Mary Ann Liebert, Inc.

cDNA Cloning of Biologically Active Chicken Interleukin-18

KIRSTEN SCHNEIDER,[1,*] FLORIAN PUEHLER,[1,*] DANIELA BAEUERLE,[1] SANDRA ELVERS,[1] PETER STAEHELI,[1] BERND KASPERS,[2] and KIRSTEN C. WEINING[1]

ABSTRACT

By searching a chicken EST database, we identified a cDNA clone that appeared to contain the entire open reading frame (ORF) of chicken interleukin-18 (ChIL-18). The encoded protein consists of 198 amino acids and exhibits approximately 30% sequence identity to IL-18 of humans and various others mammals. Sequence comparisons reveals a putative caspase-1 cleavage site at aspartic acid 29 of the primary translation product, indicating that mature ChIL-18 might consist of 169 amino acids. Bacterially expressed ChIL-18 in which the N-terminal 29 amino acids of the putative precursor molecule were replaced by a histidine tag induced the synthesis of interferon-γ (IFN-γ) in cultured primary chicken spleen cells, indicating that the recombinant protein is biologically active.

INTRODUCTION

Interleukin-18 (IL-18) was originally described as an interferon-γ (IFN-γ)-inducing factor and was cloned from *Propionibacterium acnes*-treated mouse livers in 1995.[1] In the ensuring years, IL-18 has been characterized in great detail, as summarized in some recent reviews.[2–4] IL-18 belongs to the IL-1 family of cytokines. Like IL-1β, it is synthesized as a precursor molecule that lacks a typical signal peptide and is cleaved by caspase-1 into a bioactive cytokine.[5,6] Computer modeling studies predict that IL-18 contains many β-sheets, as does IL-1β.[7] IL-18 binds to a heterodimeric receptor complex in which both the binding and the signaling chain are members of the IL-1 receptor family.[8,9] The signaling cascades of IL-18 and IL-1β are identical, and both lead to activation of NF-κB.[10,11]

The biologic properties of mammalian IL-18 are complex.[4] It shares properties with IL-12, as both cytokines can induce Th1 immune responses.[4,10] The observed synergistic effects of IL-18 and IL-12 on IFN-γ induction seem to result from IL-18 receptor upregulation by IL-12[12] and a concerted activation of the IFN-γ promoter.[13] Other functions ascribed to IL-18 include induction of IL-1β, tumor necrosis factor-α (TNF-α), and several chemokines,[14] as well as enhancement of natural killer (NK) cell cytotoxicity.[15] An important role for IL-18 has been shown in several models of infectious, inflammatory, and autoimmune diseases, as well as in tumor models.[4] IL-18 mRNA and precursor synthesis is constitutive,[16] indicating that IL-18 activity is regulated at the level of cytokine precursor processing and secretion. The *in vivo* activity of IL-18 is further regulated by a secreted IL-18-binding protein.[17,18] Interestingly, several poxviruses also encode soluble factors that bind and neutralize IL-18, thereby interfering with the host defense system.[19,20]

It has been unclear to date whether birds have a cytokine with structural and functional properties of mammalian IL-18. Here, we report the identification of a cDNA encoding a chicken homolog of IL-18 (ChIL-18). We further show that bacterially expressed ChIL-18 is a potent inducer of IFN-γ in primary chicken spleen cells.

MATERIALS AND METHODS

Cell culture

HD-11 cells[21] were grown in Dulbecco's modified essential medium (DMEM) supplemented with 8% fetal bovine serum (FBS) and 2% chicken serum. Chicken spleen cells were prepared as described[22] and maintained in RPMI 1640 medium supplemented with 8% FBS and 2% chicken serum at 40°C in a CO_2 incubator.

cDNA cloning

Analysis of BLAST search results of a chicken bursal EST database (*http://genetics.hpi.uni-hamburg.de/dt40.html*) indicated that clone dkfz426 3m9r1 contained sequence informa-

[1]Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene, University of Freiburg, 79008 Freiburg, Germany.
[2]Institut für Tierphysiologie, University of Munich, 80539 Munich, Germany.
*These two authors contributed equally to this work.

