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Identification and Molecular Cloning of Functional Chicken IL-12

Winfried G. J. Degen,* Nancy van Daal,* Hanneke I. van Zuilekom,* Joan Burnside,† and Virgil E. J. C. Schijns*  

By a combination of large-scale sequencing, bioinformatics, and traditional molecular biology, we identified the long-searched-for cDNA sequences encoding the homologues of the chicken IL-12p35 and IL-12p40 chains. These molecules are the first discovered nonmammalian IL-12 subunits. The homologies of the chicken IL-12p35 and IL-12p40 proteins to the corresponding known subunits of various species, i.e., humans, sheep, horse, cat, bovine, mouse, and woodchuck, ranged between 21 and 42%, respectively. The expression of IL-12 subunits was observed in lymphoid cells and proved to be dependent on the cell type and stimulus, while expression was not detected in stimulated primary chicken embryo fibroblast cells. Following transient expression of both molecules in COS-7 cells, we confirmed the necessity of heterodimerization into IL-12p70 to yield bioactivity as was also shown for its mammalian counterparts. The chicken IL-12p70 molecule, generated either by transient coexpression of monomeric IL-12p35 and monomeric IL-12p40 or as a fusion protein (as in a fusion linker construct), induced IFN-γ synthesis and proliferative activity of freshly exposed chicken splenocytes. The high degree of functional similarity between chicken IL-12 and IL-12 of higher mammalian vertebrates, despite their poor sequence homology, illustrates the conservation and vital importance of the IL-12 molecule since the evolutionary dichotomy of birds and mammals >300 million years ago. In this article, we describe the first nonmammalian IL-12 molecule and show that this chicken IL-12 molecule is bioactive.  

The importance of the Th1/Th2 effector subsets and the key role of IL-12 in directing Th1-associated cell-mediated immunity explains the necessity to clone and characterize this molecule for species of veterinary relevance, where it can be used as a tool for prophylactic/therapeutic vaccination or for tumor therapy. To date bovine (15), cat (16–18), dog (19, 20), goat (J. C. Beyer, W. P. Cheevers, and N. M. Kumpula-McWhirtier, unpublished observations), horse (21), pig (22), and sheep (23–25) homologues of IL-12p40 and IL-12p35 subunits have been cloned. Some of them are currently being tested in clinical applications. Unfortunately, no avian IL-12 subunit has been cloned yet. In fact, no nonmammalian IL-12 molecule has been identified or isolated so far. It is also very remarkable that there is no evidence for a balanced Th1/Th2 system in nonmammalian species. Yet, the existence of a Th1-like cytokine network in birds was, in part, evidenced some years ago by the identification of chicken IFN-γ (26) and the recent discovery of chicken IL-18 (27). One of the major reasons for the failure to identify avian IL-12 thus far is the low sequence homology, usually restricted to ~30–50%, between avian and mammalian cytokine sequences. This explains the disaster of classical approaches to identify avian homologues of mammalian cytokines. The identification by PCR amplification using primers based on mammalian sequences proved to be very difficult and unpredictable (28, 29). As a result of this poor homology, cloning and sequencing of avian cytokines lags dramatically behind similar work achieved in mammals. So far only a limited number of avian cytokines have been identified (for two recent reviews, see Refs. 28 and 29).  

By using a combination of large-scale sequencing, bioinformatics, and traditional molecular biology, we succeeded in the isolation and characterization of an avian (chicken (Ch)3) IL-12p40 (ChIL-12p40) and IL-12p35 (ChIL-12p35) subunit. Despite the limited

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3 Abbreviations used in this paper: Ch, chicken; CEF, chicken embryo fibroblast; EST, expressed sequence tag; Fe, feline; IBV-N, N protein of avian infectious bronchitis virus; Mu, mouse; ODN, oligodeoxynucleotide; SPF, specific pathogen free; CD40L, CD40 ligand; semi-Q, semiquantitative.
sequence homology, they show remarkable functional similarity to
the group of known mammalian IL-12 molecules, underscoring the
evolutionary conserved functional importance of this molecule.

Materials and Methods

Cell culture

The mammalian cell line COS-7 (African green monkey kidney cells) and the
chicken DT-40 B cell line (a kind gift from P. Staeheli, University of
Freiburg, Freiburg, Germany) were grown in DMEM (supplemented
with 10% FCS, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin,
and 1 mM pyruvate), whereas the chicken HD-11 (macrophage origin) and
the CU91 (T cell origin) cell lines (a kind gift from T. Schat, Cornell
University, Ithaca, NY) were grown in RPMI 1640 (supplements as for
DMEM). Chicken embryo fibroblasts (CEF) were isolated using 3 U/ml
trypsin, 1 mM EDTA, and 0.5% triton X-100 from embryos of 10-day-old SPF
(species pathogen-free) chicken embryos. CEF cells were grown as monolayers
in DMEM (supplemented with 5% FCS). All cell lines were maintained in a
humidified atmosphere of 5% CO2 at 41°C described before (30) and grown in RPMI
1640 (see above) in a humidified atmosphere of 5% CO2 at 41°C.

