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Title: Surfactant protein D suppresses lung cancer progression by downregulation of epidermal growth factor signaling

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Running title: SP-D binds to EGFR and suppresses EGF signaling

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Abstract

Surfactant protein D (SP-D) is a member of the collectin family that plays an important role in maintaining pulmonary homeostasis. In this study, we demonstrated that SP-D inhibited the proliferation, migration and invasion of A549 human lung adenocarcinoma cells. We found that SP-D suppressed epidermal growth factor (EGF) signaling in A549 cells, H441 human lung adenocarcinoma cells and human EGF receptor (EGFR) stable expression CHO-K1 cells. A binding study using ^{125}I-EGF demonstrated that SP-D downregulated the binding of EGF to EGFR. A ligand blot indicated that SP-D bound to EGFR, and a lectin blot suggested that EGFR in A549 cells had both high-mannose type and complex type N-glycans. We purified the recombinant extracellular domain of EGFR (soluble EGFR = sEGFR), and demonstrated that SP-D directly bound to sEGFR in a Ca^{2+} dependent manner. The binding of SP-D to sEGFR was suppressed by EDTA, mannose or N-glycopeptidase F treatment. Mass spectrometric analysis indicated that N-glycans in domain III of EGFR were of a high-mannose type. These data suggest that SP-D reduces EGF binding to EGFR through the interaction between the carbohydrate recognition domain of SP-D and N-glycans of EGFR, and downregulates EGF signaling. Our finding suggests the novel type of regulation system of EGF signaling involving lectin-to-carbohydrate interaction and downregulation of ligand binding.

Keywords:

epidermal growth factor receptor (EGFR)/ glycosylation/ mass spectrometry/ pulmonary collectin/ surfactant protein

Introduction

Pulmonary surfactant is a mixture of lipids and proteins that covers alveolar surfaces.[1, 2] Four specific proteins have been identified designated surfactant protein A (SP-A), SP-B, SP-C and SP-D. They are produced by alveolar type II cells and Clara cells, which are the major progenitor cells of peripheral airways cells. SP-B and SP-C are hydrophobic proteins, which are essential for reducing surface tension. SP-A and SP-D are hydrophilic proteins and belong to the collectin subgroup of the C-type lectin superfamily. They are mosaic proteins, consisting of collagen-like domains and carbohydrate recognition domains (CRD) (lectin domain). Each monomeric protein (SP-A, 26-36 kDa; SP-D, 43 kDa) is assembled to form a trimeric helix at the collagenous domain, and further multimerized to form an octdecamer for SP-A and a dodecamer for SP-D.

SP-D has been implicated in the regulation of innate immune responses in the lung.[3, 4] For example, SP-D prevents dissemination of infectious microbes by their biological activities including agglutination and growth inhibition.[5-7] SP-D also promotes clearance of microbes by enhancing phagocytosis in macrophages.[8, 9] In addition, SP-D interacts with the other pattern-recognition molecules including Toll-like receptors (TLRs) and TLR-associated molecules CD14 and MD-2, and regulates inflammatory responses.[10-12]

It has been suggested that SP-D expression levels are inversely correlated with lung cancer progression. The genetic abnormalities in SP-D were associated with lung cancer pathogenesis.[13, 14] Reduced SP-D expression in bronchoalveolar lavage (BAL) fluid was linked to subsequent lung cancer risk.[15] Although these studies suggested that SP-D suppressed lung cancer progression, the particular

mechanisms remain unknown.

Epidermal growth factor receptor (EGFR) is a member of the ErbB family. By binding to epidermal growth factor (EGF), conformational rearrangement occurs, which gives rise to an "extended form," in which the dimerization arm mediates homo- and heterodimers, followed by the activation of downstream signaling.[16] The EGF signaling is involved in a wide variety of cellular events and aberrant expression or dysregulation of EGFR has been implicated in cell transformation and cancer.[17] EGFR tyrosine kinase inhibitors (EGFR-TKIs) gefitinib and erlotinib show anti-tumor activity in patients with advanced non-small cell lung cancer, however, there are clinical problems in that EGFR-TKIs have a limited degree of benefit to patients with a non mutant EGFR and patients with a mutant EGFR develop disease progression by acquiring resistance.[18, 19]

In the present study, we found that SP-D downregulated EGF signaling and suppressed the progression of lung cancer cells. We demonstrated that SP-D bound to EGFR via lectin activity and reduced EGF binding. We also determined the structure of the relevant N-glycans. The results indicated that SP-D exerts tumor suppressive effects by interfering with the binding of EGF to EGFR.

Results

SP-D suppresses the proliferation, migration and invasion of A549 cells

First, we examined the effects of SP-D on the proliferation of lung cancer cells. A549 human lung adenocarcinoma cells were incubated with 1 or 10 μg/ml of SP-D and the cell proliferation was assayed after 24, 48 and 72 h. As shown in Fig. 1A, left panel, SP-D suppressed the proliferation of A549 cells. Dose dependency was also confirmed (Fig. 1A, right panel).

We evaluated the effects of SP-D on the migration and invasion of A549 cells. When SP-D was added, the number of EGF-induced migration and invasion cells was significantly decreased (Fig. 1B and 1C). It was revealed that SP-D suppressed the invasive ability of A549 cells more effectively than it did the migration ability.

SP-D suppresses EGF signaling in A549 cells, H441 cells and human EGFR stable expression CHOK1 cells

Next, we assessed the effects of SP-D on EGF signaling in A549 cells. As shown in Fig. 1D, SP-D suppressed the phosphorylation of EGFR, Erk and Akt in a dose dependent manner. The same results were obtained with H441 cells (data not shown) and human EGFR stable expression CHO-K1 cells (Fig 1E).

