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# Identification of a Critical Ig-Like Domain in IL-18 Receptor $\alpha$ and Characterization of a Functional IL-18 Receptor Complex<sup>1</sup>

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Steady state mRNA levels in various human tissues reveal that the proinflammatory cytokine IL-18 is constitutively and ubiquitously expressed. However, limited IL-18R  $\alpha$ -chain (IL-18R $\alpha$ ) expression in tissues may restrict ligand-acting sites and contribute to a specific response for IL-18. To study the IL-18R complex, [<sup>125</sup>I]IL-18 was studied for binding to the cell surface receptors of IL-18-responsive NK and macrophagic KG-1 cells. After cross-linking, [<sup>125</sup>I]IL-18 formed three IL-18R complexes with sizes of approximately 93, 160, and 220 kDa. In KG-1 cells, Scatchard analysis revealed the presence of 135 binding sites/cell, with an apparent dissociation constant ( $K_d$ ) of 250 pM; in NK cells, there were 350 binding sites per cell with an apparent  $K_d$  of 146 pM. Each domain of extracellular IL-18R $\alpha$  was cloned and individually expressed in *Escherichia coli*. An mAb specifically recognized the membrane-proximal third domain; this mAb blocked IL-18-induced IFN- $\gamma$  production in NK cells. Furthermore, deletion of the membrane-proximal third domain of IL-18R $\alpha$  prevented the formation of IL-18R ternary complex with IL-18R  $\beta$ -chain. The present studies demonstrate that the biologically active IL-18R complex requires the membrane-proximal third Ig-like domain in IL-18R $\alpha$  for the formation of IL-18R ternary complex as well as for signal transduction involved in IL-18-induced IFN- $\gamma$  in NK cells. *The Journal of Immunology*, 2003, 171: 6574–6580.

Interleukin-18 is a member of the IL-1 family and shares the common structure of IL-1 family members (1–3). IL-18 also shares cell signaling with IL-1 family members, recruiting IL-1R-associated kinases, the formation of the IL-1R-associated kinase complexes with the TNF receptor-associated factor-6, and activation of the cascade of I $\kappa$ B $\alpha$ /NF- $\kappa$ B (4–6). Like most members of the IL-1 family, IL-18 lacks a signal peptide. Recently, several new members of the IL-1 family were identified by sequence homology, some of which have a determined biological function, whereas others remain without a known activity (7–9). One of the members, IL-1H4, was found to bind IL-18R $\alpha$ , but its biological function has yet to be determined (7, 10, 11).

Most cytokines are transiently expressed shortly after exposure of the host to pathogens or injury. IL-18, unlike other cytokines, is constitutively expressed and pre-exists as a precursor molecule before becoming an active molecule. Both caspase-1-dependent (12–15) and caspase-1-independent (processing through Fas ligand stimulation) (16) result in activating intrinsic IL-18 pathways.

In general, cytokines have at least two receptor chains that collaborate during ligand-induced signaling. IL-18 has two known receptor chains, a ligand-binding IL-18R  $\alpha$ -chain and a signal-transducing IL-18R  $\beta$ -chain. Both chains of the IL-18R belong to

the IL-1R family and consist of three Ig-like domains in the extracellular region (17–19). IL-18-binding protein (IL-18BP)<sup>3</sup> is not a part of the IL-18 signaling complex, but, rather, antagonizes IL-18 activity (2, 20). It is a unique, secreted receptor-like molecule and consists of a single Ig-like domain. IL-18BP shares a significant homology with poxvirus proteins and a limited homology with the IL-1R family. Viral IL-18BP, like mammalian IL-18BP, neutralizes the biological activities of human IL-18 (21). Human IL-18BP is secreted constitutively in healthy subjects, with circulating concentrations of 2–5 ng/ml (22). IFN- $\gamma$  increases gene expression for IL-18BP in renal mesangial cells, which functions as a feedback loop and reduces IL-18-induced IFN- $\gamma$  (23).

An IL-18R $\alpha$  polymorphism has been identified (24). This polymorphism is generated by alternative splicing of the coding region of IL-18R $\alpha$  rather than by a genomic mutation. The deletion of three bases at position +950 to +952 (CAG) in the IL-18R $\alpha$  cDNA results in the deletion of alanine 317 in the membrane-proximal third domain of IL-18R $\alpha$ . The deletion of alanine 317 reduces IL-18-induced IFN- $\gamma$  production in PBMC of affected individuals (24). Xu et al. (25) reported that an Ab directed against the peptide corresponding to residues 247–266 of murine IL-18R $\alpha$  inhibited IL-18-induced IFN- $\gamma$  in Th1 cells, and this Ab also reduced LPS-induced mortality in mice.

