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The carbohydrate recognition by cytokines modulates their physiological activities

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A variety of cytokines have been reported to be able to recognize specific carbohydrate moieties. To date, the role of carbohydrate recognition in cytokine function has been analyzed for several cytokines, including fibroblast growth factor (FGF), tumor necrosis factor (TNF)-α, and interleukin (IL)-2. The FGF family and their receptors have been found to recognize a heparan sulfate proteoglycan, which generates rigid complexes that induce signal transduction. We have found that IL-2 recognizes a high-mannose type glycan on the α subunit of the IL-2 receptor as well as a peptide portion of this subunit. Blocking this carbohydrate-IL-2 interaction diminished IL-2-induced signaling and T-cell proliferation. We have also shown that TNF-α recognizes the second mannose 6-phosphate diester of the glycan portion of glycosylphosphatidylinositol (GPI)-anchored glycoproteins. Blocking this GPI-anchored glycan-TNF-α interaction abrogates TNF-α-induced apoptosis. We aim to increase the number of cytokines which modulate their functions through the unique carbohydrate recognition, and open the way to systematically elucidate the biological functions of cytokine-carbohydrate interaction in immune system.
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Abbreviations: **TNF, tumor necrosis factor; IL-2, interleukin-2; IL-2R, IL-2 receptor; GPI, glycosylphosphatidylinositol; FGF, fibroblast growth factor; hAP, human alkaline phosphatase; GlcNAc, *N*-acetylglucosamine; Man, mannose; PVL, *Psathyrella velutina* lectin; GNA, *Garanthas nivalis* agglutinin; LC/ESI/MS, liquid column chromatography/ electrospray ionization/mass spectrometry; PI-PLC, phosphatidylinositol specific phospholipase C; TNFR, TNFα receptor; EtN, ethanolamine.**

Introduction

Cytokines orchestrate immune systems such as immune responses, inflammation, cell proliferation and apoptosis. These hormone-like molecules can act in a pleiotropic, redundant, autocrine or paracrine manner. Although there are many reports about the mechanisms of the signal transduction that occurs after cytokines bind to their receptors, it remains unclear how cytokines can trigger multiple biological activities in various target cells. It has been noted that many cytokines bind specifically to particular types of glycoconjugates and we are interested in clarifying whether the expression of the physiological activities of cytokines depend on their carbohydrate recognition. The known carbohydrate recognition specificities of cytokines are summarized in Table 1 [1]. Although the physiological roles of these carbohydrate recognition abilities remain unclear in most cases, they have been analyzed for several cytokines. One relatively well-understood case is that of fibroblast growth factor (FGF)-2. X-ray crystallographic analysis has shown that FGF-2 signaling involves the mutual interaction between FGF-2 dimer, FGF-2 receptor dimer, and a heparan sulfate proteoglycan at the cell surface [2]. This interaction generates a rigid heparan sulfate-(FGF-2)$_2$-(FGF-2 receptor)$_2$ complex that can now associate with downstream signaling molecules, thereby inducing intracellular signaling (Figure 1).

In our own research examining the contribution of the lectin properties of cytokines to their biological functions, we recently found that tumor necrosis factor (TNF)-α recognizes the mannose 6-phosphate diester of the glycan portion of glycosylphosphatidylinositol (GPI) anchored glycoproteins and that this recognition is involved in the induction of cell apoptosis by TNF-α [1]. Moreover, we have analyzed the carbohydrate recognition of interleukin (IL)-2 and have shown that IL-2 binds to the high-mannose type glycan on the α-subunit of the IL-2 receptor as well as to a peptide part of the receptor, and that this dual binding is required for the IL-2 signal transduction that

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Table 1. Lectin properties of cytokines

Cytokine	*Oligosaccharide ligand*
FGF family	heparin
IL-1α	glycans of uromodulin disialylated biantennary
IL-1β	glycans of uromodulin GM4 GPI anchor glycan
IL-2	high mannose-type glycan heparin
IL-3	heparin
IL-4	mucin heparin
IL-5	heparin
IL-6	HNK-1-like epitope heparin
IL-7	sialyl Tn antigen heparin
IL-8	heparin
IL-12	heparin

leads to cell proliferation [3,4]. This research is summarized below.

The GPI anchor glycan recognition of TNF-α is involved in U937 cell apoptosis

TNF-α binds to GPI anchored glycoproteins

It was found that recombinant human (rh)TNF-α can bind to human placental alkaline phosphatase (hAP), the carcinoembryonic antigen (CEA), and the Tamm-Horsfall glycoprotein by using a solid-phase binding assay. All of these molecules carry a GPI anchor. In contrast, rhTNF-α did not bind to *N*-linked glycans, glycolipids, proteoglycans and mucin. These observations suggested that rhTNF-α recognizes GPI anchor glycans (Figure 2).