Chickens

Three-week-old normal White Leghorn SPF chickens were derived from the
Intervet Animal Facilities and housed under SPF conditions. The animals re-
ceived water and food ad libitum. All experiments were conducted according
to protocols approved by the Intervet Animal Welfare Committee.

Treatment of cells with various stimulants

Approximately 700,000 cells were incubated overnight in 3-cm dishes with
1 μl of cell culture medium and one of the following stimulants: 10 μg
Con A (Miles Scientific, Naperville, IL), 10 μg of an immunostimulating
chicken CpG-oligodeoxynucleotide (ODN), 10° C, Naperville, IL), 10 μg
Aldrich). After incubation, cells were isolated, washed three times with
atmosphere of 5% CO2 at 41°C, sequenced in both 5′ and 3′ directions using a DNA
sequencing kit (BigDye Terminator V3.0 Cycle Sequencing Ready Reac-
tion with the conditions as described above. To determine the optimum
reaction conditions with the as described by Mengonagle et al. (32). The following primers were
used to amplify Chl-12p35 without the putative 35-aa signal peptide se-
quence (as determined by the SPScan program from the Wisconsin Package
10.2; Genetics Computer Group) and that introduced a 5′-NotI and
3′-HindIII restriction site: 5′-TGGGATCCGGTGGCGGCGGATCTCTGC
GACCACTGAGTACG-3′ and 5′-CAGCTCTGCCCCAC-3′ and 5′-CAGCTCTGAGTACG-3′. For Chl-12p40 the following primers were used that introduced a 5′-NotI and a 3′-HindIII restriction site: 5′-TGGCCGGCAG
CATGCTCTACCTGATTTGGGTTCTTCTTC-3′ and 5′-TGGGATCCGGTGGCGGCGGATCTCTGC
GACCACTGAGTACG-3′ and 5′-HindIII. For Chl-12p35 fragment was also cloned into pcDNA3.1+ (In vitro Life Technologies) to obtain an antisense construct.

Chl-12p40. Full-length Chl-12p40, present in a cDNA library con-
structed from pooled T and B cells isolated from vaccinated chickens
and cloned into pcDNA3.1+ (Invitrogen Life Technologies) and sequenced.
Chl-12p60 was excised from pcDNA3.1+ vector (Invitrogen Life Technologies),
and extensively sequenced. Chl-12p60 was excised from pcDNA3.1+
vector (Invitrogen Life Technologies).

FeFlexi-IL-12. A single-chain chicken IL-12 molecule was generated by a
strategy described by McNamane et al. (32). The following primers were
used for RT-PCR or stored at 0°C for RT-PCR or stored at 70°C.

PCR and sequence reactions

Plasmid cDNA template (10–20 ng) was mixed with 0.5 μl of 1 U/μl Supertaq
HT Biotechnology, Cambridge, U.K.), 1 μl of 10 ng/μl of each primer.
1.6 μl of 2 mM dNTPs, and 2 μl of 10× ST PCR buffer (HT Biotechnology)
in a final volume of 20 μl. The reaction cycling conditions were:
5 min 94°C, 30 cycles (30 s 94°C, 1 min 55°C, 1 min 72°C), and 5 min
72°C for a final extension. For cloning purposes, PCR products were
gel purified using the Qiaquick Gel Extraction kit (Qiagen, Valencia, CA)
and ligated into the pDrive (Qiagen PCR Cloning kit; Qiagen) or the
pcDNA3.1-TOPO vector (TA Cloning kit; Invitrogen Life Technologies).
Plasmid DNA was purified using the Qiagen Plasmid Midi kit. All clones
were extensively sequenced in both 5′ and 3′ directions using a DNA
sequencing kit (BigDye Terminator V3.0 Cycle Sequencing Ready
Reaction; Applied Biosystems, Foster City, CA) and suitable sequencing
primers. Sequences were analyzed with the Sequencher 4.0 software (Gene
Codes, Ann Arbor, MI).  