Although IL-18 is studied to reveal its broad biological effects, regulation, and structure, little is known about its functional receptor complex. The present study identifies the critical domain of IL-18R $\alpha$  for IL-18-induced IFN- $\gamma$  as well as characterizes the biologically active IL-18R complex of IL-18-responsive cells.

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<sup>3</sup> Abbreviations used in this paper: IL-18BP, IL-18-binding protein; DSS, disuccinimidyl suberate; ECD, extracellular domain; His<sup>6</sup>, six histidine; ICE, IL-1 $\beta$ -converting enzyme; IL-1Ra, IL-1R antagonist; IL-18R $\alpha$ -ECD- $\Delta$ D3, soluble IL-18R $\alpha$ -deleted membrane-proximal third domain; IL-18R $\alpha$ - $\Delta$ D3, transmembrane IL-18R $\alpha$ -deleted membrane-proximal third domain.



then washed with DMEM/10% FBS, plated in DMEM/10% FBS for 4 h, washed with serum-free DMEM, and incubated for 4 days in serum-free DMEM. Culture supernatants were concentrated 20-fold in boiled dialysis bags submerged in saturated polyethylene glycol (8 kDa), followed by dialysis in Talon buffer. The concentrated supernatants were applied to a Talon column, and the His<sup>6</sup> tag recombinant proteins were eluted with imidazole buffer according to the manufacturer's instructions. The different IL-18R recombinant proteins were resolved by 10% reducing SDS-PAGE, followed by Western analysis.

#### Luciferase assay

The transmembrane IL-18R $\alpha$  or IL-18R $\alpha$ - $\Delta$ D3 was transiently transfected into A549 cells in the presence of IL-18R $\beta$  and pTK-NF $\kappa$ B-Luc (26) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The transiently transfected cells were stimulated with IL-18 (50 ng/ml) for 18 h, then supernatant and cells were harvested separately for IL-8 and luciferase assays, respectively. The cell pellet was lysed with the cell lysis buffer from luciferase report kit (Promega), then samples were read with Luminescence (monolight 2010 Luminometer; Analytical Luminescence Laboratories, San Diego, CA).

#### RT-PCR

The transiently transfected A549 cells were examined for different IL-18R constructs expression. Total RNA was isolated with Tri-Reagent (Sigma-Aldrich, St. Louis, MO), and the cells were treated as described for the luciferase and IL-8 assays. The first-strand cDNA was synthesized with SuperScript II (Invitrogen), then PCR was performed with following primers: T7 (sense) for IL-18R $\alpha$  and IL-18R $\beta$ , TAATACGACTCACTATAG GGCGAATTC; IL-18R $\alpha$  (reverse), TAGGACAATGATTAGTCTTCGGC; IL-18R $\beta$  (reverse), TCCTGAATGATGTGAGGATAGC; GAPDH (sense), ACCACAGTCCATGCCATCAC; and GAPDH (reverse), TCCACCAC CCTGTTGCTGTA. PCR reaction was performed at 94°C for 45 s, 70°C for 2 min, and 59°C for 1 min for 30 cycles.

#### Analysis of cytokines

The liquid phase electrochemiluminescence method was used to measure IFN- $\gamma$  and IL-8 (30) in cell culture medium. The amount of electrochemiluminescence was determined using an Origen analyzer (Igen, Gaithersburg, MD).

#### Radioiodination of human IL-18

Mature human IL-18 (10  $\mu$ g) in borate buffer was radiolabeled with <sup>125</sup>I-labeled Bolton-Hunter reagent (NEN Life Science Products) according to the manufacturer's recommendations. The reaction was stopped with an excess of glycine. Unbound <sup>125</sup>I was removed by chromatography on Sephadex G-25 equilibrated in PBS containing 0.25% gelatin and 0.02% sodium azide. Each fraction was counted, and the peak fractions were pooled. The pooled radiolabeled [<sup>125</sup>I]IL-18 contained  $\sim 4.2 \times 10^7$  cpm/ $\mu$ g and was used for competition binding assays and cell surface receptor cross-linking.

