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Identification and Molecular Cloning of Functional Chicken IL-12

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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 To date bovine (15), cat (16–18), dog (19, 20), goat (J. C. Beyer, W. P. Cheevers, and N. M. Kumpula-McWhirter, unpublished observations), horse (21), pig (22), and sheep (23–25) homologues of IL-12p40 and IL-12p35 subunits have been cloned.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 lag 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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1 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 evolutionarily 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 3.5 U/ml trypsin treatment and homogenization of 10-day-old SPF (specific pathogen-free [SPF]) 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 37°C. Chicken primary splenocytes were isolated from 3-wk-old normal White Leghorn SPF chickens as 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 received 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 ml of cell culture medium and one of the following stimulants: 10 ng/ml LPS (Sigma-Aldrich, St. Louis, MO), or 5 ng/ml PMA (Sigma-Aldrich, St. Louis, MO), and 5 nM CSA (Cytokine Receptor Antagonist, Promega, Madison, WI). For ChIL-12 the following primers were used to amplify ChIL-12p35 without the putative 35-aa signal peptide sequence (as determined by the SPScan program from the Wisconsin Package Version 10.2; Genetics Computer Group) and that introduced a 5′-NotI restriction site: 5′-CTGATCATCTAGGAAAGGTGTAC-3′ and 5′-GGCCGCCTGGTCACCAGGGCTGCC-3′. For ChIL-12p40 the following primers were used: 5′-TTGATCCACTCTTCGGCATCTTAGAG-3′ and 5′-CTTCTTCCTCCGAGGCA-3′. For ChFlexi-IL-12 the following primers were used: 5′-TTGATCCACTCTTCGGCATCTTAGAG-3′ and 5′-CTTCTTCCTCCGAGGCA-3′. For FeFlexi-IL-12 the following primers were used: 5′-TTGATCCACTCTTCGGCATCTTAGAG-3′ and 5′-CTTCTTCCTCCGAGGCA-3′.

**RNA isolation**

Total RNA was isolated using TRIZol reagent (Invitrogen Life Technologies, Grand Island, NY) as described by the manufacturer. RNA quality was evaluated on 1.2% agarose gels. RNA samples were used subsequently for RT-PCR or stored at −70°C.

**PCR and sequence reactions**

Plasmid DNA template (10–20 ng) was mixed with 0.5 µl of 1 U/µl SuperTag (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 Qiagen Gel Extraction kit (Qiagen, Valencia, CA) and ligated into the pDrive (Qiagen PCR Cloning kit; Qiagen) or the pCR2.1-TOPO vector (TA Cloning kit; Invitrogen Life Technologies). The ChIL-12p35 was excised from pCR2.1-TOPO (Invitrogen Life Technologies) and extensively sequenced. ChIL-12p40 was excised from pCR2.1-TOPO as a NotI/BamHI fragment and cloned into the pcDNA3.1+ vector (Invitrogen Life Technologies). The ChIL-12p35 was excised from pCR2.1-TOPO as a BamHI/HindIII fragment and cloned into the ChIL-12p40 (pcDNA3.1+) construct downstream of the p40 fragment. This resulted in a single-chain p40-p35 heterodimeric construct in which the p40 chain is linked to the p35 chain by an in-frame (Glu-lys) linker; this molecule was designated ChFlexi-IL-12 (for chicken Flexi-IL-12).

