Cloning and expression of interleukin-18 binding protein

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Abstract Interleukin-18 binding protein is a novel glycoprotein that we successfully cloned and expressed. First, murine interleukin-18 binding protein was purified from the sera of mice with endotoxin shock using ligand affinity chromatography. The murine interleukin-18 binding protein cDNA was cloned after RT-PCR using mixed primer pair sequences based on partial murine interleukin-18 binding protein amino acid sequence analysis. Subsequently, human interleukin-18 binding protein cDNA was cloned from cDNA libraries of normal human liver using murine interleukin-18 binding protein cDNA as a probe. Next, we transiently expressed recombinant human and murine interleukin-18 binding proteins in COS-1 cells and purified them from culture supernatants. Both recombinant interleukin-18 binding proteins did not exhibit species specificity and prevented interleukin-18 binding to its receptor. In addition, they inhibited interleukine-18 dependent IFN-γ production from KG-1 cells effectively. These results suggest that the interleukin-18 binding protein may possess interleukine-18 antagonist activity.

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Key words: Interleukin-18 binding protein; Interleukin-18; Interleukin-18 receptor; Complex; Antagonist

1. Introduction

Murine interleukin-18 (mIL-18) is a pleiotropic cytokine consisting of 157 amino acids and was purified from murine liver extracts in 1995 [1]. Human interleukin-18 (hIL-18) was cloned from human liver cDNA libraries in 1996, using mIL-18 cDNA as a probe [2]. The human interleukin-18 receptor (hIL-18R/IL-1Rrp) was next purified and characterized in 1997 [3] and apparently, another IL-18 receptor subunit, AcPL, has been cloned very recently [4].

Originally, mIL-18 was described as an interferon gamma (IFN-γ)-inducing factor (IGIF), which was produced by liver Kuppfer cells. The activity was identified in the sera of mice with an endotoxin shock previously induced by injecting *Proprionibacterium acnes* followed a week later by bacterial lipopolysaccharide [1,5]. Later, the cytokine was also found to be produced by keratinocytes stimulated with a sensitizing agent [6] and was also observed in the sera of humans with haematologic malignancies [7] and autoimmune conditions such as adult onset Still's disease (AOSD) and rheumatoid arthritis [8]. It was also reported that the excessive IL-18 production may be involved in the pathogenesis of insulin dependent diabetes mellitus (IDDM) in the NOD mouse model of the disease [9] and that IL-18 augments Fas dependent natural killer (NK) activity which may also result in autoimmune reactions [10]. These lines of evidence suggested that the excessive production of IL-18 may be the cause of serious chronic inflammatory conditions or that it may exacerbate disease states.

Therefore, we hypothesized that an IL-18 regulatory factor might exist which neutralizes the effects of an excessive and potentially deleterious level of IL-18 in conditions where IL-18 is implicated in disease. We attempted and succeeded in detecting a murine IL-18 binding protein (mIL-18BP) from the sera of mice suffering from endotoxin shock and which contained an excessive level of IL-18 [5].

In this report, we describe the cloning and expression of the cDNA for murine and human IL-18 binding proteins (mIL-18BP and hIL-18BP) and demonstrate some of their biologic properties.

2. Materials and methods

2.1. Cell culture and reagents

The L428 (human Hodgkin's disease) cell line, which constitutively expresses hIL-18R (hIL-1Rrp) [3] and the human myelomonocytic cell line KG-1 were maintained at 37°C with 5% CO_2, in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, BioWhittaker, Walkersville, MD, USA). COS-1 cells were also maintained at 37°C with 5% CO_2, in Hybridoma-SFM medium (Gibco BRL, Grand Island, NY, USA).

The CHO-K1 (Chinese Hamster Ovary) cell line transfected with a mIL-18R (mIL-1Rrp) expression vector plasmid was named CHO-K1/mIL-18R and was maintained at 37°C with 5% CO_2, in Ham's F12 medium (Nissui, Tokyo, Japan) supplemented with 5% heat-inactivated FBS. Recombinant human and murine $[^{125}I]IL-18s$ ($[^{125}I]hIL-18/[^{125}I]mIL-18$) were radioiodinated using the Bolton-Hunter reagent (ICN, Costa Mesa, CA, USA) according to the manufacturer's instructions [11]. Recombinant human IL-1β (R and D System, Minneapolis, MN, USA) and $[^{125}I]hIL-1β$ (Amersham Pharmacia Biotech., Uppsala, Sweden) were obtained commercially.