SP-D reduces the binding of EGF to EGFR in A549 cells

To determine the mechanisms by which SP-D suppressed EGF signaling in A549 cells, a binding analysis of ^{125}I-EGF to EGFR in the presence and absence of SP-D was performed. SP-D significantly reduced the saturation level of bound EGF in A549 cells (Fig. 2A). When suppression patterns were examined, SP-D suppressed

EGF binding in a dose dependent manner up to 75 μg/ml (Fig. 2B). These data suggested that SP-D blocked the binding of EGF and, consequently, suppressed EGF signaling.

SP-D binds to EGFR in A549 cells

Next, we examined whether SP-D bound to EGFR in A549 cells by using a ligand blot. The whole cell lysate of A549 cells was immunoprecipitated with anti-EGFR monoclonal antibody Ab-11, subjected to SDS-PAGE and finally a ligand blot analysis was performed. As shown in Fig. 3A, SP-D bound to EGFR of A549 cells on the PVDF membrane. To examine which type of N-glycans existed in EGFR of A549 cells, lectin blot analysis using concanavalin A and DSA was performed. Both concanavalin A and DSA reacted with EGFR (Fig. 3B), suggesting that EGFR of A549 cells had both high-mannose type and complex type of N-glycans.

SP-D binds to N-glycans of EGFR in a Ca^{2+} dependent manner

To examine the particular mechanisms by which SP-D directly interacts with EGFR, we prepared recombinant sEGFR. Recombinant sEGFR with or without N-glycopeptidase F treatment was subjected to SDS-PAGE and lectin blot analysis was performed. It was found that the molecular mass was decreased (Fig. 4A) and the reactivity against concanavalin A and DSA was diminished (Fig. 4B) by N-glycopeptidase F treatment, suggesting that both a high-mannose type and a complex type of N-glycans were successfully removed from sEGFR.

We next examined whether SP-D bound to sEGFR with or without N-glycopeptidase F treatment by using ligand blotting. As shown in Fig. 4C, the

binding of SP-D to sEGFR was inhibited by N-glycopeptidase F treatment.

We further examined whether SP-D bound to sEGFR coated onto microtiter wells and conversely whether sEGFR bound to coated SP-D. SP-D exhibited a concentration-dependent binding to coated sEGFR in the presence of Ca^{2+} (Fig. 4D, left panel). The binding of sEGFR to coated SP-D indicated the same result (Fig. 4D, right panel). Inclusion of 5 mM EDTA instead of Ca^{2+} inhibited the binding, indicating that the binding of SP-D to sEGFR was Ca^{2+} dependent (Fig. 4E). It was also observed that excess mannose blocked the binding of SP-D to sEGFR (Fig. 4F). When N-glycans of sEGFR were cleaved by N-glycopeptidase F, the binding of SP-D to sEGFR was suppressed, indicating that SP-D binds to sEGFR via its N-glycans (Fig. 4G).

We further determined the binding parameters of SP-D with sEGFR using surface plasmon resonance analysis. The passage of SP-D at various concentrations over immobilized sEGFR on a sensor chip yielded an association rate constant of $k_a = 4.8 \times 10^4\ M^{-1}\ S^{-1}$ and dissociation rate constant of $k_d = 1.7 \times 10^{-3}\ S^{-1}$, for a consequent dissociation constant of $K_D\ (k_d/k_a) = 3.2 \times 10^{-8}$ M. The injection of 10 mM EDTA resulted in the complete dissociation of SP-D (Fig. 4H). When an excess-mannose containing running buffer was used, there was no detectable interaction of SP-D and sEGFR (Fig. 4I). When sEGFR treated with N-glycopeptidase F was used, the interaction was also completely suppressed (Fig. 4J). These results suggested that the CRD of SP-D is involved in the interaction with N-glycans of sEGFR.

Structures of N-glycans of sEGFR

Since SP-D is known to preferentially bind to mannose,[3] we assumed that a

high-mannose type of N-glycans of EGFR was involved in the interaction of SP-D and EGFR. We examined the structures of N-glycans of EGFR by MS analysis. Purified sEGFR was subjected to enzymatic proteolysis, and the glycosylated peptides were isolated by reversed phase chromatography. Amino acid sequencing of the peptide backbone and the site of glycosylation were determined by CID multi-stage tandem MS. We found that Asn328 and Asn337 had high-mannose type N-glycans but all other Asn in N-X-T/S N-glycosylation consensus sequences and Asn32 in N-X-C atypical N-glycosylation motif had complex type N-glycans. The structural analysis indicated that the dominant population of N-glycans on Asn328 of EGFR were high-mannose types composed of 5 mannose (so called Man5) or Man6, whereas those on Asn 337 were Man5, Man6, Man7 or Man8 (Fig. 5A and supplementary Fig. 1A and 1B). Furthermore, any unglycosylated peptides containing Asn328 (*m/z* 1175.6 for monoisotopic mass) or Asn337 (*m/z* 1900.0) were not found in a peptide mixture, indicating that both Asn328 and Asn 337 are nearly 100% glycosylated. These results are summarized in Fig. 5B.

Discussion

In this study, we examined the mechanisms by which SP-D suppresses lung cancer progression. SP-D inhibited the proliferation, migration and invasion of A549 lung human adenocarcinoma cells. We found that SP-D suppressed EGF signaling in A549 cells, H441 cells, and human EGFR stable expression CHO-K1 cells. A binding study using ^{125}I-EGF suggested that SP-D blocked the binding of EGF to EGFR. Ligand and lectin blots suggested that SP-D bound to EGFR of A549 cells, and N-glycans of EGFR in A549 cells were both high-mannose and complex types. We purified recombinant sEGFR, and proved that SP-D directly bound to sEGFR in a Ca^{2+} dependent manner. Surface plasmon resonance analysis revealed that the dissociation constant of SP-D and sEGFR was $K_D = 3.2 \times 10^{-8}$ M. In the presence of EDTA or mannose, the binding of SP-D to sEGFR was completely suppressed and N-glycopeptidase F treatment of sEGFR also inhibited the binding of SP-D to sEGFR. These data suggested that the CRD of SP-D and N-glycans of EGFR were involved in the interaction between SP-D and EGFR.