Semiquantitative RT-PCR (semi-Q-RT-PCR)

Two micrograms of total RNA was reverse-transcribed into cDNA using the
Superscript II RT protocol (Invitrogen Life Technologies) in a 20-μl
reaction. One microliter of cDNA was used as a template for PCR amplifi-
cation with the conditions as described above. To determine the optimum
number of cycles (assuming linear relationship between the amount of RNA and amplified DNA band intensity, a variable number of
cycles was performed for each marker. The following internal primers were
used: 5′-GGGGCACTGACCACTCTGGC-3′ and 5′-CATTCTGGCAGT
GAGGGC3′-3′ for Chl-12p35 (151-bp fragment; 30 and 35 PCR cycles); 5′-GAACGGATGACACACACCTGAGT
GAGGGCCAC-3′ and 5′-TTACCTCCGAAAGCTTGTG
CACCACTCCTGGC-3′ for Chl-12p40 (444-bp fragment; 35 PCR
cycles); 5′-GGACATCTGCAAGTCTCC-3′ and 5′-GCCACGAGTCAT
GATATCTT-3′ for Chl-12p35 (pat.pk0055.c11), originally cloned in pcDNA3 (Invitrogen Life Technologies) (31), was excised from pcDNA3 using EcoRI and NotI and cloned into the eukaryotic expression vector pcDNA3.1+ (Invitrogen). The EcoRI/NotI Chl-12p35 fragment was
also cloned into pcDNA3.1+ (Invitrogen Life Technologies) to obtain an antisense construct.

Results

The full-length Chl-12p35 (pat.pk0055.c11), originally cloned in pcDNA3 (Invitrogen Life Technologies) (31), was excised from pcDNA3 using EcoRI and NotI and cloned into the eukaryotic expression vector pcDNA3.1+ (Invitrogen Life Technologies).

FeFlexi-IL-12. A constructor similar to the ChlFlexi-IL-12, was
a kind gift from L. Nicolson (University of Glasgow Veterinary
School, Glasgow, U.K.) and was cloned into the eukaryotic pCI-neo vector
(Promega, Madison, WI).  

Transient expression of cDNA clones in COS-7 cells

COS-7 cells were transfected with 1.5 μg each of cDNA construct using
the Invitrogen Life Technologies Lipofectamine reagent (as described by
the manufacturer) and cultured in 3-cm dishes with DMEM (without FCS
and penicillin/streptomycin). After 8 h, transfected cells were washed and
cultured in DMEM with penicillin/streptomycin and 10% FCS. After
a 72-h incubation at 37°C in 5% CO2, the cell culture supernatants were
harvested and centrifuged at 13,000 rpm for 10 min at 4°C to remove cell
debris. The supernatants were analyzed via Western blotting and used im-
ediately or stored at −70°C.

Western blot analysis

Cell culture supernatants from transfected COS-7 cells were size fraction-
ated using 4–12% Nu-PAGE (Invitrogen Life Technologies) and blotted
onto nitrocellulose filters (Schleicher & Schuell, Keene, NH). Western
blots were blocked in MBPS (3% skimmed milk in PBS) and subsequently
incubated with a polyclonal Ab that was raised against a FeL-12p40 pep-
tide (32) diluted 1/300 in MBPS. After extensive washing, blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG Abs (Sanbio, Uden, The Netherlands) diluted 1/1000 in MBPS. After washing, bound alkaline phosphatase-labeled secondary Abs were visualized via
staining. Blocking and Ab incubations were performed for 1 h at room
temperature.
Bioactivity assays for ChIL-12

**NO assay for the induction of splenic ChIFN-γ by ChIL-12.** Ch primary spleen cells (splenocytes) were seeded in triplicate in a 96-well plate at a density of 0.5 × 10⁶ cells/well in 100 μl and incubated with 50 μl of serial dilutions of cell culture supernatants from COS-7 cells transfected with cDNA clones encoding ChIL-12p40. ChIL-12p40 mixed with ChIL-12p35, ChFlexi-IL-12, FeFlexi-IL-12, or with an empty pcDNA3.1 plasmid (mock). Forty-eight hours after the addition of proteins, supernatants (75 μl) were collected and analyzed for the presence of biologically active ChIFN-γ. For this, 100 μl of 1.5 × 10⁶/ml HD-11 cells were incubated with 75 μl of the collected supernatants for 24 h at 37°C in 5% CO₂, in 96-well plates. Activation of HD-11 cells by ChIFN-γ was measured as a function of nitrite accumulation in the culture supernatants using the Griess assay (33, 34).

**Assay for spleen cell proliferation by ChIL-12.** After removing 75 μl of the supernatants (see above) 50 μl of medium and 18.5 kBq [methyl-³²H]thymidine were added to the remaining 75 μl in the 96-well plate and incubated for 18–20 h at 41°C in 5% CO₂. After incubation, the incorporated radioactivity was measured as a function of nitrite accumulation in the 96-well plate and incubated for 18–20 h at 41°C in 5% CO₂. After incubation, the incorporated radioactivity was counted using an LKB Betaplate beta counter (LKB Instruments, Gaithersburg, MD).