2.2. Purification of IL-18BP

The sera of mice suffering from endotoxin shock were prepared as described previously [5]. These sera were applied to a Wheat Germ Lectin (WGL) sepharose (Amersham Pharmacia Biotech.) column equilibrated with PBS. After washing with PBS, the crude product was eluted with PBS containing 0.5 M N-acetyl-D-glucosamine. Several eluted fractions (OD at $280 > 0.1$) were pooled and dialyzed against 20 mM PB, pH 7.2. The dialyzed material was then fractionated on a DEAE-5PW (TOSOH, Tokyo, Japan) column with a linear gradient from 0 to 0.5 M NaCl in 20 mM PB. The active fractions were pooled and concentrated using an Ultrafree-15 centrifugal concentrator (Millipore, Bedford, MA, USA). The concentrate was incubated for 2 h at 4°C with biotinylated IL-18 which was prepared from a mixture of rmIL-18 and NHS-Biotin (Bio-Rad, Hercules, CA, USA). In this step, mIL-18BP bound biotinylated IL-18 to form a biotinyl-IL-18/IL-18BP complex. The complex was then introduced into a streptavidin agarose (Calbiochem, San Diego, CA, USA) affinity column and eluted with 5 mM biotin in PBS. Eluted fractions were analyzed by SDS-PAGE.

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2.3. N-terminal amino acid sequencing and peptide mapping

The purified IL-18/IL-18BP complex was electrophoresed on a 7.5–15% gradient gel (Bio-Rad) and the gel was electroblotted onto a PVDF membrane (Pro Blott, Applied Biosystems, Cambridge, UK) for direct N-terminal amino acid sequencing according to the methods of Matudaira et al. [12].

Another similarly electrophoresed gel was stained with CBB for peptide mapping, which was performed by in gel digestion according to the method of Hellman et al. [13], except that the enzymes used were different (first digestion: trypsin (Promega, Madison, WI, USA) and second digestion: pepsin (Sigma, St. Louis, MI, USA)). The blotted membrane and other peptides of interest obtained by in gel digestion were analyzed by protein sequencer 473A (Applied Biosystems).

2.4. IL-18BP cDNA cloning using RT-PCR

Total RNA was isolated from the livers of CD-1 mice suffering from endotoxin shock in accordance with the standard method [14]. The total RNA was reverse-transcribed using random hexamers and amplified by PCR. The PCR was performed using mixed primer pairs that were deduced from partial mIL-18BP amino acid sequences. The sequences of the primers used were: sense primer: 5′-GCN-GTNCCNACNAA-3′, antisense primer: 5′-GTYTTNARNCCRTC-3′.

The partial mIL-18BP cDNA cloned by RT-PCR was subjected to complete nucleotide sequencing using the 3′ and 5′ RACE system (Gibco BRL). The gene-specific sense primer sequences of the 3′RACE system were: gene-specific sense primer: 5′-GATCCTGGA-CAAGTGGCC-3′. The gene-specific antisense primer sequences of the 5′RACE system were: gene-specific antisense primer 1: 5′-TG-CAGGCAGTACAGGACAAGG-3′, gene-specific antisense primer 2: 5′-GTGCTGGGTACTGCTTAGTTG-3′.

Both RACE systems were performed according to the manufacturer's instructions. Similarly, hIL-18BP was cloned from the cDNA libraries of normal human liver, using mIL-18BP cDNA as a probe. All the nucleotide sequences for IL-18BP cDNA obtained were confirmed by the DNA sequencer 373A (Applied Biosystems).

2.5. Northern blot analysis

Northern blot analysis on various normal human tissues was performed using human multiple Northern blot membrane (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Partial fragments of the hIL-18BP and β-actin cDNA coding regions were [^{32}P]-labelled using a Ready To Go DNA labelling kit (-dCTP) (Amersham Pharmacia Biotech.) and [α-^{32}P]dCTP (Amersham Pharmacia Biotech.) as a probe.

