High Level Expression of Recombinant Chicken Interferon-α Using Baculovirus

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ABSTRACT. Bioactive recombinant chicken interferon-α (ChIFN-α) was expressed in a baculovirus system. For easy purification, it was expressed as ChIFN-α bearing histidine hexamer (His-tag) at C-terminal, designated ChIFN-αHis. The expressed proteins were detected by SDS-PAGE analysis with Coomassie brilliant blue staining as around 23 and 19 kDa bands thought to be immature and matured ChIFN-αHis respectively. The purified ChIFN-αHis with a nickel chelated column showed anti-viral activity in vitro.

KEY WORDS: baculovirus, chicken, expression, interferon-α, purification.

The existence of chicken interferon (ChIFN) was first shown by Isaacs and Lindermann in 1957 [6] through its ability to induce viral infection resistance to cells, but the first ChIFN cDNA was not cloned until 1994 by Sekellick et al. [17]. To date the genes encoding chicken IFN-α/β and IFN-γ have been cloned and expressed in E. coli, COS cell or baculovirus [3, 10, 16–19].

Like mammalian counterparts, ChIFN-α is a powerful antiviral agent and has high Mx promoter-inducing activity [16]. ChIFN-γ is the major macrophage-activating factor (MAF) which stimulates secretion of nitric oxide and enhances expression of major histocompatibility complex II in macrophages [21]. Although ChIFN-α lacks these activities, the induction of an antiviral state in cells with ChIFN-α is much higher than that with ChIFN-γ.

Recently a work has shown that IFN-α/β could directly induce Th1 development in T cells and induce IFN-γ production independently without interleukin-12 (IL-12) in humans [13]. In human T cells, IL-12 or IFN-α up-increases surface expression of IL-12Rβ2 receptor (IL-12Rβ2) in the absence of T cell receptor (TCR) stimulation [12]. IFN-α was also found to synergize with IL-18 to induce IFN-γ production in human T cells and mouse splenocytes [5, 8, 11, 14]. These studies suggested that IFN-α, in addition to IL-12, could provide pathways for innate regulation of adaptive immunity. In chickens, however, such studies have not been done yet. Quite recently the existence of chicken IFN-α has been shown by the cloning and expression of the genes for chicken IL-12 subunits p35 and p40 [2]. So we can now investigate ChIFN-α in the innate regulation of adaptive immunity in avian systems.

In order to obtain the bioactive recombinant ChIFN-α in large amounts for in vitro and in vivo use, we selected a baculovirus expression system because of its high expression level [9, 19]. Here we describe cloning of ChIFN-α gene, modification of the gene, expression, and purification of ChIFN-α with a baculovirus system.

MATERIALS AND METHODS

Cloning of ChIFN-α gene: Spleen cells dispersed from a normal healthy White leghorn chicken about 2 months old were incubated in 5 × 10⁶ cells/ml for 6 hr in RPMI-1640 tissue culture medium (Nissui Seiyaku, Tokyo) containing 10% fetal calf serum (FCS) and 10 µg/ml of concanavalin A (Con A). Then the cells were harvested and total RNA was isolated from 1 × 10⁶ cells with phenol/guanidine thiocyanate solution (ISOGEN; Nippon Gene, Tokyo, Japan) and resuspended in 10 µl of distilled water. Primer ChIFNa-F (5′-CGAGATCTCCCACCATGGCTGGCTGCTGCTGAG-3′) and ChIFNa-RHis (5′-CGAGATCTAAAATGATGATGATGATGATGAGTGAGTGATGATGATGCTGGCTGCTGCTGAG-3′) to amplify the cDNA of the ChIFN-α gene were designed from the sequence data by Sekellick et al. (GenBank U07868). These primers contained a Bgl II site at both 5′ prime ends (underlined). RT-PCR was performed with a GeneAmp RNA PCR core Kit (Perkin Elmer Applied Biosystems Japan, Tokyo, Japan) with the primers and 2 µl of the total RNA as templates. After RT-PCR, the amplified products were ligated into a plasmid pCR2.1 with a TA cloning kit (Invitrogen, San Diego, CA, U.S.A.). The cloned cDNA was sequenced by the dyeoxy method with a Dye terminator Cycle Sequencing FS Ready Reaction Kit (Perkin Elmer Applied Biosystems Japan).

Construction of a transfer vector: The cloned cDNA in pCR2.1 was digested with Bgl II and the fragment containing the cDNA was ligated into the BamHI site of a transfer vector pAcYM1 [9] to place the gene under the control of the polyhedrin promoter. The resulted recombinant plasmid was named pAcYM1ChIFNaHis. The sequences were verified by dideoxy chain termination sequence analysis as described above.

