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A chicken homolog of mammalian interleukin-1*β*: cDNA cloning and purification of active recombinant protein

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Upon induction with lipopolysaccharide (LPS) the chicken macrophage cell line HD-11 secretes an activity that stimulates the synthesis of a CXC chemokine in the chicken fibroblast cell line CEC-32. We used a cDNA expression cloning strategy in COS cells to characterize this activity. The isolated cDNA clone codes for a polypeptide of 267 amino acids which lacks a hydrophobic N-terminal domain that could serve as secretory signal. Sequence homology and structural features indicate that this protein is the chicken homolog of mammalian interleukin-1*β* (ChIL-1*β*). Northern blot analysis showed that ChIL-1*β* RNA is quickly induced in blood monocyte-derived macrophages reaching maximal levels within one hour after onset of LPS treatment. To test for biological activity of putative mature ChIL-1*β*, a cDNA fragment comprising amino acids 106 to 267 of the open reading frame was expressed in *Escherichia coli* so that the resulting polypeptide carried a histidine tag at its N-terminus for easy purification by nickel chelate affinity chromatography. Purified His-ChIL-1*β* potently induced CXC chemokine RNA synthesis in CEC-32 cells. When injected intravenously into adult chickens, it quickly induced a transient increase in serum corticosterone levels.

Keywords: chicken; lipopolysaccharide inducibility; interleukin-1*β*; CXC chemokine; cDNA expression cloning.

Interleukin-1 (IL-1) is an important pro-inflammatory cytokine that exhibits pleiotropic activities on a wide range of target cells [1]. It is secreted by many different cell types, with stimulated macrophages being the main producers of IL-1. The diverse biological effects of IL-1 include induction of fever, elevation of serum corticosterone levels, activation of the cytokine network, triggering of the acute-phase response in the liver and activation of vascular endothelium [2]. It further stimulates T-cell proliferation via interleukin-2 induction, and it induces B-cell maturation and antibody production. IL-1 also stimulates collagenase and prostaglandin production by fibroblasts and exerts stimulating effects on cells engaged in developmental, differentiation and repair processes [2]. Furthermore, IL-1 induces the synthesis of IL-8 and other CXC chemokines in human fibroblasts [3] and other cell types [4, 5].

In mammals the IL-1 gene family comprises three members: IL-1*α*, IL-1*β* and the IL-1 receptor antagonist (IL-1Ra) [6]. IL-1*α* and IL-1*β* are only distantly related to each other with 45% similarity at the nucleic acid level and 26% similarity at the amino acid level in humans [7]. Despite low sequence similarity, both molecules bind the same receptor [8, 9] and induce nearly indistinguishable biological responses [10]. Furthermore, both molecules are similar with regard to their overall structures. Though secreted, both IL-1*α* and IL-1*β* lack a typical hydrophobic signal sequence [7, 11]. In humans the primary translation products of the IL-1*α* and IL-1*β* genes consist of 271 and 269 amino acids, respectively. By removing 112 and 116 N-terminal amino acid residues, respectively, these precursors are converted into mature proteins of approximately 17-kDa [7]. In the case of IL-1*β*, the mature 17-kDa form exhibits most powerful biological activity [12]. Cleavage of the IL-1*β* precursor is carried out by the IL-1*β* converting enzyme (ICE), a cysteinyl aspartate-specific proteinase that belongs to the caspase family of proteases [13, 14]. The mechanism of IL-1*β* secretion is not fully understood. The normal secretory pathway involving transport through the endoplasmic reticulum (ER) and Golgi apparatus seems not to be used, as inhibitors of this pathway do not affect IL-1*β* secretion [15]. Consistent with this finding is the suggestion that IL-1*β* is not glycosylated in spite of potential N-glycosylation sites being present [15]. In contrast to IL-1*β* which is mostly released from the producer cell, IL-1*α* remains predominantly cell-associated. Cleavage of the IL-1*α* precursor is not performed by ICE but involves a different protease [16, 17].

The third IL-1 family member, IL-1Ra, possesses no intrinsic biological activity. Rather it fulfills an important regulatory function by binding the same cell surface receptor as IL-1*α* and IL-1*β*, thus preventing IL-1-mediated signal transduction [18]. Human IL-1Ra consists of 176 amino acids and in its mature form shows 19% and 26% similarity to IL-1*α* and IL-1*β*, respectively [19]. In contrast to IL-1*α* and IL-1*β*, the IL-1Ra harbours a typical signal peptide and seems to be secreted via the ER/Golgi route [19].