2.6. Expression and purification of recombinant human and murine IL-18BPs

IL-18BPs cDNA with a 6xHis tag at the C-terminal end were subcloned into the transient expression vector pcDNAI/Amp (Invitrogen, Carlsbad, CA, USA) and the nucleotide sequences and correct alignments were confirmed by DNA sequencing. For the expression of recombinant IL-18BPs (rIL-18BPs), the constructs were electroporated into COS-1 cells and the transfectants that secreted rIL-18BPs were cultured in serum-free hybridoma-SFM medium (Gibco BRL) for 3 days. The supernatants were used for the purification of rIL-18BP using Ni-NTA affinity column chromatography (Qiagen, Chatworth, CA, USA).

2.7. [^{125}I]IL-18 binding inhibition assay

L428 cells or CHO-K1/mIL-18R cells were suspended in binding medium (RPMI 1640 medium containing 0.1% bovine serum albumin, 0.1% NaN$_3$ and 100 mM HEPES, pH 7.2). The binding reactions were performed on 3×10^6 L428 cells or 5×10^5 CHO-K1/mIL-18R cells, respectively, for 1 h at 4°C in 150 μl of binding medium containing various concentrations of IL-18BP and 1.0 nM [^{125}I]IL-18 with or without 500 nM unlabelled IL-18 as a competitor. After incubation, the reaction mixtures were layered over 200 μl of phthalate oil (dibutylphthalate:dioctylphthalate = 1:1), centrifuged at $6000 \times g$ for 5 min at 15°C and the radioactivity counts of the cell pellets, which represent the amounts of cell-bound [^{125}I]IL-18, were measured using a gamma counter. The binding inhibition rate was calculated by the following equation:

$$\text{Binding inhibition rate (\%)} = \frac{(\text{T.B.C.}) - (\text{S.B.C.})}{(\text{T.B.C.}) - (\text{N.B.C.})} \times 100$$

Where, T.B.C.: total binding count (cpm), N.B.C.: non-specific binding count (cpm), S.B.C.: sample binding count (cpm).

The T.B.C. was measured in the absence of IL-18BP and the N.B.C. was obtained when the cold competitor was added to the mixture.

2.8. Chemical cross-link

rhIL-18BP or rmIL-18BP were added to 8 μl of PBS containing 1.0 nM [^{125}I]hIL-18 or [^{125}I]hIL-1β with or without 500 nM of unlabelled competitive ligand, respectively, and incubated for 1 h at 4°C. After the addition of 1 μl PBS containing 10 mg/ml bis(sulfosuccinimidyl) suberate (BS3: Pierce, Rockford, ILL, USA), the mixtures were incubated for 30 min on ice and the chemical cross-link reaction was terminated with 1 μl of 1 M Tris-HCl (pH 7.2). The reaction mixtures were treated with 2.5% SDS, 10% glycerol and 2 mM DTT and were electrophoresed on a 4–20% SDS-PAGE (Daiichi Chemical, Tokyo, Japan) under reducing conditions. After electrophoresis, the gel was dried and exposed to a X-ray film for 2 days.

2.9. IL-18BP bioassay

The bioactivity of rIL-18BP was assessed by measuring the inhibition of the IFN-γ inducing activity of hIL-18 in KG-1 cells [18]. Briefly, KG-1 cells were seeded at the concentration of $3 \times 10^5/100$ μl/well in a 96 well microplate (Sumitomo Bakuite, Tokyo, Japan). The 100 μl samples, which contain various concentrations of IL-18BP and a constant concentration of hIL-18 (4 ng/ml), were added to the wells and incubated at 37°C for 24 h. RPMI 1640 medium containing 10% FCS was used for the cell preparation and sample dilutions. Then, IFN-γ activity induced in the culture supernatants was assessed by ELISA.