Isolation of recombinant baculovirus: To obtain recombinant viruses that would express ChIFN-αHis, the recombinant transfer vector pAcYM1ChIFNaHis was transfected into Spodoptera frugiperda (Sf) 21 cells with infectious AcRP23- lacZ DNA [7] by using Lipofectin (Gibco BRL, Gaithersburg, MD, U.S.A.). The cultures were harvested,
plaque-assayed with neutral red and X-gal, and white plaques were selected as recombinant viruses. The resulting virus was named AcChIFN-αHis. The recombinant baculovirus was grown and assayed in Sf 21 insect cells in TC-100 medium (Gibco BRL) containing 10% FCS. On occasion the virus was grown in SF9 cells (Protein Sciences Co., Meriden, CT, U.S.A.) in ESF921 medium (Expression System LLC, Woodland, CA, U.S.A.) without FCS.

Purification of ChIFN-αHis: AcChIFN-αHis was inoculated into SF 9 cells in a spinner flask with serum free ESF921 medium. The infected culture supernatants harvested at 4 days post-inoculation (dpi) were ultra-centrifuged with SW28SA (Beckman, Tokyo, Japan) at 80,000 × g for 1 hr and the resulting supernatant was filtered with a Diaflow Ultrafilter XM300 (Amicon, Beverly, MA, U.S.A.) to remove debris. The filtrate was concentrated by filtration with a Diaflow ultrafilter YM10 (Amicon), and then the expressed protein, ChIFN-αHis, was purified on a nickel chelated column of an Xpress System Protein Purification kit (Invitrogen) according to the manufacturer’s manual under native conditions. The affinity purified protein was gel filtered with a PD10 column (Amersham Pharmacia Biotech, Tokyo, Japan) in phosphate buffered saline (PBS) to remove imidazole which was used in elution of the protein from the nickel chelated column. The purity was confirmed by SDS-PAGE and Coomassie brilliant blue staining. The protein concentration was determined with a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IN, U.S.A.).

Chicken embryonic fibroblast (CEF) IFN assay: The antiviral activity of ChIFN-αHis was determined as described previously [19]. In brief, serial five-fold dilutions of the purified ChIFN-αHis were made in triplicate in 96 microplate wells of CEF cells in Eagle’s minimum essential medium (Nissui Seiyaku, Tokyo, Japan) containing 0.3% of L-glutamine, and after overnight incubation the cells were challenged with vesicular stomatitis virus (VSV) at a 10^5 50% tissue culture infectious dose (TCID50). The IFN titer, arbitrary units per ml (AU/ml), was calculated as a reciprocal of the highest dilution in the well in which 100% of the monolayer was protected from the cytopathic effect (CPE) of the virus.

Glycosylation assay: To detect glycosylation of the expressed proteins, a DIG Glycan Detection Kit (Roche, Tokyo, Japan) was used according to the manufacturer’s manual. In brief, the purified ChIFN-αHis sample was applied to SDS-PAGE and blotted onto nitrocellulose membrane. Then the glycosylated polypeptides were detected with the kit.

RESULTS

Cloning and sequencing of the ChIFN-αHis gene: With RT-PCR, about a 600 bp band was amplified and the band was cloned into the pCR2.1 vector. With M13 forward and M13 reverse primers, the sequence of the cloned cDNA was determined. The inserted DNA was 619 bp, including the BglII sites in the primers. The open reading frame was 597 bp, and 199 amino acids were encoded. Comparison of the sequences for Sekellick’s [17] and our cloned ChIFN-αHis cDNA revealed three nucleotide differences: nucleotides at positions 287 (A to C), 417 (G to A) and 543 (C to T). The nucleotide change at 543 made one amino acid change at position 96 (K to T). The amplified gene also contained the extra histidine hexamer (His-tag) at the C terminal.

Expression and purification of ChIFN-αHis by recombinant baculovirus: The cDNA was subcloned into the BamHI site of pAcYM1 and transfected into SF 21 cells with the infectious AcRP23-lacZ DNA. After two plaque purifications, one cloned recombinant virus was named AcChIFN-αHis and further grown in SF 9 cells in spinner flasks to get high titered virus stock.