In the chicken, an IL-1-like activity was demonstrated in conditioned medium from stimulated splenocytes [20]. As in murine macrophages, maximal IL-1 release by the chicken macrophage cell line HD-11 requires calmodulin-dependent kinase

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Abbreviations. DMEM, Dulbecco's modified essential medium; Gra*P*-DH, glyceraldehyde-3-phosphate dehydrogenase; ICE, interleukin-1*β*-converting enzyme; IL-1, interleukin-1; IL-1Ra, interleukin-1 receptor antagonist; LPS, lipopolysaccharide.

and protein kinase C [21]. Partially purified chicken IL-1 preparations were shown to exhibit weak cross-reactivity in murine IL-1 bioassays and to increase glucocorticosterone levels in chickens [22−24]. Whereas chicken IL-1 had not been characterized at the molecular level, the chicken type I IL-1 receptor was cloned more than five years ago [25]. It shows 64% overall amino acid sequence similarity to the human receptor. Conservation is even higher (79%) in the cytoplasmic domain.

We now report the cloning of a cDNA for the chicken homolog to mammalian IL-1β using a functional assay. Recombinant chicken IL-1β purified from *Escherichia coli* exhibited potent biological activity in cell culture and *in vivo*.

MATERIALS AND METHODS

Cell culture. HD-11 cells [26] and CEC-32 cells [27] were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 8% fetal calf serum and 2% chicken serum. COS-7 cells were grown in DMEM supplemented with 10% fetal calf serum. Primary macrophages were prepared from chicken blood and cultured as described [28].

cDNA library and screening system. A cDNA expression library was prepared from HD-11 cells that were stimulated for 5 h with 5 µg/ml LPS. cDNA prepared from poly(A)-rich RNA was unidirectionally cloned into the eukaryotic expression vector pcDNA1. The resulting cDNA library was divided into eight samples and the plasmids were amplified in *E. coli*. Each pool represented about 500 independent plasmids. Samples were used to transfect COS-7 cells in 6-well plates. COS cell supernatants were harvested at 48 h post transfection, diluted twofold with fresh medium and incubated for 18 h with chicken CEC-32 cells in 6-well plates. Total RNA was extracted from the cells of each well and analyzed by northern blotting using radiolabeled chicken K60 cDNA (EMBL/GenBank accession no. Y14971) which codes for a chicken CXC chemokine (Sick, C. and Staeheli, P., unpublished results) as a hybridization probe. A cDNA pool that yielded a clear K60 signal in this assay was further divided into eight subpools which were again used to transfect COS cells. The resulting COS cell supernatants were again assayed for K60-inducing activity as above. cDNA pools were subdivided until a single positive clone was identified.

RNA analysis. Total RNA was prepared by the guanidine thiocyanate/acid phenol method [29]. RNA was size-fractionated by electrophoresis through a 1.2% formaldehyde agarose gel and blotted onto a nylon membrane. The membranes were sequentially hybridized with the indicated cDNA probes which were radiolabeled with ^{32}P. Stripping between subsequent hybridizations was performed by quickly rinsing the membranes in a boiling solution of 10 mM sodium phosphate at pH 7.6. The various hybridization probes were: chicken K60 cDNA (EMBL/GenBank accession no. Y14971), chicken glyceraldehyde-3-phosphate dehydrogenase (Gra*P*-DH) cDNA [30] and the chicken IL-1β cDNA identified by the screening assay described above.

Purification of histidine-tagged ChIL-1*β* from *E. coli*. To facilitate subsequent purification of ChIL-1β from *E. coli* by affinity chromatography, it was N-terminally tagged with histidines. The coding sequence for putative mature ChIL-1β, starting at alanine 106 (see Fig. 2), was amplified by PCR using the sense primer 5′-GAGAGGATCCGCGCCCGCCTTCCGCTAC-3′ and the antisense primer 5′-GAGAGGATCCTCAGCGCC-CACTTAGCTT-3′, thus introducing *Bam*HI restriction sites near both ends of the PCR product. PCR was performed for 30 cycles (94 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min) in a total reaction volume of 100 µl with 50 ng of template, 50 pmol of each primer and 2.5 units *Taq* polymerase (Boehringer). The PCR product was digested with *Bam*HI and ligated into the *Bam*HI-restricted prokaryotic expression vector pQE9 (Qiagen), yielding construct pQE9-ChIL-1β. Recombinant protein (His-ChIL-1β) resulting from expression of this construct has a N-terminal extension comprising the amino acids Met-Arg-Gly-Ser-(His)$_6$-Gly-Ser. His-ChIL-1β was purified by affinity chromatography on a nickel chelate agarose column. Purification conditions were exactly as described for chicken interferon-γ [28]. Peak column fractions were pooled and samples were frozen at −20 °C. Yields were about 0.75 mg of His-ChIL-1β per liter of *E. coli* culture. Purified His-ChIL-1β was at least 95% pure.