**Statistical analysis**

The significance of the differences between the means of NO production or between the means of cell proliferation was analyzed using the Student’s t test. Differences were considered significant at a confidence level of 95% (p < 0.05).

**Results**

**Isolation of the putative ChIL-12p35 cDNA clone**

Cloning and sequencing of avian cytokines lags behind similar work done in mammals and so far only a few avian cytokines have been identified. Because of the low sequence homology to mammalian cytokines, classical molecular approaches to characterize avian cytokine homologues are usually not successful. The identification of these “missing” avian cytokines will therefore mainly come from 1) large-scale sequencing projects resulting in expressed sequence tags (ESTs), 2) the availability of EST clones in databases, and 3) sophisticated bioinformatics.

From a high throughput sequencing project, in which a cDNA library was used constructed from an enriched pool of T cells stimulated with Con A (31), only 2 of the 5500 cDNA clones isolated showed homology to the IL-12p35 DNA sequence of several species. One of the two clones (pat.pk0055.c11) appeared to be full length and was used for further studies. Database searches with this cDNA clone did not result in the identification of more copies.

**Sequence analysis of the putative ChIL-12p35 subunit**

Clone pat.pk0055.c11 is 618 nt long (from start to stop) and encodes a protein of 205 aa (Fig. 1A). Analysis of the pat.pk0055.c11 cDNA sequence containing the open reading frame (nt 1–618) showed an overall nucleotide homology to the IL-12p35 cDNA sequences of human, sheep, horse, cat, bovine, mouse, and woodchuck of 43, 45, 48, 43, 45, 42, and 45%, respectively (Score Table Nucleic Acid PAM 65; results not shown). A multiple alignment of the predicted pat.pk0055.c11 protein to human, sheep, horse, cat, bovine, mouse, and woodchuck IL-12p35 proteins yields an overall amino acid similarity of 27, 25, 30, 24, 25, and 21%, respectively (Score Table Blosum 62; Fig. 2A). The differences in similarity between pat.pk0055.c11 and the IL-12p35 protein of various species are the result of substitutions, insertions, and deletions of amino acid residues. Striking are the two large deletions in pat.pk0055.c11 of 13 and 15 aa when comparing this sequence to the other IL-12p35 sequences. The predicted ChIL-12p35 protein is a precursor protein with a signal sequence of 62 aa (nt 1–62) and a mature protein of 170 aa. The precursor form contains a potential N-glycosylation site at amino acid position 48.

**Isolation of the putative ChIL-12p40 cDNA clone**

The coding sequence, i.e., nt 35–1042, of the mouse IL-12p40 subunit (Mull-12p40; EMBL/GenBank accession number M86671) was used to search the Biotechnology and Biological Sciences Research Council (Manchester, U.K.) Chicken EST Project Database (http://www.chick.umist.ac.uk/) (35) using the tblastx program. A chicken EST sequence (clone ID ChEST582p2, EST name 603603708F1, derived from chicken heart) was retrieved that showed 51% identity with aa 251–
279 and 66% identity with aa 310–327 of the MuIL-12p40 sequence. At that moment, no GenBank accession number was assigned to this ChEST582p2 clone and the sequence was not annotated. A database search in the same chicken EST database with this ChEST582p2 clone did not result in a longer or full-length clone, indicating that this database at that moment contained only one incomplete copy of this molecule. A similar database search in the UD Chicken EST database (http://www.chickest.udel.edu/) with the coding sequence, i.e., nt 35–1042, of MuIL-12p40 (EMBL/GenBank accession number M86671) did not result in a significant hit using the BlastN program nor did a search with the ChEST582p2 clone result in a longer or full-length clone. Although the ChEST582p2 clone is 848 nt long, only the first 233 nt contain an open reading frame encoding a peptide of 76 aa (including the stop codon). To obtain the full-length ChIL-12p40 cDNA clone, a cDNA library was screened that was constructed from pooled T and B cells isolated from vaccinated chickens. In this library cDNA molecules are unidirectionally cloned between the NotI and EcoRI sites of the eukaryotic expression vector pBlueScript (Stratagene, La Jolla, CA). In a PCR with a 5’end pBlueScript vector primer, ~120 nt upstream of the NotI restriction site, and the 3’ end ChEST582p2 primer (5’-TTATCTG CAAAGGATGACACTCACTCCAGGAT-3’; nucleotide positions 915–948 in Fig. 1B), a PCR fragment of ~1000 nt was obtained that was cloned into pDrive (Qiagen). This clone was designated pND89, ChIL-12p40.

