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Truncated Chicken Interleukin-1β with Increased Biologic Activity

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ABSTRACT

Chicken interleukin-1β (ChIL-1β) is synthesized as a precursor molecule that unlike its mammalian counterpart, lacks a typical caspase-1 cleavage site. Therefore, it was unclear if proteolytic cleavage of ChIL-1β can occur and if cleavage might modulate the biologic activity of this cytokine. Using an avian indicator cell line that carries an NF-κB-regulated luciferase reporter gene, we established a sensitive and highly specific bioassay for ChIL-1β. Experiments with a rabbit antiserum indicated that the NF-κB-stimulating activity in supernatants of lipopolysaccharide (LPS)-treated chicken HD-11 macrophages is largely due to IL-1β and that proteolytic processing of natural and recombinant ChIL-1β is not very efficient. Functional analyses further revealed that cDNAs for either full-length or N-terminally truncated chicken ChIL-1β yielded active cytokine. A truncated molecule that closely resembled putative mature ChIL-1β exhibited more than 100-fold enhanced biologic activity after expression in mammalian cells, indicating that precursor cleavage is indeed of critical importance for maximal activity.

INTRODUCTION

Mammalian interleukin-1β (IL-1β) is an important proinflammatory cytokine that exhibits pleiotropic activities on a wide range of target cells.[1] Biologic effects of IL-1β include induction of fever, activation of the cytokine network, triggering of the acute-phase response in the liver, and activation of vascular endothelium. IL-1β further induces the synthesis of CXC chemokines in fibroblasts and other cell types.[2] It is secreted by many different cell types, with stimulated macrophages being the main producers.[1] After binding to a specific cell surface receptor, IL-1β activates a signal cascade that causes nuclear localization of activated transcription factor NF-κB and, consequently, enhanced transcription of a large number of responsive genes.[3] IL-1β lacks a typical hydrophobic signal sequence at the N-terminus.[4] The primary translation product is converted into mature cytokine by caspase-1, a cysteinyl aspartate-specific proteinase.[5,6] The mechanism of IL-1β secretion is not fully understood.

Chicken IL-1β (ChIL-1β) is synthesized as a precursor molecule that exhibits significant homology to mammalian IL-1β in the C-terminal moiety.[7] Interestingly, the caspase-1 cleavage site is not conserved in the chicken precursor. Nevertheless, bacterially produced recombinant ChIL-1β lacking the nonconserved N-terminal part of the precursor molecule is biologically active, demonstrating that the N-terminal moiety is dispensable.[7] Expression of the complete ChIL-1β cDNA in mammalian cells yielded active cytokine, but the titers of such preparations were low.[7] We reasoned that inefficient processing of the ChIL-1β precursor limited the yields of active protein, but research aimed at proving this hypothesis was slowed down because the previously available bioassay was laborious and impractical. It used quail CEC-32 cells, which were stimulated with IL-1β before cytokine-mediated accumulation of mRNA encoding the CXC chemokine K60 was measured by Northern blotting.[7]

Here, we introduce a much simpler bioassay for ChIL-1β that exhibits high sensitivity and high specificity. Using this assay, we show that N-terminal truncation of the ChIL-1β precursor near the putative protease cleavage site dramatically enhances cytokine activity.

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MATERIALS AND METHODS

Cells

Human 293T cells and quail CEC-32 cells,[8,9] permanently transfected with an IL-1β responsive luciferase reporter gene (CEC-NF-κB-LUC), were maintained in DMEM/NUT.Mix.F-12 medium (Invitrogen, San Diego, CA) supplemented with Glutamax and 10% fetal bovine serum (FBS).

Plasmids

The NF-κB-LUC reporter construct (PathDetect NF-κB) was purchased from Stratagene (La Jolla, CA). The cDNA library used for screening of clones encoding novel cytokines capable of activating the CEC-NF-κB-LUC indicator cell line was constructed from mRNAs of lipopolysaccharide (LPS)-treated chicken HD-11 cells as described.[7] Site-directed mutagenesis of plasmid DNA was performed using the QuickChange kit (Stratagene) and appropriate oligonucleotide primers as recommended by the manufacturer.