***In vivo* activity of ChIL-1*β*.** Adult chickens were given intravenous injections (10 µg protein/kg body mass) of either purified recombinant His-ChIL-1β or His-MxA [31]. Immediately before and 0.5, 1, 2 and 4 h after injection, blood was taken from each animal and the serum corticosterone content was determined.

RESULTS

Supernatants of LPS-treated HD-11 cells contain CXC chemokine-inducing activity. In cells from mammals, IL-8 and other CXC chemokines are strongly induced by IL-1 and other pro-inflammatory cytokines [3−5]. We therefore treated chicken HD-11 cells for various times with 5 µg/ml of LPS and tested the resulting supernatants for the presence of a secreted substance that would induce CXC chemokine RNA in the chicken fibroblast cell line CEC-32. Two chicken cDNAs have been cloned that might code for the chicken homolog of the CXC chemokine IL-8: 9E3/CEF4 [32, 33] and K60 (Sick, C. and Staeheli, P., personal communication). Supernatants from LPS-induced HD-11 cells were found to induce transcripts in CEC-32 cells that hybridized to the K60 cDNA probe in northern blots (Fig. 1 A). Enhanced levels of K60 RNA were detected in CEC-32 cells exposed to supernatant from HD-11 cells that were induced for 5 h with LPS, while maximal levels of this RNA were observed with supernatant from cells induced for 12 h. Supernatant of uninduced HD-11 cells did not result in the induction of K60 RNA in CEC-32 cells (Fig. 1A). Similarly, LPS by itself did not induce K60 RNA (Fig. 1A). Further experiments showed that tenfold dilutions of supernatants from HD-11 cells that were stimulated for 17 h with 5 µg/ml of LPS still induced maximal levels of K60 RNA, whereas higher dilutions were less effective (data not shown). It thus seemed that induction of K60 RNA in CEC-32 cells could be used as a biological assay for expression cloning of the critical pro-inflammatory cytokine secreted by LPS-induced HD-11 cells.

Cloning of a chicken cDNA that encodes CXC chemokine-inducing activity. To clone the K60-inducing cytokine, we used poly(A)-rich RNA from HD-11 cells treated for 5 h with 5 µg/ml of LPS to construct a cDNA library in the eukaryotic expression vector pcDNA1. cDNA from eight sublibraries, each representing some 500 independent plasmids, was transfected into COS cells. The resulting cell supernatants were collected at 48 h post transfection and assayed for K60-inducing activity. To do this, CEC-32 fibroblasts were incubated for 18 h with twofold diluted COS cell supernatants before RNA was extracted and analyzed by northern blotting for the presence of K60 transcripts. A faint hybridization signal was observed with one of the eight COS cell supernatants, suggesting that the corresponding plasmid pool contained a cDNA encoding the critical cytokine. This plasmid pool was reamplified in *E. coli* and divided into eight samples, which were then retested individually. Two of these subpools turned out to be positive and one of them was

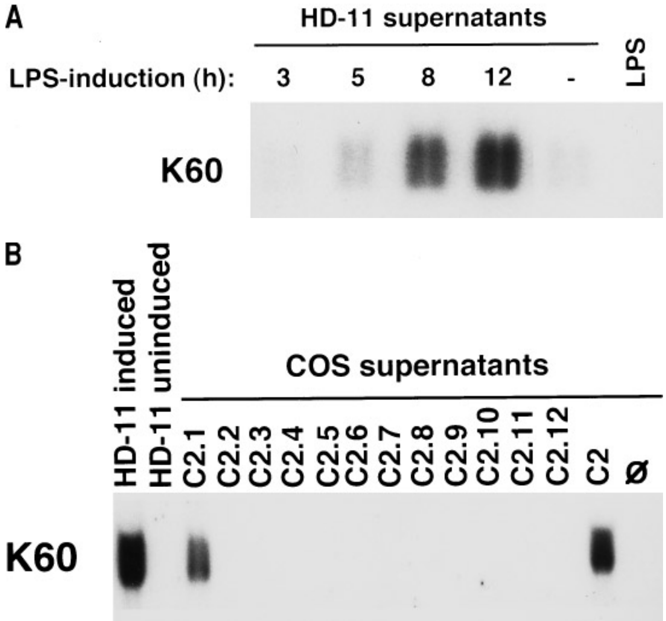

Fig. 1. Detection of K60 CXC chemokine transcripts in chicken fibroblasts. (A) Treatment of HD-11 cells with LPS results in secretion of an activity that induces K60 transcripts in CEC-32 cells. HD-11 cells were incubated in the presence or absence of 5 µg/ml of LPS for 1 h before the cells were washed and fresh medium lacking LPS was added. After the indicated times, the supernatants were collected and incubated with CEC-32 cells for 18 h. As a control for possible direct effects of LPS, one culture of CEC-32 cells was incubated with medium containing 5 µg/ml of LPS. RNA was prepared from the various cultures and subjected to northern blot analysis. The membrane was hybridized to radiolabeled K60 cDNA that codes for a chicken CXC chemokine. (B) The product of plasmid C2.1 induces the synthesis K60 transcripts in CEC-32 cells. CEC-32 cells were treated with twofold-diluted supernatants from COS cells transfected with individual plasmids (numbered C2.1 through C2.12) or a mixture of plasmids (pool C2). Tenfold-diluted supernatants of HD-11 cells incubated with or without 5 µg/ml of LPS for 17 h served as positive and negative control, respectively. RNA analysis was done as in (A).