#### Binding of [<sup>125</sup>I]IL-18 to human KG-1 and NK cells

Human KG-1 and NK cells ( $1 \times 10^6$ , 0.25 ml) were suspended in binding solution (cell culture medium containing 2% FBS and 0.02% sodium azide) containing increasing concentrations of [<sup>125</sup>I]IL-18. Eppendorf tubes were mixed occasionally for 1 h at 4°C. The mixtures were then washed three times at 4°C with the binding solution, and cell-bound [<sup>125</sup>I]IL-18 was determined using a gamma counter. The affinity constants and the number of receptors were calculated using Scatchard analysis (31).

#### Cross-linking and immunoprecipitation of intrinsic IL-18R complexes with NK cells

For chemical cross-linking, cells ( $0.6\text{--}2 \times 10^7$ ) were incubated with [<sup>125</sup>I]IL-18 ( $4.5 \times 10^6$  cpm/125 ng) for 1 h at 4°C in the presence or the absence of a 100-fold molar excess of IL-18 or IL-18BP, respectively. DSS was added for a final concentration of 1 mM and placed on ice for 20 min. After chemical cross-linking, the cells were washed twice in PBS, and then membrane proteins were prepared. Briefly, 0.5 ml of PBS was added to each cell pellet and then homogenized on ice. The homogenates were centrifuged for 10 min at  $500 \times g$ . The supernatants were transferred into new Eppendorf tubes and centrifuged for 15 min at 14,000 rpm. For immunoprecipitation of IL-18R complexes, [<sup>125</sup>I]IL-18 was cross-linked and processed in same manner as described above. The membrane pellet was lysed with 1% Triton X-100 for 30 min, then incubated with biotinylated goat anti-IL-18 (3  $\mu$ g/ml) for 2 h at 4°C. Immunoprecipitation was performed with avidin-coated magnetic beads (Igen) by centrifugation. The resulting

pellets containing membrane proteins and immunoprecipitated IL-18R complex were analyzed by 10% SDS-PAGE under reducing conditions and autoradiographed.

#### Cross-linking of different IL-18R $\alpha$ domains with IL-18R $\beta$

For chemical cross-linking, different domains of IL-18R $\alpha$ -ECDs expressed in COS-7 were preincubated alone, in the presence of IL-18, or in the presence of IL-18 plus IL-18R $\beta$ -ECD for 1 h at room temperature. Then DSS was added for the final concentration of 1 mM, followed by 20 min of incubation at room temperature. The individual IL-18R-ECDs (150 ng in each lane) were analyzed by 10% SDS-PAGE under reducing conditions, followed by Western blotting with anti-His<sup>6</sup> tag mAb.

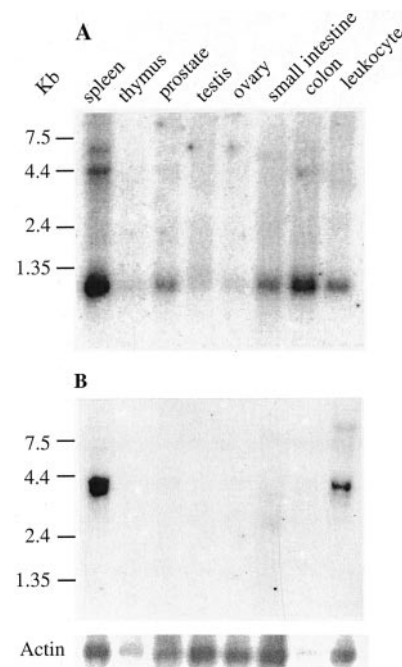
#### Western blot analysis of E. coli IL-18R $\alpha$ individual domains

Each of the six individual IL-18R $\alpha$  domains was resolved by 10% SDS-PAGE under reducing conditions. Gels were transferred to nitrocellulose membranes and then probed with the primary mAb anti-His<sup>6</sup> tag. After 24 h, goat anti-mouse IgG peroxidase was added, and the blot was developed using ECL.