Antisera

Rabbits were immunized repeatedly with a bacterially produced, C-terminal fragment of ChIL-1β that roughly corresponds to the putative mature form of this cytokine. This histidine-tagged recombinant protein was produced and purified as described[7] and emulsified in Freund's incomplete adjuvant (FIA).

cDNA transfections

Plasmid DNA was purified from bacteria using a miniprep kit (Peqlab, Erlangen, Germany). All transfections into 293T cells were performed with Metafectene (Biontex, Munich, Germany) following the protocol of the manufacturer. At 12 h post-cDNA transfection, fresh medium with reduced content of FBS (usually 2%) was added to the cultures, and supernatants were harvested 48 h later. Where indicated, cell lysates were prepared to analyze intracellular and extracellular ChIL-1β simultaneously. For this purpose, cells were lysed in the culture medium by two cycles of freezing and thawing before cell debris was removed by centrifugation.

Partial purification of natural ChIL-1β

Supernatant of HD-11 cells stimulated with LPS (1 μg/ml) for 1 h and then cultivated in serum-free medium for another 3 h served as starting material for purification. Assuming that ChIL-1β, like interferons (IFNs) and many growth factors, would efficiently bind to controlled pore glass (CPG), HD-11 supernatant containing NF-κB-activating activity was loaded onto a CPG column (0.7 g CPG per 100 ml supernatant). Subsequently, the column was extensively washed with 50 mM Tris-HCl, pH 7.2, containing 1 M NaCl before bound activity was eluted with 500 mM Tris-HCl, pH 7.2, containing 1 M NaCl. Further purification was achieved by ion exchange chromatography on MonoQ columns. Protein was loaded in 50 mM Tris-HCl, pH 7.2, buffer, and contaminating proteins were eluted with an increasing salt gradient. The NF-κB-activating activity eluted at 110 mM NaCl. Molecular sieve chromatography (Superdex 75 or TSK-Gel SW200) (Supelco, Bellefonte, PA) showed a major protein peak of 50–60 kDa, frequently accompanied by a minor peak of approximately 20–25 kDa.

Bioassay for ChIL-1β

In standard assays, serial dilutions of cell lysates or cell culture supernatants containing biologically active IL-1β were added for 6 h to approximately 5×10^4 CEC-NF-κB-LUC cells in 24-well plates. The medium was then removed, and the cells were lysed with 100 μl lysis buffer (Promega, Madison, WI). Samples were assayed for luciferase activity using a kit (Promega).

The culture supernatants were depleted of biologically active IL-1β as follows. The IgG fraction in 30-μl samples of preimmune or hyperimmune serum was first immobilized on protein A Sepharose beads. The washed beads were incubated overnight with 300-μl samples of diluted, cytokine-containing cell culture medium. After removal of the beads by centrifugation, the cleared samples were added to CEC-NF-κB-LUC indicator cells for 6 h before luciferase activity was determined.

Western blotting

Proteins in 50–100-μl samples of cell culture supernatants or cell lysates were precipitated by adding ethanol to a final concentration of 50% (v/v) and centrifuging. The dried pellet was dissolved in SDS-PAGE sample buffer and heated to 95°C for 5 min in the presence of 2-mercaptoethanol, and proteins in the sample were subjected to 10% SDS-PAGE. Western blots were probed with 0.1% rabbit hyperimmune serum in phosphate-buffered saline (PBS) containing 1% dry milk and developed using peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL) chemistry (Pierce, Rockford, IL).