3. Results and discussions

3.1. Purification of mIL-18BP

Primarily, we identified an activity in the sera of mice suffering from endotoxin shock by a [^{125}I]mIL-18 binding inhibition assay and revealed that the activity could be attributed to a protein bound to mIL-18 by chemical cross-linking (data not shown). We termed this protein mIL-18BP. The [^{125}I]mIL-18/mIL-18BP complex was observed to migrate as a 60–70 kDa broad band by chemical cross-linking, which suggested the possibility that mIL-18BP was glycosylated (data not
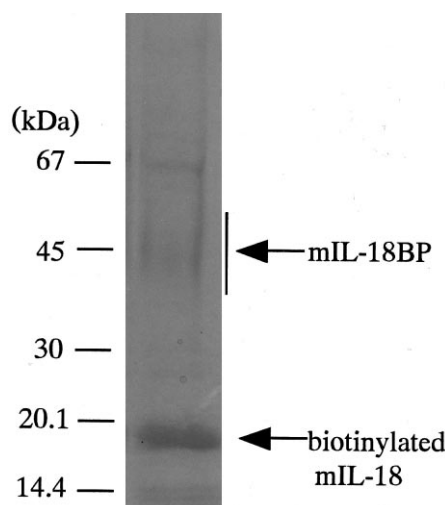

Fig. 1. Analysis of the purified mIL-18BP by SDS-PAGE. The mIL-18BP purified by a streptavidin agarose column elution was electrophoresed on a 7.5–15% gradient polyacrylamide gel under reducing conditions. Subsequently, the gel was stained with CBB. The molecular size is indicated on the left side of the figure (in kDa). The upper arrow shows the mIL-18BP and the lower arrow indicates the biotinylated mIL-18, respectively.
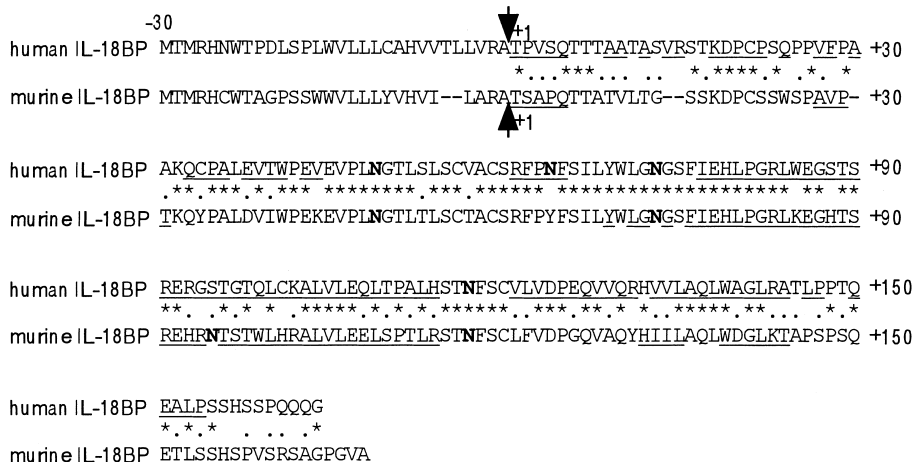
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                   -30                                      ↓+1
human IL-18BP   MTMRHNWTPDLSPLWVLLLCAHVVTLLVRATPVSQTTTAATASVRSTKDPCPSQPPVFPA +30
                                               *...**...  .. *.****.* .*. *
murine IL-18BP  MTMRHCWTAGPSSWWVLLLYVHVI--LARATSAPQTTATVLTG--SSKDPCSSWSPAVP- +30
                                               ↑+1


human IL-18BP   AKQCPALEVTWPEVEVPLNGTLSLSCVACSRFPNFSILYWLGNGSFIEHLPGRLWEGSTS +90
                .**.***.*.*** ********.*** .****** ****************** ** **
murine IL-18BP  TKQYPALDVIWPEKEVPLNGTLTLSCTACSRFPYFSILYWLGNGSFIEHLPGRLKEGHTS +90


human IL-18BP   RERGSTGTQLCKALVLEQLTPALHSTNFSCVLVDPEQVVQRHVVLAQLWAGLRATLPPTQ +150
                **. .*.* * .*****.*.*.*.****** ..***.**.* *...*****.**... .*.*
murine IL-18BP  REHRNTSTWLHRALVLEELSPTLRSTNFSCLFVDPGQVAQYHIILAQLWDGLKTAPSPSQ +150