## Results

#### Expression of IL-18 and IL-18R $\alpha$ in different human tissues

The expression of human IL-18 and IL-18R $\alpha$  was determined using MTN Blot II (Clontech, Palo Alto, CA) containing resolved poly(A)<sup>+</sup> RNA from different human tissues. Both IL-18 and IL-18R $\alpha$  mRNA were highly expressed in the spleen, similar to IL-18BP expression patterns (20). Analysis of the IL-18 Northern blot showed a major 1.3-kb transcript band in several human tissues, whereas high molecular mass transcript bands between 4.4–7.5 kb were observed only in the spleen (Fig. 1A, lane 1). A moderate level of IL-18 mRNA expression was observed in prostate, small intestine, colon, as well as leukocytes; however, thymus, testis, and ovary showed only weak expression (Fig. 1A). In contrast, a high intensity 4.2-kb mRNA transcript for IL-18R $\alpha$  was seen primarily in spleen and leukocytes. The 4.2-kb mRNA transcript was barely detectable in other tissues (Fig. 1B). A high molecular mass transcript of IL-18R $\alpha$  mRNA ( $>7.5$  kb) was seen only in leukocytes



**FIGURE 1.** Expression of IL-18 and IL-18R $\alpha$  mRNA in human tissues. Equal amounts of poly(A)<sup>+</sup> RNA containing human MTN blot II was probed with <sup>32</sup>P-radiolabeled human IL-18 and IL-18R $\alpha$  cDNA. Each lane is labeled for different tissues. The numbers on the leftmost lane indicate the size of mRNA. A, IL-18; B, IL-18R $\alpha$ .

(Fig. 1B). The actin probe revealed that the different degrees of IL-18 and IL-18R $\alpha$  expression are not due to the amount of mRNA in each lane.

#### Scatchard analysis of [ $^{125}$ I]IL-18 binding to both KG-1 and NK cells

IL-18R complex was studied in KG-1 and NK cell lines. Table I depicts the results of Scatchard analysis in KG-1 and NK cells. KG-1 cells showed specific binding and a dissociation constant ( $K_d$ ) of  $2.5 \times 10^{-10}$  (250 pM) with 135 binding sites/cell. The binding of [ $^{125}$ I]IL-18 to NK cells yielded a  $K_d$  of  $1.46 \times 10^{-10}$  (146 pM) with 350 binding sites/cell (Table I).

#### Characterization of a functional IL-18R complex in NK cells

A functional IL-18R complex was characterized in NK cells with the aid of chemical cross-linking. The [ $^{125}$ I]IL-18 was bound to NK cells ( $5 \times 10^6$ ), followed by cross-linking with the divalent lysine-directed cross-linker, DSS. Cross-linking products were subjected to SDS-PAGE. The membrane protein fraction yielded complexes with molecular masses of  $\sim 93$  (R3), 160 (R2), and 220 kDa (R1; Fig. 2A). The R3 complex may consist of [ $^{125}$ I]IL-18 and IL-18R $\alpha$ , and the R2 complex probably contains [ $^{125}$ I]IL-18, IL-18R $\alpha$ , and IL-18R $\beta$ , which is the functional complex. The R1 band most likely consists of a multicomplex consisting of more than one ternary complex or participation of an unknown IL-18R component.

We next examined the specificity of the three bands using a 100-fold molar excess of nonradiolabeled IL-18 or IL-18BP added before incubation with [ $^{125}$ I]IL-18. These bands were completely abolished in the presence of the nonradiolabeled IL-18 (Fig. 2A, lane 3) or IL-18BP (Fig. 2A, lane 4). The unbound [ $^{125}$ I]IL-18, as found in the cell-free supernatant, was examined in the next three lanes. The [ $^{125}$ I]IL-18 appears to be a dimer rather than a monomer, and this dimer (Fig. 2A, lanes 5 and 6) was absent when cross-linking was performed in the presence of the nonradiolabeled IL-18; lane 7, the supernatant (10  $\mu$ l) of [ $^{125}$ I]IL-18 after cross-linking in the presence of a 100-fold molar excess of nonradiolabeled IL-18BP. [ $^{125}$ I]IL-18 and nonradiolabeled IL-18BP form a complex between 45–69 kDa (Fig. 2A, lane 7).

We next studied the IL-18R complex using an anti-IL-18 Ab. In the immunoprecipitation of IL-18R complex, anti-IL-18 Ab precipitated R1 and R3 complexes, but not R2. This may be due to the possibility that IL-18 epitopes are masked in the complex containing both IL-18Rs (Fig. 2B).