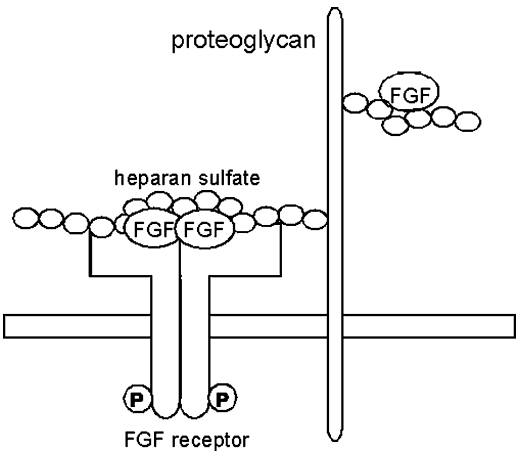

Figure 1. A schematic model for interaction of FGF, FGF receptor and heparan sulfate.

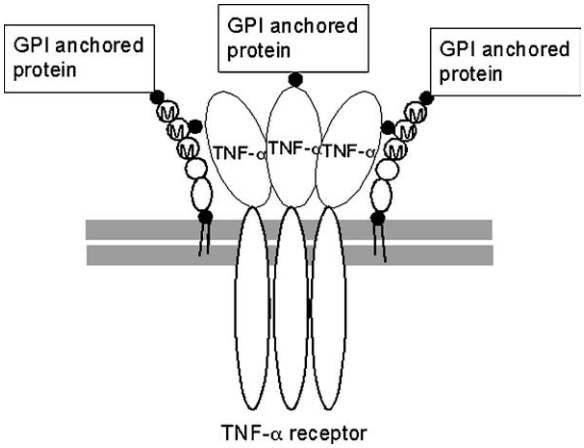

Figure 2. A schematic model for interaction of TNF-α, TNFR and GPI anchor glycan.

We then analyzed inhibitor activity of various haptenic sugars derived from GPI anchor glycans in the binding assay with TNF-α and hAP, and found that only mannose 6-phosphate inhibited the binding at a concentration of 10^{-6} M in terms of IC_{50} value. In contrast, ethanolamine phosphate, inositol phosphate, *N*-acetylglucosamine 1-phosphate, mannose 6-sulfate, mannose 1-phosphate, glucose 6-phosphate and mannitol 6-phosphate did not cause any inhibition, even at concentrations of 1 mM, indicating that a mannosyl residue substituted with phosphate at the C-6 position is required for the sugar-binding ability of TNF-α.

N-Acetylglucosamine (GlcNAc)β1 $\rightarrow$ phosphate $\rightarrow$ 6 is attached to the second mannose residue of the GPI anchor glycan of hAP

We recently determined that a β-*N*-acetylglucosaminyl phosphate diester residue is attached to the C-6 position of the second mannosyl residue in the GPI anchor glycan of hAP by methods including a *Psathyrella velutina* lectin (PVL)-Sepharose column, which binds β-GlcNAc residues, chromatofocusing gel analysis, liquid chromatography/electrospray ionization mass spectrometry (LC/ESI/MS), nitrous acid deamination, and periodate oxidation-Smith degradation, as summarized in Figure 3 [5].

TNF-α specifically recognizes the second mannose 6-phosphate diester of GPI anchor glycans

We then investigated whether the GlcNAc β1 $\rightarrow$ phosphate $\rightarrow$ 6mannose residue is involved in the binding of TNF-α to hAP. hAP treated with mild acid, which hydrolyzes the β-GlcNAc residue, retained its ability to bind to rhTNF-α. In contrast, mild acid and phosphatase digestion, which specifically releases the GlcNAcβ1-phosphate, abrogated the binding of hAP to TNF-α. These results indicate that TNF-α specifically recognizes the second mannose 6-phosphate diester of GPI anchor glycans.

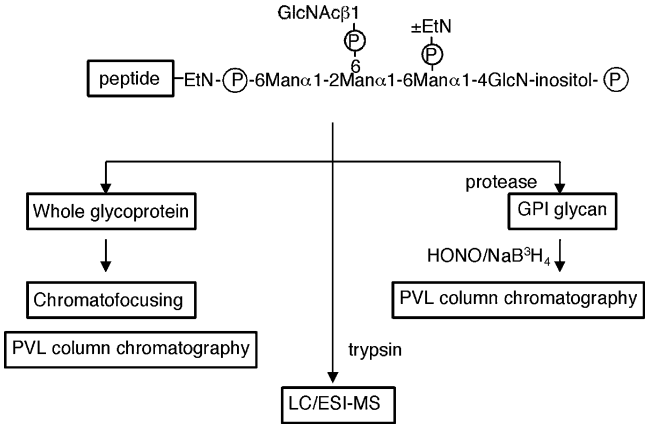

Figure 3. The scheme of structural analysis for GPI anchor glycan derived from hAP.

