Functional Role Played by the Glycosylphosphatidylinositol Anchor Glycan of CD48 in Interleukin-18-induced Interferon-γ Production

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Interleukin (IL)-18 induces T cells and natural killer cells to produce not only interferon-γ but also other cytokines by binding to the IL-18 receptor (IL-18R) α and β subunits. However, little is known about how IL-18, IL-18Rα, and IL-18Rβ form a high-affinity complex on the cell surface and transduce the signal. We found that IL-18 and IL-18Rα bind to glycosylphosphatidylinositol (GPI) glycan via the third mannose 6-phosphate diester and the second β-GlcNAc-deleted mannose 6-phosphate of GPI glycan, respectively. To determine which GPI-anchored glycoprotein is involved in the complex of IL-18 and IL-18Rα, IL-18Rα of IL-18-stimulated KG-1 cells was immunoprecipitated together with CD48 by anti-IL-18Rα antibody. More than 90% of CD48 was detected as β-GlcNAc-deleted GPI-anchored glycoprotein, and soluble recombinant human CD48 without GPI glycan bound to IL-18Rα, indicating that CD48 is associated with IL-18Rα via both the peptide portion and the GPI glycan. To investigate whether the carbohydrate recognition of IL-18 is involved in physiological activities, KG-1 cells were digested with phoshatidylinositol-specific phospholipase C before IL-18 stimulation. Phoshatidylinositol-specific phospholipase C treatment diminished intracellular signaling. These observations suggest that the complex formation of IL-18-IL-18Rα-CD48 via both the peptide portion and GPI glycan triggers the binding to IL-18Rβ, and the IL-18-IL-18Rα-CD48IL-18Rβ complex induces cellular signaling.

Interleukin (IL)-18 is a cytokine that induces T cells and natural killer cells to produce interferon (IFN)-γ (1). It also has some IFN-γ-independent pro-inflammatory activities because it induces T cells and natural killer cells to synthesize tumor necrosis factor-α, granulocyte macrophage colony-stimulating factor, nitric oxide, and chemokines (2). It has been reported that IL-18 binds to IL-18 receptor (IL-18R) α and β and that this induces signal transduction pathways that may involve nuclear factor-κB (3), p56(lck) (4), and the mitogen-activated protein kinase (MAPK) (4). However, the mechanism by which the formation of IL-18-IL-18Rα-IL-18Rβ complex leads to intracellular signal transduction remains unclear.

Our laboratory has been interested in the carbohydrate recognition activities of various cytokines because we speculate that sugar chains recognized by these cytokines may function as immunomodulators (5). We previously reported that IL-1β specifically recognizes GPI anchor glycan (6). IL-18 has been classified as a member of the IL-1 family on the basis of its structural similarity to IL-1β (7). Thus, here we investigated whether IL-1β also has carbohydrate binding ability. We found that like IL-1α, IL-18 recognizes GPI anchor glycans and that it specifically binds to the third mannose 6-phosphate diester, and this binding is inhibited by the addition of mannose 6-phosphate. Furthermore, it was found that IL-18Rα also recognizes GPI anchor glycans and that IL-18Rα specifically binds to the exposed second mannose 6-phosphate. To elucidate the physiological significance of the GPI anchor glycan binding ability of both IL-18 and IL-18Rα, we investigated the effect of phoshatidylinositol-specific phospholipase C (PI-PLC) treatment on the IL-18-stimulated tyrosine-phosphorylation of KG-1 cells. We found that PI-PLC treatment diminished intracellular signaling and IFN-γ production. It seems that GPI anchor glycan recognition by IL-18 and IL-18Rα induces formation of the IL-18-IL-18Rα-GPI anchor glycan complex, and this complex immediately binds to IL-18Rβ and induces intracellular signal transduction. Analysis of the IL-18Rα or IL-18Rβ immunoprecipitates of IL-18-treated KG-1 cells revealed the presence of a single 47-kDa GPI-anchored protein, CD48, in the complex, and CD48 bound to IL-18Rα via both GPI anchor glycan and the specific peptide sequence. Thus, it appears that IL-18, IL-18Rα, and CD48 complex formation via GPI glycan and the specific peptide sequences triggers binding to IL-18Rβ and thereby induces intracellular signal transduction and IFN-γ production.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—rhIL-18 was purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan). L-[35S]Methionine (1175 Ci/mmol) was purchased from MP Biomedicals, Inc. Ribonuclease B, thyroglobulin, ovalbumin, transferrin, fetuin, mannose 6-phosphate, mannose 1-phosphate, glucose 6-phosphate, myo-inositol 1-phosphate, mannose 6-sulfate, N-acetylglycosamine 1-phosphate, and ethanolamine-phosphate were purchased from Sigma. Mannitol 6-phosphate was prepared by NaBH₄ reduction of mannose 6-phosphate. The soluble form of human placental alkaline phosphatase (shAP) was purified according to the previously reported methods (8). T-H glycoprotein was prepared from pooled urine, as described previously (9). Carcinoembryonic-
oronic antigen (CEA) was kindly provided by Dr. M. Kuroki (Fukoku University, Fukuoka, Japan). Galβ1→4GlcNAcβ1→2Manα1→6-(Galβ1→4GlcNAcβ1→2Manα1→3Manβ1→4GlcNAc was purified from the urine of patients with GM1 gangliosidosis (10).

