-Try3 with a single N-glycan, is shown in Fig. 7. P23-T11-G1 exhibited no effect on hG9Null-induced suppression of degranulation up to 20 μM. In addition, P23-T11-G2 was also ineffective at 20 μM in the assay (data not shown).

Structural Analysis of N-Glycans—Total N-glycans of P23-T1 and P23-T11 were liberated by hydrazinolysis and then pyridylaminated. The PA-N-glycans were then subjected to affinity chromatography on a G9Null-immobilized column. PA-N-glycans recovered in the flow-through fraction (P23-T1-FT and P23-T11-FT), and those eluted from the column with lactose (affinity-purified fraction, P23-T1-LE and P23-T11-LE) were first analyzed and compared by size fractionation HPLC and RP-HPLC (Fig. 8). The size distribution of PA-N-glycans in P23-T1/T11-LE was slightly higher than that of those in P23-T1/T11-FT. Although the amount of PA-N-glycans derived from P23-T11 was significantly smaller than that from P23-T1, the size distribution patterns of PA-N-glycans in P23-T1-LE and P23-T11-LE resembled each other. In addition, analysis of P23-T1-LE and P23-T11-LE by RP-HPLC indicated that P23-T11-LE is simpler than P23-T1-LE in the glycan repertoire. The PA-N-glycans in P23-T1-LE and P23-T11-FT were purified successively by anion exchange HPLC, size fractionation HPLC, and RP-HPLC and then subjected to consecutive glycosidase digestion to determine the entire structure (supplemental Fig. S3). P23-T11-LE and P23-T11-FT were not subjected to structural analysis because of the low yield of PA-N-glycans from P23-T11. The identified glycan structures are summarized in Table 1. The most abundant N-glycan in P23-T1-FT was a disialylated biantennary complex type oligosaccharide with a core fucose residue (Table 1, FT-5), which amounted to about 45% of the glycans recovered in the flow-through fraction (about 23% of total glycan). Monosialylated biantennary complex type N-glycans with a core fucose residue were other major components in the fraction (FT-1 to FT-4). On the other hand, monosialylated triantennary complex type oligosaccharides (LE-4 to LE-6) and non-sialylated biantennary complex type oligosaccharides (LE-1 to LE-3) with a core fucose residue were abundant in P23-T1-LE. Some glycans in P23-T1-LE contained a galactose residue(s) in an 1–3 linkage at the non-reducing end (Table 1, LE-2 and LE-3). N-Glycans with a bisecting GlcNAc residue were not identified in either fraction.

Discussion

IgE is a key molecule in the initiation of an allergic reaction. Each allergic reaction is triggered by a specific antigen (allergen), which cross-links IgE bound to its high affinity Fc receptor expressed mainly on mast cells. Mast cells release various pro-inflammatory mediators upon activation. These mediators are released as a consequence of immediate degranulation. β-Hex, a lysosomal enzyme, is released together with the mediators and is used as a marker of mast cell degranulation. We have previously shown that hGal-9 suppresses degranulation of mast cell model rat basophilic leukemia (RBL-2H3) cells induced by Spe7 (mouse anti-dinitrophenyl monoclonal IgE) and the anti-
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A

H-Lys13-Try1  H-Lys13-Try2  H-Lys13-Try3  H-Lys13-Try4
↓  ↓  ↓  ↓

H-Lys13: NFTCHTVPSPFNRSLTVRNPVNETPLELHSSCDPNAFHSITIQLYCFIYGHILNDVSWSLDDREITDLAQTVL

B

Absorbance at 215 nm

Time (min)

0  10  20  30  40

HPLC peak  N-terminal amino acid sequence
P23-T1 : XFTCHV
P23-T7 : EITDLA
P23-T11 : PVXITEP
P23-T12 : not detected

C

H-Lys13-Try9-Glu1  H-Lys13-Try3-Glu2
↓

H-Lys13-Try3: PVXITEPTELHSSCDPNAFHSITIQLYCFIYGHILNDVSWSLDDOR

D

Absorbance at 215 nm

Time (min)

0  10  20  30  40

HPLC peak  N-terminal amino acid sequence
P23-T11-G1 : PVXITEP
P23-T11-G2 : LLHSSCD

FIGURE 6. RP-HPLC chromatograms of P23 digested with trypsin and P23-T11 digested with endoproteinase Glu-C. A and C, amino acid sequences of H-Lys13 (A) and H-Lys13-Try3 (C). Arrows, potential cleavage sites for trypsin (A) or endoproteinase Glu-C (C). H-Lys13-Try1 to -Try4, predicted tryptic fragments of H-Lys13, H-Lys13-Try3-Glu1 and -Glu2, predicted endoproteinase Glu-C fragments of H-Lys13-Try3. #, potential N-glycosylation sites. B and D, the trypsin digest of the P23 preparation (derived from 4.2 nmol of PE-TIB-141) (B) and the endoproteinase Glu-C digest of P23-T11 preparation (derived from 8.8 nmol of PE-TIB-141) (D) were analyzed on a C18 column. The column was developed with a linear gradient of 0 – 80% (v/v) acetonitrile in 0.1% (v/v) TFA.