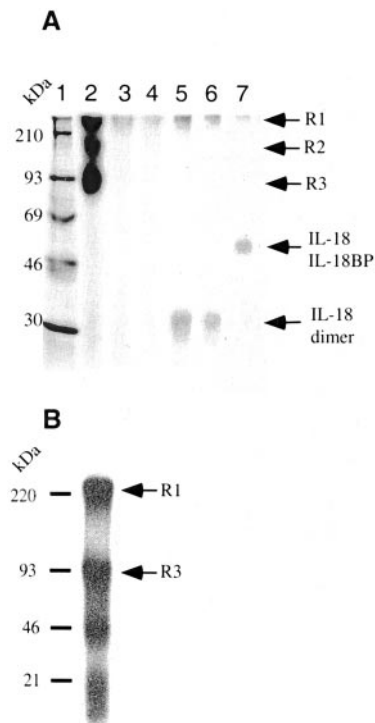
#### The third domain of IL-18R $\alpha$ is important for IL-18 activity

IL-18R $\alpha$ -ECD was divided into three domains according to homology between IL-1RI and IL-18R $\alpha$ . Determination of the three domains of IL-1RI-ECD was identified by x-ray crystallography (17, 18). In the present study individual IL-18R $\alpha$ -ECDs were expressed separately in *E. coli* as described in *Materials and Methods* (Fig. 3A). An equal amount of each IL-18R $\alpha$ -ECD was resolved on SDS-PAGE under reducing conditions and probed with an anti-IL-18R $\alpha$  mAb (Fig. 3B). The anti-IL-18R $\alpha$  mAb specifically recognized the third domain of IL-18R $\alpha$ -ECD (Fig. 3B, lanes 3, 5,

Table I. Scatchard analysis of specific binding of IL-18 to KG-1 and NK cells<sup>a</sup>

Cell Lines	$K_d$	Affinity (pM)	Binding Sites/Cell
KG-1	$2.5 \times 10^{-10}$	250	135
NK	$1.46 \times 10^{-10}$	146	350

<sup>a</sup> KG-1 and NK cells were incubated for 1 h at 4°C with increasing concentrations of [ $^{125}$ I]IL-18 as described in *Materials and Methods*.

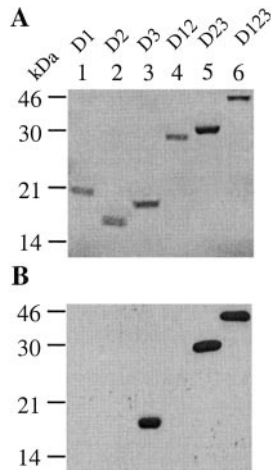


**FIGURE 2.** Analysis of IL-18R complex with NK cells. **A**, A 100-fold molar excess of nonradiolabeled IL-18 or IL-18BP was preincubated for 20 min before adding [ $^{125}$ I]IL-18 (2 nM). Lane 1, Molecular mass makers; lane 2, [ $^{125}$ I]IL-18 was cross-linked to membrane proteins; lane 3, [ $^{125}$ I]IL-18 was cross-linked to membrane proteins in the presence of a 100-fold molar excess of nonradiolabeled IL-18; lane 4, [ $^{125}$ I]IL-18 was cross-linked to membrane proteins in the presence of a 100-fold molar excess of nonradiolabeled IL-18BP; lane 5, the supernatant (10  $\mu$ l) of [ $^{125}$ I]IL-18 after cross-linking; lane 6, the supernatant (10  $\mu$ l) of [ $^{125}$ I]IL-18 after cross-linking in the presence of a 100-fold molar excess of nonradiolabeled IL-18; lane 7, the supernatant (10  $\mu$ l) of [ $^{125}$ I]IL-18 after cross-linking in the presence of a 100-fold molar excess of nonradiolabeled IL-18BP. **B**, The immunoprecipitation of IL-18R complexes by anti-IL-18 as described in *Materials and Methods*. Samples were directly subjected to 10% SDS-PAGE under reducing conditions, and the gel was dried and autoradiographed.

and 6). The same anti-IL-18R $\alpha$  mAb was used in the biological assay of IL-18 on NK cells. The third domain-specific anti-IL-18R $\alpha$  mAb neutralized IL-18-induced IFN- $\gamma$  in a dose-dependent manner (Fig. 4).

