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Camelid type I interferons: Identification and functional characterization of interferon alpha from the dromedary camel (Camelus dromedarius)

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A B S T R A C T

Investigations into the molecular immune response of dromedary camel, a key livestock species of the arid, have been limited due to the lack of species-specific reagents. Here we describe for the first time, the identification and characterization of type I IFNs of dromedary camel, which are the most important cytokines in the innate host immune response against viruses. We cloned camel IFN-α coding sequences and identified a total of eleven subtypes. The canonical IFN-α subtype designated as IFN-α1 contained a 555-bp Open Reading Frame encoding a protein of 184 amino acids. Recombinant IFN-α1 protein was produced in E. coli and purified from inclusion bodies. Recombinant camel IFN-α1 induced the mRNA expression of interferon-stimulated genes (ISGs) in camel kidney cells. The purified protein also showed potent in-vitro antiviral activity against Camelpox Virus in kidney cells. The identified camel IFN-α protein and the subtypes will facilitate a better understanding of the host immune response to viral infections in camel and the development of potential antiviral biologicals for zoonotic diseases for which camel act as a reservoir.

1. Introduction

The interferons (IFNs) of vertebrates constitute the primary line of defense of host immune response against invading pathogens. These helical proteins are classified into type I (viral interferons), type II (immune interferon or IFN-γ) and type III (IFN-λ) interferons based on the amino acid sequence, chromosomal location and the specific receptors used for signaling (Pestka et al., 2004). Type I IFNs form the primary line of defense in the host immune response against viral pathogens which comprise of IFN-α, IFN-β, IFN-ω, IFN-ε, IFN-κ, IFN-τ and IFN-δ (de Weerd and Nguyen, 2012d). In the human genome, type I IFN genes are intronless and are arranged in tandem from IFNB to IFNE spanning a region of ~ 400 kb on the short arm of chromosome 9 (Díaz et al., 1994; Roberts et al., 1998). The first primordial IFN gene is believed to have originated 500 million years ago and eventually diverged into IFNA and IFNB around 400 million years ago. The IFNA gene evolved again and duplicated many times to produce the multiple IFNA subtypes present in human and other animals (Gillespie and Carter, 1983). In mammals, IFN-α is one of the major type I IFN proteins modulating the host immune response against viruses and is the only type I IFN used in therapeutic practice (Antonelli et al., 2015). Thirteen functional IFN-α subtypes have been reported in human (Kuruganti et al., 2014). These are produced as precursor proteins with a 23 amino acid hydrophobic signal polypeptide at the N-terminus which is cleaved off before mature IFN-α proteins are secreted from the cells. Four conserved cysteine residues are present in the mature proteins of all IFN-α subtypes and are located at 1, 29, 99 and 139 positions in the protein sequence. Multiple IFNA genes have been reported in other domestic animals as well. Fourteen IFNA genes have been reported from porcine and the recombinant proteins exhibited antiviral activities against porcine viruses in-vitro (Cheng et al., 2006). Recombinant bovine IFN-α showed antiviral activity to vesicular stomatitis virus (HSV) and Bovine Viral Diarrhea Virus (Li et al., 2010; Yanagida et al., 2004). IFNA genes have been identified from the horse and bacterially expressed recombinant equine IFN-α exhibited antiviral effect against equine herpesvirus (Himmler et al., 1986; Steinbach et al., 2002).
Thirteen IFN-α subtypes were identified in mink, a fur producing animal and antiviral activity tested against vesicular stomatitis virus and canine distemper virus (Zhang et al., 2015). In wild animals, twelve IFNA genes have been identified in the giant panda, and the recombinant proteins tested for antiviral activity with VSV (Tan et al., 2007).