**Western blot analysis**

Cell culture supernatants from transfected COS-7 cells were size fractionated 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 FeIL-12p40 peptide (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 × 10^6 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-expressing supernatants were mixed with ChIL-12p35, ChFlexi-IL-12, Flexi-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 × 10^6/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 the assayed supernatants (above) 50 µl of medium and 18.5 kBq [methyl-³H]thymidine (25 µl/well) 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 counted using an LKB Bepatlane 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 cDNA 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 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.
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.chickenest.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'-TTATCTGCAAAGCGTGGACCACTCACTCCAGGAT-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 EST clone was annotated in the above-mentioned Biotechnology and Biology Sciences Research Council 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

FIGURE 2. Multiple protein alignments of ChIL-12p35 (A) and ChIL-12p40 (B). Multiple alignments of the ChIL-12p35 and ChIL-12p40 proteins to the IL-12p35 and IL-12p40 proteins of several species was performed using the GeneDoc program (Score Table Blosum 62). Gaps (−) are introduced to allow maximal alignment. Conserved cysteines are indicated with asterisks (*), whereas putative N-glycosylation sites in the ChIL-12 subunits are indicated with arrows (↓). The proteins used for the multiple alignments have the following EMBL/GenBank accession numbers for p35/p40: human, M65271/M65272; sheep, AF173557/AF004024; horse, Y11130/Y11129; cat, Y07761/Y07762; bovine, U14416/U11815; mouse, M86672/M86671; woodchuck, X970189/X97019. 55.c11, ChIL-12p35 (clone pat.pk0055.c11); pND89, ChIL-12p40.
general nucleotide homology to the IL-12p40 cDNA sequences of human, sheep, horse, cat, bovine, mouse, and woodchuck of 57, 55, 56, 35, 57, and 57, respectively (Score Table Nucleic Acid PAM 65; results not shown). 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 (Score Table Blosum 62; Fig. 2B). The differences in similarity between pND89 and the IL-12p40 protein of various species are the result of substitutions, insertions and deletions of amino acid residues. 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 WSXWS 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 IL-12p35 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, nondenaturing 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

![FIGURE 3](http://www.jimmunol.org/) Semi-Q-RT-PCR analysis of ChIL-12p35 and ChIL-12p40 mRNA in several chicken cell types. Chicken cells were treated overnight with several stimulants, RNA was isolated and used for semi-Q-RT-PCR analysis. PCR products were separated on agarose gel, stained with ethidium bromide, and visualized under UV light. ChGAPDH was used for RT-PCR control and semiquantitative comparison for ChIL-12p35 and ChIL-12p40 mRNA expression. A typical result obtained in several experiments is shown. The number of PCR cycles is indicated for each marker in parentheses. Chicken cells used: freshly isolated splenocytes and CEF cells, macrophage-derived HD-11 cell line, B cell-derived DT-40 cell line, and T cell-derived CU91 cell line. CpG, immunostimulating chicken CpG-ODN; DNA, DNA plasmid encoding p35, p40, or ChGAPDH; H2O, water control; medium, cell culture medium; p35, ChIL-12p35; p40, ChIL-12p40.
cotransfection of ChIL-12p40 cDNA with antisense ChIL-12p35 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-12p40 and ChIL-12p35 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-12p70 (a cotransfection of ChIL-12p40 with ChIL-12p35) 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-12p40 alone, could induce ChIFN-γ secretion to a level comparable to the heterodimeric ChIL-12p70. 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 spleenocytes to various proteins, isolated after transient (co-)expression in COS-7 cells, was measured by a cell proliferation assay. Only heterodimeric ChIL-12p70 (a cotransfection of ChIL-12p40 with ChIL-12p35) is 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-12p40 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-12p40 with single-chain ChIL-12p35 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-12p40 chain is linked to the ChIL-12p35 chain by an in-frame (G4 S)-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-12p70 (a cotransfection of ChIL-12p40 with ChIL-12p35) molecule (see Fig. 5B).

To directly compare the potency of ChFlexi-IL-12 vs ChIL-12p70 (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)
chicken splenocytes at 48 h after incubation with undiluted cell culture tested for ChIFN-
COS-7 cells. After 48 h of incubation, supernatants were collected and with serial dilutions of various cell culture supernatants of transfected chicken splenocytes were treated as described for IL-12-dependent proliferation of chicken spleen cells. Freshly isolated treatment.