RESULTS

A simple bioassay for ChIL-1β

Assuming that ChIL-1β can activate a signaling cascade in avian cells that causes the activation of transcription factor NF-κB, we generated a stable CEC-32 quail cell clone that expresses a firefly luciferase reporter gene under the control of an NF-κB regulatory element. Incubation for 6 h of CEC-NF-κB-LUC cells with supernatants of human 293T cells transfected with an expression construct for ChIL-1β caused 10–20-fold enhanced luciferase activity (Fig. 1A). Luciferase activity was gradually lost when increasing dilutions of supernatant were used. Comparable luciferase induction rates were measured if cytokine exposure was varied between 6 and 16 h (data not shown). When 293T supernatants containing recombinant ChIL-1β were depleted with a rabbit antiserum raised against a purified, bacterially expressed C-terminal ChIL-1β fragment, luciferase activity was reduced to background levels (Fig. 1A), strongly suggesting that the active factor was IL-1β. This view was further supported by the observation that supernatants of 293T cells transfected with the empty vector contained no detectable NF-κB-stimulating activity (data not shown).

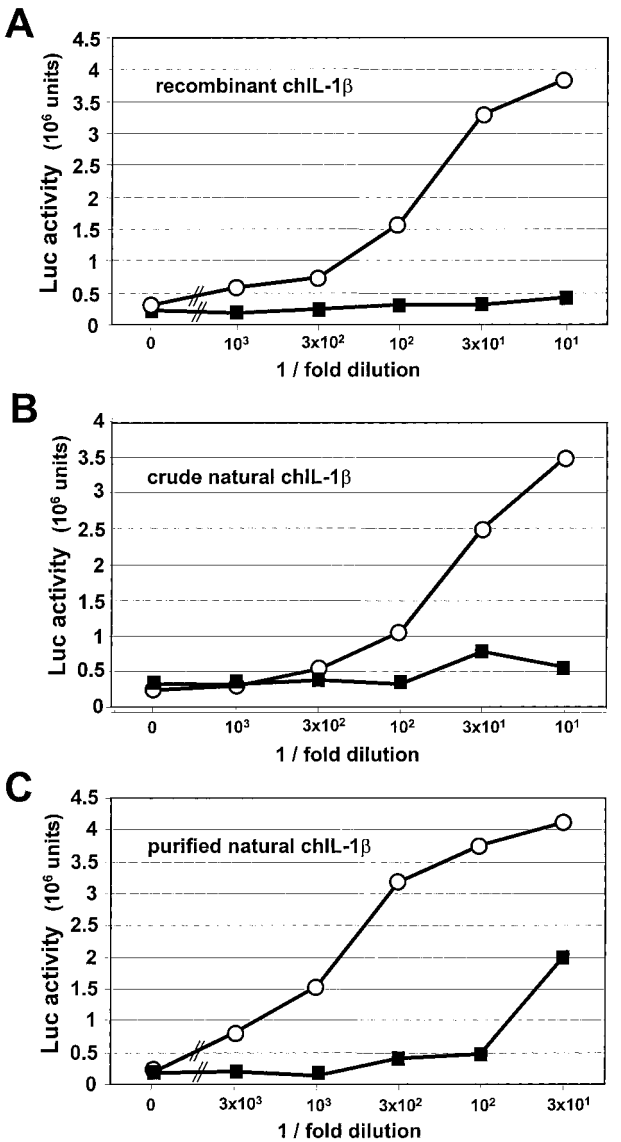

FIG. 1. Rabbit antiserum against a bacterially produced ChIL-1β fragment blocks the activation of a luciferase reporter gene in CEC-NF-κB-LUC indicator cells by (**A**) recombinant ChIL-1β from transfected 293T cells, (**B**) crude natural ChIL-1β, or (**C**) partially purified natural ChIL-1β. IgG in 30-μl samples of preimmune serum (circles) or hyperimmune serum (squares) was immobilized on protein A Sepharose beads. Washed beads were incubated overnight with 300-μl samples of cell culture medium containing the indicated dilutions of either (**A**) recombinant ChIL-1β derived from lysates of transfected 293T cells or (**B**) natural ChIL-1β partially purified from supernatants of LPS-treated HD-11 cells. After removal of the beads by centrifugation, the samples were added to CEC-NF-κB-LUC indicator cells for 6 h before luciferase activity was determined.