Camelids are a unique group of mammals with distinct immunology, the most important being the single-chain antibodies. Camelids originated in North America during the Eocene period and the present-day members have evolved to adapt to diverse challenging habitats ranging from hot deserts of Asia to cold high altitudes of South America. They are the only surviving members of the mammalian suborder Tylopoda and are represented by the Old World Camelids (the dromedary and the Bactrian camel) the New World camels (alpaca, llama, guanaco, and vicuña). Though considered a hardy species, the dromedary camel is still susceptible to many viral, bacterial and protozoan pathogens. One of the most contagious and infectious diseases affecting the dromedary camels is Camelpox. It is enzootic to every region of the world (except Australia), where camels are reared, and severely impact the economy of communities dependent on camels for milk, meat, transportation, and sport. This disease is caused by Camelpox virus (CMLV), belonging to the genus Orthopoxvirus of the Poxviridae family. Like many members of the poxviruses, CMLV is also host-restricted to a single host – Camelus species (Duraffour et al., 2011b). However recent reports of human infections in India, the Middle East, and Africa have pointed out the zoonotic threat of the camelpox virus (Bera et al., 2011; Khalafalla and Abdelazim, 2017). Although the CMLV vaccine has been developed, the disease is still prevalent since the vaccination is not widely used and only calves infected a few times. The Middle East Respiratory Syndrome coronavirus (MERS-CoV) and as a reservoir of another major zoonotic disease – the Middle East Respiratory Syndrome coronavirus (MERS-CoV) and as a host-restricted to a single host – Camelus species (Duraffour et al., 2011b). Dromedary camels are also reported to be the reservoir of another major zoonotic disease – the severe acute respiratory syndrome coronavirus (SARS-CoV-2) – the origin of the COVID-19 pandemic (Wheeler et al., 2020). Dromedary camels are also reported to be the reservoir of another major zoonotic disease – the severe acute respiratory syndrome coronavirus (SARS-CoV-2) – the origin of the COVID-19 pandemic (Wheeler et al., 2020).

2. Methods

2.1. PCR amplification and cloning of dromedary IFNA

The IFN-α coding region in camel was identified in the camel genome by BLAST sequence analysis of porcine IFN-α1 sequences. We identified a few Camelus dromedarius whole-genome sequence contigs in NCBI GenBank (JQ0001000073, JQ0001000674, and LSZX01007849.1) which contained putative IFNA like sequences. Primers CIFNa1vFF 5’-CAG GTC TTC AGA GAA CCT TCA G-3’ and CIFNa1vFR 5’-GCA GAG AAC GTG ATG TTG-3’) were designed based on the regions flanking the IFN-α protein-coding region.

Genomic DNA was isolated from dromedary camel blood using Wizard genomic DNA purification kit (Promega). PCR amplification was carried out using Phusion High-Fidelity DNA Polymerase (Thermo Life Sciences) using 50 ng of genomic DNA as the template. Amplicons of the expected size were cloned into pGEM-T Easy vector and sequenced using an ABI 3130xl Genetic Analyzer (Applied Biosystems).

2.2. 5’ RACE to identify the complete cDNA

Primers for 3’ RACE were designed based on the cloned sequence to identify the cDNA sequence. RNA was isolated from Camelpox virus-infected camel kidney cells (Premrj et al., 2015) using TRIzol reagent (Thermo) and cDNA made using FirstChoice RLM-RACE Kit (Thermo). For 5’ RACE, two rounds of PCR was carried out using the RLM-RACE Kit primers and IFNA specific primers IFNa5P R1 (5’-CAA AAG CTG CGT CTG TCC TTC AGG CAG CAG-3’) and IFNa5P R2 (5’-AGC ACC AAG GTC CTC GTG GTG GCC AG-3’) primers.

2.3. Identification of the promoter region

Gene walking was done using the Genome Walker Universal Kit (Thermo) to identify the 5’ promoter region. Camel genomic DNA was digested with EcoRV, PvuII, DraI, and Stul enzymes and each of these digests ligated separately with the Genome Walker adapter to create Genome walker libraries. Using these libraries as the template, PCR was carried out with camel IFN-a1 specific primers IFNaSPR1 / IFNaSPR2 to amplify 5’ region of the gene. Amplicons were cloned and sequenced.

