BIOLOGICAL ACTIVITIES AND DETECTION OF CYNOMOLGUS IFN-α SUBTYPES

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Abstract

Cynomolgus monkeys (Macacca fascicularis) have become valuable animal models due to their genetic similarity to humans. They have become useful for the evaluation of pharmacodynamic, pharmacokinetic and toxicity parameters of different immune response modifiers currently in development. In addition, they are frequently used as a surrogate to study human virus infections. One hallmark of many of these compounds and viruses is the ability to induce Type I interferon (IFN). **Therefore the identification , biological characterization of endogenous Cynomolgus IFN-α subtypes and their accurate detection are of utmost importance for predicting the clinical outcome of effective human therapeutics.** We have isolated full length IFN-α coding sequences from Cynomolgus genomic DNA using PCR methods. Sequence analysis revealed 11 unique IFN-α subtypes which were transiently expressed in Vero cells. The conditioned supernatants were tested in a variety of assays which represent hallmark activities of IFN. All samples tested on Cynomolgus cells exhibited antiviral activity. Additionally, the biological activities of the IFN proteins secreted in the tissue culture supernatants were characterized in several human cell-based assays including A549/EMCV cytopathic protective effect, OVCAR-3 antiproliferative and caspase-3/7 activation assays. All subtypes displayed activity on these separate human cell lines suggesting reasonable species cross-reactivity. Separately, we analyzed the activity in a human cell-based reporter assay (iLite™ Human IFN-α Kit) including the use of an anti-IFN-α receptor neutralizing antibody. This assay correlated with the other human cell-based assays, providing an additional, more rapid measure of activity. Moreover, the activity could be potently neutralized by the anti-human IFNAR monoclonal antibody further suggesting similarities between Cynomolgus and human IFN-α receptor proteins. **Therefore these reagents should prove useful in the detection and biological evaluation of Cynomolgus IFN-α proteins used for the better understanding of human viral diseases and their treatments.**

Methods

Cell culture. African green monkey kidney Vero, human lung carcinoma A549, rhesus LLC-MK2, and human ovarian adenocarcinoma OVCAR-3 cell lines were obtained from American Type Culture Collection (ATCC # CCL-81, CCL-185, CCL-7, and HTB-161, respectively). JTC12 cells were obtained from Health Science Research Resource Bank (Osaka, Japan). Vero cells were maintained in MEM, A549 and JTC12 in DMEM media supplemented with 10% FBS. OVCAR-3 were grown in RPMI 1640 medium, supplemented with 20% FBS and bovine insulin (0.01 mg/ml,Sigma, Cat.# I0516). All media and FBS were purchased from Invitrogen.

Cynomolgus IFN-α subtype gene cloning and sequence determination. Full length IFN-α subtypes were isolated from Cynomolgus genomic DNA using PCR methods and cloned into pcDEF3-based mammalian expression vector which utilizes the human polypeptide chain elongation factor 1α promoter (EF-1α). Nucleotide sequence identity was determined by fluorescent-based DNA sequencing (IDT DNA). Phylogeny analysis was done by the software at http://www.phylogeny.fr. Homologues of human sequences were determined by BLAST at NCBI using the reference sequences for Human and Rhesus IFN. Percent identity was the primary criteria and percent relatedness was used as a secondary criteria.

Mammalian expression. Plasmid DNA containing full length individual Cynomolgus IFN-α subtypes was isolated with EndoFree Plasmid Maxi Kit (Qiagen, Cat. #12362) and transfected in Vero cells using Lipofectamine and PLUS reagent (Invitrogen, Cat. #P/N50470 and 10964-021, respectively) according to the manufacturer' s protocol. Briefly, one day before transfection, cells were plated at 250,000 cells per well in 6-well plates and incubated overnight at 37ºC to 70-80% confluence. Five μg plasmid DNA were mixed with 10μl PLUS reagent in a serum-free MEM media, incubated at room temperature for 15 min. Lipofectamine (5μl) was added to the DNA-PLUS complexes, incubated for additional 15 min and overlayed the cell monolayer. Cells were transfected for 6 hr at 37ºC with 5% C02, then the transfection mixture was replaced with fresh growth media and further incubated at 37ºC. Tissue culture supernatants were collected 24 hr post-transfection, aliquoted and stored at -20ºC until use.