We demonstrated that SP-D treatment significantly decreased the saturation level of EGF binding (Fig. 2). These results are consistent with the data indicating that SP-D treatment downregulated EGF signaling in A549 cells (Fig. 1). Together with the fact that SP-D directly binds to the extracellular domain of EGFR (Fig. 4), probably via N-glycans in domain III of EGFR (Fig. 4-5), it is possible that SP-D binding to EGFR affects conformational changes in EGFR or EGFR dimerization, and subsequently alters the binding characteristics of EGF to EGFR.

Recent studies have revealed that binding of EGF to EGFR shows negative cooperativity;[20, 21] EGF binds with high affinity to the first site on the dimer, induces

formation of an asymmetric dimer and binds with substantially lower affinity to the second site on the dimer. Structural analysis of SP-D and the EGFR heterodimer would be of help toward further understanding of the mechanisms of interaction of SP-D and EGFR.

The functional regulation of EGFR by N-glycans has been investigated.[22-29] Some of the regulation mechanisms include the interaction of extracellular molecules and N-glycans of EGFR. It has been reported that galectin-3, which is secreted to the cell surface, interacts with poly-N-acetyl lactosamine in the N-glycans of EGFR, and delays the endocytosis of EGFR.[27] It was also demonstrated that the binding of ganglioside GM3 to GlcNAc termini of N-glycans of EGFR by carbohydrate-to-carbohydrate interaction results in the inhibition of EGF signaling without affecting EGF binding to EGFR.[28, 29] In the present study, we have demonstrated that SP-D directly binds to EGFR via its N-glycans. SP-D binding decreases the EGF binding to EGFR and subsequently downregulates EGF signaling, suggesting the novel type of EGFR regulation involving lectin-to-carbohydrate interaction and downregulation of ligand binding.

In this study, the assignment of the structures of N-glycans of EGFR was conducted by MS (Fig. 5). Although there are many differences with the data previously reported,[30] which were based on susceptibility to N-glycopeptidase F and Endo H digestion, we believe that the accuracy has been improved.

At present, SP-D is often implicated in lung cancer progression. The genetic abnormalities in SP-D are associated with lung cancer pathogenesis.[13, 14] Reduced SP-D expression in BAL fluid has relevance to subsequent lung cancer risk.[15] The level of SP-D in BAL fluid of patients with idiopathic pulmonary fibrosis (IPF) is

decreased[31] and the incidence of lung cancer is increased in patients with IPF.[32-34] As we have suggested, SP-D potentially interacts with EGFR or other receptors with N-glycan(s), and modulates those functions. The similar mechanisms might be involved in the antitumor effects of SP-D observed in the previous studies.

It has been reported that some primary lung cancers expressed SP-D.[35] The levels of SP-D are increased in the pleural effusion and serum from some patients with lung cancer.[36, 37] Murine lung tumors express SP-D[38] and serum levels of SP-D are increased in mice with lung tumors.[39] Primary lung adenocarcinoma originates from the progenitor cells of peripheral airways cells that have potential to produce SP-D. It is possible that these lung cancers are regulated by SP-D by autocrine mechanisms.

Since SP-D is a constituent of a pulmonary surfactant that exists in the epithelial lining fluid of the alveolus, it is difficult to determine its exact concentration in vivo. Nonetheless, its concentration can be estimated based on the recovery of the proteins in bronchoalveolar lavage fluids and the extrapolated hypophase volume, and the concentration of SP-D was calculated as ~60 μg/ml in healthy volunteers.[31, 40-43] Although its concentration appears to vary in a disease state, the SP-D concentrations used in this study are within the best estimates of the physiological ranges.

EGFR is one of the most important therapeutic targets in cancer. SP-D plays a role in suppressing lung cancer progression by direct binding to an extracellular domain of EGFR; therefore, it is possible that they are effective for patients without EGFR gene mutation and resistant to EGFR-TKI treatment. Elucidating the antitumor effects of SP-D may provide important clues for establishing new

therapeutic strategies for lung cancers, especially those resistant to existing therapies.

Materials and methods

Reagents and antibodies

Human recombinant EGF was purchased from Sigma Aldrich (St. Louis, MO) and ^{125}I-EGF was purchased from PerkinElmer (Waltham, MA). The polyclonal antibodies to Akt and Erk and phospho-specific polyclonal antibodies to EGFR (Tyr 1068), Akt and Erk were purchased from Cell Signaling Technology (Danvers, MA). The polyclonal antibody to EGFR was from Millipore (Billerica, MA). The monoclonal antibody to EGFR Ab-11 was from NeoMarkers (Fremont, CA). The polyclonal antibody to His-tag was from MBL (Nagoya, Japan). HRP (horseradish peroxidase) conjugated anti-rabbit and anti-mouse IgG were from Promega (Madison, WI). To produce the polyclonal antibody against human SP-D, purified recombinant human SP-D was injected into New Zealand White rabbits intramuscularly and the antiserum was purified.[10, 12] Hybridomas producing anti-SP-D monoclonal antibody 7C6 were established and 7C6 was prepared as described previously.[44] The anti-SP-D polyclonal and monoclonal antibodies reacted with SP-D purified from human alveolar lavage fluids or recombinant human SP-D but not with SP-A.[12] All other chemicals and reagents were purchased from Wako Pure Chemicals (Osaka, Japan) unless otherwise noted.