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CTTCACCCTCAGCTTTCACGCTGGGCACAGAGATGGCGTTCGTTCCCGACCTGGACGTGC   60
                                  M  A  F  V  P  D  L  D  V
                                                          9
TGGAGAGCAGCAGCCTCAGCGAAGAGACCTTCTACGGCCCCTCCTGCCTCTGCCTGCAGA  120
L  E  S  S  S  L  S  E  E  T  F  Y  G  P  S  C  L  C  L  Q
                                                         29
AGAAGCCTCGCCTGGATTCTGAGCACACCACAGTGGACGTGCAGGTGACGGTGCGGAAGG  180
K  K  P  R  L  D  S  E  H  T  T  V  D  V  Q  V  T  V  R  K
                                                         49
GACGTGGTGCCCGGAGCTTTCGGCGGGCCGCCGTGCTGGTGGTGGCCATGACCAAACTGC  240
G  R  G  A  R  S  F  R  R  A  A  V  L  V  V  A  M  T  K  L
                                                         69
TGCGGAGGCCGAGGAGCAGGGACTTTGCTGACAGCGACCTGAGCGCGCTCCTGGAGGAGG  300
L  R  R  P  R  S  R  D  F  A  D  S  D  L  S  A  L  L  E  E
                                                         89
TTTTTGAGCCCGTCACCTTCCAGCGGCTGGAGAGCAGCTACGCCGGGGCGCCCGCCTTCC  360
V  F  E  P  V  T  F  Q  R  L  E  S  S  Y  A  G  A  P  A  F
                                                        109
GCTACACCCGCTCACAGTCCTTCGACATCTTCGACATCAACCAGAAGTGCTTCGTGCTGG  420
R  Y  T  R  S  Q  S  F  D  I  F  D  I  N  Q  K  C  F  V  L
                                                        129
AGTCACCCACCCAGCTGGTGGCCCTGCACCTCCAGGGGCCCTCCTCCAGCCAGAAAGTGA  480
E  S  P  T  Q  L  V  A  L  H  L  Q  G  P  S  S  S  Q  K  V
                                                        149
GGCTCAACATTGCGCTGTACCGGCCCCGAGGCCCACGGGGCAGCGCTGGAACTGGGCAGA  540
R  L  N  I  A  L  Y  R  P  R  G  P  R  G  S  A  G  T  G  Q
                                                        169
TGCCAGTGGCACTGGGCATCAAGGGCTACAAGCTCTACATGTCGTGTGTGATGAGCGGCA  600
M  P  V  A  L  G  I  K  G  Y  K  L  Y  M  S  C  V  M  S  G
                                                        189
CCGAGCCCACACTGCAGCTGGAGGAAGCCGACGTCATGCGGGACATCGACAGCGTCGAGC  660
T  E  P  T  L  Q  L  E  E  A  D  V  M  R  D  I  D  S  V  E
                                                        209
TGACCCGCTTCATCTTCTACCGCCTGGACAGCCCGACTGAGGGCACCACGCGCTTCGAGT  720
L  T  R  F  I  F  Y  R  L  D  S  P  T  E  G  T  T  R  F  E
                                                        229
CGGCCGCCTTCCCCGGGTGGTTCATCTGCACCTCCCTGCAGCCCCGGCAGCCCGTGGGCA  780
S  A  A  F  P  G  W  F  I  C  T  S  L  Q  P  R  Q  P  V  G
                                                        249
TCACCAACCAACCCGACCAGGTCAACATCGCCACCTACAAGCTAAGTGGGCGCTGACTGC  840
I  T  N  Q  P  D  Q  V  N  I  A  T  Y  K  L  S  G  R
                                                  267
CCGCTCCAACCCAAACCGGCCCGTTGCTGGTTTCCATCTCGTATGTACCGAGTACAACCC  900
CTGCTGCCCCGCCACGCTGTTCCACGTGGAATAGCCGCTCTGGGCCCTGTGGGCCTGAGT  960
CATGCATCGTTTATGTTTCATTACCGTCCCGTTGCTTTTAACTTATATATATATATTTTT 1020
TACCGTAATTATGATGGTTTTATTTCATCTCCCCTTGTCGGGGTGGAGGTATTTATTGTG 1080
ACTGGAGAAATAAAAGCTTCCCTGTGGAn                                1108
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Fig. 2. cDNA sequence (EMBL/GenBank accession no. Y15006) and predicted amino acid sequence of ChIL-1*β*. Nucleotide numbering is given at the right. An ATTTA sequence motif in the 3′-noncoding region which might mediate RNA instability is highlighted. The sequence of the ORF is given in the single letter code. Amino acid numbers are indicated below the sequence. Biologically active recombinant His-ChIL-1*β* included all amino acids downstream of alanine at position 106 (printed in bold).