Preparation of 35S-rhIL-18—DNA encoding human IL-18 was kindly provided by Hayashibara Co. Ltd. The pET3a plasmid (Novagen, Inc., Madison, WI) was used as the T7 expression plasmid. A Sall-EcoRI fragment corresponding to the human IL-18 gene was inserted between the Sall and EcoRI sites of pET3a to produce the expression plasmid pET3a-IL-18. This was used as a template for in vitro transcription and translation in a Puresystem (10) in the presence of [35S]methionine. The in vitro transcription and translation was accomplished as described in the manufacturer's instructions. An aliquot of the translation products was subjected to SDS-PAGE using 15% polyacrylamide gels and autoradiographed. The translation products were separated from free [35S]methionine using a PD-10 column (Amersham Biosciences) with PBS and used immediately. Reactions using 1 pmol of plasmid DNA template and 30 μCi of [35S]methionine reproducibly provided 150 pmol of 35S-rhIL-18.

Binding Assays of IL-18 or IL-18R to Glycoproteins—The binding of 35S-rhIL-18 to various glycoproteins was measured by solid-phase binding assays. Thus, enzyme-linked immunosorbent assay plates (Corning, Inc., Corning, NY) were coated with glycoproteins at 1 μg/ml in PBS at 4 °C overnight. The plates were washed with PBS containing 0.05% Tween 20 and 0.1% BSA. Bound IL-18Rα or IL-18Rβ subunits of IL-18-stimulated KG-1 cells were immunoprecipitated by using goat anti-IL-18Rα and goat anti-IL-18Rβ antibodies (R&D System) and protein G-immobilized resin (Amersham Biosciences). After SDS-PAGE using a 10.5% acrylamide gel and blotting onto a nitrocellulose filter, the GPI-anchored protein involved in the high-affinity complex of IL-18 was first adjusted to pH 8 followed by incubation with a 20-μl slurry of bovine intestine alkaline phosphatase-immobilized beads (Sigma) at 37 °C for 2 h. After washing with 0.05% Tween 20 in PBS, the bound 35S-rhIL-18 was released by treatment with 100 μl of 1% SDS, and the radioactivity was measured by means of a liquid scintillation counter. The protein concentration used to coat the plates was determined by experiments using various concentrations up to 1 mg/ml.

To hydrolyze the GlcNAc-phosphodiester linkage and the Neu5Acα2→3Gal linkages of shAP, shAP was treated with acid as follows (11): 10 μg of shAP was incubated in 50 μl of 0.01 N HCl at 100 °C for 30 min, after which the pH was adjusted to 7, and de-GlcNac-shAP was prepared. An aliquot of de-GlcNac-shAP was also treated with acid (pH 8.0) containing 0.05% Tween 20, which was first adjusted to pH 8 followed by incubation with a 20-μl slurry of bovine intestine alkaline phosphatase-immobilized beads (Sigma) at 37 °C for 1 h. The beads were then removed by centrifugation (1000 × g, 30 s) to generate de-GlcNac-phosphate-shAF.

