Involvement of Caspase-1 and Caspase-3 in the Production and Processing of Mature Human Interleukin 18 in Monocytic THP.1 Cells

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Recently, human interleukin 18 (hIL-18) cDNA was cloned, and the recombinant protein with a tentatively assigned NH2-terminal amino acid sequence was generated. However, natural hIL-18 has not yet been isolated, and its cellular processing is therefore still unclear. To clarify this, we purified natural hIL-18 from the cytosol extract of monocytic THP.1 cells. Natural hIL-18 exhibited a molecular mass of 18.2 kDa, and the NH2-terminal amino acid was Tyr77. Biological activities of the purified protein were identical to those of recombinant hIL-18 with respect to the enhancement of natural killer cell cytotoxicity and interferon-γ production by human peripheral blood mononuclear cells. We also found two precursor hIL-18 (prohIL-18)-processing activities in the cytosol of THP.1 cells. These activities were blocked separately by the caspase inhibitors Ac-YVAD-CHO and Ac-DEVD-CHO. Further analyses of the partially purified enzymes revealed that one is caspase-1, which cleaves prohIL-18 at the Asp35-Tyr site to generate the mature hIL-18, and the other is caspase-3, which cleaves both precursor and mature hIL-18 at Asp71-Ser72 and Asp77-Asn77 to generate biologically inactive products. These results suggest that the production and processing of natural hIL-18 are regulated by two processing enzymes, caspase-1 and caspase-3, in THP.1 cells.

Interleukin (IL)1-18 (originally called IGIF, interferon-γ-inducing factor) is a novel cytokine with multiple biological functions. In 1995 we purified murine IL-18 from the liver extract of mice sensitized with Propionibacterium acnes followed by elicitation with lipopolysaccharide (1). The cDNA of murine IL-18 was cloned from cDNA libraries prepared from the livers of mice with endotoxin shock (2). Using this as a probe, human IL-18 was cloned from cDNA libraries prepared from the livers of mice with endotoxin shock (2). Using this as a probe, human IL-18 cDNA was also cloned from a human normal liver cDNA library (3). The recombinant human IL-18 with a tentatively assigned NH2-terminal amino acid based on its homology with the natural murine IL-18 sequence was expressed in Escherichia coli, and its biological activities were examined (3).

IL-18 has an interleukin 1 (IL-1) signature-like sequence (3) as reported and is similar to the IL-1 family and fibroblast growth factor in terms of their trefoil structures (4, 5). Despite their similarities, IL-18 and IL-1β exhibit different biological activities (2, 3, 6), transmitted through their specific receptors.2 Genetic information suggested that IL-18 is synthesized as an inactive precursor form (prohIL-18) and that this prohIL-18 has no known signal peptide sequence. Therefore, proteolytic cleavage is required for its maturation like IL-1β (2, 3, 7). Gu et al. (7) reported that IL-1β-converting enzyme (ICE/caspase-1-cleaved murine proIL-18 at the authentic processing site, Asp35-Asn36, to generate biologically active mature murine IL-18. However, natural hIL-18 had not yet been isolated, and its maturation site remained unclear.

In this report, we screened for hIL-18 mRNA-expressing cell lines and purified natural hIL-18 from the cell-free extract of positively expressing cells. Furthermore, we identified two hIL-18-processing enzymes in the same cellular extract. One enzyme is ICE/caspase-1, which acts on prohIL-18 to generate the mature active form of hIL-18, and the other is CPP32/caspase-3, which acts on both the prohIL-18 and the mature hIL-18 to generate biologically inactive degraded products. This is the first report on the identification of natural hIL-18 and its processing enzymes existing in the same cells.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies

Tetrapeptidyl ICE-like protease inhibitor Ac-YVAD-CHO and CPP32-like protease inhibitor Ac-DEVD-CHO, and ICE- and CPP32-like protease fluorogenic substrates Ac-YVAD-MCA and Ac-DEVD-MCA, respectively, were purchased from the Peptide Institute (Osaka, Japan). Neutralizing and non-neutralizing anti-hIL-18 murine monoclonal antibodies (mAbs) 125-2H (IgG) and 25-2G (IgG) were raised against the recombinant protein in our laboratory. Human IL-1β precursor and anti-precursor human IL-1β rabbit polyclonal antibody (pAb) were obtained from Cistron (Pine Brook, NJ). Anti-human ICE-p20 subunit, anti-human CPP32 anti-human poly(ADP-ribose)polymerase goat pAbs, and anti-human ICE-p10 subunit rabbit pAb were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Recombinant purified human (His)6-tagged CPP32, (His)6-tagged CMH-1, 2Torigoe, K., Ushio, S., Okura, T., Kobayashi, S., Tanai, M., Kuni-kata, T., Murakami, T., Sanou, O., Kojima, H., Fujii, M., Ohta, T., Ikeda, M., Ikegami, H., and Kurimoto, M. (1997) J. Biol. Chem., in press.