#### Lack of the third Ig-like domain in IL-18R $\alpha$ prevents formation of the ternary complex of IL-18 with its receptors

IL-18R $\alpha$  and IL-18R $\beta$  require glycosylation in the extracellular segment, and this glycosylation is important for IL-18 binding to its receptors, whereas *E. coli*-expressed IL-18R is not appropriate for IL-18 binding (data not shown). Therefore, the entire IL-18R $\alpha$ -ECD, a third-domain deletion (IL-18R $\alpha$ -ECD- $\Delta$ D3), and the entire IL-18R $\beta$ -ECD were transiently expressed in COS-7 cells for glycosylation of IL-18R-ECDs. Each IL-18R-ECD was fused with His<sup>6</sup> tag at the C terminus. An anti-His<sup>6</sup> tag mAb detected the expression of glycosylated IL-18R-ECDs (Fig. 5). Chemical cross-linking and Western blot showed COS-7 IL-18R-ECDs in the presence of DSS only (Fig. 6, lanes 1–3). IL-18 binds to IL-18R $\alpha$ -ECD, but not to IL-18R $\alpha$ -ECD- $\Delta$ D3 or IL-18R $\beta$ -ECD (lanes 4–6). A ternary complex was only observed in the presence of the complete IL-18R $\alpha$ -ECD and IL-18R $\beta$ -ECD (lane 8), but not in the presence of IL-18R $\alpha$ -ECD- $\Delta$ D3 and IL-18R $\beta$ -ECD (lane 7).



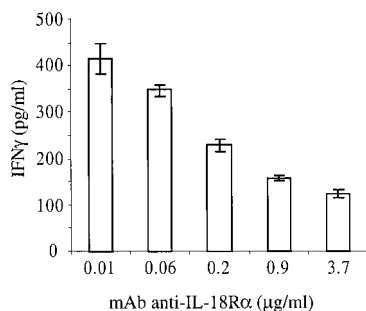
**FIGURE 3.** Affinity-purified separate IL-18R $\alpha$ -ECDs. *A*, Coomassie Blue stain; *B*, Western blot with a neutralizing anti-IL-18 mAb. The numbers in the left lane indicate molecular masses. Each lane is indicated by domain 1 (D1), domain 2 (D2), domain 3 (D3), domains 1 and 2 (D12), domains 2 and 3 (D23), and the entire ECD (D123) in both *A* and *B*.

#### Deletion of the third Ig-like domain in transmembrane IL-18R $\alpha$ loses its function

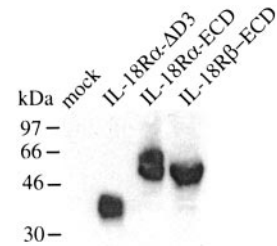
The third Ig-like domain-deleted transmembrane IL-18R $\alpha$  (IL-18R $\alpha$ - $\Delta$ D3) was created by two-step PCR as described in *Materials and Methods*. The RT-PCR data exhibit transient expression of IL-18R $\alpha$  with IL-18R $\beta$  or IL-18R $\alpha$ - $\Delta$ D3 with IL-18R $\beta$ , but not in mock transfection (Fig. 7*A*). The difference between IL-18R $\alpha$  and IL-18R $\alpha$ - $\Delta$ D3 was shown in the upper panel that is 350 bp for the 117 aa of the third domain of IL-18R $\alpha$ . The same transfectants were stimulated with human IL-18 and measured for NF- $\kappa$ B-Luc or IL-8 induction. As shown in Fig. 7, *B* and *C*, IL-18R $\alpha$  with IL-18R $\beta$  induced NF $\kappa$ B-Luc and IL-8 at 3-fold that of the mock transfection with NF $\kappa$ B-Luc alone. IL-18R $\alpha$ - $\Delta$ D3 with IL-18R $\beta$  failed to induce NF $\kappa$ B-Luc and IL-8, although IL-18R $\alpha$ - $\Delta$ D3 expression was as great as with IL-18R $\alpha$ .

#### Discussion

These studies characterize the functional receptor complex for IL-18 activity using cell lines responding to IL-18. We also identified a critical domain of IL-18R $\alpha$  for IL-18-induced IFN- $\gamma$  and compared the expression of steady state IL-18 and IL-18R $\alpha$  mRNA in different human tissues. The limited expression of IL-18R $\alpha$  presumably controls the activity of constitutively expressed



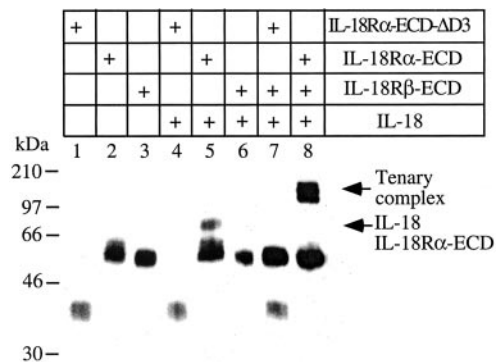
**FIGURE 4.** The effect of mAb anti-IL-18R $\alpha$  on IL-18-induced IFN- $\gamma$  in human NK cells. The mAb anti-IL-18R $\alpha$  neutralizes IL-18 (20 ng/ml)-induced IFN- $\gamma$  in the presence of IL-12 (0.5 ng/ml) on human NK cells. The horizontal axis indicates the concentration of mAb anti-IL-18R $\alpha$ . The data represent the mean  $\pm$  SEM of three separate experiments.