Antiserum to ChIL-1β blocks NF-κB-stimulating activity in supernatants of Chicken HD-11 cells

Chicken HD-11 macrophages treated with LPS secrete IL-1β and presumably several other cytokines.[10] Crude supernatant of LPS-treated HD-11 cells strongly activated the NF-κB-regulated luciferase reporter gene in CEC-NF-κB-LUC cells. The material remained active if subjected to exhaustive depletion with preimmune serum, but it was completely inactivated even at low dilutions if subjected to depletion with antiserum raised against ChIL-1β (Fig. 1B). We next purified the NF-κB-stimulating activity by affinity chromatography on CPG and showed that the activity of the purified material was also efficiently neutralized by our rabbit antiserum but not by the preimmune serum (Fig. 1C). From the titration curve, it can be seen that the titer of the purified natural ChIL-1β preparation dropped about 30-fold in the presence of antiserum, indicating that either IL-1β alone or together with some other as yet uncharacterized, antigenically related proteins accounted for >96% of the luciferase activity.

Poor cleavage of ChIL-1β precursor

Western blot analysis showed that the vast majority of ChIL-1β in supernatants of transfected 293T cells was present as uncleaved 30-kDa precursor (Fig. 2A, lane 1). The two minor immunoreactive bands at approximately 15–17 kDa may represent either correctly processed, mature ChIL-1β or inactive degradation products. When partially purified, natural ChIL-1β was analyzed by Western blotting, we observed two prominent immunoreactive bands at approximately 30 kDa and one at approximately 16 kDa (Fig. 2A, lane 2). They may represent the uncleaved and caspase-cleaved forms of natural ChIL-1β, respectively. It should be noted that the rather low amounts of purified protein loaded in lane 2 were about 100-fold more active on CEC-NF-κB-LUC cells than the comparably high amounts of recombinant protein loaded in lane 1, suggesting that the specific biologic activity of natural ChIL-1β was at least 1000-fold higher than that of the recombinant cytokine. To determine if the rChIL-1β precursor would be processed more efficiently in avian cells, we performed cDNA transfections into chicken HD-11 cells. Western blot analysis showed that precursor processing was not more efficient in HD-11 cells than in human 293T cells (Fig. 2B).

Specific response of CEC-NF-κB-LUC cells to ChIL-1β

To further characterize the NF-κB-stimulating cytokines secreted by LPS-treated HD-11 cells, we employed the bioassay described to perform a functional screen of an appropriate cDNA expression library.[7] Approximately 18,000 cDNAs, represented by 150 pools comprising about 120 individual library plasmids each, were transfected into 293T cells before the resulting supernatants were used to stimulate CEC-NF-κB-LUC cells. About one third (52 of 150) of the cDNA pools were positive in the initial screen. PCR with a primer pair that matched ChIL-1β mRNA sequences flanking the initiation codon revealed that 44 of the 52 positive pools contained at least one ChIL-1β cDNA with complete coding information. These pools were not characterized further. Seven of the eight remaining pools contained at least one 5′-terminally truncated ChIL-1β plasmid similar to clone 16-16-9 (Fig. 3A). These cDNAs seemed to employ a second in-frame ATG codon at amino acid position 46 for initiation of protein synthesis. Western blot analysis (Fig. 2C, lane 3) confirmed that cDNA 16-16-9 can direct the synthesis of an immunoreactive protein of ap-