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§ The abbreviations used are: IL, interleukin; pro, precursor; h, human; ICE, interleukin-1β-converting enzyme; mAb, monoclonal antibody; pAb, polyclonal antibody; PCR, polymerase chain reaction; PB, phosphate buffer; IFN, interferon; NK cell, natural killer cell; ICA, human IL-18-converting activity; IDA, human IL-18-degrading activity; hIL-18-CE, human IL-18-converting enzyme; hIL-18-DE, human IL-18-degrading enzyme; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonic acid; DTT, dithiothreitol; Bis-Tris, 2,2-bis(2-hydroxyethyl)aminoo]-2-(2-hydroxyethyl)propane-1,3-diol; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; PBMC, peripheral blood mononuclear cell.
and anti-CMH-1 rabbit antisemur (antibody 25) were prepared at Ver-
tex Pharmaceuticals Inc. (Cambridge, MA).

Cell Culture
42 hematopoic cells were cultured for the screening of hIL-18 mRNA in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Whittaker, Walkersville, MD), 100 units/ml peni-
cillin, and 100 μg/ml streptomycin. Cells were cultured under standard
conditions in a humidified 5% CO2 and air mixture at 37 °C. To obtain
large amounts of THP.1 cells, an in vivo cell propagation method using
immunosuppressed hamsters was applied as described previously (9, 10).
Briefly, 5 × 107 THP.1 cells were transplanted subcutaneously into
newborn hamsters and injected intraperitoneally with 0.1 ml of a rabbit
anti-murine THP.1 cell serum (4 R & D Inc., Woodland, CA). Every 3 or 4
days after the transplantation, these hamsters were given
0.1 ml of the cell intraperitoneally. After 3 weeks, the solid tumor
masses of THP.1 cells were harvested, rinsed thoroughly with RPMI
1640 medium, and small tumor masses were dispersed by scissors and
then passed through stainless steel meshes to obtain a single cell
suspension.

hIL-18 mRNA Screening Using Reverse Transcription-PCR
Total RNAs were isolated from various cell lines by ULTRASPEC-3
(Biotecx, Houston, TX) in accordance with the supplier’s instructions.
The RNAs were reverse transcribed and amplified by PCR. The ther-
mocycle conditions were 30 or 35 cycles of 94 °C for 1 min, 55 °C for 1
min, and 72 °C for 1 min, for denaturation, annealing, and extension,
respectively. The sequences of the primer pair for hIL-18 were 5′-
GCTTGAATCTAAATTATTATCAGTC-3′ and 5′-CAAATTGCATCT-
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buffer. Next, the sample was eluted with 24 bed volumes of an increas-
ing acetonitrile linear gradient (0–90% acetonitrile). The main peak
fraction eluted at about 40% acetonitrile and was used for peptide
mapping of natural hIL-18.

Biological Analysis of Natural hIL-18
INF-γ production assay and NK cell cytotoxicity assay were per-
formed as described previously (3).

Separation of hIL-18-converting Activity (ICA) and
hIL-18-degrading Activity (IDA)
As in the previous procedures, all purification steps were performed
at 4 °C except for DEAE-5PW chromatography, which was carried out
at room temperature.

Step 1: Preparation of a Cell-free Extract of THP.1—
A THP.1 cell-free extract was prepared by hypotonic lysis (11). Briefly, the cell pellets
from 5 × 108 THP.1 cells were washed once with 10 volumes of hypotonic buffer
(20 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 2Na) and were collected by centrifugation at 1,000
g/min. The resulting cell pellets were resuspended in 3 volumes of hypo-
tonic buffer and frozen at

-0.1% trifluoroacetic acid, and the column was washed with the same
buffer.

-0.1 M NaCl at pH 2.5. Eluted
fractions were neutralized immediately with 1.0 M Tris.

fractions containing ICA was loaded onto a 0.1-ml Mono S column and equilibrated with TGCD buffer and washed with the same buffer. Then the sample was eluted in three batches of 25-μl fractions with an HGCD buffer containing 0.5 M KCl. Fractions 3–5 containing ICA were pooled and used in the next process.

Step 3: Superdex 200 Gel Filtration—One-third of the pooled
fractions from step 2 was loaded onto a 2.4-ml Superdex 200 column (Pharmacia) equilibrated with HGCD buffer. The sample was eluted in three batches of 80-μl fractions with the same buffer. ICA was eluted in fractions 11–14. Biologically active fractions were pooled and subjected to NH2-terminal analysis of the polypeptides.