Bacterial expression and purification. The mature open reading frame for Cynomolgus IFN-a2 was subcloned into a standard bacterial expression vector, transformed in E. coli, and expressed by heat shock induction. The inclusion bodies were prepared by lysis of the E. coli and centrifugation, denatured in 7 M guanidine hydrochloride and refolded using standard ox/red conditions in the presence of 0.5 M arginine. The IFN protein was precipitated with ammonium sulfate (3 M final) followed by centrifugation. Precipitated IFN was resuspended in buffer and purified through standard chromatographic techniques (gel filtration and ion exchange chromatography).

Cytopathic Effect Inhibition Assay (CPE). Human A549, Rhesus LLC-MK2, or Cynomolgus JTC12 cells were seeded in 96-well tissue culture plates at 10,000 to 29,000 cells per well in 0.1 ml of media. Cells were allowed to adhere for 1-4 hr and then serial dilutions of test IFN were added in 0.1 ml to each well. After incubation for 18-24 hr at 37ºC with 5% C02 in a tissue culture incubator, cells were challenged with EMCV. Cytopathic effect was allowed to develop for 24 to 48 hr, cells were stained with crystal violet and excess stain was washed off with tap water. After drying the plates for at least 2 hr, the dye was solubilized with 70% methanol and absorbance was determined at 562 nm in a Molecular Devices UVMax plate reader. Inhibition of CPE was calculated by setting 100% inhibition as cells incubated without virus (6 wells) and 0% inhibition as cells incubated with virus and no IFN. Generally the cell control was 6-10 times the virus control. All sample data points were run in duplicate in at least two independent assays.

Growth Inhibition Assay. OVCAR-3 cells were plated in 96-well plates at 1000 cells per well, allowed to adhere for 1-4 hr and then serial dilutions of test IFN were added to each well. After 6 days incubation at 37ºC , the conditioned media was removed, 0.1 ml of fresh media containing MTS/PMS reagent [1:6 v/v, Promega, CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Cat.# G5430] was added. The amount of soluble formazan produced by the viable cells was measured at 490 nm in an ELISA plate reader. The 50% effective dose of growth inhibition effects [EC50] was determined by data analysis using a 4 parameter logistic equation in GraphPad Prism software. Curves were constrained between 0% and 100%.

Caspase 3/7 Activation Assay. OVCAR-3 cells were plated in 96-well black wall tissue culture plates at 10,000 cells per well in 0.1 ml media and incubated for 24 hr at 37ºC. Serial dilutions of test IFN were added to the cells and further incubated for 24 hr. Caspase 3/7 activity was determined using Caspase-Glo reagent (Promega, Cat. #G8091). The luminescent signal proportional to the amount of caspase activity present in the treated cells was measured in Victor-3 luminometer and recorded with Wallac 1420 workstation. The number of viable cells was determined by CellTiter-Glo Luminescent Cell Viability Assay (promega, Cat.# G7570) in a duplicate plate which was treated with the same test IFN concentrations. The Caspase 3/7 activity was normalized per viable cells at each IFN concentration.

Reporter Cell-based Assay. Quantitative determination of the activity of Cynomolgus IFN-α proteins secreted in the tissue culture supernatants was carried out by using the iLite human IFN-α gene-reporter cell-based assay kit (PBL, Cat.# 51100-1). Briefly, appropriate volumes of cell culture supernatants were mixed with sample buffer, added to the reporter cells in 96-well test plates and incubated for 17 hr at 37ºC. The assay detects the amount of active Luciferase enzyme molecules which is directly proportional to the number of IFN-α molecules that have bound to the cell receptor. The luminescent sustrate for the luciferase enzyme is added and the reaction product measured in Victor-3 luminometer. The level of the luciferase enzyme activity observed in the particular sample is compared to the standard curve using the human IFN-α2 protein calibrated to the International Units (IU) of IFN-α standard or Cynomolgus IFN-α2 purified protein (PBL, Cat.#16100-1).

