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# Molecular cloning and sequence analysis of feline interferon-stimulated gene 15

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**Abstract**

The interferon-stimulated gene 15 (ISG15) is induced by type I interferon (IFN). Recent studies have revealed that like ubiquitin, ISG15 is conjugated with target proteins. In this study, the feline ISG15 (FeISG15) gene was cloned from feline IFNω (FeIFNω)-stimulated feline kidney epithelial (CRFK) cells. According to gene sequence results, cDNA was 474 bp long and encoded a protein of 157 amino acids. The putative amino acid sequences showed 62.5–72.1% identity with those of other mammalian ISG15s. Similar to human and mouse ISG15, FeISG15 included tandem ubiquitin-like domains; its homology with feline ubiquitin was 36.3–39.5%. The LRLRGG conjugating motif was located only in the carboxyl terminal ubiquitin-like domain. FeISG15 also lacked the carboxyl terminal extension after the LRLRGG motif, which is present in mouse and human ISG15. Recombinant FeISG15 protein was expressed as a His-tagged fusion protein in *Escherichia coli* and purified by ion-exchange chromatography followed by affinity chromatography. Monoclonal anti-FeISG15 antibodies revealed free FeISG15 and FeISG15 conjugated with target proteins in cells after IFNω stimulation by Western blotting analysis. Furthermore, mRNA of IFNγ was detected from peripheral blood mononuclear cells (PBMCs) after stimulation with rFeISG15 extracellularly by RT-PCR. Taken together, these results suggested that FeISG15 had ubiquitin- and cytokine-like activity, as in other species.


*Keywords:* ISG15; Ubiquitin-like protein; Interferon; Feline

## 1. Introduction

Interferon-stimulated gene 15 (ISG15), long known as one of the interferon (IFN)-induced gene products (Farrell et al., 1979), is up-regulated by stimulation of type I IFN. ISG15 contains two tandem ubiquitin-like domains, connected by proline. Ubiquitin is a highly conserved 76-residue amino acids and regulates a broad range of eukaryotic cell functions, initiated by conjugation with target proteins through its carboxyl terminal (C-terminal) LRLRGG motif. Similar to ubiquitin, ISG15 includes an LRLRGG motif at the C-terminal and conjugates to target cellular proteins through its C-terminal LRLRGG motif (Loeb and Haas, 1992). Recently, studies have identified several proteins that are utilized in this cascade, such as UBE1L (E1) (Yuan and Krug, 2001), Ubc8 (E2) (Kim et al., 2004), estrogen-responsive finger protein and Herc5 (E3 ligase) (Dastur et al., 2006) and UBP43 (deconjugating enzyme) (Malakhov et al., 2002). ISG15 can be induced by viral infection as well as by type I IFNs. Lenschow et al. (2005) reported that ISG15 may act as an antiviral module against Sindbis virus, herpes virus type 1 and

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influenza virus infection in mice, and Okumura et al. (2006) reported that ISG15 inhibits HIV-1 replication and release of the HIV-1 virion. These findings suggest that ISG15 in posttranslational modification might play an important role in host response to pathogens.

ISG15 exists in free or conjugated form in cells. Interestingly, free ISG15 is released from cells that are monocytes and lymphocytes after stimulation by a signal sequence-independent mechanism (Knight and Cordova, 1991; D'Cunha et al., 1996a,b). Released free ISG15 acts as a cytokine, which induces IFNγ in T cells and stimulates NK cells (Recht et al., 1991). Therefore, like intracellular ISG15, released ISG15 may play a part in the immune system. However, the role of ISG15 in the immune system has not been clarified.

A type I IFN product for cats is commercially available and widely used. However, its mechanism has not been studied thoroughly, unlike the case of humans and mice. ISG15 from cats might be induced during IFN treatment and somehow assist their immune system. Therefore, the study of ISG15, which is one of defense mechanisms in type I IFN, might clarify a more effective IFN treatment for cats.

In this study, we cloned and characterized the FeISG15 gene. The putative amino acid sequences were then analyzed and compared with published sequences from other mammals.

## 2. Materials and methods

### 2.1. Cell lines and reagents

Feline kidney epithelial (CRFK) cells were maintained in growth medium, which was a mixture of equal amounts of Eagle's minimum essential medium and L-15 supplemented with 10% fetal bovine serum, 100 IU/ml Penicillin G, 100 μg/ml streptomycin sulfate and 7.5% sodium carbonate.

Rabbit anti-His antibodies were from Bethyl Laboratories (Montgomery, TX), and horseradish peroxidase-conjugated anti-rabbit IgG goat serum was from Seikagaku Co. (Tokyo, Japan). For production of anti-rFeISG15 monoclonal antibody, purified His-rFeISG15 (50 μg) was emulsified with TiterMax Gold (Sigma, St. Louis, MO) following the manufacturer's procedure. The samples were injected into Balb/c mice every week for 3 weeks. The fusion was carried out as based on the method described by De StGroth and Scheidegger (1980). After immunization, spleen cells were collected and fused with FO mouse myeloma cells using polyethylene glycol. When the hybridomas were semi-confluent, their supernatants were tested by ELISA. Positive hybrids were cloned by limiting dilution at least two times. The monoclonal antibodies from the supernatants of the hybridomas were purified on HiTrap Protein G HP (GE Healthcare UK Ltd., England, UK).

Intercat (Kyoritsu Seiyaku Co., Tokyo, Japan) was used as feline IFNω (FeIFNω).

### 2.2. RNA isolation and cDNA synthesis

CRFK cells were sheeted into 25 $cm^2$ flasks and stimulated with $10^3$ U/ml of FeIFNω for 6 h at 37 °C in 5% $CO_2$. Total RNA was isolated with ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) following the manufacturer's procedure. Total RNA was reverse transcribed to cDNA using Oligo$(dT)_{20}$ – M13 forward (−40) primer with SuperScript III (Invitrogen Co., Carlsbad, CA).

### 2.3. Cloning of feline ISG15

The FeISG15 gene was amplified from the cDNA by PCR using a forward consensus primer (5′ ATGGGCTGGGACCTGACGGTGAAGA 3′), which was developed using a sequence alignment of human, mouse and bovine ISG15 sequences, and M13 forward (−40) primer as the reverse primer. After amplification, the PCR product was cloned into pCR2.1-TOPO (Invitrogen Co.) and sequenced. To identify the 5′ end sequence, another primer (5′ TTGTGGTTCCTCACCAGGAT 3′) was developed using the partially identified FeISG15 sequence. 5′ RACE methods were performed with the 5′/3′ RACE kit (Roche Diagnostics Co., Indianapolis, IN) as described by the manufacturer. An amplified product was cloned into pCR2.1-TOPO and sequenced.

For expression of FeISG15, the FeISG15 gene was amplified by PCR using primers that amplified the full coding sequence and added a *Bam*HI restriction site at the 5′ end and a Sal I restriction site at the 3′ end (forward primer 5′ TTACGGATCCTATGGGCGGGAC 3′, reverse primer 5′ ATAGTCGACCCTCACCCACCCCG 3′). After amplification of the target gene, PCR products were digested with restriction enzymes and then inserted into pQE-81L (Qiagen GmbH, Hilden, Germany), which added 6xHis to the 5′ end of FeISG15. The plasmid was transformed to *Escherichia coli* strain XL-1-Blue MRF′. After the sequence was confirmed, that transformant was used for expression and purification of FeISG15.