Gen dinitrophenyl-human serum albumin (10). Several lines of evidence suggest that hGal-9 binds to IgE via a glycan(s), leading to inhibition of IgE-antigen complex formation through a steric hindrance mechanism. TIB-141, a mouse anti-TNP monoclonal IgE, was used in the present study instead of Spe7, because TIB-141-producing mouse hybridoma clone IGELb4 was available from ATCC. TIB-141 in combination with the antigen TNP-BSA was capable of inducing degranulation of RBL-2H3 cells as effectively as Spe7 plus dinitrophenyl-human serum albumin. The suppressive action of hGal-9 on the degranulation was also closely similar to that observed in the previous study (Fig. 2).

It is well known that a single N-glycosylation site of a glycoprotein can be modified with a wide variety of oligosaccharides, including high mannose type, hybrid type, and complex type ones with different degrees of sialylation and fucosylation. More than 10 distinct types of oligosaccharides are usually identified at a single site (30). Meanwhile, site-specific N-glycosylation (i.e. site-specific differences in the N-glycan repertoire) has been reported for cellular and serum glycoproteins, including immunoglobulins (31–33). The protein structure and the oligosaccharide moiety at a neighboring site(s) may influence maturation of the N-glycans at a given site. Based on this possibility, we designed experiments to identify the glycosylation site(s) to which oligosaccharides with high affinity for Gal-9 are attached and also to determine their structures. It is likely that efficient inhibition of IgE-antigen complex formation by Gal-9 depends not only on the affinity of Gal-9 for IgE glycans but also on the spatial arrangement of the glycans in relation to the antigen-binding sites in an IgE molecule. Therefore, high affinity for Gal-9 is not a sufficient condition to conclude that the IgE glycan(s) is responsible for the Gal-9-induced suppression of degranulation, but it would be a necessary condition for the function.

IgE is a highly glycosylated protein; there are nine potential N-glycosylation sites in the constant region of the H chain of TIB-141 (supplemental Fig. S1). The CH1 domain contains...
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FIGURE 7. Effects of P23 and tryptic peptides derived from P23 on the suppressive action of hGal9Null on degranulation. P23-T1, -T11, and -T11-G1 were examined for their ability to antagonize Gal9Null. Molar concentrations were calculated using the molecular weights of the peptide moieties of H-Lys13-Try1 (P23-T1), H-Lys13-Try3 (P23-T11), and H-Lys13-Try3-Glu1 (P23-T11-G1). The assay method is described in the legend to Fig. 4. The filled triangle represents the activity of P23-T1 treated with PNGase F (peptide moiety of P23-T11). The horizontal broken line represents the mean value for a control sample (without TIB-141 and TNP-BSA). The data are derived from a single representative experiment of three independent experiments. The results are presented as means ± S.D. (error bars) (n = 3).

four of the nine sites (Asn\(^{181}\), Asn\(^{210}\), Asn\(^{222}\), and Asn\(^{233}\)), and the Fc domain (CH2, CH3, and CH4 domains and C-terminal tail) contains the remaining five sites (Asn\(^{304}\), Asn\(^{376}\), Asn\(^{399}\), Asn\(^{503}\), and Asn\(^{553}\)). Three N-glycosylation sites in the CH1 domain, Asn\(^{210}\), Asn\(^{222}\), and Asn\(^{233}\), are located in relatively close proximity to each other. Digestion of pyridylethylated TIB-141 with lysyl endopeptidase is expected to generate seven glycopeptides, H-Lys10/13/16/19/22/28/33 (supplemental Table S1). Among them, the largest predicted peptide (H-Lys13) contains three sites (Asn\(^{210}\), Asn\(^{222}\), and Asn\(^{233}\)). The lysyl endopeptidase digest of TIB-141 was separated into about 40 peaks, including minor ones, on RP-HPLC (Fig. 3). Only two peak fractions, P22 and P23, exhibited neutralizing activity toward Gal-9-induced inhibition of degranulation. H-Lys13 was identified as the predominant component in the P23 fraction on N-terminal amino acid sequence analysis, SDS-PAGE, and MALDI-TOF MS analysis after treatment with PNGase F. Therefore, among the seven predicted glycopeptides, only H-Lys13 was found to possess neutralizing activity toward Gal-9 under the assay conditions used, because the predominant component in the P22 fraction was suggested to be H-Lys13 with an elongated C-terminal end (H-Lys13 + H-Lys14 + H-Lys15 + H-Lys16). MS analysis of deglycosylated P23 also suggested that all of the three potential N-glycosylation sites are glycosylated. Among the seven peptides with a potential N-glycosylation site(s), H-Lys22 and H-Lys33 were not found in the affinity-purified fraction of the digest. It is most probable that these sites are not glycosylated or modified with N-glycans that exhibit low or negligible affinity for Gal-9. In the case of human IgE, Plomp et al. (33) reported that one of the seven potential N-glycosylation sites is unoccupied and that the most C-terminally located site is modified with only high mannose type N-glycans, which do not show affinity for hGal-9 (4, 34).