2.4. Bioinformatic analysis

Sequence contig for each clone was made using the Sequencer 4.9 (Gene Codes, Ann Arbor, MI). We used BioEdit 7.0. software (Hall, 1999) to identify the Open Reading Frame (ORF) and the predicted amino acid sequence of the cloned IFNA-α sequences. Multiple sequence alignment was done using the ClustalW program (Thompson et al., 1994). We used the SignalP (http://www.cbs.dtu.dk/services/SignalP/) and ProtParam (http://web.expasy.org/protparam/) servers to predict the signal peptide sequence and protein properties respectively. A phylogenetic tree was made with MEGA4 software (Tamura et al., 2007) using the amino acid sequences of the generated dromedary IFN-α amino acid sequences, previously reported and predicted mammalian IFN-α sequences available in the NCBI Genbank. We made the 3-D homology models of the mature dromedary IFN-α1 protein using the I-TASSER server (Yang and Zhang, 2015).

2.5. Expression of recombinant dromedary IFN-α1 protein in E.coli and purification

We used the primers IFNA-MPF (5’ GAA TTC TGT GAC CTG CCT CAA ACC - 3’) and IFNA-NR (5’TCA TTC TTG TGT CCT CAA TGT 3’) to amplify the mature protein-coding sequence of the dromedary camel IFN-α. The amplified fragment was cloned into the pET28 vector (Novagen) to create an N-terminal His6-tagged recombinant protein expression construct. The expression construct was sequence confirmed and transformed into BL21(DE3)pLysS (Promega) / Rosetta 2(DE3) (Novagen) expression strains of E.coli. E. coli cells harboring the pET28-IFN-α1 constructs were grown in LB Kanamycin broth at 37 °C to an optical density of ~0.5 (600 nm). Isopropyl β-D-1-thiogalactopyranoside (IPTG, Promega) was added to the culture medium (1 mM concentration) to induce recombinant protein expression and cells grown for four hours at 30 °C. Induced cells
were harvested by centrifugation (5000 G at 4 °C for 10 min), and the cell pellet was resuspended in lysis buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) containing 1X Protease Inhibitor Cocktail (Thermo Pierce) and lysed by repeated freeze-thaw cycles. The inclusion bodies were separated from the soluble fraction of the lysate by centrifugation at 10,000 G for 10 min and resuspended in Inclusion Body Lysis Buffer (6 M Guanidine hydrochloride, 300 mM NaCl, 50 mM NaH₂PO₄ (pH 8.0)). The solubilized inclusion bodies were incubated for 16 h at 4 °C and the cell debris was removed by centrifugation at 15,000 G for 30 min at 4 °C. Ten ml of supernatant was loaded onto a 1 ml HisTrap FF Crude (GE Life Sciences) affinity chromatography column (equilibrated with 6 M Guanidine hydrochloride, 300 mM NaCl, 50 mM NaH₂PO₄ (pH 8.0) connected to an ÄKTA Avant 25 FPLC system (GE Life sciences). The column was washed initially with Inclusion Body Wash Buffer (8 M Urea, 10 mM imidazole, 500 mM NaCl, 20 mM Tris HCl, pH 8.0). On-column refolding of the recombinant proteins bound to the column was performed by applying a slow linear gradient of 8 M to 0 M urea refolding buffer (10 mM imidazole, 20 mM Tris HCl, 500 mM NaCl, pH 7.5). After refolding, the protein bound to the column was washed with wash buffer (40 mM imidazole, 20 mM Tris HCl, 500 mM NaCl, pH 7.5) and finally eluted with a linear gradient of 100 to 500 mM imidazole containing elution buffer (20 mM Tris HCl, 500 mM NaCl, pH 7.5). Fractions containing the recombinant protein were analyzed by SDS-PAGE. Pooled recombinant protein-containing fractions were desalted using PD-10 desalting columns (GE healthcare) and endotoxin removed using Pierce High Capacity Endotoxin removal columns (Thermo Fisher). Purified proteins were sterilized using a 0.22 μm Millex GP filter unit (Merck Millipore, Cork, Ireland) and stored in phosphate-buffered saline (PBS, pH 7.4 with 50 % glycerol).