Several months after its initial discovery we discovered that the ChEST582p2 clone was annotated in the above-mentioned Biotechnology and Biology Sciences Research Council (35) database as an IL-12p40 subunit with the highest homology (57%) to the IL-12p40 subunit of Marmota monax (woodchuck; X97019 in EMBL/GenBank). ChEST582p2 was also deposited in the EMBL/GenBank database with accession number BU291084 (release date, November 28, 2002) but without the IL-12p40 annotation.

Sequence analysis of the putative ChIL-12p40 subunit

Clone pND89 was extensively sequenced and revealed that the cDNA clone (from start to stop) is 948 nt long and encodes a protein of 315 aa (Fig. 1B). Analysis of the pND89 cDNA sequence containing the open reading frame (nt 1–948) showed an
overall nucleotide homology to the IL-12p40 cDNA sequences of human, sheep, horse, cat, bovine, mouse, and woodchuck of 57, 56, 57, 55, 56, 57, and 57%, respectively. A multiple alignment of the predicted pND89 protein to human, sheep, horse, cat, bovine, mouse, and woodchuck IL-12p40 proteins yields an amino acid homology of 41, 40, 40, 39, 36, and 41%, respectively. Sequence analysis further revealed the presence of a signal peptide with the cleavage site between aa 20 and 21 (as determined by using SPScan from the Wisconsin Package Version 10.2) resulting in a mature protein of 295 aa acids. The precursor form of ChIL-12p40 contains nine cysteines, of which six are conserved between all analyzed species (Fig. 2B). Next to this, five putative N-glycosylation sites are present (amino acid positions 84, 134, 215, 239, and 304). The presence of a WXXWXS box (aa 305–311), an Ig-like C2-type domain (aa 41–94), and a fibronectin-type III domain (aa 228–308), which are both characteristic for IL-12p40, were confirmed by similarity. Based on these homologies we conclude to have cloned the putative chicken IL-12p40 subunit.

**Cellular source of ChIL-12p35 and ChIL-12p40 mRNA**

In mammals, IL-12 expression is generally restricted to APCs such as macrophages, dendritic cells, and B cells (for a recent review, see Ref. 36). To examine cell type-associated expression of ChIL-12p35 and ChIL-12p40 mRNA, several available immunorelevant chicken cell types were tested as candidate producers in a preliminary study using semi-Q-RT-PCR. mRNA expression was examined in freshly isolated chicken splenocytes and CEF cells and in several chicken cell lines including HD-11 (macrophage derived), DT-40 (B cell derived), and CU91 (T cell derived). Cells were exposed overnight to Con A, PMA, an immunostimulating chicken CpG-ODN, recombinant ChCD40L, or LPS (Fig. 3). Unstimulated cells, cultured in medium only, showed minimal or hardly detectable ChIL-12 subunit expression levels, suggesting a low basal ChIL-12p35 and ChILp40 mRNA expression. In general, in stimulated cells detection of ChIL-12p40 mRNA proved to be easier than ChIL-12p35 mRNA, in which case we had to increase the number of PCR cycles from 30 to 35 to obtain visible bands.

Up-regulated ChIL-12p40 mRNA expression was noted in splenocytes exposed to Con A, CpG-ODN, ChCD40L, and LPS (Fig. 3, lanes 1 and 3–5), but not following PMA treatment (lane 2). ChIL-12p35 mRNA was only detected after 35 cycles; expression was noted faintly in most cell types (Fig. 3, lanes 1–6). In HD-11 cells, ChIL-12p40 mRNA expression was clearly induced by CpG-ODN, ChCD40L, and LPS (Fig. 3, lanes 9–11), while ChIL-12p35 mRNA levels were hardly detectable even at 35 PCR cycles. In contrast, for DT-40 cells up-regulated expression of ChIL-12p40 mRNA was noted only after LPS induction (Fig. 3, lane 17). Notably, this stimulus mainly stimulated ChIL-12p35 mRNA expression in DT-40 cells. Analysis of the T cell line CU91 however showed marked ChIL-12p40 up-regulation after exposure to PMA and to a lesser extent to ChCD40L (Fig. 3, lanes 20 and 22). ChIL-12p35 mRNA was also up-regulated by PMA and ChCD40L (Fig. 3, lanes 20 and 22). Nonlymphoid primary CEF cells showed no detectable expression levels of ChIL-12p35 and ChIL-12p40 mRNA with or without LPS treatment, suggesting that also ChIL-12 is mainly a product of inflammatory cells (Fig. 3, lanes 25 and 26). Collectively, if produced at all, these preliminary results show that ChIL-12p40 mRNA was expressed in large excess over the ChIL-12p35 mRNA, which is in line with mammalian IL-12 results (36). The expression of ChIL-12 subunits may vary depending on the cell type and its stimulus. It should be kept in mind that the available cell lines tested may have altered characteristics in terms of stimulus-induced IL-12 expression when compared with nontransformed cells.