In the case of IL-18Rα, the plates were blocked with PBS containing 0.05% Tween 20 and 1% bovine serum albumin (BSA) and then incubated with various concentrations of the shAP-Fc chimera (R&D Systems) in PBS containing 0.05% Tween 20 and 0.1% BSA. Bound IL-18Rα or IL-18Rβ was treated with mouse anti-human IgG Fc antibody (Sigma) and anti-mouse IgG antibody-conjugated horseradish peroxidase (HRP) (Amersham Biosciences) and detected with TMB (tetramethylbenzidine) solution (Wako Chemicals). The GPI-anchored glycoproteins on KG-1 cells were released by digestion with proaerolysin, mouse anti-proaerolysin antibodies (R&D System) and HRP-conjugated goat anti-mouse IgG antibody (Amersham Biosciences) and detected with TMB solution. To investigate the inhibitory effect of anti-CD48 antibody, the plates were treated with mouse anti-CD48 antibody after blocking with BSA.

Separation of GPI-anchored Proteins by PVL Column Chromatography—GPI-anchored glycoproteins on KG-1 cells were released by digestion with PI-PLC (5 milliunits/ml PBS) at 37 °C for 1 h. Released GPI-anchored proteins were applied to a PVL-Sepharose column. PVL was obtained from Wako Chemicals. PVL-Sepharose (3 mg/ml) was prepared from PVL and CNBr-Sepharose (Amersham Biosciences). After flow-through components were collected, bound components were eluted with PBS containing 0.5 mM GlcNAc. Both components were applied to a PD-10 column (Amersham Biosciences) and freeze-dried. After SDS-PAGE using a 10.5% acrylamide gel and blotting onto nitrocellulose membrane, CD48 was detected using mouse anti-CD48 antibody and HRP-conjugated goat anti-mouse IgG antibody. Before blotting, total protein was stained with SYPRO® Orange (Bio-Rad).

RESULTS

IL-18 Binds to the GPI-anchored Protein shAP—We previously reported that IL-18 is able to bind to carbohydrates and that it recognizes the GPI anchor glycan (6). Because IL-18 shares amino acid sequence homology with IL-18, we investigated whether IL-18 also has carbohydrate binding activity. First, we performed binding assays using plates coated with 10
investigated the inhibitory effects on the binding of 35S-rhIL-18. Consequently, we used this protein to more precisely determine the binding in the presence of [35S]methionine followed by separation from excess [35S]methionine by PD-10 column chromatography. We prepared 35S-rhIL-18 by high nonspecific binding, we used human serum albumin as the blocking reagent. We prepared 35S-rhIL-18 using in vitro translation in the presence of [35S]methionine. A, shAP; B, T-H glycoprotein; C, CEA; D, transferrin. Fetuin, orosomucoid, ribonuclease B, ovalbumin, and thyroglobulin showed the same results as transferrin. These data represent the average of four experiments (p < 0.05).

Inhibitory Effects of Haptenic Sugars Derived from the GPI Anchor Glycan of shAP on the Carbohydrate Binding Activity of 35S-rhIL-18—The N-glycans and GPI anchor glycan structures of shAP have been determined, as summarized in Fig. 2A (11). Consequently, we used this protein to more precisely determine the carbohydrate binding specificity of IL-18. To do this, we investigated the inhibitory effects on the binding of 35S-rhIL-18 to shAP of haptenic sugars derived from the GPI anchor glycan of shAP. The saccharides tested and their inhibitory effects are summarized in Table I. The biantennary sugar chain of shAP, which is an asialo-N-linked sugar chain, was not inhibitory at concentrations of up to 1 mM. In contrast, mannose 6-phosphate, which is a constituent of the GPI anchor glycan of shAP, was an effective inhibitor. However, other constituents of the GPI anchor glycan of shAP, such as ethanolamine phosphate, inositol phosphate, and GlcNAc-1-phosphate, were not inhibitory up to 1 mM. In addition, mannose 6-sulfate, mannose 1-phosphate, glucose 6-phosphate, and mannitol-6-phosphate were not inhibitory at concentrations up to 1 mM, which indicated that mannose substituted with phosphate at the C-6 position is necessary for the carbohydrate binding of IL-18.