human IL-18BP   EALPSSHSSPQQQG
                *.*.*  . ... .*
murine IL-18BP  ETLSSHSPVSRSAGPGVA
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Fig. 2. Deduced amino acid sequences of human and murine IL-18BPs. The alignment of amino acid sequences for human and murine IL-18BPs and their homology are shown. Identical amino acid residues are indicated by asterisks (*). Homologous amino acid residues are indicated by periods (.). The N-terminal amino acid sites for human and murine IL-18BPs are indicated by vertical arrows. Potential *N*-glycosylated binding sites are shown in bold characters. Partial amino acid sequences of human and murine IL-18BPs, we purified from human urine and the sera of mice, respectively, are shown underlined.

shown). Therefore, we employed WGL affinity chromatography for the partial purification of mIL-18BP. Separation of sera on a WGL-Sepharose column resulted in a 100-fold purification as estimated by the binding inhibition assay (data not shown). After concentrating mIL-18BP on a DEAE-5PW column, biotinylated mIL-18 was added to the eluate to construct a biotinyl-mIL-18/mIL-18BP complex. Further purification of this complex was performed by streptavidin agarose column chromatography and the purified sample was revealed to produce two major bands, corresponding to biotinylated mIL-18 (20 kDa) and mIL-18BP (40 kDa–55 kDa) by SDS-PAGE (Fig. 1). These results suggested that the purified mIL-18BP (40 kDa–55 kDa) was the same protein that was detected in the murine sera by chemical cross-linking.

3.2. Nucleotide and deduced amino acid sequences of human and murine IL-18BPs

Using RT-PCR cloning, we obtained complete cDNA sequences for human and murine IL-18BPs, which were 585 bp and 582 bp in size, respectively. (DDBJ/EMBL/GenBank nucleotide database, accession numbers: AB019504 (for hIL-18BP) and AB019505 (for mIL-18BP)).

According to the amino acid sequences deduced from their cDNAs, hIL-18BP consists of 164 amino acids with a 30 amino acid signal peptide and four potential *N*-glycosilated sites and, correspondingly, mIL-18BP consists of 165 amino acids with a 28 amino acid signal peptide and four potential *N*-glycosilated sites. There is 60.8% homology between the human and murine IL-18BP amino acid sequences (Fig. 2).

From a hydrophilisity/hydrophobicity plot of IL-18BP, IL-18BP is predicted not to have a transmembrane region.

3.3. Properties of human and murine IL-18BPs

We performed Northern blot analysis using a partial fragment of a hIL-18BP cDNA coding region as a probe. Among the normal human tissues examined, hIL-18BP mRNA was expressed strongly in the heart, lung and placenta, and appeared as a 1.6 kb single band (Fig. 3). According to the data reported previously, hIL-18 mRNA is strongly expressed in skeletal muscle, kidney and pancreas [2] and hIL-18R (hIL-

1Rrp) mRNA is expressed strongly in the lung and liver [3]. The mRNAs for these three factors have different expression patterns and we could not find a clear correlation between these patterns.

Next, using rhIL-18BP and rmIL-18BP, a chemical cross-linking reaction was performed with [^{125}I]hIL-18. The results
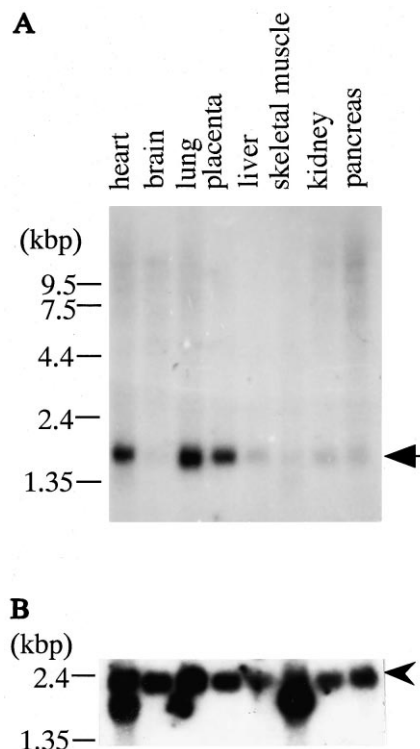