further subdivided and retested until a single positive cDNA clone (designated C2.1) was identified (Fig. 1B).

The K60-inducing cytokine is the chicken homolog of mammalian IL-1*β*. The cDNA insert of clone C2.1 consists of 1107 nucleotides followed by a poly(A) tail. A short 5′-noncoding region of 32 nucleotides is followed by a long open reading frame that codes for a polypeptide of 267 amino acids (Fig. 2). The 3′-untranslated region consists of 271 nucleotides excluding the poly (A) tail. It harbors one ATTTA sequence element, typically found in cytokine mRNAs. This element is believed to confer RNA instability [34]. Database searches revealed significant similarity of the encoded polypeptide to human IL-1*β* (25% similarity) and the IL-1Ra (30% similarity) (Fig. 3A). Similarity to human IL-1*α* was 13% (data not shown). The overall structure of the novel chicken cytokine suggested that we have cloned the chicken homolog of mammalian IL-1*β* rather than IL-1Ra. The primary translation product of the chicken cDNA is similar in length to the IL-1*β* precursor. It is about 100 amino acids longer than the uncleaved form of IL-1Ra. Like IL-1*β*, but unlike IL-1Ra, the protein encoded by cDNA clone C2.1 lacks a typical signal peptide. Instead, it contains a N-terminal region that resembles that of the IL-1*β* propeptide. Stretches of basic amino acids and stretches of negatively charged residues are found at corresponding positions in the propeptides of human IL-1*β* and in the putative propeptide of the chicken cytokine (Fig. 3A). Based on these structural features, the product of cDNA clone C2.1 was designated chicken IL-1*β* (ChIL-1*β*).

In mammals the propeptide of IL-1*β* is removed by proteolytic cleavage carried out by the IL-1*β* converting enzyme (ICE). Cleavage by ICE occurs after a highly conserved aspartic acid residue (Fig. 3B). Sequence alignments showed that ChIL-1*β* lacks the critical aspartic acid residue, complicating predictions regarding the N-terminus of mature ChIL-1*β* (Fig. 3B).

ChIL-1*β* transcripts in LPS-treated chicken macrophages. To study the induction of the ChIL-1*β* gene, we treated primary blood-derived chicken macrophages and the macrophage cell line HD-11 for various times with 5 µg/ml of LPS. RNA was extracted from these cultures and 20 µg-samples were analyzed for the presence of ChIL-1*β* transcripts by northern blot analysis. A prominent LPS-induced transcript of about 1.5 kb and a minor transcript of approximately 3 kb hybridized to the radiolabeled ChIL-1*β* cDNA probe (Fig. 4). Induction of ChIL-1*β* RNA was very fast in both HD-11 cells and primary macrophages: maximal levels of ChIL-1*β* transcripts were observed within 1 h of LPS induction (Fig. 4). While ChIL-1*β* RNA levels remained high for at least 12 hours in HD-11 cells, ChIL-1*β* transcripts accumulated only transiently in primary macrophages.

Histidine-tagged recombinant ChIL-1*β* from *E. coli* is biologically active. Although supernatants from COS cells

A

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1    MAFVPDL--DVLESSSLSEETFY----GPSCLCLQKK---PRLDSEHTTVDVQVTVRKGRGARSFRRAAVLV   chicken IL-1β
1    MAEVPKLASEMMAYYSGNEDDLFFEADGPK----QMKCSFQDLDLCPLDGGIQLRISDHHYSKGFRQAASVV   hu IL-1β
1    M---------------------------------------EIC---------------RGLR------   hu IL-1Ra

64   VAMTKLLRR--PRSRDFADSDLSALLEEVFE--PVTFQR-LESSYAGAPAFRYTRSQSFDIFDINQKCFVLE   chicken IL-1β
69   VAMDKLRKMLVPCPQTFQENDLSTFFPFIFEEEPIFFDTWDNEAYVHDAP---VRSLNCTLRDSQQKSLVMS   hu IL-1β
9    ------------------SHLITLLLFLFH--SETICR-PSGR----KS---SKMQAFRIWDVNQKTFYLR   hu IL-1Ra

