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Novel plant-derived recombinant human interferons with broad spectrum antiviral activity

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ABSTRACT

Type I interferons (IFNs) are potent mediators of the innate immune response to viral infection. IFNs released from infected cells bind to a receptor (IFNAR) on neighboring cells, triggering signaling cascades that limit further infection. Subtle variations in amino acids can alter IFNAR binding and signaling outcomes. We used a new gene crossbreeding method to generate hybrid, type I human IFNs with enhanced antiviral activity against four dissimilar, highly pathogenic viruses. Approximately 1400 novel IFN genes were expressed in plants, and the resultant IFN proteins were screened for antiviral activity. Comparing the gene sequences of a final set of 12 potent IFNs to those of parent genes revealed strong selection pressures at numerous amino acids. Using three-dimensional models based on a recently solved experimental structure of IFN bound to IFNAR, we show that many but not all of the amino acids that were highly selected for are predicted to improve receptor binding.

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1. Introduction

Type I interferons (IFNs), including IFN-α and IFN-β, play critical roles in the antiviral innate immune response and are induced in response to unique components of viral infection such as double-stranded RNA. After secretion from infected cells, IFN binds to a receptor (IFNAR) on nearby cells, initiating signaling cascades that enhance cellular resistance to viral infection [reviewed in (Platanias, 2005; Samuel, 2001)]. IFN-α/β are the best studied of the type I human IFNs, and IFN-α therapy has been successful in treating viral infections in people, most notably hepatitis C (Carreno et al., 1987; Fried et al., 2002). Advances in IFN-α therapy include: (1) the addition of a branched 40 kDa polyethylene glycol molecule (pegylation) to synthetic IFN-α, which enhances the effective half-life of the IFN when compared to its native form (Glue et al., 2000); and, (2) use of a synthetic IFN-α (IFN-alfacon-1), which contains the most frequently observed amino acids among several natural IFN-α subtypes (Ozes et al., 1992). These synthetic IFNs are currently manufactured using bacterial expression methods.

Most viruses, including highly pathogenic viruses such as Venezuelan equine encephalitis (VEEV), Rift Valley fever (RVFV), Ebola (EBOV), and monkeypox (MPXV) viruses, have developed methods to counteract the antiviral effects of IFNs (Bouloy et al., 2001; Fernandez de Marco Mdel et al., 2009; Mateo et al., 2010; Simmons et al., 2009; Weaver and Isaacs, 2008; Yin et al., 2009). Treatment options for these viruses are limited and can include ribavirin (Jahrling et al., 1980), small interfering RNAs (Geisbert et al., 2010), and double-stranded RNA activated caspase oligomerizers (Rider et al., 2011). Potent IFNs, especially those that could overcome the viral IFN antagonistic mechanisms, would provide additional broad spectrum options for prophylaxis and therapy.

Here we report studies aimed at generating novel type I IFNs with broad spectrum activity against VEEV, RVFV, EBOV, and MPXV. We used a new gene crossbreeding method termed Genetic ReAssortment by MisMatch Resolution (GRAMMR™), which combines a mismatch endonuclease with a proofreading polymerase and ligase to resolve unpaired bases in heteroduplexes, which are produced by melting and annealing genes within an expression

Abbreviations: IFNs, interferons; IFNAR, interferon-α receptor; VEEV, Venezuelan equine encephalitis virus; RVFV, Rift Valley fever virus; EBOV, Ebola virus; MPXV, monkeypox virus; GRAMMR™, Genetic Reassortment by MisMatch Resolution; BSA, bovine serum albumin; GFP, green fluorescent protein; PSPP, protein structure prediction pipeline; PDB, Protein Data Bank.

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vector. We chose plants rather than bacteria to express the hybrid IFNs because of advantages they offer such as ease of use, high yield of expression products, ability to produce secreted proteins, and mechanisms for oxidative cross-linking of cysteines for proper folding (Giritch et al., 2006; Ma et al., 2003). Also, hundreds of plants can be individually inoculated in parallel, making it possible to rapidly produce and evaluate numerous potential candidate expression products. Our results demonstrate that it is possible to create new human IFNs with improved activity against very diverse viruses and that the improvements can be partially traced to subtle amino acid changes that are predicted to influence their binding to IFNAR.

2. Materials and methods

2.1. Production of the hybrid IFN library

IFN-α2a reference standard (Gxa01-901-535) was obtained from the NIAID Reference Reagent Repository operated by Braton Biotech, Inc. in Gaithersburg MD. Pharmaceutical grade consensus IFN-α (IFN-alfacon-1) reference standard (Infergen; Valeant) was obtained by prescription from a pharmacy. IFN-α1, -α2a, -α4, -α5, -α8, -α10, -α21, IFN-β, IFN-ε, IFN-κ, and IFN-ω genes and genes for the controls IFN-α2a, green fluorescent protein (GFP), and alfacon-1 were codon-optimized for plant expression (see Fig. 4 for IFN amino acid sequences) and inserted into the tobamovirus vector pLSBC-DN15 (O'Keefe et al., 2009) containing a N-terminal extensin secretory signal peptide from *Nicotiana plumbaginifolia* (De Loose et al., 1991) and a C-terminal 6xHis tag with the KDEL endoplasmic retention sequence (Munro and Pelham, 1987). Hybrid libraries were generated using GRAMMR™ (US Patents; 7056,740, 7217,514, and 7833,759). Plasmid heteroduplexes were generated by linearizing the pLSBC-DN15-interferon genes with *Stu*I or *Sma*I, heating to 95 °C to dissociate the DNA strands, and cooling to allow strands to anneal. Heteroduplex DNAs were incubated with *Res*I, a mismatch endonuclease from *Selaginella lepidophylla* (US Patents; 7056,740, 7217,514, and 7833,759), T4 DNA polymerase, and *Escherichia coli* DNA ligase. After one h at 25 °C, DH5-α cells (Invitrogen, Carlsbad, CA) were transformed to isolate individual hybrid plasmids. Infectious viral RNA transcripts from these plasmids were generated using the mMessage mMachine kit (Ambion, Austin, TX).