The purified recombinant proteins were characterized by western blot using a rabbit polyclonal anti-human IFN-alpha antibody (Abcam, ab168449) as the primary antibody and a monoclonal anti-rabbit IgG alkaline Phosphatase conjugated antibody (Sigma, A2556) as the secondary antibody. The membrane was developed with CDP-Star Chemiluminescent Substrate (Sigma) and visualized on a G:BOX Chemi XX9 imaging system (Syngene, Cambridge, UK). To detect the His₆-tag in the recombinant protein, western blot was carried out with an anti-His₆ antibody (Sigma) and developed with NBT BCIP reagent (Promega).
2.6. In-vitro induction of interferon-stimulated genes (ISGs) in Camel kidney cells

Camel kidney cells were cultured in 25 cm² flasks in DMEM medium (Life Technologies) supplemented with 10 % FCS. Before the assay, the growth medium was removed and cells were incubated for 2 h in plain DMEM without serum. Cells were treated with recombinant camel IFN-α1 (500 ng/ml) in assay medium (DMEM with 1 % FCS) for 4 h. We also incubated cells with a His 6-tagged control protein of similar size (20.4 kDa His6-tagged Trx fusion protein expressed from an intact pET32a expression vector) at the same concentration as camel IFN-α1 and medium without any other additives as experimental controls. Post-treatment, the cells were harvested and total RNA was isolated using TRIzol reagent (Thermo Fisher) as per the manufacturer’s recommendations. Approximately 2 μg of the total RNA after DNase treatment was reverse transcribed using Maxima First Strand cDNA Synthesis Kit (Thermo). We performed real-time PCR to quantify the expression of IFN responsive genes like ISG15, Mx1, RIG1 and Viperin using an Applied Biosystems 7500 Fast Real-time PCR and GoTaq qPCR Master Mix (Promega). All samples were analyzed in triplicate and the relative expression of each sample was normalized to that of GAPDH as calculated as 2^−ΔΔCT.

2.7. Antiviral assay of recombinant camel IFN-α1 in-vitro

We assayed the antiviral activity of the purified recombinant IFN-α1 as the reduction in cytopathic effect (CPE) on Camelpox virus infected Camel Kidney Fibroblast cell lines. Camel kidney cells cultured in 24-well plates were treated with 500, 125, 31 or 7.8 ng/ml of recombinant CdIFN-α1 or His-tagged control protein for 4 h. After treatment, the medium was removed and cells infected with Camelpox virus at a multiplicity of infection (MOI) of 0.1 and incubated for 1 h at 37 °C. The virus inoculum medium was then removed and cells were grown in growth medium for another 24 h. Cells were observed using a Nikon Eclipse Ti-S inverted microscope and images were captured with NIS-Elements D 3.10 Software (Nikon).

2.8. Real-time quantitative PCR analysis of the reduction in Camel Pox Virus (CMLV) DNA as a measure of antiviral activity

Camel kidney cells were treated with 500 ng/ml of IFN-α1 or His-tagged control protein and infected with camelpox virus (MOI of 0.1) as described earlier and incubated for 4 h. The cells along with super-natant were collected and viral DNA was isolated using DNeasy DNA isolation Kit (Qiagen). Viral DNA in the samples was estimated by real-time PCR using previously reported primers and probes targeting the CMP48L gene (Durauffour et al., 2011a) of the camelpox virus (Forward Primer CMLV-qTF (5′-CAACACCTCATTATAGACGCTATG3′), reverse Primer CMLV-qTR (5′-CTGATTGCATTCTCAATACATACGCTT3′) and CMLV QT Probe (5′-GAGGATTAATGATTAGGGAGGCTCTG-3′). The qPCR was performed in an ABI 7500 Fast Real-time PCR machine using the following conditions – initial denaturation at 95 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 30 s and annealing and extension at 60 °C for 30 s. The amount of viral DNA was estimated using a standard curve made from 10-fold serial dilutions of viral DNA standard run under the same conditions in qPCR.
Fig. 3. Multiple amino acid sequence alignment of the eleven dromedary camel IFN-α subtypes with human IFN-α2 and Porcine IFN-α1: Multiple sequence alignment of the IFN-α protein sequences were performed using the ClustalW program. Identical sequences in all sequences are marked by a dot (.). Gaps in the sequence alignment are marked by dashes (-). Predicted eukaryotic signal peptide cleavage sites in the sequences are marked by a downward arrow. Conserved cysteine residues are marked in bold. Putative IFNAR-1 and IFNAR-2 binding sites in the camel predicted by the Conserved domain search (CD-Search at NCBI) are marked in single underline and double underline respectively. The Arginine-33 and Tyrosine-123 residues conserved in all human and murine IFN-α sequences are also marked. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).
3. Results