Partial Purification of ICA

Partial Purification of ICA

Step 1: Preparation of a Cell-free Extract of THP.1—
A THP.1 cell-free extract was prepared by hypotonic lysis (11). Briefly, the cell pellets
from 5 × 108 THP.1 cells were washed once with 10 volumes of hypotonic buffer
(20 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 2Na) and were collected by centrifugation at 1,000
g/min. The resulting cell pellets were resuspended in 3 volumes of hypo-
tonic buffer and frozen at

-0.1% trifluoroacetic acid, and the column was washed with the same
buffer.

-0.1 M NaCl at pH 2.5. Eluted
fractions were neutralized immediately with 1.0 M Tris.

fractions containing ICA was loaded onto a 0.1-ml Mono S column and equilibrated with TGCD buffer and washed with the same buffer. Then the sample was eluted in three batches of 25-μl fractions with an HGCD buffer containing 0.5 M KCl. Fractions 3–5 containing ICA were pooled and used in the next process.

Step 3: Superdex 200 Gel Filtration—One-third of the pooled
fractions from step 2 was loaded onto a 2.4-ml Superdex 200 column (Pharmacia) equilibrated with HGCD buffer. The sample was eluted in three batches of 80-μl fractions with the same buffer. ICA was eluted in fractions 11–14. Biologically active fractions were pooled and subjected to NH2-terminal analysis of the polypeptides.

Partial Purification of IDA

Partial Purification of IDA

Step 1: Preparation of a Cell-free Extract of THP.1—
A THP.1 cell-free extract was prepared by hypotonic lysis (11). Briefly, the cell pellets
from 5 × 108 THP.1 cells were washed once with 10 volumes of hypotonic buffer
(20 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 2Na) and were collected by centrifugation at 1,000
g/min. The resulting cell pellets were resuspended in 3 volumes of hypo-
tonic buffer and frozen at

-0.1% trifluoroacetic acid, and the column was washed with the same
buffer.

-0.1 M NaCl at pH 2.5. Eluted
fractions were neutralized immediately with 1.0 M Tris.

fractions containing ICA was loaded onto a 0.1-ml Mono S column and equilibrated with TGCD buffer and washed with the same buffer. Then the sample was eluted in three batches of 25-μl fractions with an HGCD buffer containing 0.5 M KCl. Fractions 3–5 containing ICA were pooled and used in the next process.

Step 3: Superdex 200 Gel Filtration—One-third of the pooled
fractions from step 2 was loaded onto a 2.4-ml Superdex 200 column (Pharmacia) equilibrated with HGCD buffer. The sample was eluted in three batches of 80-μl fractions with the same buffer. ICA was eluted in fractions 11–14. Biologically active fractions were pooled and subjected to NH2-terminal analysis of the polypeptides.

Partial Purification of IDA

Partial Purification of IDA

Step 1: Preparation of a Cell-free Extract of THP.1—
A THP.1 cell-free extract was prepared by hypotonic lysis (11). Briefly, the cell pellets
from 5 × 108 THP.1 cells were washed once with 10 volumes of hypotonic buffer
(20 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 2Na) and were collected by centrifugation at 1,000
g/min. The resulting cell pellets were resuspended in 3 volumes of hypo-
tonic buffer and frozen at

-0.1% trifluoroacetic acid, and the column was washed with the same
buffer.

-0.1 M NaCl at pH 2.5. Eluted
fractions were neutralized immediately with 1.0 M Tris.

fractions containing ICA was loaded onto a 0.1-ml Mono S column and equilibrated with TGCD buffer and washed with the same buffer. Then the sample was eluted in three batches of 25-μl fractions with an HGCD buffer containing 0.5 M KCl. Fractions 3–5 containing ICA were pooled and used in the next process.

Step 3: Superdex 200 Gel Filtration—One-third of the pooled
fractions from step 2 was loaded onto a 2.4-ml Superdex 200 column (Pharmacia) equilibrated with HGCD buffer. The sample was eluted in three batches of 80-μl fractions with the same buffer. ICA was eluted in fractions 11–14. Biologically active fractions were pooled and subjected to NH2-terminal analysis of the polypeptides.
out CHAPS) at pH 7.4. IDA was eluted in fractions 29–35. Fractions containing IDA were pooled and used in next process.

**Step 4: Hydroxypatite Chromatography**—The pooled fractions from step 3 were loaded in two batches onto a 4.4-mL hydroxypatite column (Tomen, Tokyo) equilibrated with HGD buffer at pH 7.4. The sample was then eluted in 0.5-mL fractions with 5 bed volumes of a linear phosphate gradient (0–0.5 M). Fractions containing IDA eluted at 500 mM phosphate in HGD buffer in fractions 54 and 55, which were pooled and subjected to NH2-terminal analysis of the polypeptides.