The above finding that only H-Lys13 exhibited detectable activity is not surprising because the neutralizing activity of a glycopeptide toward Gal-9 depends not only on the affinity of individual glycans for Gal-9 but also the multivalency (avidity) of glycans. Indeed, clustering of glycans promotes glycan-protein interactions that are often of relatively low affinity. We examined this by digesting H-Lys13 (P23) with trypsin, which resulted in the formation of two glycopeptides, H-Lys13-Try1 with two N-glycans and H-Lys13-Try3 with a single N-glycan. H-Lys13-Try1 and H-Lys13-Try3 were recovered in P23-T1 and P23-T11, respectively, on RP-HPLC (Fig. 6). Contrary to our expectation, the dose-response curves showed that the specific activity of P23-T1 was less than one-tenth that of P23-T11 (Fig. 7). P23-T11 retained about one-third of the P23 activity. H-Lys13-Try1 is a 16-amino acid-long peptide, whereas H-Lys13-Try3 comprises 48 amino acid residues. Although the peptide moiety of H-Lys13-Try3 (i.e. P23-T11 deglycosylated with PNGase F), was inactive in the assay at 20 \(\mu\)M (Fig. 7), there is still the possibility that the peptide and oligosaccharide moieties of H-Lys13-Try3 cooperatively interact with Gal-9. The drastic decrease (more than 90%) in the activity of H-Lys13-Try3 caused by removal of the C-terminal 38 amino acid residues with endoproteinase Glu-C suggests that this is the case (Fig. 7). It is possible that the position of the oligosaccharide chain in H-Lys13-Try3 (near the N terminus) permits a weak and possibly nonspecific interaction between the peptide part of H-Lys13-Try3 and Gal-9, leading to an increase in the overall affinity. The avidity effect may explain the finding that H-Lys13-Try1 (a 16-amino acid-long peptide with two N-glycans) exhibited low but significant activity at 20 \(\mu\)M, whereas H-Lys13-Try3-Glu1 (a C-terminally truncated form of H-Lys13-Try3, a 10-amino acid-long peptide with a single N-glycan) was inactive at that concentration.

We prepared PA-N-glycans from H-Lys13-Try1 (P23-T1) and H-Lys13-Try3 (P23-T11) to determine and compare the structures of N-glycans of the glycopeptides. In accordance with the number of N-glycosylation sites, the PA-N-glycans derived from H-Lys13-Try1 showed more complex patterns than those from H-Lys13-Try3 in RP-HPLC (Fig. 8). However, the low yield of PA-N-glycans from H-Lys13-Try3 did not permit detailed structural analysis based on serial enzymatic digestion. Accordingly, we carried out structural analysis of PA-N-glycans only derived from H-Lys13-Try1. PA-N-glycans derived from H-Lys13-Try1 were first separated into two groups, adsorbed (P23-T1-LE) and nonadsorbed (P23-T1-FT) fractions, comprising nearly equal amounts, by Gal-9 affinity chromatography (Table 1). The N-glycan composition of the unadsorbed fraction was relatively simple, and no glycan with more than one non-sialylated lactosamine unit at the non-reducing end was found in it. This is in sharp contrast to the major
glycans found in the adsorbed fraction, having two non-sialylated lactosamine (or α-galactosylated lactosamine) units at the non-reducing end. This finding is consistent with the binding specificity of Gal-9 revealed on frontal affinity chromatography (4, 34). The α-Gal epitope (Galα1–3Galβ1–4GlcNAc-R) found in some glycans in the adsorbed fraction is not present in humans, but it is known to be present in many mammals, including mice.