Fig. 3. RNA blot analysis of hIL-18BP mRNA expression. Northern blot analysis of hIL-18BP mRNA in normal human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas was performed using hIL-18BP cDNA as a probe (A). β-Actin was analized similarly for standardization of the blot (B). Each lane contains 2 μg of poly(A)$^+$RNA. The molecular size is indicated on the left side of the figure (in kb). The arrow shows the hIL-18BP mRNA and the arrow head indicates β-actin mRNA, respectively.
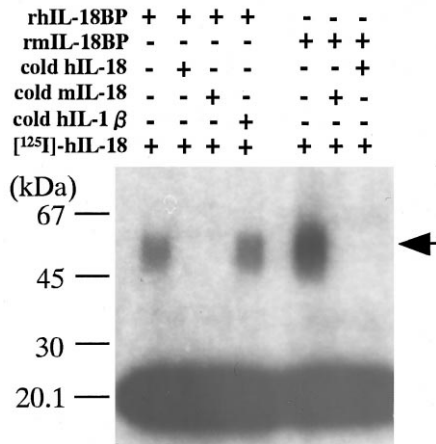
Fig. 4. Analysis of the binding abilities of rIL-18BPs with [^{125}I]hIL-18 by chemical cross-linking. rhIL-18BP or rmIL-18BP were incubated with 1.0 nM of [^{125}I]hIL-18 in the presence or absence of a 500-fold molar excess of unlabelled hIL-18, mIL-18 or hIL-1β. Chemical cross-linking reactions and electrophoresis were performed as described under Section 2. Subsequently, the gel was subjected to autoradiography. The molecular size is indicated on the left side of the figure (in kDa). The arrow shows the position of the hIL-18/rhIL-18BP and hIL-18/rmIL-18BP complexes.

showed that rhIL-18BP and rmIL-18BP singly bound to hIL-18 and mIL-18, respectively, and bound to their inverse ligands and an excess of cold hIL-1β, which was the ligand structurally similar to hIL-18, could not prevent hIL-18BP binding to [^{125}I]hIL-18 (Fig. 4). Similarly, by another chemical cross-linking, we revealed that rIL-18BPs could not bind to [^{125}I]hIL-1β (data not shown).

Furthermore, we also performed a [^{125}I]IL-18 binding inhibition assay and measurement of the IL-18 neutralizing ef-

fect of rIL-18BP using KG-1 cells. The results suggested that rIL-18BP dose dependently prevented the binding of the ligands to their respective receptors (Fig. 5) and inhibited the activity of IL-18 as an inducer of IFN-γ in KG-1 cells (Fig. 6). Therefore, IL-18BP may function as an IL-18 antagonist. These results indicate that IL-18BP may be expected to have the similar function as an anti-IL-18 antibody reported to inhibit IL-18 activity [1,15].

Recently, Novik D. et al. reported on the isolation and purification of a soluble IL-18 receptor present in human urine [16,17]. This soluble human IL-18R was found to be a 38 kDa protein that did not exhibit species specificity. Corre-
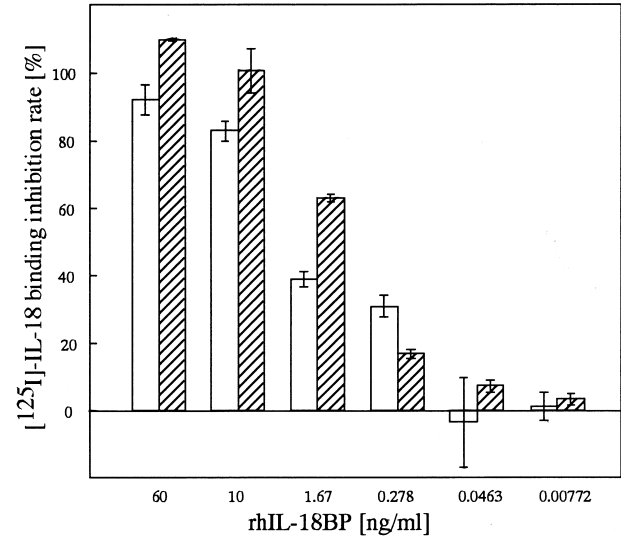