MHC Class I up-regulation. A549 cells were plated at 50,000 cells per well in 6-well tisue culture plates, allowed to adhere for 1-4 hr and treated with individual Cyno-IFN- α subtypes in tissue culture supernatants (300 μl) for 72 hr. Cells were washed with cold PBS, trypsinized and resuspended in complete DMEM media. MHC I antigen were stained with mouse monoclonal [W6/32] antibody conjugated with FITC (Abcam, Cat. # ab20313) and analyzed by flow cytometry (FACS SCAN, BD Flow Cytometry).

Figure 1. Antiviral activities of purified human and Cynomolgus IFN-α2 proteins on human lung carcinoma A549 cells.

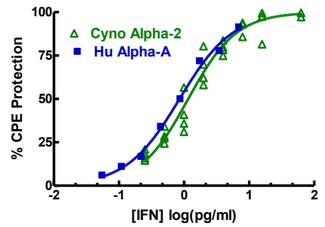

Figure 2. Antiviral activities of purified human and Cynomolgus IFN-α2 proteins on Rhesus LLC-MK2 cells.

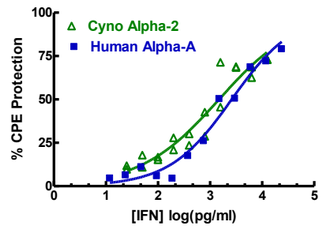

Figure 3. Antiviral activities of purified human and Cynomolgus IFN-α2 proteins on Cynomolgus fibroblast JTC-12.

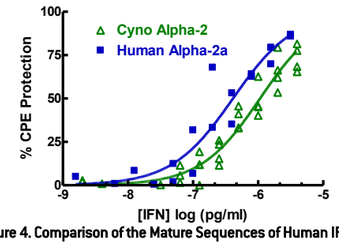

Figure 4. Comparison of the Mature Sequences of Human IFN Alpha 2b and Cynomolgus IFN Alpha 2

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          1
Cyno    CDLPQTHSLG NRRTLILLAQ MRRISLFFCL KDRHDFEFPQ
Human   .......... S........ ........S. .......G...

         41
Cyno    EEFGNQFQKA QTIPVLHEMI QQTFNLFSTK DSSAAWDETL
Human   .......... E..........I....... ..........

         81
Cyno    LNKFYTELYQ QLNDLEACVM QEMGVTETPL MNKNSILAVR
Human   .D........ .........I .GV....... .KED......

        121
Cyno    KYFQRITLYL KEKKYSLCAW EVVRAEIMRS FSLSTNLQES
Human   .......... ......P... .......... ..........

Cyno    LRSKE
Human   .....
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Figure 5. Phylogeny of Human Rhesus and Cynomolgus IFNs.

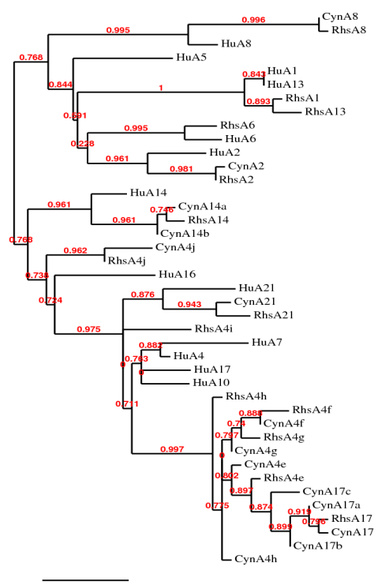


Table 1. Screening the biological activities of Cynomolgus IFN- α subtypes secreted in Vero cell culture supernatants.