**NH2-terminal Analysis and Peptide Mapping**

Amino acid sequences of the NH2-terminal portions of purified natural hIL-18 and processed forms of hIL-18 (p18, p16, and p15) and of partially purified hIL-18-processing enzymes were determined with an automated protein sequencer model 473A (Applied Biosystems, Foster City, CA). Briefly, proteins of interest were subjected to electrophoresis in 15% SDS-PAGE gels and transferred onto ProBlott™ membranes (Applied Biosystems). The proteins were visualized by Coomassie Brilliant Blue staining and excised for direct NH2-terminal sequencing. For automated protein sequencer model 473A (Applied Biosystems). The proteins were visualized by Coomassie Brilliant Blue staining and excised for direct NH2-terminal sequencing. For peptide mapping, purified natural hIL-18 was digested with chymotrypsin (Sigma), and the resulting peptide fragments were separated using a 2.5-mL ODS-120T column (Tosoh) with 20 bed volumes of increasing acetonitrile linear gradient (0–70% acetonitrile) in 0.1% trifluoroacetic acid. Eluted fragments detected at a wavelength of 214 nm were separately collected and analyzed.

**Preparation of Precursor hIL-18**

A 0.8-kilobase cDNA encoding precursor hIL-18 (3) was ligated into the expression vector BCMGSNeO (13). The hIL-18 expression vector was transfected into CHO-K1 cells by electroporation, and transfectants were harvested after 3 days. The expressed protein was analyzed by immunoblotting using a hIL-18-specific mAb (25-2G).

**Preparation of Recombinant Human ICE (rhICE)**

Human ICE cDNA was cloned from THP.1 cells using the PCR method. PCR primers were designed based on the published hICE nucleotide sequence (14). COS-1 cells were transfected by electroporation with an expression construct containing hICE cDNA into pCDSM expression vector (Invitrogen, San Diego) and harvested after 3 days. Proteins from the cells were extracted with hypotonic buffer using a Dounce homogenizer. The supernatant of this homogenate was incubated at 37 °C for 1.5 h to activate ICE precursor and was partially purified by ammonium sulfate fractionation followed by DEAE-5PW and Superdex 75 column chromatographies.

**hIL-18 Assay**

The quantities of hIL-18 were assessed by a two-site sandwich ELISA using mAbs prepared in our laboratory (15). The bioactivity of hIL-18 assessed by its IFN-y-inducing activity on the myelomonocytic cell line KG-1. Briefly, 3 × 10⁵ KG-1 cells suspended in RPMI 1640 medium containing 10% fetal bovine serum were seeded in a 96-well microplate. Samples were added to the wells and incubated for 24 h, and IFN-γ activity induced in the culture supernatants was assessed by ELISA.

**Measurements of ICE-like and CPP32-like Activities**

ICE-like and CPP32-like activities were measured using the fluorogenic substrates Ac-YVAD-MCA and Ac-DEVD-MCA, respectively (12, 14, 18). Briefly, 1 mM substrate and samples (10 µL) were incubated at 37 °C in the presence of 20 mM Hepes, 10% glycerol, and 2 mM DTT in 5% SDS-PAGE gels and transferred onto ProBlott™ membranes (Applied Biosystems). The proteins were visualized by Coomassie Brilliant Blue staining and excised for direct NH2-terminal sequencing. For peptide mapping, purified natural hIL-18 was digested with chymotrypsin (Sigma), and the resulting peptide fragments were separated using a 2.5-mL ODS-120T column (Tosoh) with 20 bed volumes of increasing acetonitrile linear gradient (0–70% acetonitrile) in 0.1% trifluoroacetic acid. Eluted fragments detected at a wavelength of 214 nm were separately collected and analyzed.

**RESULTS**

**hIL-18 mRNA Is Predominantly Detected in Myelomonocytic Cell Lines**—To identify hIL-18-producing cell lines, we first examined the level of constitutive expression of hIL-18 mRNA by reverse transcription-PCR analysis using hIL-18-specific primers. 42 hematopoietic cell lines of various lineages were screened. hIL-18 mRNA was highly expressed in myelomonocytic cell lines such as THP-1, KG-1, HBL-38, and HL-60, and some non-lymphocytic and non-myelomonocytic cell lines such as MEG-01 and TS9-22. In some B cell and non-T, non-B cell lines such as BJAB, MOLP-2, and NALM-20, weak expression of hIL-18 mRNA was observed. Interestingly, no expression was observed in T cell lines (Table I).