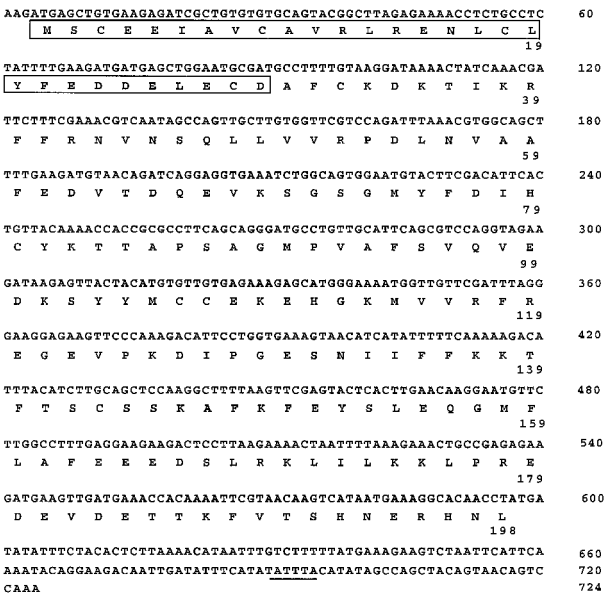

FIG. 1. cDNA sequence (EMBL/GenBank accession No. AJ277865) and predicted amino acid sequence of ChIL-18. Nucleotide numbering is at right. An ATTTA sequence motif in the 3′-noncoding region, which presumably mediates mRNA instability, is underlined. The protein sequence is given in the single letter code. Amino acid numbers are indicated below the sequence. The propeptide that is probably cleaved off by caspase-1 after the conserved aspartate residue at position 29 is boxed.

tion for ChIL-18. To isolate a cDNA fragment containing the complete open reading frame (ORF) for this protein, PCR was performed with primers 5′-CCCAAGCTTATGAGCTGT-GAAGAGATCGCTGTG-3′ and 5′-TGCTCTAGATCATAG-GTTGTGCCTTTCATTATG-3′, which introduced HindIII and XbaI restriction sites near the ends of the amplification product. cDNA from HD-11 cells stimulated for 5 h with 5 μg/ml lipopolysaccharide (LPS)[23] was used as template. The PCR product was directionally cloned between the HindIII and the XbaI restriction sites of vector pcDNA3 (Invitrogen, Groningen, Netherlands).

Purification of histidine-tagged ChIL-18 from Escherichia coli

A fragment of ChIL-18 cDNA (nucleotide positions 91–600 in Fig. 1) was amplified by PCR using primers 5′-CGCG-GATCCGCCTTTTGTAAGGATAAAACT-3′ and 5′-CCCA-AGCTTTCATAGGTTGTGCCT-3′, which introduced unique BamHI and HindIII restriction sites near the ends of the fragment, and the PCR product was cloned between the BamHI and HindIII restriction sites of prokaryotic expression vector pQE9 (Qiagen, Chatsworth, CA). Expression of this construct in *E. coli* strain M15 yielded His-ChIL-18, which corresponds to putative mature ChIL-18 with an N-terminal extension consisting of the peptide Met-Arg-Gly-Ser-$(His)_6$-Gly-Ser. His-ChIL-18 was purified by affinity chromatography on a nickel chelate agarose column. Purification conditions were as described for ChIFN-γ[24] with slight modifications. Induction was done at $OD_{600} = 0.8$ for a period of 3 h using an isopropylthiogalactose (IPTG) concentration of 0.5 mM. We checked for purity of recombinant His-ChIL-18 by standard SDS-PAGE (15% gel) and subsequent Coomassie blue staining.

Induction of IFN-γ synthesis by His-ChIL-18 in chicken spleen cells

Chicken spleen cells were prepared as described,[22] seeded at a density of 2.5×10^5 cells per well of a 96-well plate, and incubated with the indicated amounts of recombinant His-ChIL-18, His-ChIL-18 that has been heat-inactivated for 10 min at 100°C, or the control protein His-MxA. At 48 h after onset of cytokine treatment, cell supernatants were assayed for the presence of biologically active IFN-γ. For this purpose, HD-11 cells were incubated for 24 h with samples of the various supernatants. Activation of HD-11 cells by IFN-γ was measured as a function of nitrite accumulation in the culture supernatants using the Griess assay.[25,26] To confirm that the observed nitric oxide (NO) production was induced by IFN-γ, supernatants of spleen cells stimulated with the indicated amounts of His-ChIL-18 were preincubated for 2 h at 37°C with a polyclonal neutralizing antiserum to ChIFN-γ[24] at a dilution of 1:200 and subsequently transfered to HD-11 cells.