131  SPTQLVALHLQGPSSSQKVRLNIALYRPRGPRGSAGTGQMPVALGIKGYKLYMSCVMSGTEPTLQLEEADVM   chicken IL-1β
138  GPYELKALHLQGQDMEQQVVFSMSF-----VQGEESNDKIPVALGLKEKNLYLSCVLKDDKPTLQLESVDPK   hu IL-1β
52   N-NQLVAGYLQGPNVNLEEKIDVVP-----IEPHA------LFLGIHGGKMCLSCVKSGDETRLQLEAVNIT   hu IL-1Ra

203  R-DIDSVELTRFIFYRLDSPTEGTTRFESAAFPGWFICTSLQPRQPVGITNQPD-QVNIATYKLS-GR.   chicken IL-1β
205  N-YPKKKMEKRFVFNKIEI--NNKLEFESAQFPNWYISTSQAENMPVFLGGTKG-GQDITDFTMQFVSS   hu IL-1β
112  DLSENRKQDKRFAFIRSDS--GPTTSFESAACPGWFLCTAMEADQPVSLTNMPDEGVMVTKFYFQ-EDE   hu IL-1Ra
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B

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84  S A L L E E V F E - - P V T F Q R L E S S - - - - Y A G - A P A F R Y T R S Q S F D I F D I N Q K C F   chicken IL-1β
91  S T F F P F I F E E E P I F F D T W D N E A - - - Y V H D A P - - - - V R S L N C T L R D S Q Q K S L   human IL-1β
91  S T L I P F I F E E E P V F L D T R N N D A - - - C V H D A P - - - - V R S L H C T L R D A Q L K S L   makaque IL-1β
90  S T F F S F I F E E E P I L C D S W D D D - D N L L V C D V P - - - - I R Q L H Y R L R D E Q Q K S L   mouse IL-1β
89  R S I L S F I F E E E P V I F E T S S D E - - - - L L C D A A - - - - V Q S V K C K L Q D R E Q K S L   sheep IL-1β
90  K S I F S F I F E E E P I I L E T C N D D - - - - F V C D A N - - - - V Q S M E C K L Q D K D H K S L   sus IL-1β
91  K S L F C C I F E E E P I I C D T W D D G - - - - F V C D A A - - - - I Q S Q D Y T F R D I S Q K S L   cat IL-1β
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Fig. 3. Comparison of (A) protein sequences of ChIL-1*β*, human IL-1*β* and human IL-1Ra, and (B) amino acid sequences near the cleavage sites of IL-1*β* from various mammalian species and the corresponding sequence of ChIL-1*β*. (A) Alignment was done using the Clustal method. Conserved stretches of basic (boxed) and acidic (underlined) residues of the human IL-1*β* propeptide and the putative propeptide of ChIL-1*β* are marked. (B) Alignment was done using the J. Hein method. All mammalian IL-1*β* propeptides are cleaved (arrow) after a highly conserved aspartate residue.

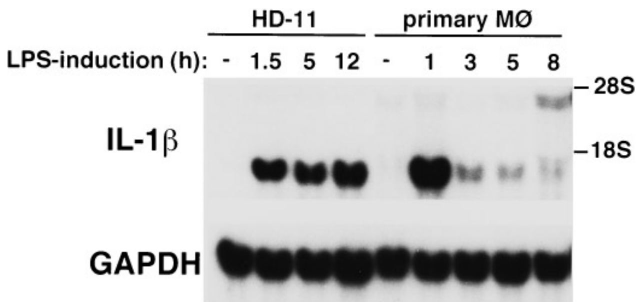

Fig. 4. Detection of ChIL-1*β* transcripts in LPS-stimulated HD-11 cells and in primary chicken macrophages. HD-11 cells or primary chicken macrophages were cultured for the indicated times in the presence or absence of 5 μg/ml of LPS. RNA was prepared from the various cultures and 20 μg-samples were subjected to northern blot analysis. The membrane was sequentially hybridized to radiolabeled ChIL-1*β* and Gra*P*-DH (GAPDH) cDNA probes.

transfected with clone C2.1 contained biologically active ChIL-1*β*, its concentration was rather low. To produce recombinant ChIL-1*β* for future *in vivo* experiments, we set out to produce biologically active ChIL-1*β* in *E. coli*. Sequence comparisons yielded no clear indications regarding the N-terminus of mature ChIL-1*β* (Fig. 3B). Since addition of a histidine-tag to the N-terminus of mature porcine IL-1*β* did not interfere with biological activity [35], we tested whether a fragment of ChIL-1*β* that includes all residues downstream of the alanine at position 106 was functional by cloning the truncated ChIL-1*β* cDNA into the bacterial expression vector pQE9 so that the resulting translation product carried a histidine tag at its N-terminus. A large portion of histidine-tagged ChIL-1*β* (His-ChIL-1*β*) produced in *E. coli* was soluble. It efficiently bound to nickel chelate agarose, and it could be eluted from the matrix under nondenaturing conditions. Peak column fractions were pooled and diluted to a concentration of 1 mg protein per ml. His-ChIL-1*β* was at least 95% pure and migrated on SDS/PAGE as a prominent band at approximately 23 kDa (Fig. 5).