The stimulation by TNF-α of U937 cell apoptosis and tyrosine phosphorylation is inhibited by treatment with mannose 6-phosphate or phosphatidyl inositol-specific phospholipase C (PI-PLC)

The stimulation of the apoptosis of human lymphoma U937 cells by TNF-α was inhibited when the cells were cultured with TNF-α in the presence of 1 μM mannose 6-phosphate at 37°C for 18 h. The apoptosis was detected by flow cytometry after staining the cells with a MEBSTAIN direct apoptosis kit, which labels the 3′OH ends of fragmented DNA with fluorescein-dUTP.

Treatment of U937 cells with TNF-α for 20 min at 37°C resulted in the marked tyrosine phosphorylation of three proteins in the cells, as detected by immunoblot analysis. This tyrosine phosphorylation was inhibited when the cells were cultured with TNF-α in the presence of 10^{-6} M mannose 6-phosphate or when they had been pretreated with PI-PLC. These results suggest that TNF-α binding to the GlcNAcβ1 $\rightarrow$ phosphate $\rightarrow$ 6mannose residue in the GPI anchor glycan, along with its binding to TNF-α receptor (TNFR), triggers the intracellular signaling pathway in U937 cells. Since it has been reported that active TNF-α forms a bell-like trimer, it appears that the TNF-α trimers bind to three TNFRs and at the same time each TNF-α molecule binds to the GlcNAcβ1 $\rightarrow$ phosphate $\rightarrow$ 6Man residue in a GPI anchor glycan. This results in the formation of a tight nanomeric complex $(TNFR)_3$-$(TNF\text{-}\alpha)_3$-$(GPI\ anchor\ glycan)_3$ (Figure 2).

IL-2 carbohydrate recognition is involved in CTLL-2 cell proliferation

The exogenous mannose $(Man)_5GlcNAc_2$ inhibits the CTLL-2 cell proliferation and tyrosine phosphorylation induced by IL-2

The carbohydrate recognition of IL-2 was first discovered in 1989 through the use of ELISA. This revealed that it binds to high mannose type glycans bearing mannose 5 and mannose 6. We analyzed the significance of this carbohydrate recognition in relation to the physiological activity of IL-2 by employing a murine T-cell line, CTLL-2, which proliferates in an IL-2-dependent manner. We found that if IL-2 was preincubated with $Man_5GlcNAc_2$/$Man_6GlcNAc_2$ before being added to the cell medium, the proliferation of the cells was inhibited. Other high mannose type glycans did not have any inhibitory effects. However, when the high-mannose type glycan was added at the same time or after IL-2 was added to the cells, the inhibitory effect could not be observed. This suggested that the binding of IL-2 to endogenous glycoproteins was strong enough and was not replaced by the exogenous glycan.

The IL-2 receptor (IL-2R) consists of the α, β, and γ subunits. Although these subunits do not have any intrinsic phosphorylating activity, the β and γ subunits bind to tyrosine kinases, which become phosphorylated and thereby induce intracellular signal transduction. To analyze the effect of $Man_5GlcNAc_2$ on the tyrosine kinase phosphorylation induced by the binding of IL-2, CTLL-2 cells were stimulated with IL-2 in the presence or absence of $Man_5GlcNAc_2$ and their lysates were immunoprecipitated with antibodies specific to the tyrosine kinases Jak1, Jak3, and Lck. Their IL-2-dependent phosphorylation, which was detected by immunoblotting with an anti-phosphotyrosine antibody, decreased in the presence of $Man_5GlcNAc_2$, suggesting that the signal transduction mechanism of IL-2 involves the recognition by IL-2 of a glycoprotein that bears $Man_5GlcNAc_2$.