Fig. 5. Binding inhibition activity of rhIL-18BP on IL-18 binding to L428 cells or CHO-K1/mIL-18BP cells. The binding inhibition assay on L428 cells or CHO-K1/mIL-18R cells was performed to estimate the ability of rhIL-18BP to prevent IL-18 binding to its receptor. The Y axis represents the binding inhibition rate as described under Section 2. The X axis represents the concentration of rhIL-18BP employed. The open column denotes the result with L428 cells and the hatched column denotes the result with CHO-K1/mIL-18R cells. Each value is presented as a mean ± S.D. (n = 3).
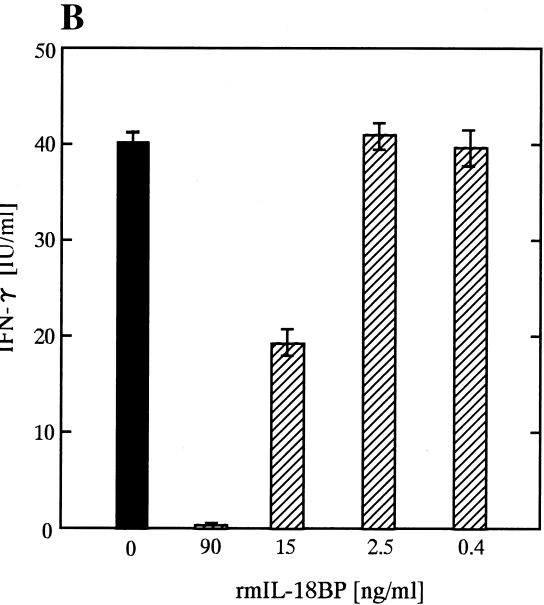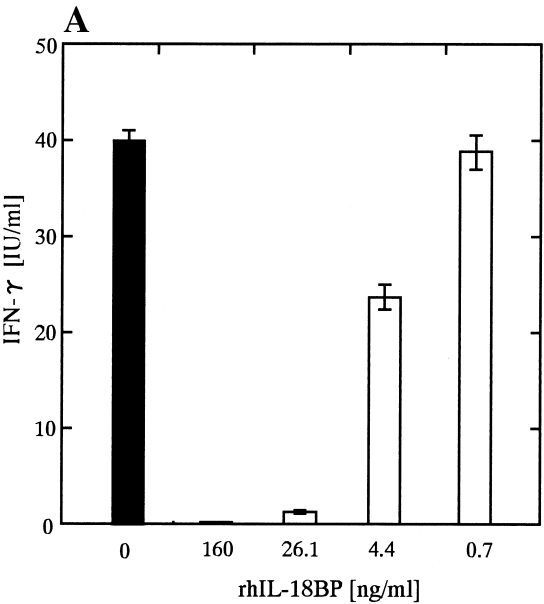

Fig. 6. The hIL-18 neutralizing effect of rIL-18BPs with the assay of IFN-γ production on hIL-18 in KG-1 cells. The Y axis represents the amount of IFN-γ induced from KG-1 cells. The X axis represents the concentrations of rhIL-18BP (A) and rmIL-18BP (B). The closed column denotes the amount of IFN-γ from KG-1 cells without rIL-18BP. Each value is presented as a mean ± S.D. (n = 3).

spondingly to this, we also purified a similar protein from healthy human donor urine by the above mentioned purification methods. By amino acid sequencing, we revealed that the purified protein was the same one as the hIL-18BP we have cloned in this report (Fig. 2). There is a possibility that the soluble IL-18R described by Novik is the same protein as the hIL-18BP we have cloned. However, we could not find any evidence that hIL-18BP had a transmembrane region and we therefore have the opinion that IL-18BP is not a soluble form of the IL-18 receptor currently.

Thus, we have clarified some biologic properties of IL-18BP, but others remain to be revealed. For example, if any correlation exists between IL-18BP expression and diseases as well as a comparison of the IL-18 binding affinities between anti-IL-18 antibody and IL-18BP. Some of these studies are now being performed and revealing these properties will become our future works.

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