To test for biological activity of the purified His-ChIL-1*β*, CEC-32 cells were incubated for 18 h with increasing dilutions of this material and RNA was analyzed for K60 transcripts. His-ChIL-1*β* from *E. coli* was biologically active: it strongly induced K60 RNA in CEC-32 cells at concentrations as low as 0.1 μg/ml (Fig. 6). To determine whether recombinant ChIL-1*β* was also active *in vivo*, we injected samples of purified His-ChIL-1*β* (10 μg/kg body weight) into the jugular vein of adult chickens and measured the corticosterone levels in the serum at various times post treatment. In all treated animals, hormone levels increased quickly after cytokine application (Fig. 7). Corticosterone concentrations reached maximal values at about 1 h post injection and decreased rapidly thereafter. No significant increase of the corticosterone levels was observed in control animals that were treated with an unrelated protein (His-MxA, [31]) that was purified by the same protocol, indicating that the observed corticosterone increase was a specific response to the IL-1*β* stimulus. These results clearly showed that His-ChIL-1*β* is also active *in vivo*.

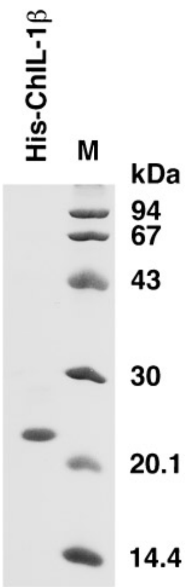

Fig. 5. Purification of histidine-tagged ChIL-1*β* from *E. coli*. Bacteria transfected with plasmid pQE9-ChIL-1*β* were lysed by sonication and the cell-free supernatant was applied on a nickel chelate agarose column. After extensive washing, bound His-ChIL-1*β* was eluted with a buffer containing 250 mM imidazole. A sample of this material was analyzed by SDS/PAGE and Coomassie blue staining. M, molecular size marker.

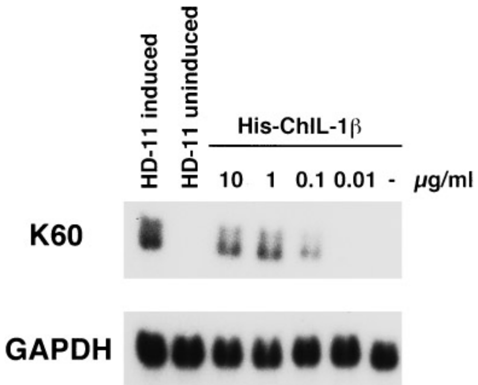

Fig. 6. Recombinant His-ChIL-1*β* from *E. coli* is active in cultured cells. CEC-32 cells were incubated for about 18 h with the indicated concentrations of purified His-ChIL-1*β*. CEC-32 cultures treated with supernatants from HD-11 cells incubated with or without 5 µg/ml of LPS for 17 h served as positive and negative controls, respectively. RNA was prepared from the various cultures and subjected to northern blot analysis. The membrane was sequentially hybridized with radiolabeled K60 and Gra*P*-DH (GAPDH) cDNA probes.

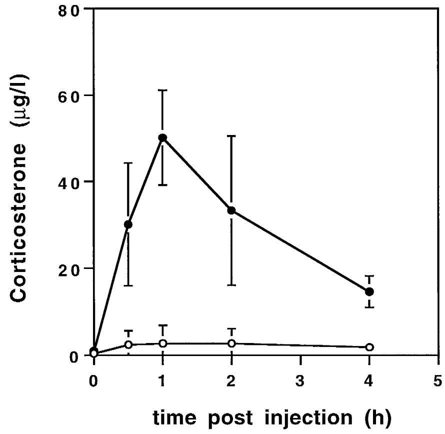

Fig. 7. Recombinant His-ChIL-1*β* from *E. coli* is active *in vivo*. Samples (10 µg/kg body mass) of purified His-ChIL-1*β* (closed circles) or an unrelated control protein (His-MxA, open circles) were injected intravenously into adult chickens. Three animals were used for each protein preparation. Blood samples were drawn from these animals directly before and at the indicated times after cytokine treatment and the serum corticosterone levels were determined.