3.1. Cloning and sequence analysis of Camelus dromedarius IFN-α cDNA

We amplified and cloned an 818 bp DNA fragment containing the dromedary camel IFNα by PCR using CdIFNaVF & VR primers. To identify the 5′ UTR of IFN-α1, RACE PCR was done with RNA from virus-infected cell lines. After two rounds of 5′ RACE PCR for IFN-α1, a 340 bp fragment was obtained. This fragment contained 220 bp of 5′ UTR and 120bp of the ORF (Fig. 1). The cloned IFN-α1 sequence contained a 555 bp Open Reading Frame (ORF) that encoded a 184 amino acid protein. The sequence of the Camel IFN-α1 has been submitted to the NCBI GenBank (Accession number MN883354). When compared to the reference sequence of other well-studied mammals, the Camel IFN-α1 shares 84 % nucleotide identity with equine IFN-α1, 82 % with porcine, 80 % with human and 73 % with murine (Supplementary Fig. S1).

At the protein level, the amino acid similarity values were 83% (porcine IFN-α1), 81% (equine IFN-α1 and IFN-α2), 79% (porcine IFN-α4) and 75% human IFN-α2. Eukaryotic signal peptide prediction analysis using the SignalP Server predicted that the first 23 N-terminal amino acids of the 184 amino acid camel IFN-α1 precursor represented the signal peptide. Hence the predicted mature protein is 161 amino acids long, with a molecular weight of 18.6 kDa and a pI of 8.37. Five cysteine residues are present in the dromedary camel IFN-α1 sequence at amino acid positions 1, 29, 86, 99 and 139 in the mature protein (Fig. 2A).

We constructed 3-D homology model with the mature protein sequences of the CdIFN-α1 protein using i-TASSER server. The mature protein is predominantly alpha-helical, comprising of five helices (Fig. 2B).

3.2. Identification of subtypes of camel interferon-alpha

A total of eleven subtypes of camel IFN-α were identified (NCBI GenBank accession numbers MN883354 to MN883364). Of the eleven IFN-α subtypes, seven subtypes including the canonical IFN-α1 had 184 aa long precursor protein. Four subtypes were 187 aa long since they had three additional amino acids at the N-terminal. This could be due to the usage of a start codon placed three amino acids upstream in these subtypes. The original methionine position in these subtypes is substituted with a valine residue (Fig. 3). No N-linked glycosylation sites were predicted in any of the eleven Cd IFN-α proteins.
3.3. Identification of the IFNA1 gene and promoter

We identified and cloned an 1132 bp fragment from a StuI digested genomic DNA library by gene walking which contained the 5′ end of the IFNA1 gene. This fragment contained 957 bp of the 5′ regulatory region of IFNA1 gene (Fig. 4). A key feature of the IFNA1 gene promoter is the presence of the virus-responsive elements (VRE) which modulate the activity of the IFN in response to viral infection. The three modules (B, C & D) of this VRE are present in the camel promoter also. Modules B and C correspond to the IRF-7 and IRF-3 binding sites. The consensus IRF3/7 binding site GAAANN is conserved in camel also. Module D corresponds to the proximal interferon regulatory domain (Fig. 4).