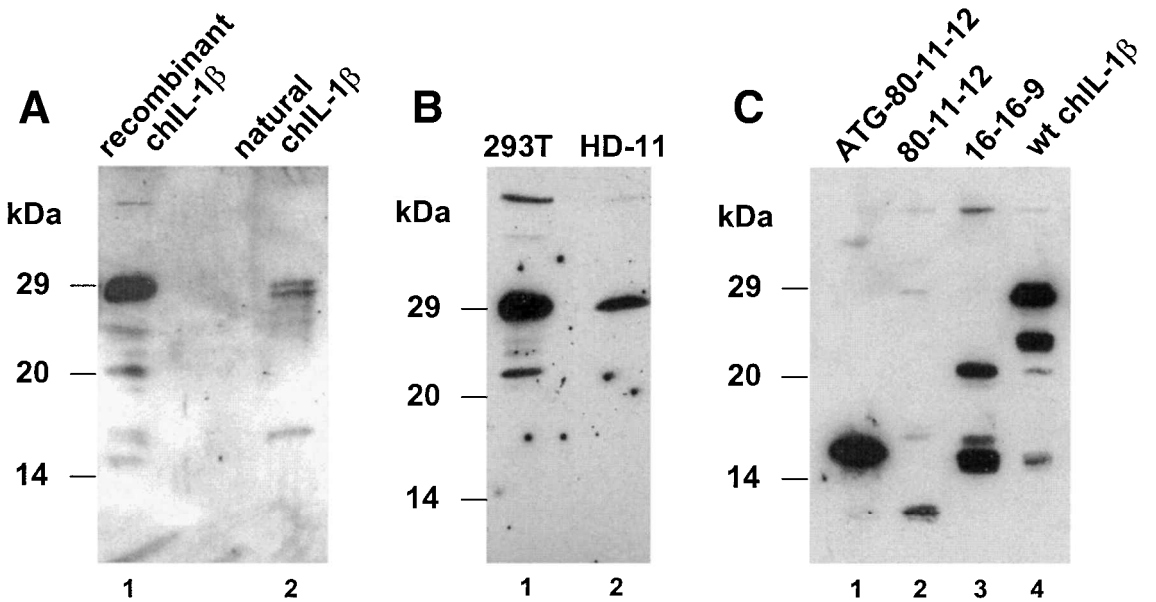

FIG. 2. Western blot analysis of natural and recombinant ChIL-1β. Proteins in the various preparations were precipitated with ethanol, dissolved in gel sample buffer, subjected to 15% SDS-PAGE, blotted to nylon membranes, and probed with rabbit antiserum to bacterially produced ChIL-1β fragment. (**A**) Comparison of recombinant ChIL-1β in the supernatant of 293T cells (lane 1) and natural ChIL-1β partially purified from supernatants of LPS-treated HD-11 cells (lane 2). (**B**) Analysis of ChIL-1β precursor processing after transfection of ChIL-1β cDNA into either human 293T cells (lane 1) or chicken HD-11 cells (lane 2). (**C**) Analysis of immunoreactive products present in 50-μl samples of lysates of 293T cells transfected with various forms of ChIL-1β cDNA (see Fig. 3A). The gel positions of molecular weight markers are indicated.

proximately 20 kDa in transfected 293T cells. It further yielded an immunoreactive product of about 15 kDa. We did not try to determine if it represents ChIL-1β that is cleaved correctly at the putative processing site. The single remaining positive cDNA pool yielded negative PCRs with all primers from regions upstream of the putative proteolytic cleavage site of the ChIL-1β precursor. After the active plasmid (80-11-12) in this cDNA pool was identified by functional tests, we found that it represented an IL-1β cDNA that was truncated immediately upstream of the putative protease cleavage site. This cDNA carried the hexanucleotide ACGAGG at its 5′-end (Fig. 3A), which was presumably introduced artificially during cDNA cloning. Thus, all biologically active cDNAs that we identified in this screen were derived from the ChIL-1β gene. No cDNAs from other chicken genes that might encode additional NF-κB-stimulating proteins were found.

Greatly enhanced bioactivity of ChIL-1β artificially processed to resemble putative mature cytokine