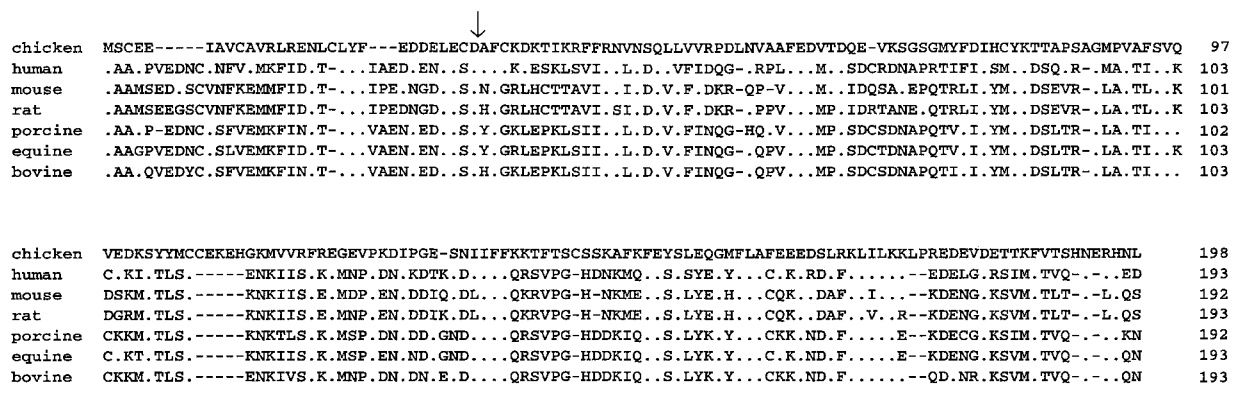

FIG. 2. Comparison of IL-18 sequences from chicken and various mammals. Alignments were done using the Jotun Hein method.[27] Dots represent identical amino acids, and alignment gaps are indicated by hyphens. The arrow marks the position of a conserved aspartate residue at which caspase-1-mediated cleavage of the precursor molecules is predicted to occur.

RESULTS AND DISCUSSION

Cloning of ChIL-18 cDNA

By analyzing the BLAST search results of a chicken EST database, we identified clone dkfz426 3m9r1, which seemed to encode a polypeptide with significant similarity to mammalian IL-18. PCR with cDNA from LPS-induced chicken HD-11 cells and primers corresponding to the EST clone resulted in the amplification of a cDNA fragment whose nucleotide sequence (EMBL/GenBank accession No. AJ277865) precisely matched the sequence of the original EST clone. This cDNA codes for a polypeptide of 198 amino acids (Fig. 1).

Alignment of ChIL-18 with IL-18 from various mammals revealed amino acid identities of approximately 30%. A comparison of chicken and mammalian IL-18 sequences is shown in Figure 2. IL-18 is synthesized as an inactive precursor in mammals that is activated by caspase-1, which cleaves the molecule after a conserved aspartate (arrow in Fig. 2). Sequence comparisons revealed that the critical aspartate residue is conserved in the ChIL-18 sequence (Fig. 2), indicating that it may also be cleaved at this residue. Mature ChIL-18 thus seems to consist of 169 amino acids.

Recombinant His-ChIL-18 from E. coli *is biologically active*

Assuming that pro-ChIL-18 is cleaved after the conserved aspartate residue at position 29 (Fig. 2), we used PCR to amplify a cDNA fragment that encodes an N-terminally truncated form of ChIL-18. After cloning into the prokaryotic expression vector pQE9, the resulting construct should yield a translation product that consists of an N-terminal histidine tag followed by the putative mature ChIL-18 sequence. This product, which we designated His-ChIL-18, was purified from *E. coli* by affinity chromatography on nickel chelate agarose beads. After elution under nondenaturing conditions, peak column fractions were pooled and diluted to a concentration of 0.5 mg protein per ml. His-ChIL-18 appeared as a prominent band on SDS-PAGE at about 23 kDa. It was more than 50% pure (Fig. 3A).

To determine whether bacterially expressed recombinant His-ChIL-18 is biologically active, we tested its ability to induce IFN-γ synthesis in cultured primary chicken spleen cells. Spleen cells were incubated with various concentrations of purified His-ChIL-18 or as negative controls with either heat-inactivated His-ChIL-18 or an unrelated protein (His-MxA). At 48 h after onset of treatment, the spleen cell supernatants were analyzed for the presence of IFN-γ by a standard bioassay that monitors IFN-γ activity as a function of NO secretion by the chicken macrophage cell line HD-11.[28] As shown in Figure 3B, only supernatants of spleen cells treated with nondenatured recombinant ChIL-18 exerted macrophage-activating activity. The minimal concentration of His-ChIL-18 that induced significant amounts of IFN-γ in spleen cells was 60 ng/ml. Treatment of spleen cells with 250 ng/ml of either His-MxA or heat-inactivated His-ChIL-18 did not cause significant secretion of NO. In addition, neither the His-ChIL-18 nor the His-MxA preparation induced NO synthesis when added directly to HD-11 indicator cells at concentrations as high as 1 μg/ml (data not shown), indicating that the observed effect was a specific activity of ChIL-18.