These observations led us to speculate that IL-18 recognizes a mannose 6-phosphate diester moiety in GPI anchor glycans. To test this, we removed the second mannose 6-phosphate diester of shAP by mild acid and phosphatase treatment and performed the IL-18-binding assay again. Because de-GlcNAc shAP and de-GlcNAcβ1→phosphate shAP maintained their ability to bind to 35S-rhIL-18 (Fig. 2B, ● and ○), it appears that IL-18 specifically binds to the third mannose 6-phosphate diester in the GPI anchor glycan.

The Binding of IL-18 to the GPI Anchor Glycan Modulates IFN-γ Production and Intracellular Tyrosine Phosphorylation of IL-18-stimulated KG-1 Cells—It is known that human leukemia KG-1 cells produce IFN-γ when they are stimulated with IL-18 (1). To determine whether the recognition of the GPI anchor glycan of shAP is necessary for the binding of 35S-rhIL-18 to shAP, we performed the IL-18-binding assay again. Because de-GlcNAc shAP and de-GlcNAcβ1→phosphate shAP maintained their ability to bind to 35S-rhIL-18 (Fig. 2B, ● and ○), it appears that IL-18 specifically binds to the third mannose 6-phosphate diester in the GPI anchor glycan.
anchor glycan by IL-18 is important for the expression of its physiological functions, we assessed the ability of mannose 6-phosphate and PI-PLC to block the stimulatory effects of IL-18 on the production of IFN-γ by KG-1 cells. First, we confirmed that incubation of KG-1 cells (1 × 10⁵ cells/well) for 24 h in the presence of rhIL-18 indeed induced them to produce IFN-γ in a dose-dependent manner (Fig. 3A). Because 10 ng/ml IL-18 was sufficient to stimulate IL-18-dependent IFN-γ production, the following experiments were performed at this concentration. As shown in Fig. 3B, mannose 6-phosphate inhibited the IFN-γ production of KG-1 cells, although 10⁻⁵ M mannose 6-phosphate was not sufficient to inhibit all the activity. These results suggest that mannose 6-phosphate competes for IL-18 binding to the anchor glycans on the cell surface and thereby suppresses the IL-18-induced physiological activity of KG-1 cells. Furthermore, removal of the GPI-anchored proteins on KG-1 cells by PI-PLC treatment inhibited the rhIL-18-stimulated IFN-γ production (Fig. 3B) and tyrosine phosphorylation (Fig. 4). These results indicate that IL-18 binding of the third mannose 6-phosphate diester in the GPI-anchor glycan is at least required to enhance IL-18-dependent intracellular signal transduction and IFN-γ production.

**IL-18Ra Binds to Both IL-18 and the GPI Anchor Glycan—** Because it is known that the first step in the signal transduction cascade induced by IL-18 involves its binding to IL-18Ra (21), we speculated that IL-18Ra may interact with the second mannose 6-phosphate diester of the GPI anchor glycan. To test this notion, we coated plates with intact shAP, de-β-GlcNAc-shAP, and de-GlcNAcβ1-phosphate-shAP and assessed the binding of the IL-18Ra-Fc chimera by using a mouse anti-human IgG Fc antibody and HRP-conjugated anti-mouse IgG antibody. Only a single 47-kDa protein was detected as a GP1-anchored protein whose tyrosine phosphorylation has been enhanced by IL-18 treatment. On the other hand, IL-18Rβ did not show any binding ability to these shAP derivatives (Fig. 5B). These results suggest that both IL-18 and IL-18Ra can recognize the GPI anchor glycan, although they have different binding specificities. Thus, IL-18 binds to the third mannose 6-phosphate diester, whereas IL-18Ra binds to the second mannose 6-phosphate.