**Characterization of recombinant ChIL-12**

To detect secreted ChIL-12p40 and ChIL-12p35 subunits after (co-)transfection of COS-7 cells, nonenrichment Western blot analysis was applied. For this, a polyclonal Ab that was raised against a peptide of the FeIL-12p40 subunit was used. McMonagle et al. (32) reported that this Ab recognized the equine IL-12p40 unit based upon a homology of >85% between the feline peptide and the corresponding equine protein sequence. Because the homology between this FeIL-12p40 peptide and the corresponding chicken protein sequence is >80%, we hypothesized that this Ab would possibly also recognize the ChIL-12p40 subunit on Western blot. Indeed, when using this anti-FeIL-12p40 peptide Ab, we were able to detect ChIL-12p40 and the ChIL-12p40 homodimer ChIL-12p80 (i.e., ChIL-12p40-ChIL-12p40) in the supernatants of transfected COS-7 cells (Fig. 4, lane 1). In the supernatants from COS-7 cells transfected with both ChIL-12p35 and ChIL-12p40, we detected the heterodimeric ChIL-12p70 (ChIL-12p35-ChIL-12p40) protein (Fig. 4, lane 4). The formation of heterodimeric ChIL-12p70 was more efficient than the formation of homodimeric ChIL-12p80 as no or only very small amounts of ChIL-12p80 could be detected. Also, formation of ChIL-12p70 is specific as...
cotransfection of ChIL-12-p40 cDNA with antisense ChIL-12-p35 cDNA or with a cDNA construct encoding an irrelevant viral protein (IBV-N) did not result in heterodimerization (Fig. 4, lanes 5 and 6). A double band was detected in the cell culture supernatant recovered from the FeFlexi-IL-12 transfection (Fig. 4, lane 7). These bands may reflect differential glycosylation states for the single-chain FeFlexi-IL-12, a phenomenon also reported for the equine Flexi-IL-12 (32). The high molecular band that was detected in all FeFlexi-IL-12 samples possibly represents protein aggregates. In conclusion, these results clearly show that the ChIL-12-p40 and ChIL-12-p35 subunits heterodimerize into the ChIL-12p70 molecule after cotransfection in COS-7 cells.

ChIL-12-dependent induction of ChIFN-γ

A hallmark of IL-12 activity in mammals is its induction of IFN-γ by T lymphocytes (5). To assess whether ChIL-12 also induces ChIFN-γ we incubated freshly isolated primary chicken spleen cells with dilutions of various proteins isolated after transient (co-)expression in COS-7 cells. Activation of macrophage-like chicken HD-11 cells by released IFN-γ was subsequently measured as a function of nitrite accumulation in the HD-11 cell culture supernatant using the (indirect) Griess assay. Since IFN-γ is the major macrophage-activating factor in chicken (37), release of NO is a generally accepted indicator for IFN-γ production. As shown in Fig. 5A, only heterodimeric ChIL-12-p70 (a cotransfection of ChIL-12-p40 with ChIL-12-p35) is able to significantly (p < 0.05) induce the production of IFN-γ in chicken spleen cells in a concentration-dependent manner. No other combination of transfection, nor mock or ChIL-12-p40 alone, could induce ChIFN-γ secretion to a level comparable to the heterodimeric ChIL-12-p70. Next to this it is clear that only species-specific IL-12 induces significant (p < 0.05) IFN-γ in chicken spleen cells as the FeFlexi-IL-12, which is bioactive in the mammalian IL-12 bioassay (data not shown), induced no significant amounts of ChIFN-γ. Theoretically, besides IFN-γ, other factors, e.g., CD40L or TNF-α, may be able to evoke NO production by HD-11 cells. Therefore, we also measured ChIFN-γ mRNA expression directly via semi-Q-RT-PCR on primary chicken spleenocytes 48 h postincubation with various proteins isolated after transient (co-)transfection in COS-7 cells. For this assay we used undiluted cell culture supernatant. The results from this semi-Q-RT-PCR analysis confirmed that ChIL-12 induces ChIFN-γ mRNA expression (Fig. 5C) which coincided with NO production by HD-11 cells after exposure to splenocyte supernatants (data not shown). Taken together, the NO and semi-Q-RT-PCR results demonstrate, as known for its mammalian counterparts, that ChIL-12 induces ChIFN-γ.