3.4. Expression of recombinant Camel IFN-α1 in E.coli and purification from inclusion bodies

To evaluate the biological activity of camel IFN-α, we generated an N-terminal His6 tagged expression construct of Camel IFN-α1 mature protein-coding region in pET28a prokaryotic expression vector. Codon analysis of the camel IFN-α1 revealed the presence of 22 E.coli rare codons in the mature protein coding region that could potentially impede the recombinant expression (Supplementary Fig. -S3). Hence, the expression construct was transformed into Rosetta 2(DE3) E.coli strains of E.coli which carried tRNAs for 7 rare codons (AGA, AGG, AUA, CUA, GGA, CCC, and CGG). Rosetta 2(DE3) E.coli carrying the CdIFN-α1 expression construct produced a specific band of the expected size of ~22 kDa upon IPTG induction (Fig. 5A). Almost all of the overexpressed recombinant CdIFN-α1 protein was found to be in the inclusion body.
3.5. Induction of interferon-stimulated genes (ISGs) expression in camel kidney cells by recombinant CdIFN-α1 protein

IFN binding to its receptor initiates a signaling cascade which ultimately results in the expression of many ISGs. To investigate the ability of the recombinant CdIFN-α1 proteins to induce the expression of ISGs, we treated camel kidney cells with CdIFN-α1 for 4 h. The mRNA expression of four candidate ISGs – Mx1, ISG15, Viperin (VIP) and RIG1 - were analyzed by qPCR. The expressions of these ISGs were significantly up-regulated in recombinant CdIFN-α1 treated samples. VIP and ISG15 mRNA expression were found to increase by more than ~750 and ~1800 fold respectively upon CdIFN-α1 treatment. Mx1 and RIG1 mRNA expression also were enhanced ~350 to 300 fold upon treatment with CdIFN-α1 protein (Fig. 6). There were no significant changes in the expression of these ISGs in cells treated with a His6-tagged control protein which was purified under similar conditions.

3.6. Antiviral activity of recombinant CdIFN-α1 against camelpox virus in-vitro

The biological activity of recombinant CdIFN-α1 protein was assayed in-vitro by induction of the antiviral activity of IFN-α against camelpox virus in Camel Kidney fibroblast cells. Pre-treatment of recombinant CdIFN-α1 conferred antiviral resistance to Camel fibroblastic cells against camelpox virus infection. The His6-tagged control protein did not show any antiviral activity as evident from the cytopathic effects of CMLV in this sample (Fig. 7A).

We also used qPCR to quantify the camelpox virus DNA reduction upon recombinant CdIFN-α1 treatment to assess the antiviral activity of CdIFN-α1. Camel Kidney fibroblastic cells were pre-treated with CdIFN-α1 or control His tagged protein for 4 h and then infected with the virus. After 24 h, the DNA in the supernatant was extracted and CMLV virus quantified by qPCR. Cells pretreated with recombinant CdIFN-α showed significantly lesser amounts (~ 56 % reduction) of viral DNA when compared with the CMLV-infected samples pretreated with control His6-tagged protein or without any pretreatment (Fig. 7B).

Cells pre-treated with recombinant CdIFN-α1 (concentration of 500 to 31 ng/ml) showed improved cell survivability in a dose-dependent manner (65 to 31 %) when compared to the control His6-tagged protein treated cells (7–9%) (Fig. 7C and D).

4. Discussion

IFN-α has been well studied and characterized in many mammalian species due to its immunomodulatory role, but not in camels. We report in this paper, the identification of IFNA genes from the dromedary camel. IFNA genes are characterized by their intronless nature and are proposed to have originated from a common ancestral gene (Capon et al., 1985; Goeddel et al., 1981). A total of 11 IFN-α subtypes were identified from the dromedary camel.

Comparative sequence analysis of the camel IFN-α to other well-studied mammalian counterparts indicate the highest homology to the porcine and equine IFN-α (~ 80 % amino acid similarity). In the phylogenetic analysis (Supplementary Fig. S2), the cameld IFN-α proteins cluster together in a distinct group like other dromedary camel immune genes we reported earlier (Premraj et al., 2015, 2013).