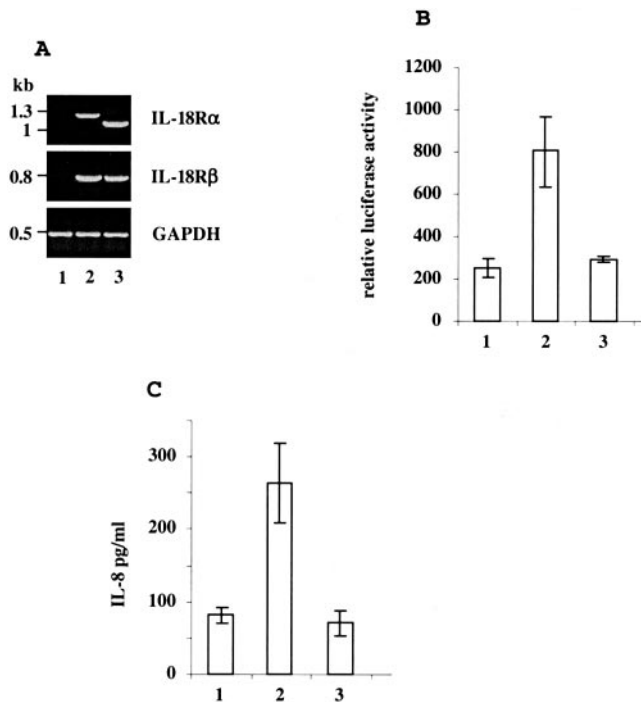
**FIGURE 5.** Western blot of separate domains of glycosylated IL-18Rs. Individual domains of IL-18R-ECDs were expressed and purified over a Talon column and then subjected to 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose. The membrane was probed with anti-His<sup>6</sup> tag mAb.

IL-18. Another participant in the control of IL-18 activity is IL-18BP (20, 22). Unlike other cytokines, cells contain pre-existing mRNA and/or protein levels of the precursor IL-18. IL-18 exists in a pro form awaiting cleavage for activation and therefore is likely to participate in the immediate host defense against infection or bodily injury. IL-18 mRNA is expressed in moderate levels in various tissues (Fig. 1*A*). However, IL-18R $\alpha$  mRNA expression is limited in spleen and leukocytes, distinct from IL-18 mRNA expression patterns (Fig. 1*B*).

Most cytokine receptors are heterodimeric with ligand-binding and signal-transducing chains that cooperate in ligand-induced signal transduction. This is unlike growth factors, which have a homodimeric conformation, or the TNF receptor, which has a trimeric conformation. To date, IL-18 has the typical cytokine receptor components; IL-18R $\alpha$  is the ligand-binding chain (32), and IL-18R $\beta$  is the signal-transducing chain (33). Usually cytokine receptors are present at low levels on cell surfaces, although these same cells are highly responsive to the ligand, as is the case for IL-1RI for IL-1 $\beta$  (34). A biological response occurs when only 2–3% of IL-1RI is occupied by the ligand (34, 35). IL-18 shares this common characteristic of cytokine receptor biology. The Scatchard analysis revealed that KG-1 cells possess 135 receptors/cell and that NK cells possess 350 receptors/cell (Table I). The affinity of IL-18-responsive cell lines (146–250 pM) is comparable to that found for IL-18BP (2), and both are 100-fold higher than the affinity of L428 cells, which may have a single receptor component, IL-18R $\alpha$  (32).



**FIGURE 6.** Western blot of IL-18R ternary complex. SDS-PAGE of IL-18 cross-linked to different receptor domains probed with mAb to anti-His<sup>6</sup> tag. Lanes 1–3 contain the different domains alone. Lanes 4–6 are the different domains in the presence of IL-18. Lanes 7 and 8 are the different domains in the presence of both IL-18 and the IL-18R $\beta$ -ECD.