ChIL-12-dependent proliferation of chicken spleen cells

Another characteristic of IL-12, shared with several other cytokines, is its induction of T cell proliferation. The growth response of freshly isolated chicken splenocytes to various proteins, isolated after transient (co-)expression in COS-7 cells, was measured by a cell proliferation assay. Only heterodimeric ChIL-12-p70 (a cotransfection of ChIL-12-p40 with ChIL-12-p35) was able to induce significant (p < 0.05) proliferation of chicken spleen cells (Fig. 5B). The relatively low proliferation data observed for the first dilutions are possibly explained by overdose effects for this parameter. This phenomenon was described before by McMonagle et al. (32). Neither ChIL-12-p40 alone nor FeFlexi-IL-12 were able to induce similar proliferative responses. These results show that ChIL-12 (p70) is able to induce the proliferation of chicken spleen cells. We therefore conclude to have cloned the fully bioactive subunits of ChIL-12.

The use of undiluted ChIL-12 COS-7 cell culture supernatants in the semi-Q-RT-PCR analysis and the subsequent positive identification of ChIFN-γ mRNA expression (Fig. 5C) is in line with the observed NO induction, but seems to contradict the low proliferation data observed at high ChIL-12 protein concentrations (Figs. 5B and 6, B and D). However, cell proliferation and IFN-γ production, as measured via NO release, are two distinct physiological processes that may both be activated by IL-12. It is clear from Figs. 5 and 6 that at high ChIL-12 concentrations proliferation can be repressed, but NO synthesis is not affected. Apparently, IFN-γ synthesis is not affected at high concentrations of IL-12 (Figs. 5A and 6, A and C). The results from the semi-Q-RT-PCR analysis for ChIFN-γ mRNA expression support this (Fig. 5C).

Bioactivity of single-chain ChFlexi-IL-12

After showing that cotransfection of single-chain ChIL-12-p40 with single-chain ChIL-12-p35 resulted in the formation of a bioactive ChIL-12 heterodimer (Figs. 4 and 5), a single-chain IL-12 molecule (ChFlexi-IL-12) was constructed. ChFlexi-IL-12 is a single-chain p40-p35 heterodimeric construct in which the ChIL-12-p40 chain is linked to the ChIL-12-p35 chain by an in-frame (Gly,Ser)3 linker. McMonagle and coworkers (32) already showed that this approach resulted in a bioactive equine IL-12 molecule. Western blot analysis showed that the expression profile of the ChFlexi-IL-12 after transfection in COS-7 cells is comparable to FeFlexi-IL-12 (data not shown). Following incubation of freshly isolated chicken spleen cells with ChFlexi-IL-12, we observed both the release of ChIFN-γ, measured via NO release and expression of ChIFN-γ mRNA via semi-Q-RT-PCR, as well as cell proliferation (Figs. 5C and 6, A and B) indicating that the ChFlexi-IL-12 construct is also bioactive. At high ChFlexi-IL-12 concentrations, the proliferation of splenocytes appeared to be limited, likely as a result of overdosing or contaminating factors, as we also noticed for the heterodimeric ChIL-12-p70 (a cotransfection of ChIL-12-p40 with ChIL-12-p35) molecule (see Fig. 5B).

To directly compare the potency of ChFlexi-IL-12 vs ChIL-12-p70 (p35 plus p40), we monitored the bioactivity of both constructs in one experiment using the splenocytes from the same individual chicken. We found no significant difference (p < 0.05)
in bioactivity between the two constructs by measuring NO release and cell proliferation (Fig. 6, C and D).

**Discussion**

In this study, we described the isolation and characterization of the first avian IL-12 molecule, namely, ChIL-12. To the best of our knowledge, this is also the first report describing a nonmammalian IL-12 molecule. The predicted sequences of ChIL-12p40 and ChIL-12p35 show homology to several mammalian IL-12 molecules. Depending on the analyzed species and subunit, the protein homology for ChIL-12p35 and ChIL-12p40 ranged between 21 and 30% and 36 and 42%, respectively. The ChIL-12p40 and ChIL-12p35 subunits, similar to their mammalian counterparts, need to heterodimerize to exert IL-12 bioactivity. Based on the homologies and the bioactivity of ChIL-12, we hereby provide clear evidence that we have cloned the first nonmammalian IL-12 molecule.