**Purification of Natural hIL-18**—Initially, we chose four myelomonocytic cell lines (THP-1, HL-60, U-937, and HBL-38) and two non-L, non-M cell lines (HEL and MEG-01) as targets for the detection of natural hIL-18 at the protein level. As a preliminary study, culture supernatants or extracts of these cell lines were analyzed using hIL-18-specific ELISA and immunoblotting. Small amounts of hIL-18 were detected in the cell extracts of THP-1 and U937 cells, but little or no hIL-18 was detected in the culture supernatants (data not shown). Furthermore, hIL-18 was found mostly in the cytosolic fraction of

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3 K. Konishi, F. Tanabe, M. Taniguchi, H. Yamauchi, T. Tanimoto, M. Ikeda, K. Orita, and M. Kurimoto, submitted for publication.
the cells. Next we employed some differentiation-inducing agents (lipopolysaccharide, retinoic acid, A23187, and phorbol myristate acetate) to investigate whether these reagents can augment the production or secretion of hIL-18. However, no significant elevation in the levels of intra- or extracellular hIL-18 was observed (data not shown). Based upon the above mentioned preliminary results, we selected the cytosolic extract of THP.1 cells as a starting material for the isolation of natural hIL-18. For this purpose, large numbers of THP.1 cells were obtained with an cell propagation method (9, 10), and a cytosolic extract was prepared by hypotonic lysis. The cell extract was subjected to DEAE anion-exchange chromatography twice (Fig. 1, A and B) for the purification of natural hIL-18. Subsequently, affinity purification on anti-hIL-18 mAb-conjugated Sepharose was employed. The purified protein had a molecular mass of 18.2 kDa on SDS-PAGE under reducing conditions and exhibited IFN-γ-inducing activity on KG-1 cells (Fig. 1C). The overall yield of natural hIL-18 after affinity purification ranged from 3 to 30 μg/10^11 cells. Direct amino acid sequencing of the affinity-purified polypeptide showed that the NH₂-terminal amino acid is Tyr⁵⁷. Furthermore, the amino acid sequences of the peptides prepared from the purified polypeptide coincided with those deduced from the cDNA (Fig. 2; underlined). These results indicate that the purified polypeptide derived from THP.1 is a mature form of hIL-18 proteolytically processed behind the Asp³⁶ residue in a manner similar to that of the murine homolog (Fig. 2; vertical arrow) (1, 2, 6).

**Biological Analysis of Natural hIL-18**—To confirm the biological activities of the protein purified from THP.1, IFN-γ-inducing ability and enhancement of NK cell cytotoxicity of the natural hIL-18 were examined on human PBMC. In the presence of anti-CD3 mAb, a concentration as low as 5.5 pm of either natural or recombinant hIL-18 significantly augmented IFN-γ production by PBMC (Fig. 3A). Pretreatment of PBMC with natural hIL-18 enhanced NK cytotoxicity against a target cell line, K562, in a manner similar to that of the recombinant hIL-18 (3). The enhancement of NK cell cytotoxicity was observed at concentrations as low as 0.55 pg (Fig. 3B). These results suggest that the purified natural hIL-18 is equivalent to the recombinant protein in its biological functions.

**THP.1 Cell Lysate Contains Two Types of ProhIL-18-processing Activities**—Besides the natural hIL-18, the THP.1 cell-free extract also contained the precursor protein as determined by immunoblotting (data not shown). This suggests that proteolytic maturation occurs in THP.1 cells or in the cell-free extract during the purification process. The amino acid sequence of the hIL-18 maturation site (Asp³⁶-Tyr³⁷) seems to be specific to THP.1 cells (16) or granzyme B (17), suggesting the presence of an IL-18-converting enzyme (hIL-18-CE) in the cytosolic extract of THP.1 cells. To confirm this, the recombinant precursor protein of hIL-18 (prohIL-18; p24) was incubated for various periods with the THP.1 cell extract. Immunoblotting using hIL-18-specific antibody (25-2G) revealed that prohIL-18 was proteolytically cleaved by the THP.1 extract to generate three cleavage products with molecular masses of 18 kDa (p18), 16 kDa (p16), and 12 kDa (p12) under reducing conditions. These results further support the existence of an hIL-18 maturation site in THP.1 cells.
Panel A, induction of IFN-γ production by PBMC in response to hIL-18. Human PBMC (5 × 10⁶ cells/ml) from healthy donors were incubated with various concentrations of natural or recombinant hIL-18 in the presence or absence of 0.1 µg/ml anti-CD3 mAb for 24 h. The amounts of IFN-γ produced in the culture supernatants were assessed by human IFN-γ ELISA. Closed circles and closed squares indicate the response to natural and recombinant hIL-18 in the presence of anti-CD3 mAb; open circles and open squares indicate the response to natural and recombinant hIL-18 without anti-CD3 mAb, respectively.