IL-2 recognizes both $Man_5GlcNAc_2$ and a specific peptide sequence in the α subunit of the IL-2R

Since IL-2 forms a high-affinity complex with IL-2R, it would be possible to co-immunoprecipitate, from IL-2-stimulated CTLL cells, the complex composed of IL-2 and the IL-2R $\alpha\beta\gamma$ subunits, along with the bound tyrosine kinases, with an antibody specific for one of the three subunits. If an IL-2-binding $Man_5GlcNAc_2$-bearing glycoprotein is involved in the high-affinity complex, it should be detectable in the immunoprecipitated IL-2-IL-2R $\alpha\beta\gamma$ complex. Indeed, the immunoprecipitated high-affinity complexes were stained with *Garanthas nivalis* agglutinin (GNA), which specifically recognizes high-mannose type glycans. Moreover, when the immunoprecipitates were fractionated by polyacrylamide gel electrophoresis, GNA only recognized a single 55 kDa band, regardless of which antibody had been used to immunoprecipitate the complex. The 55 kDa band corresponds to the IL-2R α subunit. These results indicated that the IL-2R α subunit has a high-mannose type glycan that bears $Man_5GlcNAc_2$ and that IL-2 binds to both the high-mannose type glycan and a specific peptide sequence in the IL-2R α subunit. Notably, when each of the IL-2R subunits was expressed in COS-7 cells and their binding affinity with IL-2 was determined, the α subunit had the lowest dissociation constant of the three. This suggests that IL-2 first recognizes both a high mannose-type glycan and a specific peptide sequence in the IL-2Rα subunit and that this dual recognition then induces

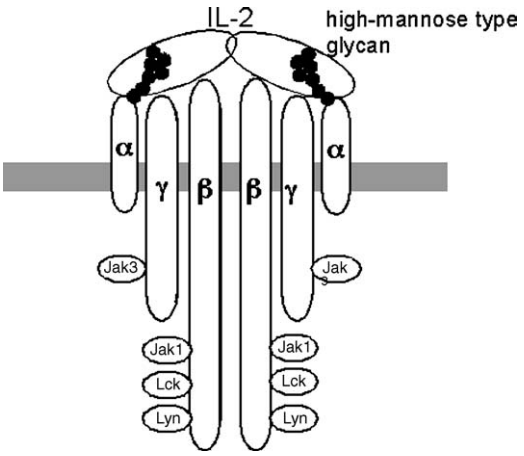

Figure 4. A schematic model for formation of the high-affinity complex (IL-2-IL-2Rα, β, $\gamma)_2$.

the conformational change of IL-2 and triggers the formation of the high-affinity IL-2-IL-2R $\alpha\beta\gamma$ complex [2].

The Asn-26 residue in IL-2 is involved in the carbohydrate recognition of IL-2

The carbohydrate-binding site in IL-2 was determined by using various mutants of *in vitro* translated [^{35}S]rhIL-2. *In vitro* translated [^{35}S]rhIL-2 was employed because it spontaneously forms a dimer, as determined by Superose-12 column chromatography, and it retains the ability to induce CTLL-2 cell proliferation and binds to the carbohydrate ligand. Among the mutants that could form dimers, a mutant with the substitution of the asparagine 26 residue with glutamine increased both the carbohydrate recognition ability and cell proliferation activity. In contrast, another mutant with the substitution of the same residue with aspartate decreased both activities. These results indicate that asparagine 26 is included in the carbohydrate-binding site of IL-2. X-ray crystallographic analyses suggest that the side chain of the asparagine 26 residue of IL-2 points toward the outside of the first α-helix, and that this residue does not overlap with the IL-2 sites that bind to three IL-2R subunits. We have proposed on the basis of these observations that IL-2 forms the octameric complex depicted schematically in Figure 4 and that once IL-2 binds to the carbohydrate on IL-2Rα, IL-2 changes the conformation, and bind to a specific peptide sequence on IL-2Rα. Once the IL-2-IL-2Rα complex is formed, it then triggers the formation of the high affinity complex (IL-2-IL-2Rα, β, $\gamma)_2$. This hetero-octamer stimulates IL-2-dependent T-cell proliferation by intensifying cellular signaling.

References

1 Fukushima K, Ishiyama C, Yamashita K, GPI-anchor recognition of TNF-α modulates apoptosis of U937 cells, *Arch Biochem Biophys* **426**, 298–305 (2004).
2 Rapraegh A, Krufka A, Olwin BB, Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation, *Science* **252**, 1705–8 (1991).
3 Fukushima K, Yamashita K, Interleukin-2 carbohydrate recognition modulates CTLL-2 cell proliferation, *J Biol Chem* **276**, 7351–6 (2001).
4 Fukushima K, Hara-Kuge S, Ideo H, Yamashita K, Carbohydrate recognition site of interleukin-2 in relation to cell proliferation, *J Biol Chem* **276**, 31202–8 (2001).
5 Fukushima K, Ikehara Y, Kanai M, Kochibe N, Kuroki M, Yamashita K, Aβ-*N*-acetylglucosaminyl phosphate diester residue is attached to the glycosyl-phosphatidylinositol (GPI) anchor of human placental alkaline phosphatase: A target of the channel-forming toxin aerolysin, *J Biol Chem* **278**, 362960-303 (2003).

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