**Identification of the GPI-anchored Protein Involved in the Complex of IL-18-IL-18Ra—** If IL-18 and IL-18Ra binding to a GPI-anchored glycoprotein triggers the formation of the IL-18-IL-18Ra-IL-18Rβ complex, a specific GPI-anchored glycoprotein should be co-immunoprecipitated with IL-18Ra or IL-18Rβ in lysates of IL-18-stimulated KG-1 cells. To identify the GPI-anchored protein recognized by IL-18 and IL-18Ra, the IL-18-IL-18Ra-IL-18Rβ complex was immunoprecipitated by using anti-IL-18Ra or IL-18Rβ antibody, and the co-immunoprecipitated GPI-anchored protein was detected by using proaerolysin, which specifically binds to GPI-anchor glycans (11). Only a single 47-kDa protein was detected as a GPI-
anchored protein in the anti-IL-18Rα antibody or anti-IL-18Rβ antibody immunoprecipitates in lysates of IL-18-stimulated KG-1 cells (Fig. 6A, lanes 3 and 5). However, the immunoprecipitates using rabbit IgG instead of anti-IL-18Rα or anti-IL-18Rβ antibody were not stained by proaerolysin (Fig. 6A, lanes 6 and 7). Because whole GPI-anchored proteins in KG-1 cells were detected as shown in lane 1 of Fig. 6A, it appears that this 47-kDa protein is specifically involved in the IL-18-IL-18Rα-IL-18Rβ complex. We also confirmed that IL-18Rβ was co-immunoprecipitated with IL-18Rα by staining the immunoprecipitates with anti-IL-18Rβ antibody in IL-18-stimulated KG-1 cells; moreover, IL-18Rα was co-immunoprecipitated with IL-18Rβ (data not shown).

It has been reported that CD48 is one of the GPI-anchored proteins on T cells and associates with protein kinase p56lck (22). We found that an anti-CD48 antibody stained the immunoprecipitates that were generated by using anti-IL-18Rα or anti-IL-18Rβ antibodies (Fig. 6B, lanes 8–11); on the other hand, the immunoprecipitates using rabbit IgG were not stained by anti-CD48 antibody (Fig. 6B, lanes 12 and 13). In the absence of IL-18, CD48 was weakly detected in the immunoprecipitate by using anti-IL-18Rα antibody or anti-IL-18Rβ antibody (Fig. 6B, lanes 8 and 10). Therefore, CD48 seems to be a GPI-anchored protein that weakly associates with IL-18Rα.

GPI Anchor Glycan of CD48 Lacks βGlcNAc—CD48 was specifically immunoprecipitated with IL-18Rα, although many GPI-anchored glycoproteins exist on the cell surface. In order to determine why CD48 specifically binds to IL-18Rα, we investigated whether CD48 has βGlcNAc-deleted GPI glycan or whether the CD48 protein portion interacts with IL-18Rα. After KG-1 cells were treated with PI-PLC, released GPI-anchored glycoproteins were subjected to PVL-Sepharose column chromatography, which specifically recognizes the βGlcNAc residue (23). Approximately half of GPI-anchored proteins flowed through the column (PVL component), and the remainder was eluted with 0.3 M GlcNAc (PVL component) (Fig. 7, lanes 1 and 2). Both PVL components were applied to SDS-PAGE, blotted, and immunostained with anti-CD48 antibody. More than 90% of CD48 molecules were PVL components (Fig. 7, lanes 3 and 4), suggesting that most CD48 molecules have βGlcNAc-deleted GPI glycan and can bind to IL-18Rα.

**CD48 Binds to IL-18Rα via Both GPI Glycan and the Peptide Portion**—Sequentially, to elucidate whether the protein portion in CD48 also interacts with IL-18Rα, soluble recombinant human CD48 (srhCD48) without GPI glycan was prepared, and we assayed the binding ability between IL-18Rα and srhCD48 without GPI glycan by using a solid-phase binding assay. IL-18Rα bound to srhCD48-coated plates dose-dependently, and the binding was inhibited by the addition of anti-CD48 antibody (Fig. 8A). On the other hand, IL-18Rβ could not bind to srhCD48 (Fig. 8B). These results suggested that only CD48 was associated with IL-18Rα because CD48 could bind to IL-18Rα via both GPI glycan and the protein portion of CD48.