Panel B, enhancement of NK cell cytotoxicity in response to natural hIL-18. Human PBMC (5 × 10⁶ cells/ml) were incubated with various concentrations of natural hIL-18 for 48 h and then assayed for lytic activities against K562 target cells. The result shown was obtained at an effector:target ratio of 6:1. Each value is presented as a mean ± S.D. (n = 3).

(p16), and 15 kDa (p15), respectively (Fig. 4A). p18 can be detected within the first 30 min and subsequently, p16 and p15 appeared sequentially. Prolonged incubation resulted in a disappearance of the prohIL-18 band and in a decreased intensity of the p18 band. In contrast, an increase in the intensity of the p16 and p15 bands was observed. No significant proteolysis of these p16 and p15 bands was observed upon prolonged incubation, suggesting that there are at least three cleavage sites in prohIL-18 acting as substrates for the processing protease(s).

In the same experiment, hIL-18 activities were measured by a hIL-18-specific ELISA (15) and by IFN-γ-inducing assay on KG-1 cells (Fig. 4B). KG-1 cells produce IFN-γ in response to hIL-18, but not to IL-12.² hIL-18 activity correlated with the appearance of the p18 molecule. p24, p16, and p15 were hardly detectable by the ELISA and show little or no IFN-γ-inducing activity on KG-1 cells (Fig. 4B). Next, to determine whether proteolytic cleavage is caused by ICE family proteases or by granzyme B, various protease inhibitors were employed. As expected, proteolysis showed features characteristic of caspases. The generation of the processed products was only completely inhibited by a thiol-alkylation reagent, iodoacetamide. Ac-YVAD-CHO inhibited the generation of p18 selectively and had no effect on that of p16. In contrast to this, Ac-DEVD-CHO inhibited the generation of p16 and had no effect on that of p18 (Fig. 4C). These results suggest that there are at least two IL-18-processing enzymes in THP.1 cell lysate, one is an ICE-like protease generating p18, the mature form of hIL-18, and the other is a CPP32-like protease generating p16 and/or p15, inactive degraded products of hIL-18.

Separation of the Converting and Degrading Enzymes—To confirm the above findings further, we tried to separate the two hIL-18-processing activities, hIL-18-converting activity (ICA) and hIL-18-degrading activity (IDA), from the cytosolic extract of THP.1 cells. Purification methods were partially based on those reportedly used for the separation of ICE (12, 14). Two activities were distinctly separated by DEAE-5PW column chromatography (Fig. 5, insets). ICA eluted sharply at a low NaCl concentration, whereas IDA eluted at a relatively high NaCl concentration as a broad peak. We also examined the ICE-like activity and CPP32-like activities (14, 18) in the DEAE elutes using the fluorogenic substrates Ac-YVAD-MAC and Ac-DEVD-MAC, respectively (Fig. 5). The ranges of activities of these two fluorogenic assays coincided with those of ICA and IDA as assessed by in vitro prohIL-18 cleavage assay, although high concentrations of IDA also exhibited Ac-YVAD-MAC cleavage activity. This cleavage of Ac-YVAD-MAC by IDA was confirmed using further purified preparations containing high concentrations of IDA (data not shown). Therefore, for further purification of ICA and IDA, we employed both ICE-like and CPP32-like cleavage assays on fluorogenic substrates and the in vitro prohIL-18 cleavage assay.

hIL-18-converting Enzyme Is ICE—hIL-18-CE was partially purified by S-Sepharose cation-exchange column chromatography, Mono S column chromatography followed by Superdex 200 column chromatography (data not shown). The fractions containing hIL-18-CE from Superdex 200 chromatography were...
hIL-18-converting enzyme may be ICE itself. To confirm this, antibodies, respectively (data not shown). This suggested that the existence of these two subunits in the preparation was also confirmed by immunoblotting using anti-p10 and anti-p20 antibodies, respectively (data not shown). This suggested that hIL-18-converting enzyme may be ICE itself. To confirm this possibility, in vitro cleavage assays of precursor human IL-1β by partially purified hIL-18-CE were performed. hIL-18-CE could cleave precursor human IL-1β to generate the 17.5-kDa mature IL-1β as well as the mature hIL-18 (Fig. 6A). These cleavage activities were inhibited by Ac-YVAD-CHO and iodoacetamide. The bioactivity of the mature proteins was confirmed by IFN-γ-inducing assay for hIL-18 and D10.G4.1 proliferation assay (19) for IL-1β (data not shown). Furthermore, recombinant hICE cleaved prohIL-18 to generate the mature hIL-18 (Fig. 6B). These results support the conclusion that hIL-18-converting enzyme is ICE.