TABLE 1
Major N-glycans identified in the P23-T1 fraction derived from IgE
PA-glycans derived from P23-T1 were first subjected to affinity chromatography on an hG9Null-immobilized column. The PA-glycans recovered in the unadsorbed fraction and the adsorbed fraction were successively purified by anion exchange chromatography, size fractionation chromatography, and RP-HPLC. FT-1-FT-5, PA-glycans purified from the unadsorbed fraction (see supplemental Fig. S3); LE-1-LE-6, PA-glycans purified from the adsorbed fraction (see supplemental Fig. S3).

| Unadsorbed fraction | Adsorbed fraction |
|---------------------|-------------------|
| Fraction No.        | Structure          | Abundance (%) | Fraction No.        | Structure          | Abundance (%) |
| FT-1                | Man1-Man1-GlcNAcβ1-GlcNAc-PA | 9.0           | LE-1                | Galβ1-GlcNAcβ1-Man1-GlcNAc-PA | 4.9           |
| FT-2                | Man1-Man1-GlcNAcβ1-GlcNAc-PA | 6.6           | LE-2                | Galβ1-GlcNAcβ1-Man1-GlcNAc-PA | 6.9           |
| FT-3                | Man1-Man1-GlcNAcβ1-GlcNAc-PA | 5.2           | LE-3                | Galβ1-GlcNAcβ1-Man1-GlcNAc-PA | 6.4           |
| FT-4                | Man1-Man1-GlcNAcβ1-GlcNAc-PA | 7.5           | LE-4                | Galβ1-GlcNAcβ1-Man1-GlcNAc-PA | 9.2           |
| FT-5                | Galβ1-GlcNAcβ1-Man1-GlcNAc-PA | 22.8          | LE-5                | Galβ1-GlcNAcβ1-Man1-GlcNAc-PA | 7.9           |

* Percentage of total glycan derived from the flow-through fraction (unadsorbed fraction) plus lactose-eluted fraction (adsorbed fraction).
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Plomp et al. (33) reported site-specific N-glycome analysis of human IgE from three different sources, the serum of a hyperimmune donor, the pooled serum of healthy donors, and the pooled serum of two patients with IgE myeloma, essentially by mass spectrometry. Their findings regarding the three N-glycosylation sites in the CH1 domain (i.e. that (i) the sites were solely occupied by complex type oligosaccharides, (ii) the vast majority of complex type IgE glycan structures were found to contain a core fucose residue, and (iii) galactose residues were present on all antennae of complex type glycans, and most structures also contained one or two terminal N-acetyleneuraminic acid residues) are mostly consistent with the present findings, although one-to-one correspondence between potential N-glycosylation sites of mouse and human IgE is not possible. In addition, the low abundance of bisecting GlcNAc-containing glycans and relatively high abundance of monosialylated triantennary complex type glycans in IgE from myeloma patients but not from other sources are also consistent with our results.

The present strategy to identify the glycosylation site(s) to which glycans with high affinity for Gal-9 are attached is based on an assumption that the peptide moiety of the glycopeptides derived from IgE has no effect or a negligible effect on oligosaccharide-lectin (Gal-9) interaction. However, it seems like this is not the case, as revealed by the effect of removal of the C-terminal amino acid residues on the activity of H-Lys13–Try3 described above. Nevertheless, the low but significant neutralizing activity of H-Lys13–Try1 (with two glycosylation sites, Asn210 and Asn222), the clustering of glycans in the CH1 domain (Asn210, Asn222, and Asn233), and the spatial relationship between the antigen-binding site(s) and the CH1 domain(s) suggest that the N-glycans in the region play an important role in Gal-9-induced inhibition of IgE-antigen complex formation.

The affinity of galectins for glycoconjugates has been almost exclusively interpreted and discussed in the context of classic lectin-oligosaccharide interactions, which have been analyzed by using isolated oligosaccharides like PA glycan. Recently, Nagae et al. (35, 36) found that CLEC-2, a member of the C-type lectin superfamily, recognizes both the sialylated O-glycans and the adjoining polypeptide of podoplanin through crystallographic studies. CLEC-2 utilizes the noncanonical side face of its CRD for binding to the polypeptide moiety of podoplanin and also to its non-glycoprotein ligand rhodocytin. Because the size of CLEC-2 CRD (about 130 amino acid residues) is nearly the same as those of members of the galectin family, it is possible that the CRDs of Gal-9 interact with the oligosaccharide and peptide moieties of IgE (and other ligands) at the same time. The present results and those for CLEC-2 indicate the possibility that cooperative binding of oligosaccharides and spatially neighboring polypeptide structures of a glycoprotein ligand to lectin CRD determines the overall affinity and specificity of the lectin-glycoprotein interactions in other lectin-ligand systems.

Author Contributions—S.N. and Y. Nakakita designed and performed the N-glycan analysis experiments. A.I. performed the molecular cloning, DNA sequencing, and biochemical analyses. Y. Nonaka, T. O., and T. N. performed the biochemical analyses and interpreted the data. N. N. conceived, designed, and performed the experiments; analyzed the data; and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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