IFN	iLite Assay	A549/EMCV	JTC12/EMCV
CynA2	6.63E+03	1.14E+04	1.26E+04
CynA8	4.88E+04	3.52E+04	1.43E+05
CynA21	1.16E+04	1.00E+04	9.73E+03
CynA14a	8.95E+03	1.47E+04	4.47E+03
CynA14b	8.04E+03	1.77E+04	1.18E+03
CynA4e	2.70E+03	5.38E+03	1.05E+03
CynA4f	2.82E+03	2.28E+03	2.69E+03
CynA4g	8.22E+02	3.50E+03	4.71E+03
CynA4j	5.15E+02	3.72E+02	<8
CynA17a	3.67E+04	9.77E+03	3.76E+04
CynA17b	1.60E+04	1.14E+04	1.26E+04
CynA17c	2.17E+03	2.78E+03	3.94E+02

Table 2. Summary of growth inhibition activities of Cynomolgus IFN-α proteins on human ovarian carcinoma cells.

IFN	Antiproliferative	Caspase
CynA2	+	+
CynA8	+	+
CynA14a	+	+
CynA14b	+	+
CynA21	+	+
CynA4e	+	+
CynA4f	+	+
CynA4g	+	+
CynA4j	+	+
CynA17	+	+
CynA17b	+	+
CynA17c	+	+

Figure 8. Upregulation of MHC class I antigen on human lung carcinoma A549 cells.

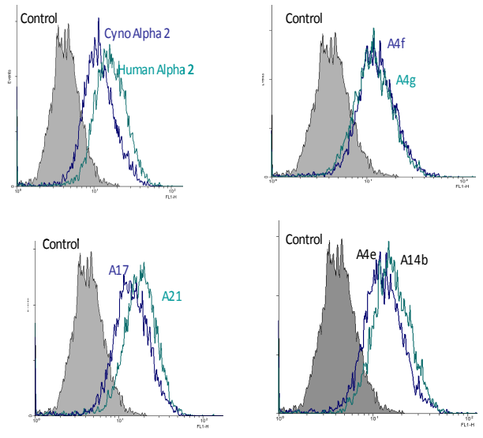


Figure 9. Detection of Cynomolgus subtypes in iLite Human IFN-Alpha Kit

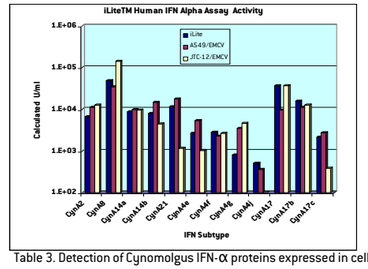
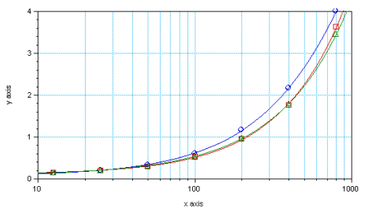


Table 3. Detection of Cynomolgus IFN-α proteins expressed in cell culture supernatants on different ELISA formats.

A Cynomolgus/Rhesus IFN-α2, currently in development at PBL. Protein concentrations are expressed in pg/ml.



Conclusions

We have identified, sequenced, cloned and expressed 12 different unique Cynomolgus IFN-α proteins.

There are good homologues for Human IFN α2, 8, 14 and 21. Both Rhesus and Cynomolgus have a cluster of sequences similar the Human α 4/17.

All Cynomolgus IFN-α proteins expressed in *E.coli* or in mammalian cell culture supernatants exhibit protection on human and non-human primate cells from the cytopathic effects of viruses.

All identified Cynomolgus IFN-α subtypes demonstrated growth inhibition properties on human OVCAR-3 cell line, they also activate caspase 3/7.

The ability to upregulate the MHC class I antigen further confirmed that all hallmark IFN activities are intrinsic to the newly identified Cynomolgus IFN-α subtypes.

Cynomolgus IFN-α2 protein expressed in *E. coli* has been used to develop an ELISA which detects this IFN between 12.5 and 800 pg/ml.

Relative and specific activities are being determined as is the subtype reactivity of the IFN-α subtypes in both Human and Cyno targeted ELISAs.