DISCUSSION

Using an expression cloning strategy in COS cells and induction of a CXC chemokine in chicken fibroblasts as bioassay, we isolated a cDNA from LPS-stimulated HD-11 cells that encodes the chicken homolog of mammalian IL-1*β*. Our successful cloning approach was based on the assumption that the regulation of mammalian and chicken cytokine genes are very similar. Our results thus support the concept that most elements of the mammalian cytokine network are conserved in birds, although the primary sequences of the protein components involved are quite dissimilar.

The isolated chicken cDNA codes for a primary translation product of 267 amino acids that has similarity to both mammalian IL-1*β* and IL-1 receptor antagonist. Several structural features of the chicken cytokine strongly indicate that it represents the mammalian homolog of IL-1*β* rather than IL-1 receptor antagonist. First, the primary translation product of the chicken cytokine and the non-cleaved precursors of mammalian IL-1*β* have nearly identical sizes [6], whereas the primary translation products of mammalian IL-1Ra are about 100 amino acids shorter [6]. Second, IL-1Ra harbors a hydrophobic signal peptide at the N-terminus [19] that is absent in mammalian IL-1*β* and in the chicken cytokine. Instead, mammalian IL-1*β* is synthesized as a precursor of which about 115 N-terminal amino acids are proteolytically removed [6]. The N-terminal domain contains conserved stretches of basic and acidic residues [7] which can also be identified in the cloned chicken cytokine. Third, AUUUA de-stabilization signals are present in mammalian IL-1*β* trancripts [11] as well as in the chicken cytokine mRNA. Such elements are not found in transcripts of IL-1Ra of all species investigated to date [18]. Finally, the chicken cytokine was cloned on the basis of its biological activity, whereas mammalian IL-1Ra lacks intrinsic biological activity [18].

Regarding the regulation of ChIL-1*β*, we were able to demonstrate a quick and strong response to LPS in both the chicken macrophage cell line HD-11 and primary chicken macrophages. In HD-11 cells rapid accumulation of ChIL-1*β* RNA was observed within 90 min of LPS treatment, and ChIL-1*β* transcript levels were maintained at high levels for at least 12 h. By contrast, in primary macrophages high ChIL-1*β* RNA levels were observed as early as 60 min post induction with LPS, but these transcripts disappeared rapidly. This latter picture might be a more authentic reflection of what is happening *in vivo*, as persistent high levels of IL-1*β* might have deleterious effects for the organism.

Having identified the cloned chicken cytokine as the homolog of mammalian IL-1*β* raises the question of whether biologically active ChIL-1*β* is generated by proteolytic processing of the primary translation product, like its mammalian counterpart [13]. Sequence comparisons of mammalian IL-1*β* and ChIL-1*β* revealed that this question cannot be answered easily. In mammals, cleavage of IL-1*β* by the ICE protease occurs after a highly conserved aspartate residue [14]. ChIL-1*β* shows significant similarity to mammalian IL-1*β* regions located N-terminally and C-terminally of the cleavage site, but the sequence at the mammalian IL-1*β* cleavage site is not conserved and the critical aspartic acid residue is missing. We directly tested the possibility that a C-terminal fragment of the cloned chicken cytokine, which roughly corresponds to mature mammalian IL-1β, contains all sequences required for biological activity. This fragment was expressed in *E. coli* after a histidine tag was engineered to its N-terminus to simplify subsequent purification. We indeed found that purified recombinant His-ChIL-1β exhibited biological activity: it induced the synthesis of the K60 CXC chemokine RNA in chicken fibroblasts at concentrations as low as 0.1 µg/ml, and it induced a rapid increase in serum corticosterone levels when injected into adult chickens as was previously described for crude preparations of natural ChIL-1β [36].

From these experiments we cannot conclude, however, that alanine 106 which we have arbitrarily chosen as the first residue following the histidine tag in His-ChIL-1β also represents the N-terminus of active natural ChIL-1β. As all known caspases of mammals and nematodes cleave their substrates after an aspartic acid residue [14], cleavage of ChIL-1β might occur after the aspartic acid residues located at positions 118 and 121. Alternatively, cleavage of the ChIL-1β precursor might be carried out by a protease with different substrate specificity, as is the case for mammalian IL-1α [16, 17]. Another possibility is that the unprocessed form of ChIL-1β is active, as was shown for human IL-1α [37]. However, a molecular size of 16−21.5 kDa has been determined for partially purified chicken IL-1β [36]. This size estimate is consistent with the assumption that proteolytic cleavage of ChIL-1β does occur. Although the N-terminal sequence of mature ChIL-1β remains unknown, our results demonstrate that unlimited quantities of biologically active cytokine can now be produced for *in vivo* studies aimed at determining the role of this cytokine during inflammatory processes in the chicken.

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