The biologically active cDNA clone 80-11-12 appeared to code for only the C-terminal 97 amino acids of the ChIL-1β precursor. It remained possible, however, that either the ACG or the AGG codon at the 5′-end of the plasmid insert served as an alternative initiation codon. Western blot analysis failed to provide evidence for this hypothesis (Fig. 2C, lane 2), indicating that if translation started at one of these two putative alternative sites, it did only with rather low efficacy. However, we observed that if the AGG was mutated to a canonical ATG initiation codon, a high amount of immunoreactive protein of predicted size was present (Fig. 2C, lane 1). The various ChIL-1β variants that we produced in transfected 293T cells all lacked signal sequences that would facilitate their secretion into the culture medium. Side-by-side titrations of supernatants and cell lysates indicated that the bulk (>95%) of the NF-κB-stimulating activity remained inside the 293T producer cells (data not shown). A comparison of the biologic activities of the various IL-1β variants revealed that lysates of 293T cells transfected with either full-length ChIL-1β, 16-16-9, or 80-11-12 cDNAs contained comparable levels of NF-κB-stimulating activity, whereas the activity in lysates of cells transfected with construct ATG-80-11-12 was at least 100-fold enhanced (Fig. 3B). Western blot analysis indicated that cells expressing either full-length ChIL-1β cDNA or construct ATG-80-11-12 contained comparable levels of recombinant IL-1β protein (Fig. 2C). Interestingly, 293T cells transfected with a ChIL-1β cDNA that was truncated at its 5′-end past the border of clone 80-11-12, designated C-term, also contained low levels of biologic activity, whereas control cells transfected with the empty vector did not (Fig. 3B). We concluded from these experiments that artificial truncation of the ChIL-1β precursor near the predicted protease cleavage site dramatically increased its NF-κB-stimulating activity. These data further indicated that the fairly high biologic activity of clone 80-11-12 might be explained by assuming that a nonconventional initiation codon was used at low efficacy to produce highly active ChIL-1β.