In mammals, IL-12 is a critical inducer of Th1-type immune responses typically associated with IFN-γ production. This molecule has long been searched for in birds. Although ChIFN-αβ, also known as IFN-I, was the first discovered cytokine in birds (38), it took >35 years until ChIFN-γ was discovered (26). Only recently the characterization of ChIL-18 (27) and ChIL-6 (39) has been reported. The presently discovered ChIL-12 molecule complements the set of typically Th1-associated cytokines in birds. The search for new chicken cytokines is hampered by the poor homology between avian and mammalian cytokine sequences. As such, traditional molecular biological approaches based on RT-PCR often fail. For comparison, the feline IL-12 (17) and guinea pig IL-12 (40) were cloned by using a rather standardized RT-PCR approach based on available mammalian IL-12 sequences. As a consequence for the chicken, the successful discovery of new cytokines depends on large-scale EST sequencing projects combined with sophisticated bioinformatic analysis. Using both approaches and traditional molecular biology, we succeeded in the isolation of the p40 and p35 subunits of ChIL-12. The homology of the ChIL-12 subunits to several mammalian counterparts ranges between 21 and 30% (p35) and 36 and 42% (p40). These homologies are in a range similar to other recently isolated and characterized chicken cytokines, i.e., IL-6 (average 40%) (39) and IL-18 (average 30%) (27). ChIL-6 was isolated using the suppression subtractive hybridization technology (41), whereas ChIL-18 was isolated using a database search combined with a PCR on a chicken cDNA library.

After the identification of the ChIL-12 subunit sequences, we were able in a preliminary study to monitor cell type-associated expression using semi-Q-RT-PCR. Several chicken cell types were stimulated with known mammalian IL-12 inducers such as CD40L, CpG-ODN, which triggers the Toll-like receptor 9 for immunostimulatory activity, and LPS, triggering Toll-like receptor 4 (42). Remarkably, Con A stimulation of freshly isolated splenocytes triggered ChIL-12 mRNA expression probably via a T cell-dependent pathway. However, Con A stimulation of the T cell line CU91 failed to induce ChIL-12p40 and ChIL-12p35 mRNA expression above cell culture medium levels. Strikingly, however,
FIGURE 6. A, ChFlexi-IL-12-dependent induction of IFN-γ. Freshly isolated chicken splenocytes were stimulated in triplicate with serial dilutions of various cell culture supernatants of transfected COS-7 cells. After 48 h of incubation, supernatants were collected and tested for ChIFN-γ characteristic NO induction. B, ChFlexi-IL-12-dependent proliferation of chicken spleen cells. Freshly isolated chicken splenocytes were treated as described for A but after 48 h of incubation proliferation was assessed by [3H]thymidine uptake. For clarity reasons, we omitted data below 2000 cpm. C and D, Comparison of the bioactivity between ChFlexi-IL-12 and p40 + p35 as measured via (C) the induction of IFN-γ and (D) cell proliferation. A typical response repeatedly observed in numerous experiments is shown. Data are expressed as geometric mean ± SD (vertical bars) and are representative of at least four independent experiments. *, Significantly different (p < 0.05) from mock treatment. p40 + p35, cotransfection of ChIL-12p40 and ChIL-12p35; mock, empty pcDNA3.1 plasmid.
exposure of this cell line to PMA activated the mRNA expression of both subunits. To the best of our knowledge, mammalian T cells are normally not identified as IL-12 producers (36). Although intriguing, this might also be related to the transformed phenotype of this cell line and therefore an artifact. In line with mammalian data, we noted ChIL-12 mRNA synthesis following stimulation of B cells, in particular after stimulation with LPS, while for macrophage-type HD-11 cells up-regulation was noted for known mammalian IL-12 inducers, including CpG-ODN, ChCD40L, and LPS.

IL-12 was initially recognized as an inducer of IFN-γ synthesis (5) and since all mammalian IL-12 molecules isolated thus far typically show this activity we tested whether ChIL-12 was also able to characteristically induce ChIFN-γ. Our data clearly showed that the activity of ChIL-12 is in line with this mammalian concept. This was surprising because of the limited sequence homology of the ChIL-12 subunits with the group of known mammalian IL-12 molecules. The high degree of functional similarity between ChIL-12 and higher mammalian vertebrates illustrates, therefore, the conservation and vital importance of the IL-12 molecule since the evolutionary dichotomy of birds and mammals. The conservation and vital importance of the IL-12 molecule since the discovery of ChIL-12 further provides evidence that the IL-12 molecule which is to the best of our knowledge the non-mammalian IL-12 molecule discovered. We showed that the molecule has functional similarities with mammalian IL-12 homologues despite the rather poor homology. Future experiments are necessary to examine the role of ChIL-12 in vivo.

In summary, we have isolated and characterized the chicken IL-12 molecule which is to the best of our knowledge the first nonmammalian IL-12 molecule discovered. We showed that the molecule has functional similarities with mammalian IL-12 homologues despite the rather poor homology. Future experiments are necessary to examine the role of ChIL-12 in vivo.

References