Cell culture

The A549 human lung adenocarcinoma cell line was obtained from ATCC (Manassas, VA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich) with 10% (v/v) fetal calf serum (FCS). The H441 human lung adenocarcinoma cell line was obtained from ATCC and maintained in RPMI 1640

(Sigma Aldrich) with 10% (v/v) FCS. The Flp-In CHO cell line was obtained from Invitrogen (Carlsbad, CA), and maintained in Ham's F12 medium (Sigma Aldrich) with 10% (v/v) FCS. CHO-K1 cells expressing SP-D were grown in glutamate-free Glasgow minimum essential medium (Sigma Aldrich) containing 10% (v/v) dialyzed FCS.[10, 45]

Purification of recombinant human SP-D

Recombinant human SP-D was purified as described previously.[10, 45] The physical forms of the recombinant collectins used in this study were confirmed by rotary shadow electron microscopy.[12]

Cell proliferation assay

A549 cells were plated in a 96-well plate and incubated with indicated concentration of SP-D. Cell proliferation was assayed using a WST-1 reagent (Takara, Japan).

Cell migration and invasion assays

Cell migration and invasion assays were conducted using the Transwell double chamber (BD BioCoat Matrigel Invasion Chamber. BD Bioscience, Bedford, MA). A549 cells were seeded into the upper insert in Dulbecco's Modified Eagle's Medium (DMEM) with 0.1% (v/v) FCS, and DMEM with 10% (v/v) FCS as a chemoattractant was added to the bottom wells. After fixation with 4% paraformaldehyde/phosphate buffered saline (PBS) (-), the cells were permeabilized and stained with DAPI (200 ng/ml) in PBS (-). The cells were counted under a fluorescence microscope

(KEYENCE, Osaka).

Establishment of human EGFR and the extracellular domain of EGFR (sEGFR) stable expression cells

To establish human EGFR and sEGFR stable expression cells, the Flp-In system (Invitrogen) was used. cDNA for human EGFR and the myc-His tagged sEGFR (residues 1-618 of the mature protein) were subcloned into a pcDNA5/FRT expression vector, and transfected into Flp-In CHO-K1 cells with pOG44 plasmids.

Protein sample preparation and Western blotting

Cells were harvested in a lysis buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 5 mM ethylenediaminetetraacetic acid, 1% (w/v) Nonidet P-40, 10% (w/v) glycerol, 5 mM sodium pyrophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 10 mM ß-glycerophosphate, 1 mM PMSF, 2 mg/ml aprotinin, 5 mg/ml leupeptin and 1 mM dithiothreitol) and centrifuged at 15,000 X g for 10 min at 4°C, and the resultant supernatant was used as a protein sample. The samples were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). After blocking, the blots were probed with an indicated antibody and immunoreactive bands were visualized using a chemiluminescence reagent (SuperSignal West Pico, Pierce, Rockford, IL). Densitometric analysis was performed by using a Luminous analyzer.

Binding analysis of ^{125}I-EGF to EGFR

A549 cells were seeded at a density of 7×10^4 cells/well in 24-well plate and allowed to adhere overnight. The cells were washed with DMEM containing 0.1% (w/v)

bovine serum albumin (BSA), and incubated with the indicated concentration of SP-D for 2 h at 37°C in the same medium. The medium was removed, the cells were washed with the same medium and ^{125}I-EGF was added in the presence of unlabeled EGF in order to reach the indicated concentration. Nonspecific binding was determined by adding 1,000 nM cold EGF. After incubation for 2 h at 4°C, the cells were washed three times with ice-cold PBS (-) containing 0.1% (w/v) BSA and hydrolyzed in 0.5 ml of 1 N NaOH for 30 min at room temperature. The radioactivity of the cell lysate was quantified using a γ-counter.

Immunoprecipitation

The whole cell lysate of A549 cells was precipitated with anti-EGFR monoclonal antibody Ab-11 (0.8 μg) and Protein A-Sepharose (15 μl) by gentle shaking at 4°C for 16 h. The beads were washed four times with a lysis buffer and resuspended in the SDS sample buffer. The samples were subjected to SDS-PAGE after boiling for 5 min under reducing conditions and transferred onto PVDF membranes. The membranes were used for Western blot, lectin blot and ligand blot.

Ligand blot

EGFR in A549 cells were immunoprecipitated with anti-EGFR monoclonal antibody Ab-11, electrophoresed and transferred onto PVDF membranes. sEGFR treated with or without N-glycopeptidase F was also electrophoresed and transferred onto PVDF membranes. After nonspecific binding was blocked with 10 mM HEPES (PH 7.4) containing 0.15 M NaCl, 5 mM $CaCl_2$ and 5% (w/v) BSA, these membranes were incubated with SP-D (1 μg/ml) for 16 h. After washing with 20 mM Tris-HCl (PH

7.4) containing 0.15 M NaCl, 0.05% (v/v) Tween-20, bound SP-D was detected by an anti-human SP-D polyclonal antibody or monoclonal antibody 7C6.

Lectin blot

EGFR in A549 cells were immunoprecipitated, electrophoresed and transferred onto PVDF membranes. sEGFR treated with or without N-glycopeptidase F was also electrophoresed and transferred onto PVDF membrane. These membranes were blocked with 3% (w/v) BSA in Tris-buffered saline containing 0.1% (v/v) Tween 20. The membranes were then incubated with biotinylated concanavalin A or biotinylated DSA (J-Oil Mills, Tokyo, Japan) (4 μg/ml) at room temperature for 1 h followed by incubation with HRP-labeled streptavidin.

Purification of recombinant sEGFR

sEGFR stable expression cells were cultured and the medium was collected. The expressed myc-His tagged sEGFR was purified by a series of column chromatographies on HisTrap HP5 (GE Healthcare), Mono Q (GE Healthcare) and HiLoad Superdex 200 pg (GE Healthcare) using the AKTA purifier system (GE Healthcare).[46] Purity of sEGFR was confirmed by SDS-PAGE.