Four-week old *Nicotiana benthamiana* plants were inoculated with infectious RNA by manual abrasion with diatomaceous earth. Approximately 2 g of plant tissue were homogenized [150 mM Tris–HCl buffer (pH 8.2), 0.5 M NaCl, 15 mM imidazole, 2 mM PMSF, 0.15% sodium metabisulfite] 5–7 days post-inoculation. Clarified homogenates were purified using nickel-conjugated agarose beads (Qiagen, Valencia, CA) using wash buffer [50 mM Tri–HCl (pH 8.2), 0.5 M NaCl, 10 mM imidazole, 0.01% Na metabisulfite, 0.2 mM PMSF] and elution buffer [0.4 M imidazole-0.1 M Tris–HCl (pH 8.2) with 0.2 mg/ml bovine serum albumin (BSA)]. Expressed protein was quantified by densitometry of Coomassie blue-stained SDS–PAGE gels using standards of plant-produced, untagged IFN-α2a protein. The concentration of each protein was adjusted to 75 ng/ml in phosphate-buffered saline (PBS), pH 7.4, containing 1 mg/ml BSA, and IFN concentrations were determined again using densitometry. IFNs and GFP were diluted 10-fold in RPMI 1640 supplemented with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 10% FBS for further studies. Low-yielding clones were omitted from subsequent rounds of GRAMMR.

2.2. Viruses and cells

Luciferase-tagged RVFV rMP12-rluc (Ikegami et al., 2006) virus was kindly provided by Dr. Shinji Makino of the University of Texas Medical Branch at Galveston. The GFP-tagged Zaire ebolavirus, EboZ-eGFP (Palacios et al., 2007), was kindly provided by Dr. Jonathan Towner, Centers for Disease Control and Prevention. The MPXV-GFP virus was produced as previously described (Goff et al., 2011). To generate luciferase-tagged VEEV, a *Not*I-flanked firefly luciferase expression cassette was cloned into a plasmid containing VEEV, strain Trinidad donkey, nsP3 having *Not*I restriction sites inserted at random locations (Beitzel et al., 2010). The nsP3-luciferase fusions were excised and cloned into a full-length genomic clone of VEEV, strain Trinidad donkey. Infectious RNA was transcribed and transfected into BHK cells to generate replication-competent VEEV containing the luciferase expression cassette. Luciferase activity was assessed 24 h post-infection on Vero cells using the Steady-Glo luciferase assay system (Promega, Madison, WI). Plaque-picked virus, which contained a fusion at nucleotide 5431 (GenBank Accession No. L01442), maintained cytopathic effects and luciferase activity, was selected for this study.

Vero and Vero 76 cells were used for the antiviral screening assays. Cells were maintained in either DMEM supplemented with 10% FBS (for RVFV and VEEV) or MegaVir (Hyclone, Logan, UT; for EBOV and MPXV).

2.3. Antiviral and antiproliferative assays

Confluent cells in 96-well luminometer plates were treated with IFN (approximately 60,000 to 1 pg/ml) for 24 h before being infected with a signal-optimized amount of reporter virus. The luciferase-tagged VEEV and RVFV assays were read 18 h post infection using the Steady-Glo (VEEV) or *Renilla* (RVFV) luciferase assay systems (Promega, Madison, WI) according to the manufacturer's instructions. For VEEV, Steady-Glo reagent containing both lysis buffer and luciferase substrate was added to the wells, and luciferase activity was measured. For RVFV, the media was removed from the cell monolayers, and *Renilla* lysis buffer was added to release the luciferase enzyme. Luciferase substrate was added, and luminescence was measured. The GFP-tagged viruses, MPXV and EBOV, were assayed for fluorescence 48 h after infection. Daudi cell-proliferation assays were conducted as previously described (Nederman et al., 1990). For these assays luciferase activity was recorded as relative light units (RLU), and for the GFP-based assays, fluorescence activity was recorded as relative fluorescence units (RFU). Initially, IFNs from early rounds of screening were evaluated for antiviral activity with EBOV, and promising hybrid IFNs were included in the additional rounds of GRAMMR. For subsequent evaluations, all four reporter viruses were included in the antiviral screenings.

2.4. Protein modeling

The Protein Structure Prediction Pipeline (PSPP) (Lee et al., 2009), initially developed for domain boundary detection, sequence homology search, fold recognition, homology modeling, de novo design, and model evaluation, was used to generate three-dimensional models for the high-potency IFNs from sequence. The PSPP uses the program NEST (Petrey et al., 2003) for generating homology models. Two data inputs were used to produce these models: (a) template files from the experimentally determined structures of IFN-α2a (Klaus et al., 1997) and IFN-α2 bound to an IFN-αβ receptor (Nudelman et al., 2010) [Protein Data Bank (PDB) (Berman et al., 2000)]; and (b) pair-wise alignments between each of the target IFN sequences and that of the template structures. Models based on different templates were built to assess the variability of unique parts of the structure, particularly those involving regions of the IFN molecules that were expected to be in contact with the receptor. Analysis of the final structures