**DISCUSSION**

We clearly demonstrated in this study that both IL-18 and IL-18Rα have carbohydrate recognition abilities and that each of them separately recognizes a different site in the GPI anchor glycan of CD48. Although IL-18 and IL-18Rα recognize common epitope of various GPI-anchored glycoproteins, IL-18Rα may selectively bind to CD48 via both the specific peptide...
sequence and GPI glycan. We also showed that mannose 6-phosphate or PI-PLC treatment inhibits the response of KG-1 cells to IL-18 stimulation in terms of IFN-γ secretion and intracellular tyrosine phosphorylation and that this may inhibit the binding of CD48 to IL-18Rα and IL-18. These results also suggested that CD48 binding to IL-18Rα via the peptide portion was not strong enough to retain the association after inhibition of binding via GPI glycan. As previously reported, IL-18 induces signal transduction pathways that may involve nuclear factor-κB (3), p56lck (4), and MAPK (4). It has not been shown that p56lck and MAPK associate with IL-18Rα and IL-18Rβ, but p56lck has been reported to associate with CD48 (22, 24). This in turn suggests that CD48 is an integral component of the signal-transducing IL-18 complex. Because it is known that the IL-18-IL-18R complex binds to IL-18Rβ (21), it is likely that after IL-18Rα, IL-18, and CD48 form a complex on the cell surface, this complex immediately binds to IL-18Rβ, as presented in the model shown in Fig. 9, and that this leads to signal transduction and IFN-γ production. This is the first report showing that a GPI anchor glycan of CD48 may actually be essential for the delivery of the IL-18 signal.

We found that IL-18 binds to the third mannose 6-phosphate diester, whereas IL-18Rα binds to the second exposed mannose 6-phosphate of GPI glycan. On the other hand, >90% of CD48 does not have GlcNAcβ1→phosphate→6 mannose residue (Fig. 8), although GlcNAcβ1→phosphate residue is a common epitope of GPI glycan (11). Because a β-N-acetylglucosamine residue of the GlcNAcβ1→phosphate→6 mannose residue must be removed to be recognized by IL-18Rα, the β-N-acetylglucosamine residue that protects the exposure of the second mannose 6-phosphate diester in the GPI anchor glycan may regulate the IL-18-dependent immune response. As a result, we are currently determining whether a GlcNAcβ1→phosphate-specific β-N-acetylglucosaminidase is secreted together with IL-18 by stimulated macrophages.

The inhibitory effect of mannose 6-phosphate on the IL-18-stimulated production of IFN-γ by KG-1 cells was very weak (its 50% inhibitory concentration was 10⁻⁵ M). However, we could not use more than 10⁻⁵ M mannose 6-phosphate because it changed the pH of the medium. It is likely that mannose 6-phosphate does not efficiently inhibit the recognition by IL-18 or IL-18Rα of the endogenous GPI anchor glycan in CD48 because the endogenous GPI anchor glycan is a rather strong ligand compared with exogenous mannose 6-phosphate.

Although both tumor necrosis factor-α and IL-18 recognize the GPI anchor glycan, they bind to different sites because tumor necrosis factor-α binds the second mannose 6-phosphate diester (5), whereas IL-18 binds the third mannose 6-phosphate diester. These observations indicate that different cytokines may have precise and unique carbohydrate binding specificities. IL-1β and IL-18 have moderate sequence similarity (7), and the receptors for IL-1β and IL-18 belong to the IL-1 receptor family (25). The intracellular signaling pathways of IL-1β and IL-18 also share the same downstream mediators (3). Because IL-1β also recognizes GPI anchor glycans (6), we are now investigating whether the mechanism by which IL-1β recognizes the GPI anchor glycan is equivalent to that of IL-18.

GPI-anchored proteins are widely distributed on the cell surface (26). GPI anchor glycans are essential for embryogenesis and skin development in mice (27), and GPI deficiencies cause paroxysmal nocturnal hemoglobinuria in humans (28). Moreover, GPI-anchored proteins are receptors for bacterial toxins, clostridial α-toxin (29), aerolysin (30), and the plant toxin enterolobin (31). Here we show a novel putative function of the GPI anchor glycan of CD48, namely, as an immunomodulator of the response to IL-18. Our future studies will analyze...
this potential immunomodulatory function of the GPI anchor glycan.

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