Treatment of sEGFR with N-glycopeptidase F

sEGFR was treated with N-glycopeptidase F (Takara, Japan) (10 milliunit/25 μg of protein) for 24 h at 37°C under non-reducing conditions. After incubation, the state of cleavage was confirmed by SDS-PAGE and a lectin blot. The cleavage was dialyzed against PBS (-) before being used for ligand blotting, ELISA and surface

plasmon resonance analysis.

Binding assay of SP-D to sEGFR with ELISA

sEGFR (100 ng/well) was coated onto microtiter wells, and nonspecific binding was blocked with 10 mM HEPES buffer (PH 7.4) containing 0.15 M NaCl, 5 mM $CaCl_2$ and 5% (w/v) BSA (buffer A). The indicated concentrations of SP-D in buffer A were added and incubated for 2 h. After incubation, the wells were washed with PBS (-) containing 0.05% (v/v) Tween-20 (PBST) and further incubated with an anti-SP-D polyclonal antibody (1 μg/ml) for 2 h. After washing with PBST, HRP-conjugated goat anti-rabbit IgG was added and further incubated for 1 h. Finally, a peroxidase reaction was performed by using *o*-phenylenediamine as a substrate. The interaction of SP-D with sEGFR was also analyzed with coated SP-D (500 ng/well). The binding of sEGFR to SP-D was detected by an anti-His-tag polyclonal antibody (1 μg/ml). To eliminate the effect of Ca^{2+} on the binding, in some experiments we included 5 mM EDTA instead of $CaCl_2$ in buffer A. To examine the effect of mannose, we included 200 mM mannose in buffer A.

Binding assay of SP-D to sEGFR with surface plasmon resonance analysis

sEGFR (20 μg/ml) in 10 mM sodium acetate (pH 5.0) was immobilized on a sensor chip C1 of the Biacore 3000 system (Biacore, Uppsala, Sweden), according to the manufacturer's specifications. For the running buffer, 25 mM HEPES, pH 7.4, containing 0.15 M NaCl and 5 mM $CaCl_2$ and 0.005% surfactant P-20 were used. The association rate constant (k_a) and the dissociation rate constant (k_d) were calculated according to the BIAevaluation software (Version 3.1, Biacore AB).

Isolation of glycosylated peptides

The purified sEGFR was S-carbamidomethylated and digested with 1% (w/w) each of lysyl endopeptidase (Wako Pure Chemicals, Japan) and trypsin (Promega) at 37°C for 16 h. The glycosylated peptides in the digest were enriched by the hydrophilic affinity method as described previously.[47] Reversed phase chromatography was carried out on an Inertsil WP300 C8 column (1.0 X 150 mm, 300 Å, GL Sciences, Tokyo).

Mass spectrometry

The glycan profile and amino acid sequence of glycosylated peptides were obtained by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) using a pulsed nitrogen laser (337 nm) and nanoelectrospray ionization MS. For glycan profiling, MALDI time-of-flight (TOF) measurements were performed using a Voyager DE Pro mass spectrometer (AB Sciex, Framingham, MA) in linear mode.[48] Amino acid sequencing was carried out by collision-induced dissociation (CID) and multiple-stage tandem MS using an LTQ-XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA).[49] The glycosylated peptide samples were dissolved in a 0.1% formic acid and 20% (v/v) methanol solution and directly infused by using a spray tip (New Objective, Woburn MA) for infusion mode nanoelectrospray ionization MS. The collision gas was helium.

Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Fig. 1. SP-D suppresses the cell proliferation, migration and invasion by suppressing EGF signaling

(A, left panel) A549 cells were plated in a 96-well plate (1×10^3 cells/well), maintained in DMEM with 10% (v/v) FCS, and incubated with 10 μg/ml of SP-D at 37°C. The cell proliferation was assayed after 24, 48 and 72 h using WST-1 reagent. (A, right panel) A549 cells were incubated with 1 or 10 μg/ml of SP-D, and the cell proliferation was assayed after 48 h. The data shown are the means ± S.D. (error bars) from three independent experiments. **$p < 0.01$ (compared with the control).

(B) (C) A549 cells were seeded into the upper insert of a transwell double chamber in DMEM with 0.1% (v/v) FCS and EGF (10 ng/ml) with or without SP-D (10 μg/ml) and DMEM with 10% (v/v) FCS was added to the bottom wells as a chemoattractant. A control insert was used for migration assay (B) and a matrigel insert was used for invasion assay (C). After 22 h, cells migrating or invasive cells were fixed, stained with DAPI and counted under a microscope. The data shown are the means ± S.D. (error bars) from three independent experiments. **$p < 0.01$ (compared with the control).

(D) A549 cells were serum starved overnight and incubated with various concentrations of SP-D for 2 h at 37°C. After incubation, the cells were washed in a medium without serum and stimulated with 10 ng/ml of EGF for 10 min at 37°C. The cell lysate was prepared and 15 μg protein/lane were subjected to Western blotting using indicated antibodies. The data are representative of three independent experiments. The right panel displays the densitometric evaluation

and the data shown are the means ± S.D. (error bars) from three independent experiments.

(E) The same experiment as panel (D) was performed using human EGFR stable expression CHO-K1 cells. The data are representative of three independent experiments. The right panel displays the densitometric evaluation and the data shown are the means ± S.D. (error bars) from three independent experiments.

Fig. 2. SP-D reduced the binding of EGF to EGFR in A549 cells

(A) ^{125}I-EGF binding to A549 cells in the presence and absence of SP-D. Binding of EGF to the cells was evaluated using a γ-counter as described in "Materials and Methods."

(B) Dose dependent suppression of EGF binding by SP-D. Binding of EGF to the cells was evaluated using a γ-counter as described in "Materials and Methods." The data are expressed as relative values with the binding in the absence of SP-D being 100%.