The resulting high titered recombinant baculovirus AcChIFN-αHis, was inoculated into SF 9 cells and the culture was harvested at 4 dpi. The infected culture supernatants were ultra-centrifuged and concentrated with membrane filters, then ChIFN-αHis was purified with a nickel chelated column as described in Materials and Methods. As shown in Fig. 1, the bands around 27, 23, 19 and 17 kDa were made visible by CBB staining. The main bands were 23 and 19 kDa and thought to be immature and matured ChIFN-α, respectively. With a glycan detection kit, only the 27 kDa band was detected but the other bands were not detected in this assay (Fig. 1). The quantity of purified ChIFN-αHis was estimated to be about 100 µg/ml with a Micro BCA Protein Assay Reagent Kit (Pierce).

Biological activity of ChIFN-αHis: The antiviral activity of the purified ChIFN-αHis was 4.8 × 10^7 AU/ml and the efficacy was calculated to be about 4.8 × 10^5 AU/µg. To confirm that the expressed protein is ChIFN-α, the purified ChIFN-αHis and ChIFN-γTHis [19] were heated at 65°C for
Table 1. Anti-viral activity of the purified chicken IFN-αHis and IFN-γTHis produced by baculoviruses

<table>
<thead>
<tr>
<th>IFNs</th>
<th>IFN titer (AU/ml)</th>
<th>pre-heating</th>
<th>post-heating</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChIFN-αHis</td>
<td>4.9 × 10^3</td>
<td>4.9 × 10^3</td>
<td></td>
</tr>
<tr>
<td>ChIFN-γTHis [19]</td>
<td>3.9 × 10^4</td>
<td>7.8 × 10^3</td>
<td></td>
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</tbody>
</table>

CEFs were treated overnight with fivefold serial dilutions of the purified ChIFN-αHis or ChIFN-γTHis, and were challenged with VSV at 100 TCID<sub>50</sub>. The IFN titer (AU/ml) was calculated as a reciprocal of the highest dilution in the well in which 100% of the monolayer was protected from the CPE of the virus at three days post-challenge. a) Heating at 65°C for 1 hr. After heat treatment, the antiviral activity of ChIFN-αHis was not reduced but ChIFN-γTHis was reduced from 3.9 × 10^4 AU/ml to 7.8 × 10^3 AU/ml (Table 1).

DISCUSSION

Since the ChIFN-α gene was isolated by Sekellick et al. [16], many reports on the expression of recombinant ChIFN-α have been published. Most of them used E. coli or COS cells [10, 16, 17]. To the best of our knowledge, this is the first report on a baculovirus expression system for ChIFN-α. In the present study, recombinant ChIFN-α gene was expressed by baculovirus in order to obtain the bioactive ChIFN-α in large amounts. For the purification we designed the primer to add His-tag to the C-terminal of ChIFN-α and made ChIFN-αHis, because an N-terminal signal sequence is important for secretion of a recombinant protein in a baculovirus expression system. From the deduced amino acids sequence, the molecular weights were estimated to be 23 kDa for an immature ChIFN-α and 19 kDa for a matured one that is cleaved from an immature precursor. After purification of ChIFN-αHis, 27, 23, 19 and 17 kDa bands were made visible by CBB staining and the main bands were 23 and 19 kDa that were thought to be immature and mature ChIFN-αHis, respectively. There are four potential glycosylation sites in the ChIFN-α sequence [17]. With the glycan detection kit, only the 27 kDa band was detected but the other bands were not detected in this assay (Fig. 1), but glycosylation is not necessary for antiviral activity for IFN-α as described [22].

The purified product of ChIFN-αHis was able to induce antiviral activity in CEF cells. The titer of the purified ChIFN-αHis was 4.8 × 10^3 AU/ml and after heat treatment the titer was not reduced. In contrast, after heat treatment the purified ChIFN-γTHis [19] was reduced from 3.9 × 10^4 AU/ml to 7.8 × 10^3 AU/ml (Table 1). Because type I IFNs are resistant to heating and low-pH treatment [4, 22], we think this expressed protein is ChIFN-α.

Recent studies in man and mouse have shown that the induction of IFN-γ by IFN-α and IL-18 proceeds via a Stat4-dependent signaling pathway and that the activation of Stat 4 is directly linked to IFN-α [5, 8, 11, 14]. In the present study, bioactive ChIFN-αHis was expressed and purified in a large amount by baculovirus. In mammalian models, IFN-α has been used for immunomodulation in vivo [20]. The molecular mechanisms of IFN-α immunomodulation have not yet been clarified completely, but it is postulated that IFN-α initiates and sustains a Th1 response by selective up-regulation of the IL-12/IFN-γ [12, 20]. Now sequence data for chicken IL-12 and IL-18 are available [2, 15], which allow us to investigate a cytokine network in avian species.

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