IFN-α belongs to the long-chain helical cytokine subfamily which includes IFN-β and IL-10 and the protein has a left-handed four-helix bundle structure (Klaus et al., 1997). The mature dromedary IFN-α1 protein is predicted to be predominantly alpha-helical, with the helices accounting for around 56 % of the secondary structure (Fig. 2 & 3). Based on secondary structure prediction and 3-D homology modeling, the dromedary IFN-α mature proteins have the characteristic alpha-helical bundle topology. Human IFN-α1 has two disulfide bonds between Cys-1/Cys-98 and Cys-29/Cys-139. Mutational studies have revealed that these Cys-29/Cys-139 disulfide bridges are critical for the antiviral activity, whereas mutations in Cys-1/Cys-98 bridge only marginally altered antiviral activity (Beilharz et al., 1986). In addition to these four cysteines, human IFN-α1 has an additional cysteine residue (Cys-86), which is not present in human IFN-α2. This additional cysteine (Cys-86) is conserved in all the eleven camel IFN-α subtypes, the porcine IFN-α1, porcine IFN-α4, and mouse IFNα1 (Fig. 2A). No additional cysteine residues were noted in any of the cameld IFN-α subtypes (Fig. 3).

The major putative IFNAR-1 binding residues are located on the helices A and C. These residues are fully conserved across the IFN-α subtypes. The putative IFNAR-2 binding residues are distributed at two regions in the mature protein; the extended loop region between helix A-B and in helix D region. These residues are also entirely conserved (Fig. 3). The Arg-33 and Tyr-123 residues in the mature murine and human IFN-α1 protein are reported to be critical for antiviral activity (Kerry et al., 1988). The stringent conservation of these two residues in all the IFN-α sequences indicates their significance in antiviral action (Fig. 2A and Fig. 3).

Although the different mammalian mature IFN-α protein sequences show a high level of structural and sequence conservation, they differ in the total length of the mature protein (Fig. 2). The mature camel IFN-α1 (161 aa) is shorter compared to human IFN-α1/murine IFN-α1 (166 aa) or human IFN-α2 (165 aa). Both the equine IFN-α1 and IFN-α2 have the
same size as the camel IFN-α mature protein. Porcine IFN-α1 (158aa) seems to 8 amino acids shorter than porcine IFN-α4 (166 aa). The variation in size of these mature mammalian IFN-α proteins is due to the unstructured C-terminal tail and is presumed to contribute to differential receptor binding and biological activity (Slutzki et al., 2006). Interestingly all the eleven predicted camel IFN-α mature proteins are of the same size (161 aa) and have identical C-terminal tail (Fig. 3).

No N-linked glycosylation sites are predicted in any of the camel IFN-α mature proteins. N-linked glycosylation is predicted in a few murine IFN-α, four porcine IFN-α, two mink interferons and only human IFN-α14, and none of the other IFN-α subtypes. Since IFN-α is intronless and does not need glycosylation for its activity, prokaryotically produced recombinant IFN-α has been the first choice for commercial production. However prokaryotic expression of interferons in heterologous expression systems like E.coli poses other technical challenges like the presence of rare codons and formation of insoluble inclusion bodies. There were 22 rare codons in the mature protein-coding sequence of camel IFN-α which severely hampered recombinant expression. Recombinant expression of the protein was improved after the usage of Rosetta 2 (DE3) strain of E.coli which supplemented the tRNAs for 19 of the 22 rare codons present (Supplementary Fig. S3 - A, B & C). When overexpressed in E.coli, recombinant human IFN-α
Control His-tag protein treated & CMLV infected

500ng/ml

125ng/ml

31ng/ml

7.8ng/ml

Medium Control - Uninfected

Medium Control - CMLV infected

D

His Protein treated

Cd-IFNa1 treated

Uninfected Control

CMLV infected Control

Percentage of cells survived

0 10 20 30 40 50 60 70 80 90 100

Protein (ng/ml)

500 125 31 7.8

Fig. 7. (continued)
tends to form insoluble protein aggregates termed inclusion bodies, which are insoluble and do not exhibit biological activity (Babu et al., 2000). Prior to recombinant expression, we analyzed the mature protein sequence of the dromedary camel IFN-α1 in PROSO-II (http://mips.helmholtz-muenchen.de/prosoII/), a sequence-based protein solubility evaluator. Recombinant camel IFN-α1 was predicted to be insoluble when expressed in E.coli upon PROSO-II analysis and was later confirmed by small-scale expression studies. We used a purification strategy in which His6-tagged CdIFN-α1 proteins in inclusion bodies were initially solubilized using 6 M GuHCl as a denaturing agent, captured on a His-affinity column and finally eluted out after on-column refolding (Supplementary Fig. 3D & E). Before use in in-vitro assays, to confirm the identity of recombinant protein as IFN-α, we analyzed it by western blot with an anti-human IFNα antibody. For animals like camels which have limited species-specific immunological reagents, we have to rely on antibodies targeted against proteins of other well-studied organisms. The anti-human IFN-α antibody recognized the recombinant camel protein, but the sensitivity seemed lower especially at low target concentration of the target protein (Fig. 5B). This may be due to the lesser amino acid identity between the human and camel IFN-α proteins. For better sensitivity, anti-camel IFNα specific antibodies need to be generated, keeping in mind of the role of camel as a potential reservoir of zoonotic diseases like MERS.