A

FIGURE 5. A, Chicken heterodimeric IL-12-dependent induction of IFN-γ. Freshly isolated chicken splenocytes were stimulated in triplicate with serial dilutions of various cell culture supernatants or Con A (positive control). For clarity reasons, we omitted data below 2000 cpm. A typical response 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, 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.

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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 (300 million years ago). Species specificity of ChIL-12 function was evidenced by the failure of bioactive FeIL-12 to induce sufficient ChIFN-γ in freshly isolated chicken spleen cells. Because ChIL-12p40 alone exhibits no ChIFN-γ inducing capacity, we conclude that biologically active ChIL-12 depends on the interaction of the p40 and p35 subunits covalently linked to each other into the 70-kDa heterodimer p70. Gubler et al. (1) showed already in 1991 that bioactive IL-12 is a heterodimeric cytokine composed of disulfide-linked p35 and p40 subunits.

Another characteristic feature of IL-12 is its capacity to induce cell proliferation. The growth response of splenocytes to various forms of ChIL-12 showed that only the ChIL-12p40 with ChIL-12p35 combination (heterodimeric ChIL-12p70) was able to induce the proliferation of chicken spleen cells. These results showed that proliferation of freshly isolated chicken spleen cells is readily induced upon exposure to ChIL-12 (p70) only. Using a single-chain IL-12 molecule (ChFlexi-IL-12), consisting of a single chain ChIL-12p40 linked to the ChIL-12p35 chain by an in-frame (G4S) linker, we showed proliferation, NO-inducing activity, and ChIFN-γ mRNA expression in freshly isolated chicken splenocytes.

From human and mouse studies, it is known that homodimeric p40 (p80) competes with heterodimeric p40-p35 (p70) for binding to the IL-12R which results in an inhibition of cell proliferation and IFN-γ synthesis in vitro (43, 44). To investigate whether homodimeric chicken p40 (p80) also has this antagonizing effect, an in vitro experiment was set up in which the 10-fold concentrated supernatant of COS-7 cells transfected with ChIL-12p40 was preincubated for 2 h with splenocytes. After that, serial dilutions of ChFlexi-IL-12 protein were added and IFN-γ release was measured. In contrast to Gillessen et al. (43) and Ling et al. (44), we did not observe a clear decrease in IFN-γ synthesis (data not shown). This might be in line with the results found for human p80 (45). Human p80, in contrast to mouse, has only a minor ability to antagonize IL-12 and might therefore work differently when compared with the mouse system. Our preliminary data suggest that this might also be true for chicken p80.

The discovery of ChIL-12 further provides evidence that the chicken Th1 cytokine pathway was already functional before the avian and mammalian lineages separated 300 million years ago (46). The conservation of this pathway in vertebrate evolution illustrates its importance for species fitness. Remarkably, within the chicken far only Th1-type cytokines have been isolated and clearly characterized. The absence of Th2-associated IgE, eosinophils, and allergies suggests no or limited Th2 responses in the chicken and possibly nonmammalian vertebrates in general. However, using Ag delivery through scavenger receptors on avian macrophages, Vandaveer et al. (47) were able to manipulate Th1/Th2-associated responses in chickens. Interestingly, within several chicken EST databases sequences are present which are annotated as the ChIL-10 R, indicating the presence, although only in part, of a putative Th2 pathway in the chicken. Recently, P. Kaiser and coworkers presented evidence for the presence of the IL-4, IL-5, and IL-13 genes which have to be functionally analyzed (48). More research will be necessary to identify components of the avian Th2 pathway. Combinations of ongoing large-scale sequencing projects (a draft of the chicken genome sequence is expected to be completed by the end of 2003) and bioinformatic approaches will therefore be necessary to isolate and characterize novel avian cytokines. Both techniques are definitively necessary to explore the existing cytokine gap between birds and mammals. These investigations may also help to unravel the evolutionary development of the immune system of birds and mammals.

In summary, we 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