Treatment of supernatants from His-ChIL-18-treated chicken spleen cells with a polyclonal antiserum that neutralizes ChIFN-γ[24] blocked their NO synthesis-inducing activity (Fig. 3B), demonstrating that the factor secreted by spleen cells after treatment with ChIL-18 is indeed IFN-γ.

Our discovery that chickens have a cytokine with structural and functional similarities to mammalian IL-18 further supports the view that most parts of the cytokine network had already been in place when the avian and mammalian lineages separated about 300 million years ago. In this context, it is of in-

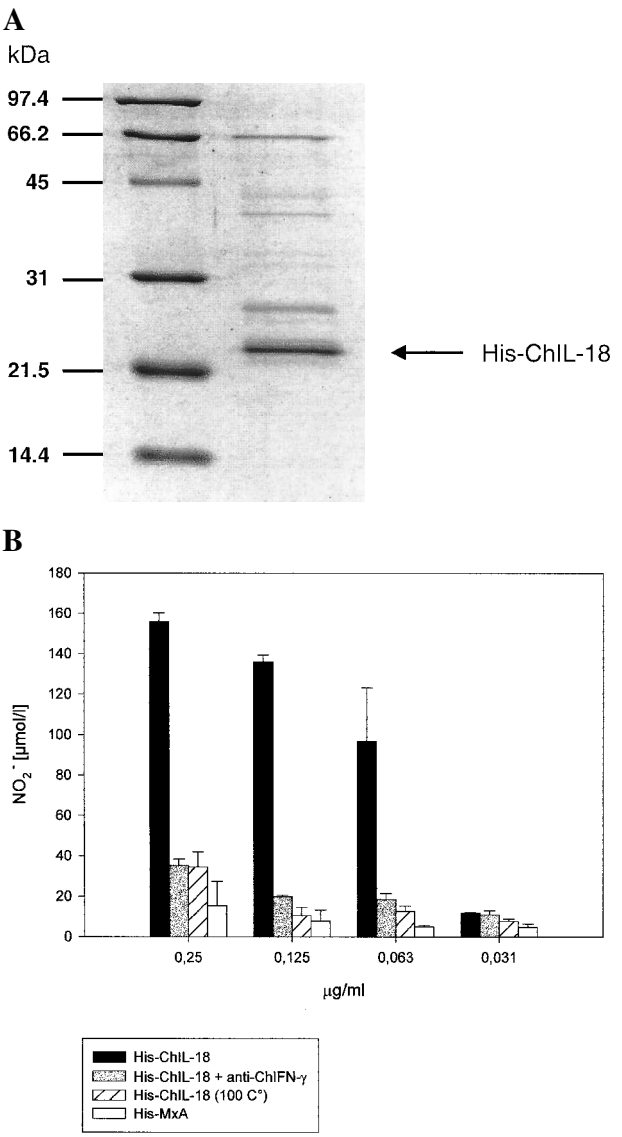

FIG. 3. Purified His-ChIL-18 from *E. coli* is biologically active. (**A**) Coomassie blue-stained gel showing a sample of purified His-ChIL-18. The molecular sizes of marker proteins are indicated. (**B**) Chicken spleen cells were treated for 48 h with the indicated concentrations of either active His-ChIL-18, heat-inactivated His-ChIL-18, or the control protein His-MxA. Spleen cell supernatants were added to HD-11 cells either directly or after preincubation with a polyclonal antiserum that neutralizes ChIFN-γ in order to monitor IFN-γ-induced NO secretion.

terest to note that the various chicken cytokines for which cDNA have to date been cloned (type I IFN,[29,30] IFN-γ,[24,31] IL-1β,[23] IL-2,[32] IL-18 [this report]) are all considered to drive Th1 immune responses. Such cytokines as IL-4, IL-5, or IL-10, which characterize Th2 immune responses, have to date not been found in the chicken. It is unclear at present whether the latter cytokines are absent from birds or were simply missed because of the lack of appropriate assay systems.

In summary, the unlimited availability of biologically active recombinant ChIL-18 now makes it possible to test if this cytokine has the potential to modulate vaccine-induced immune responses in chickens.

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Address reprint requests to:
Dr. Peter Staeheli
Department of Virology
University of Freiburg
Hermann-Herder-Strasse 11
D-79008 Freiburg, Germany

Tel: +49-761-203-6579
Fax: +49-761-203-6562
E-mail: staeheli@ukl.uni-freiburg.de

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