Primary evaluation of the biological activity of the purified recombinant CdIFN-α1 protein was performed by qRT-PCR analysis of the mRNA expression of different interferon responsive genes in camel kidney cells in vitro. The recombinant IFN-α1 protein induced the mRNA expression of key interferon-responsive genes like ISG15, Mx1, Viperin and RIG1 (Fig. 6). Hundreds of interferon-stimulated genes (ISGs) have been identified so far and they form the major interferon-induced effectors contributing to antiviral activity. These IFN effecter genes work in combination to induce a potent antiviral state (Schoggins and Rice, 2011). Mx1 effectively blocks viral replication, whereas viperin inhibits the early stages of viral entry and replication. ISG15 is a ubiquitin-like protein that has been reported to inhibit viral release and also modify viral as well as host proteins to elicit an antiviral response. Pre-treatment of the camel kidney cells with the recombinant CdIFN-α1 is presumed to induce a signaling cascade leading to the expression of these ISGs which further confer antiviral activity.

We evaluated the antiviral activity of the recombinant Camel IFN-α1 using Camelpox Virus (CMLV), one of the common contagious orthopoxviruses infecting dromedary camels. Pre-treatment of recombinant protein prokaryotically expressed Camel IFN-α1 conferred resistance to Camel kidney cells in vitro against the cytopathic effects of CMLV. Preliminary evaluation of antiviral activity was conducted by visual observation of the reduction in cytopathic effects of CMLV in IFN-α1-treated cells (Fig. 7A). The antiviral effect of IFN-α pretreatment in vitro could be confirmed by quantitative real-time PCR estimation by showing reduction of the CMLV viral load and also by measuring the cell survivability (Fig. 7B and D). As the most abundant subtype of IFN-α present among the clones screened and having identical protein sequence to that from a potential IFNA gene identified in a few Camelus whole-genome contigs, we selected CdIFN-α1 for preliminary recombinant expression and bioactivity testing. It would be interesting to compare the antiviral activity of all the cameld IFNα subtypes against different camel viruses.

Immune evasion strategies of other orthopoxviruses like Variola virus and cowpox virus have been well studied compared to CMLV. Many of these viruses encode multiple proteins to antagonize or modify the host immune response by interfering with the interferon response, pro-inflammatory cytokine and chemokine pathways. CMLV has been reported to encode a few proteins that inhibit IFN-γ, TNF-α, and chemokines. CMS-252, a protein similar to VACV B19R secreted by CMLV is reported to bind to IFN-α with low inhibitory potency (Duraflour et al., 2011b). Recombinant camel IFN-α proteins hence could be useful both in understanding the immune/counter-immune strategies of host/virus, as well as for the development of potential biological antivirals for CMLV. The IFN-α1 protein we generated seems to be the first recombinant antiviral camelid-specific type I interferon. Considering the high level of sequence similarity with the predicted IFN-α from other species, the dromedary IFN-α recombinant proteins could be used in other camelds also.

In conclusion, we identified eleven functional IFN-α subtypes from the dromedary camel by cloning and sequencing. Prokaryotically expressed recombinant IFN-α1 protein induced the expression of ISGs and showed antiviral activity against camelpox virus in-vitro. To our knowledge, these proteins represent the first antiviral recombinant type-I cameld interferons and could be used in better understanding of the immune system of dromedary camels which has been reported to be a reservoir of zoonotic viruses like MERS coronavirus.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

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