**hIL-18-degrading Enzyme Is CPP32—**hIL-18-degrading enzyme (hIL-18-DE) was partially purified by S-Sepharose cation-exchange column chromatography, Mono P chromatofocusing, Superdex 200, followed by hydroxyapatite column chromatographies (data not shown). Some members of the caspases, particularly those of the CPP32 subfamily, are reported to cleave poly(ADP-ribose)polymerase efficiently (18, 20, 21). Therefore, we tested this cleavage ability using partially purified hIL-18-DE. THP.1 cell-derived hIL-18-DE cleaved endogenous poly(ADP-ribose)polymerase to generate an NH2-terminal 30-kDa fragment as well as degraded prohIL-18 (Fig. 7A). The preparation containing partially purified hIL-18-DE was also subjected to NH2-terminal amino acid sequence analysis. From this analysis, both the large and small subunits of CPP32 (p17 and p12) and the small subunit of CMH-1 (p12) were detected (Table II). The quantities of these two enzymes were estimated to be comparable. Immunoblotting analyses also showed that both CPP32 and CMH-1 do indeed exist in the preparation containing hIL-18-DE (data not shown). These results suggested that the candidate for the hIL-18-degrading enzyme was either CPP32 or CMH-1. Cleavage assays using recombinant enzymes showed that effective degradation of prohIL-18 is caused by CPP32 but not by CMH-1 (Fig. 7B). CPP32 cleaved prohIL-18 >125-fold and >25-fold more efficiently than CMH-1 at pH 6.5 and pH 7.2, respectively (data not shown). From these results, we conclude that hIL-18-DE found in THP.1 cell lysate is CPP32.

**DISCUSSION**

Natural hIL-18 and its processing enzymes were identified in the cytosolic extract of monocytic THP.1 cells. It has been reported that murine IL-18 is produced by activated Kupffer cells (2) or intestinal epithelial cells (22). Fully activated Kupffer cells are considered to be able to secrete murine IL-18 efficiently with the aid of ICE (7). At the mRNA level, IL-18 is constitutively expressed in Kupffer cells, but the levels of IL-18 mRNA failed to elevate after injection of *P. acnes* intraperitoneally. In our study, to identify hIL-18-producing cell lines, constitutive expression of hIL-18 mRNA in 42 hematopoietic cell lines was examined. High levels of mRNA expression were detected predominantly in myelomonocytic and non-L, non-M cell lines. Some B cell and non-T, non-B lines showed low expression of IL-18 mRNA, whereas T cell lines did not show any expression at all. These results strongly support the notion that major IL-18-producing cells are macrophage-like cells.

During purification of natural hIL-18, endogenous precursor protein or probable degraded products were detected by immunoblotting analysis, from which the mature hIL-18 was isolated selectively by specific affinity chromatography (Fig. 1C). In respect of the enhancement of NK cell cytotoxicity and IFN-γ production by human PBMC, purified natural hIL-18 possessed the same biological functions as those of the recombinant protein that had an NH2-terminal sequence tentatively assigned based on homology with the murine natural IL-18 (Fig. 2). In fact, the maturation site of natural hIL-18 was in accordance with that of the murine homolog. hIL-18 has no putative N-linked glycosylation site, and the theoretical molec-
ular mass deduced from the genetic information is 18,217 Da. The molecular mass of natural hIL-18 determined by SDS-PAGE analysis is 18.2 kDa (Fig. 1C). These results suggest that THP.1-derived natural hIL-18 is a monomeric 18-kDa protein without a sugar chain or post-transcriptional modification.

Natural prohIL-18 is detected as a 24-kDa band by SDS-PAGE analysis under reducing conditions, although the theoretical molecular mass of prohIL-18 is 22,326 Da. Moreover, it seems likely that the NH₂ terminus of natural prohIL-18 is blocked by unknown modification from the finding that the NH₂-terminal amino acid could not be analyzed by the direct sequencing method (data not shown). These results suggest that prohIL-18 may be modified to facilitate its constitutive presence in the cell. This further implies that specific secretion mechanisms may be active in macrophage-like cells to transport mature IL-18 into the extracellular space similarly to IL-1β. However, preliminary studies on hIL-18 mRNA-expressing cell lines failed to detect extracellular hIL-18 even under the same conditions that IL-1β secretion is observed (data not shown). This might be due only to the lack of accumulation of the precursor protein in the cytoplasm. Another possibility is that the regulatory mechanisms for secretion may be different between hIL-18 and IL-1β. The latter assumption is supported by the hypothesis on the multistage regulation of IL-1β secretion proposed by Siders and Mizel (23).