**FIGURE 7.** Deletion of the third Ig-like domain in transmembrane IL-18R $\alpha$ . A549 human lung carcinoma cells were transiently transfected with various constructs. Lane 1, pTK-NF $\kappa$ B-Luc with pTARGET (Mock); lane 2, pTARGET-IL-18R $\alpha$  with pTARGET-IL-18R $\beta$  in the presence of pTK-NF $\kappa$ B-Luc; lane 3, pTARGET-IL-18R $\alpha$ - $\Delta$ D3 with pTARGET-IL-18R $\beta$  in the presence of pTK-NF $\kappa$ B-Luc. A, RT-PCR analysis of transiently transfected A549 cell line. The data represent one of three separate experiments. B, The relative induction of NF $\kappa$ B-Luc after stimulation with IL-18 (50 ng/ml) for 18 h. C, The induction of IL-8 in cell culture supernatant. The data in B and C represent the mean  $\pm$  SEM of three separate experiments.

Chemical cross-linking of [ $^{125}$ I]IL-18 using the biologically responsive NK cells revealed IL-18R complexes of the whole membrane. These complexes were also revealed using immunoprecipitation with anti-IL-18 Ab. The major complex band was 93 kDa (R3) and consisted of [ $^{125}$ I]IL-18 and IL-18R $\alpha$ . The band at 160 kDa (R2) is probably the functional complex comprised of [ $^{125}$ I]IL-18, IL-18R $\alpha$ , and IL-18R $\beta$ . This 160-kDa (R2) complex was not observed in COS cells (32), and an affinity-purified polyclonal anti-IL-18 failed to precipitate R2 complex. The failure of R2 complex immunoprecipitation may due to masking IL-18 epitopes in the complex containing both IL-18Rs. Although most cell lines will bind the IL-18 to form the 95-kDa (R3) complex, they do not respond to IL-18 because they lack the IL-18R $\beta$  required for a ternary complex (160 kDa; R2). The finding of a molecular mass complex of 220 kDa (R1) may represent a multimeric formation with more than one of each IL-18R component, or possibly this complex contains an unknown receptor component.

The determination of the interaction of IL-1 $\beta$  with the three Ig-like domains in IL-1RI-ECD was resolved by x-ray crystallography (17, 18). The x-ray crystallography of the complex of IL-1 $\beta$  or IL-1Ra with IL-1RI-ECD illustrated the differences in their interactions. The crystal structure revealed that IL-1RI-ECD consists of three Ig-like domains, which wrap around IL-1 $\beta$  in a manner similar to those of other cytokine receptor complexes. There are two receptor binding regions on IL-1 $\beta$  identified by site-directed mutagenesis, both making contact with the receptor: one binds to the first two domains of the receptor, whereas the other binds exclusively to the third domain (18). Unlike IL-1 $\beta$ , IL-1Ra interacts

solely with the first and second domains of IL-1RI, but not with the third domain.

In the present study the third domain-specific anti-IL-18R $\alpha$  neutralized IL-18-induced IFN- $\gamma$  in a dose-dependent manner. This third Ig-like domain in IL-18R $\alpha$  is essential for IL-18 binding and formation of the ternary complex. In addition, deletion of third Ig-like domain in soluble IL-18R $\alpha$  abrogated IL-18 binding to IL-18R $\alpha$ , and the formation of ternary complex (Fig. 6) as well as deletion of third Ig-like domain in transmembrane IL-18R $\alpha$  (IL-18R $\alpha$ - $\Delta$ D3) caused loss of function (Fig. 7). Other investigators have reported data that support our findings. Xu et al. (25) have shown that a rabbit anti-mouse IL-18R $\alpha$ <sub>247–266</sub>, a peptide residing in the third domain of mouse IL-18R $\alpha$ , specifically neutralizes mouse IL-18 biological activity in vivo. Thus, the x-ray crystallography of IL-1/IL-1RI complex as well as the human and mouse anti-IL-18R $\alpha$  third-domain-specific Abs support the role of the third domain in the IL-1R family. It is important to note that anti-cytokine receptor Abs can bind to receptors, but do not interrupt the biological activity of cytokines (36, 37). This excludes the possibility of stereographic interruption by epitope-specific Abs.

The consistency of IL-1 $\beta$ - and IL-18-induced signal transduction with a role for the third Ig-like domain in the IL-1R family is an important for understanding the biology of the growing family of IL-1 ligands and receptors.

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