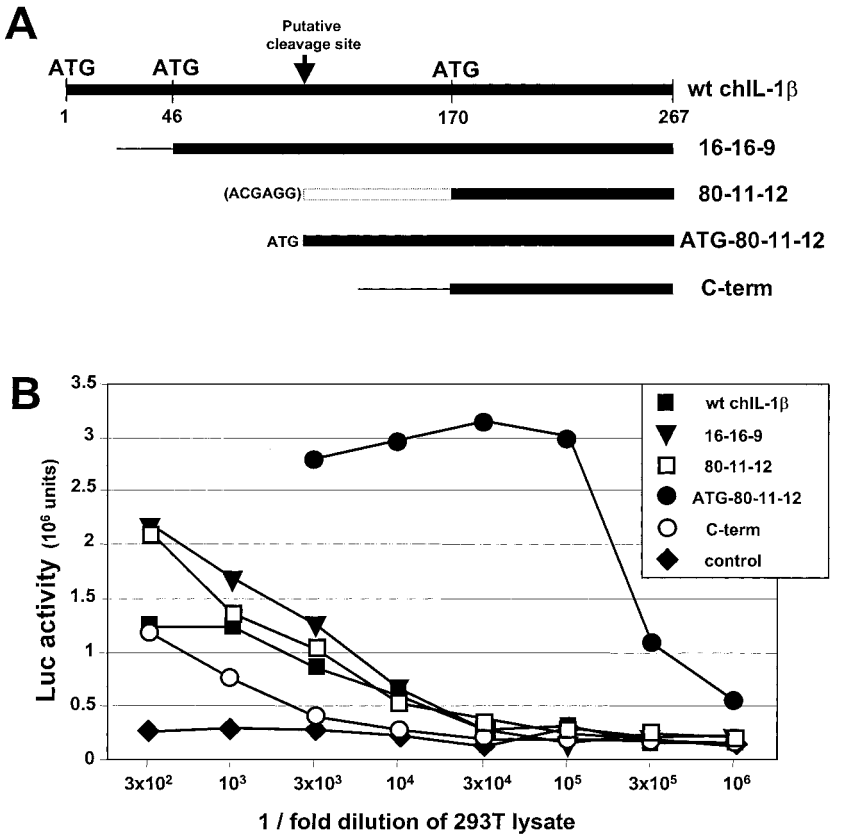

FIG. 3. cDNA structures and biologic activities of several ChIL-1β variants. (**A**) Full-length ChIL-1β cDNA encodes a precursor protein of 267 amino acids. The putative protease cleavage site at position 107 and two in-frame translation initiation codons are indicated. cDNA clones 16-16-9 and 80-11-12 were isolated in a functional screen. Clone ATG-80-11-12 was made by converting the ACGAGG hexanucleotide at the 5′-end of clone 80-11-12 to ACGATG, thus creating a canonical in-frame translation initiation codon. The location of the 5′-end of cDNA clone, C-term, is indicated. (**B**) 293T cells carrying the indicated plasmids were lysed at 48 h posttransfection. A side-by-side comparison of the NF-κB-stimulating activity of the resulting lysates in CEC-NF-κB-LUC cells at various dilutions is shown.

DISCUSSION

The new bioassay for ChIL-1β that we introduced here is based on the observation that signaling of IL-1β through its cognate receptor results in rapid activation and nuclear translocation of transcription factor NF-κB. The indicator cell line that we used for our experiments carries an NF-κB-inducible luciferase gene that permits a quick and highly sensitive readout. This bioassay exhibits high selectivity for IL-1β. In a large number of control experiments, we noted no unwanted background activity that may result from other factors present in crude cell extracts. Importantly, our depletion experiments with specific rabbit antiserum demonstrated that >96% of the NF-κB-stimulating activity in supernatants of LPS-treated chicken HD-11 cells was due to ChIL-1β or antigenically related molecules or both.

It is well known that activated HD-11 cells secrete a wide range of cytokines, which all appeared to be inert in our assay. In particular, they secrete IL-6, which is a potent activator of NF-κB in B cells.[11] We found that ChIL-6 was not active in our CEC-NF-κB-LUC indicator cell line (unpublished results), presumably because CEC-32 cells lack IL-6 receptors. From neutralization experiments with soluble chicken type I IL-1 receptor, other researchers concluded that about 30% of the IL-1-like activity in supernatants of LPS-treated HD-11 cells is probably caused by at least one other cytokine.[10] Obviously, our new bioassay fails to pick up the activity of these other putative cytokines. Furthermore, our large functional screen of cDNAs derived from LPS-treated chicken HD-11 cells successfully identified many ChIL-1β clones but failed to identify any other cytokine that would show activity in this assay. These findings thus collectively show that the newly introduced bioassay for ChIL-1β exhibits a very high degree of specificity, and they further indicate that chickens may have no functional IL-1α homolog.

Sequence comparisons revealed that although the basic structures of chicken and mammalian IL-1β precursors are well conserved, the caspase-1 cleavage site is not conserved.[7] The data presented here nevertheless demonstrated that as in the case of mammalian IL-1β, proteolytic processing greatly enhances the biologic activity of the primary translation product of the ChIL-1β cDNA. Thus, chicken caspase-1[12] may have a different

substrate specificity. Alternatively, other proteases might be responsible for cleavage of the IL-1β precusor. This would fit with recent observations indicating that caspase-1 is also not the sole caspase involved in mammalian IL-1β precursor processing.[13] In this context, it is of interest to note that a large fraction of the partially purified, natural ChIL-1β was present in a noncleaved state (Fig 2A). This suggests that poor proteolytic processing might represent an intrinsic property of this molecule. It is tempting to speculate that this property is important for the fine-tuning of the IL-1β activity in the chicken.

A final important aspect of our work should be mentioned. We found a simple way to increase the notoriously low yields of biologically active IL-1β in eukaryotic cells. Rather than expressing the full-length cDNA, we expressed only a fragment encoding an N-terminally truncated molecule that almost precisely corresponds to the putative mature form of the cytokine. Because such artificial molecules lack secretion signals, they accumulate in the cytoplasm of the producer cells and have to be released by cell lysis. Although this strategy bears the risk of product degradation by nonspecific proteases, it obviously is beneficial if natural product processing is inefficient. Future research will need to show if this strategy can be used to increase the biologic activity of other cytokines with similar needs for posttranslational processing steps, as, for example, ChIL-18.[14] This strategy could be used to equip viral vaccine vectors with genes encoding biologically active ChIL-1β or ChIL-18.

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