### Table II

| Polypeptide | Amino acid sequence | Assigned protein |
|-------------|---------------------|------------------|
| hIL-18-CE |
| 22 kDa | D/N* P A M T - - G - E G N V K L - - L | hICE-p20 |
| 10 kDa | A I K K A H I E K D F I A F | hICE-p10 |
| hIL-18-DE |
| 17 kDa | S G I K L D N - Y K M - Y P E M - - I - I | hCPP32-p17 |
| 12 kDa | S G V D D D M A - H | hCPP32-p12 |
| 11 kDa | A - P R Y K I P V E A | hCMH-1-p12 |

*Two amino acids (Asp and Asn) were detected in the first cycle of the Edman sequencing.

### Fig. 6
**In vitro cleavage assays for the identification of hIL-18-CE.** Panel A, a preparation of prohIL-18 or prohIL-1β (about 100 ng each) were incubated at 37 °C for 3 h with partially purified hIL-18-CE (30 µl) in the presence of 20 mM Hepes, 10% glycerol, 2 mM DTT, at pH 7.4 in a total reaction volume of 50 µl. I, Y, and N denote iodoacetamide, Ac-YVAD-CHO, and without inhibitor, respectively. Cleaved products were analyzed by immunoblotting with anti-hIL-18 mAb or anti-hIL-1β pAb and visualized by enhanced chemiluminescence detection reagents.

### Fig. 7
**In vitro cleavage assays for the identification of hIL-18-DE.** Panel A, a preparation containing partially purified hIL-18-DE (5 µl) under the same conditions. PARP* denotes the degraded form of poly(ADP-ribose)-polymerase (left). A preparation containing about 100 ng of prohIL-18 was incubated at 37 °C for 2 h with partially purified hIL-18-DE (10 µl) with 20 mM Hepes, 10% glycerol, and 2 mM DTT at pH 6.5 in a total of 20 µl of reaction volume (right). D, Y, I, and N denote Ac-DEV-D-CHO (10 µM), Ac-YVAD-CHO (10 µM), iodoacetamide (650 µM), and without inhibitor, respectively. Cleaved products were analyzed by immunoblotting with anti-hIL-18 mAb (25-2G) or anti-human poly(ADP-ribose)polymerase pAb, and visualized by enhanced chemiluminescence detection reagents.
With respect to the structural similarity between IL-18 and IL-1β, the IL-1 signature-like sequence (3) and structural similarities including the lack of signal-peptide sequences have been reported (4). Moreover, involvement of ICE/caspase-1 in the maturation of endogenous hIL-18 has also been confirmed in this report. However, the biological functions of IL-18 show clear differences from those of IL-1β in terms of the augmentation of NK cell cytotoxicity and IFN-γ and granulocyte-macrophage colony-stimulating factor production by T cells and the induction of T cell proliferation (3, 24). Furthermore, although IL-1β is known to act as an accessory molecule in the activation of Th2 cells (25), IL-18 reportedly acts on Th1 cells but not on Th2 cells to augment IFN-γ production and induces proliferation and IL-2 receptor α-chain expression of Th1 cells (26). Thus, IL-18 and IL-1β exhibit different biological functions transmitted through their specific receptors.2

THP-1 is well known as an ICE- and CPP32-producing cell line (14, 18). Therefore, it is not surprising that these two enzymes were identified in THP-1 cell-free extracts. Of great interest is that a common substrate of ICE and CPP32 was found concurrently. Fig. 8 shows the schematic illustration of cleavage sites in hIL-18. A membrane-bound 24-kDa precursor proIL-18 is synthesized as a biologically inactive 24-kDa precursor polypeptide (p24). LESED^26-Y^37 is the maturation site for p24 cleaved by ICE to generate the active p18 molecule, and DXXD-X (DMDT^21-S^22 and DCRD^25-A^27) are the cleavage sites for CPP32 resulting in the generation of biologically inactive p16 and p15 products. All three cleavage sites for processing enzymes in hIL-18 were determined by NH2-terminal amino acid sequence analysis of the cleaved products.

In conclusion, the analyses on natural hIL-18 purified from monocytyic THP-1 cells have revealed the involvement of endogenous ICE/caspase-1 in its maturation. Moreover, endogenous CPP32/caspase-3 was found to degrade hIL-18 to biologically inactive forms effectively. Although further experiments are necessary to demonstrate the secretion mechanism or physiological regulation of IL-18, these findings will help to shed some light on the potential roles of IL-18 in immune responses.

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