Fig. 3. SP-D binds to EGFR in A549 cells

(A) Whole cell lysate of A549 cells was immunoprecipitated with anti-EGFR monoclonal antibody Ab-11 (0.8 μg) at 4°C for 16 h. The samples and BSA (200 ng/lane) were subjected to SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated with SP-D (1 μg/ml) for 16 h. The membranes were then incubated with an anti-human SP-D polyclonal antibody and monoclonal antibody 7C6 followed by HRP-labeled anti-rabbit IgG and anti-mouse IgG.

(B) The membranes were prepared similarly to panel (A) and subjected to lectin

blotting using biotinylated concanavalin A and DSA.

Fig. 4. Binding assay of SP-D to sEGFR

(A) sEGFR was produced in Flp-In CHO-K1 cells and purified as described in "Materials and Methods." 0.5 μg of proteins with or without N-glycopeptidase F treatment were subjected to SDS-PAGE, followed by Coomassie Brilliant Blue R-250 staining.

(B) sEGFR with or without N-glycopeptidase F treatment was electrophoresed under reducing conditions and transferred onto PVDF membranes. The membranes were subjected to lectin blotting using biotinylated concanavalin A and DSA.

(C) sEGFR with or without N-glycopeptidase F treatment and BSA was electrophoresed under reducing conditions and transferred onto the PVDF membrane. The membrane was incubated with SP-D (1 μg/ml) for 16 h. The membrane was then incubated with anti-human SP-D monoclonal antibody 7C6 followed by HRP-labeled anti-mouse IgG.

(D, left panel) The indicated concentrations of SP-D were incubated with sEGFR (100 ng/well) or BSA (100 ng/well) coated onto microtiter wells at room temperature for 2 h in the presence of 5 mM $CaCl_2$. ELISA was performed as described under "Materials and Methods." The data shown are the means ± S.D. (error bars) from three independent experiments. $*p < 0.05$, $**p < 0.01$ (compared with the control).

(D, right panel) The indicated concentrations of sEGFR were incubated with SP-D (500 ng/well) or BSA (500 ng/well) coated onto microtiter wells at room temperature for 2 h in the presence of 5 mM $CaCl_2$.

(E) The same experiment as panel (D) was performed in the presence of 5 mM EDTA

instead of $CaCl_2$.

(F) The same experiment as panel (D) was performed in the presence of 5 mM $CaCl_2$ with 0.2 M mannose.

(G) The same experiment as panel (D) was performed with sEGFR with or without N-glycopeptidase F treatment.

(H) The parameters of bindings of sEGFR to SP-D were determined by surface plasmon resonance analysis as described under "Materials and Methods." Sensorgrams for the bindings of various concentrations of SP-D to sEGFR immobilized on a sensor chip are shown. After the dissociation time, 10 mM EDTA were injected for 1 min.

(I) The same experiment as panel (H) was performed with 0.2 M mannose at SP-D injection.

(J) The same experiment as panel (H) was performed with sEGFR with N-glycopeptidase F treatment. RU, response units.

Fig. 5. Structures of N-glycans of sEGFR produced in CHOK1 cells

(A) MALDI linear TOF mass spectrum of the glycosylated peptides including Asn328 (upper panel) and Asn337 (lower panel) of sEGFR produced in CHO-K1 cells. The intensity of the signals allows a rough estimation of the relative abundances of the molecules.

(B) The summary of N-glycans of EGFR produced in CHO-K1 cells. The illustration indicates the structure of N-glycans of sEGFR. Blue square, N-acetylglucosamine; green circle, mannose; yellow circle, galactose; purple diamond, sialic acid; red triangle, fucose.

Figure 1

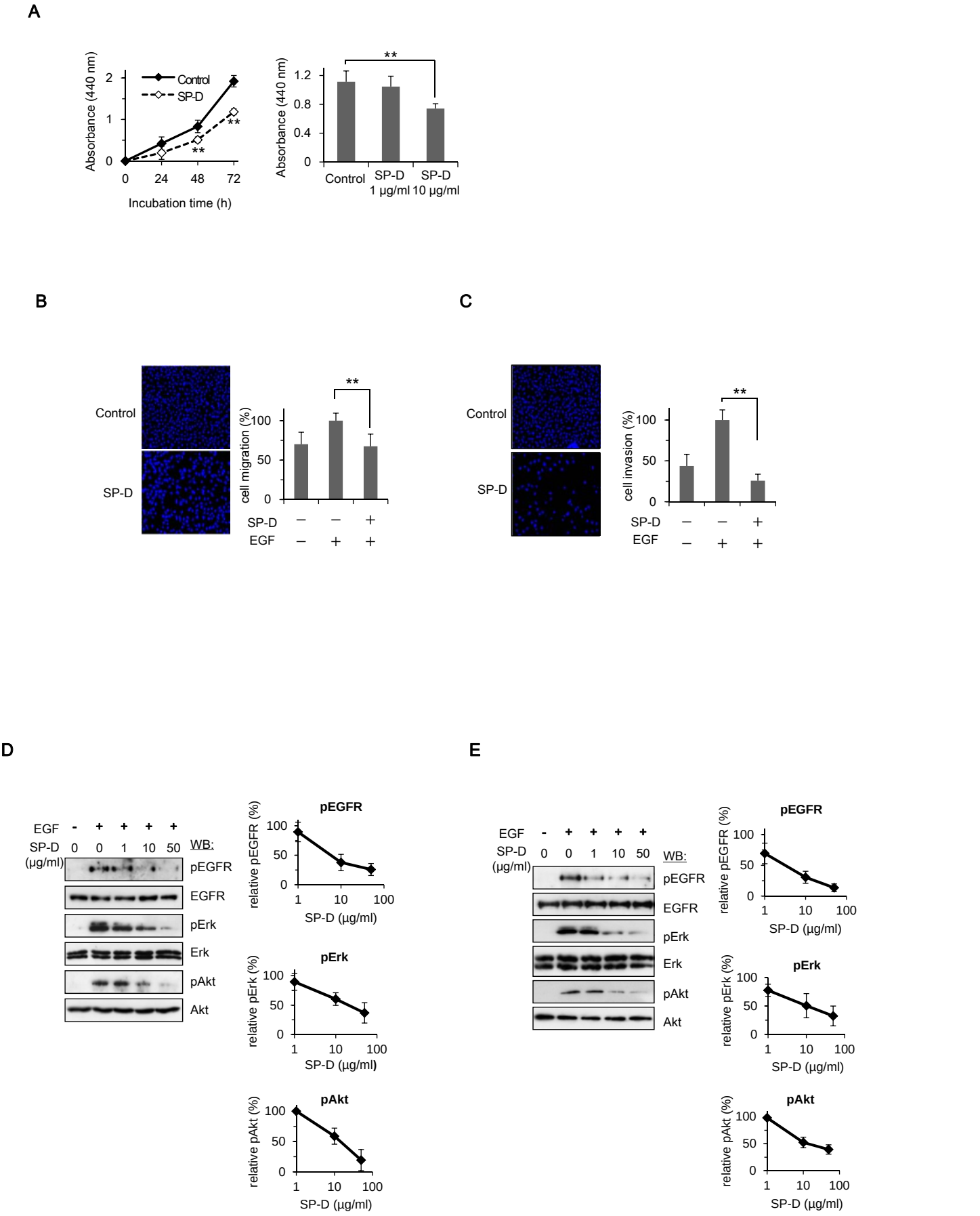
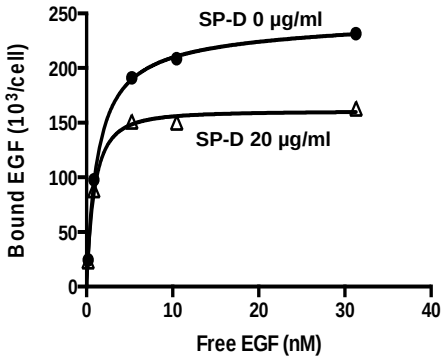

A
Absorbance (440 nm)
Control
SP-D
Incubation time (h)
Control
SP-D 1 μg/ml
SP-D 10 μg/ml
B
Control
SP-D
cell migration (%)
SP-D
EGF
C
Control
SP-D
cell invasion (%)
SP-D
EGF
D
EGF
SP-D (μg/ml)
WB:
pEGFR
EGFR
pErk
Erk
pAkt
Akt
relative pEGFR (%)
relative pErk (%)
relative pAkt (%)
SP-D (μg/ml)
E
EGF
SP-D (μg/ml)
WB:
pEGFR
EGFR
pErk
Erk
pAkt
Akt
relative pEGFR (%)
relative pErk (%)
relative pAkt (%)
SP-D (μg/ml)

Figure 2

A


SP-D 0 µg/ml
SP-D 20 µg/ml
Bound EGF (10³/cell)
Free EGF (nM)
250
200
150
100
50
0
0
10
20
30
40

B

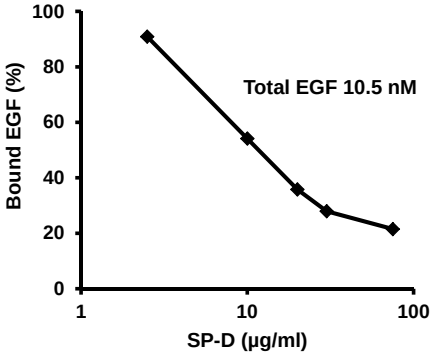
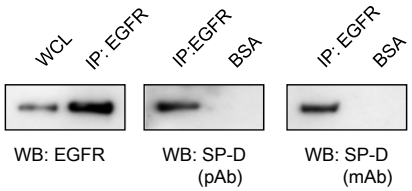

Total EGF 10.5 nM
Bound EGF (%)
SP-D (µg/ml)
100
80
60
40
20
0
1
10
100

Figure 3

A


WCL
IP: EGFR
IP:EGFR
BSA
IP: EGFR
BSA
WB: EGFR
WB: SP-D (pAb)
WB: SP-D (mAb)

B

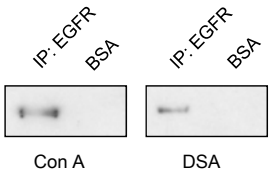
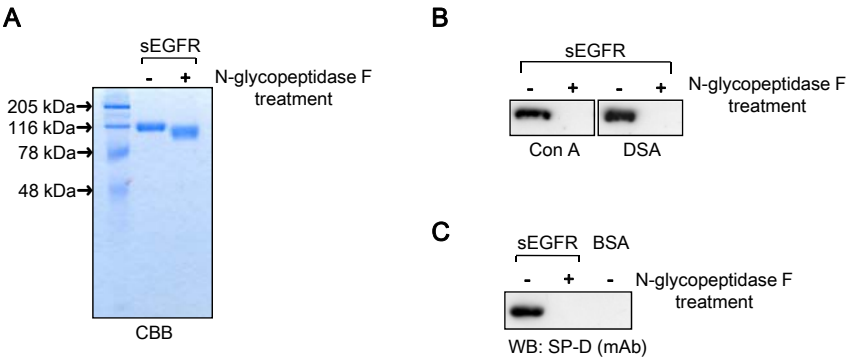
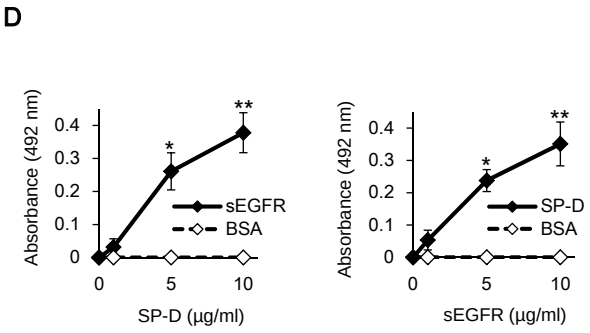

IP: EGFR
BSA
IP: EGFR
BSA
Con A
DSA

Figure 4


A
sEGFR
- +
N-glycopeptidase F treatment
205 kDa
116 kDa
78 kDa
48 kDa
CBB
B
sEGFR
- + - +
N-glycopeptidase F treatment
Con A
DSA
C
sEGFR BSA
- + -
N-glycopeptidase F treatment
WB: SP-D (mAb)


D
Absorbance (492 nm)
0.4
0.3
0.2
0.1
0
0 5 10
SP-D (μg/ml)
sEGFR
BSA
*
**
Absorbance (492 nm)
SP-D
BSA
sEGFR (μg/ml)

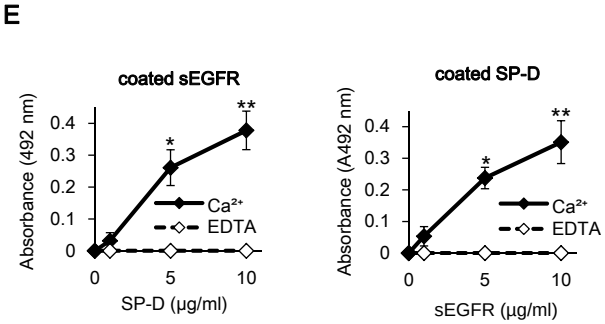
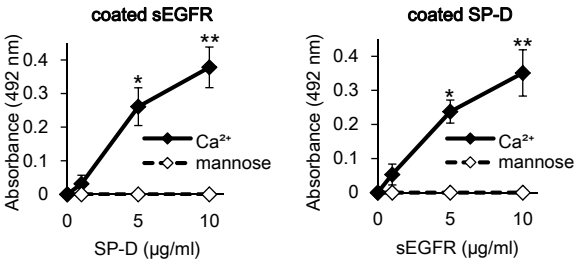
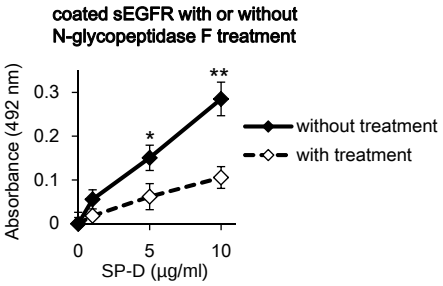

E
coated sEGFR
Absorbance (492 nm)
Ca²⁺
EDTA
SP-D (μg/ml)
coated SP-D
Absorbance (A492 nm)
sEGFR (μg/ml)

Figure 4

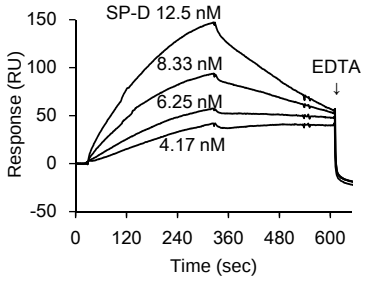
F



G



H



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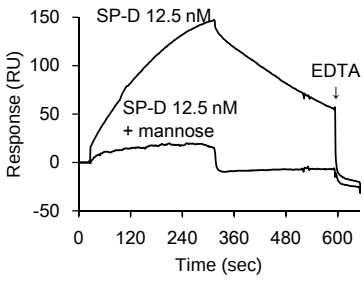

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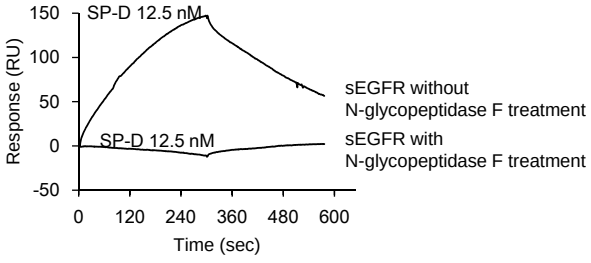
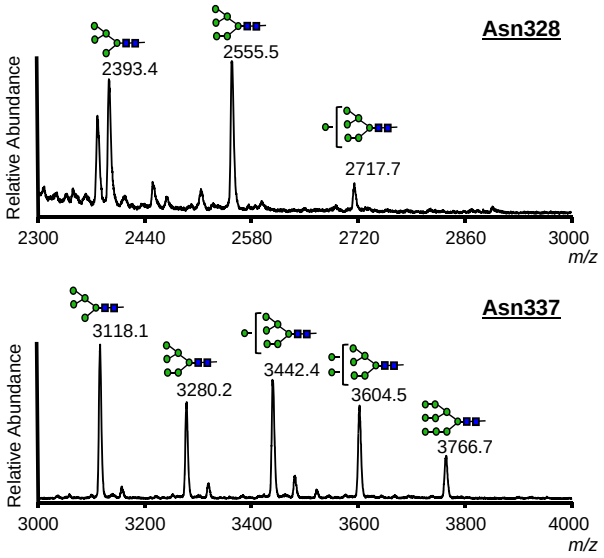


Figure 5

A



B

glycosylation site	High-mannose type or Complex type	Structure
N32	Complex	(i)
N104	Complex or (-)	(i)
N151	Complex	(i)
N172	Complex or (-)	(i)
N328	High-mannose	(ii)
N337	High-mannose	(iii)
N389	Complex	(iv)
N420	Complex	(i)
N504	Complex	(i)
N544	Complex	(i)
N579	Complex	(i)
N599	Complex or (-)	(i)