Interferon-gamma Induced 5' Alternative Splicing of Tryptophanyl-tRNA Synthetase (WaRS)

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May 17, 2006

Abstract

Interferons (IFNs) are a vital part of the non-specific immune system and lead to the induction of an "antiviral state" in cells, which is a crucial for activating cell based defenses. IFN- γ has many downstream targets, one of which is tryptophanyl-tRNA synthetase (WaRS), an essential enzyme that covalently links typtophan to its cognate tRNAs. The induction of WaRS following treatment of HeLa cells with IFN-γ was examined by RT-PCR as was the specific 5' alternative splice variants produced. IFN-γ caused a two fold induction of WaRS mRNA.

Introduction

Viruses are small obligate parasites that invade the body, and use the host's machinery to replicate and invade other cells. In response to a virus, the cell stimulates production of interferons (IFNs) which bind to high affinity cell surface receptors and activate the expression of downstream target genes (Lewis et al. 1989). IFNs are part of the non-specific immune system and lead to the induction of an "antiviral state" in cells, which is characterized by inhibition of both viral replication and cell proliferation, as well as the increased ability of natural killer cells to lyse infected cells (Pottathil et al. 1980). The first two interferons identified were IFN-α and IFN-β, which encompasses a family of structurally related cytokines called type I IFNs (Pestka et al. 1987). IFN-γ, the only type II IFN, initiates signaling by binding to a distinct receptor at the cell surface (Platanias 2005). IFN- γ is made by T-lymphocytes and natural killer cells, and provides the specific signaling that activates cell based defenses. IFN-γ triggers production of major histocompatibility complex molecules and changes the composition of

proteosomes, so abnormal proteins can be targeted for ubiquitination (Goodsell 2001). Induction of nitric oxide by macrophages via the interferon signaling pathway ensures that the amplified amount of protein aggregations does not lead to apoptosis (Goodsell 2001).

One of the downstream genes induced by IFN-γ is tryptophanyl-tRNA synthetase (WaRS). The aminoacyl-tRNA synthetases (ARS) are essential enzymes that covalently link amino acids to their cognate tRNAs (Schimmel 1987). The 20 ARS are divided into two groups of ten each; class I enzymes share a common active site architecture which includes a specific type of folded nucleotide-binding domain while class II enzymes are made up of a seven-stranded antiparallel β-sheet surrounded by α-helices. In reactions catalyzed by the class I ARS, the aminoacyl group is coupled to the 2'-hydroxyl of the tRNA. This differs from class II reactions where the 3'-hydroxyl sites are used (Cusack et al. 1990). These two variants in structure for class I and class II synthetases are thought to aid in specificity and sequence adaptions, and might contribute to the additional cellular functions that these enzymes catalyze (Ruff et al. 1991). Alternative splicing of aminoacyl tRNA synthetases occurs in the lysyl-tRNA synthetase (Targoff et al.1993), tyrosyl-tRNA synthetase (Wakasugi et al. 2002), isoleucyl-tRNA synthetase (Nichols et al. 1995), cysteinyl-tRNA synthetase (Kim et al. 2000), and tryptophanyl-tRNA synthetase (Wakasugi and Schimmel 1999). The alternative splice variants might be responsible for the housekeeping functions of the ARS (Schimmel 1987). The fragments of WaRS have anti-angiogenic activity, which provides a biological rationale for their production (Otani et al. 2002). Here I examine the induction of WaRS in response to interferon-γ and the alternative splice variants produced.

Materials and Methods

Cell Culture: HeLa cells were grown in MEM with 10% fetal calf serum, 10U/ml of penicillin and 10μg/ml of streptomycin at 37°C with 5% CO_2 and humidity. Cells were treated with 500U of IFN-γ (Sigma-Aldrich, Inc) for 8 hours (product number-I 1520).

RNA Isolation: Total RNA was isolated from HeLa cells using RNAqueous Phenol-Free Total RNA isolation kit according to manufacturer's protocol.

Primer Design: Primers were designed to specifically amplify the 5' mRNA segment of the human tryptophanyl-tRNA synthetase based on the GenBank accession sequence (number BC095453). The WaRS primers designed examined the known alternative splice sites in exons 1-3, the sequences are as follows; Forward-exon1a 5'-AGCTCAACTGCCCAGCGTGACC -3' (position 128-150), Forward-exon1b 5'-GGAGTAGGCAGTTTTGCTC -3' (position 202-221), Reverse-exon1b 5'-CAGTCAGCCTTGTAATCCTC -3' (position 398-416), and Reverse-exon3 5'-GAGGCTGAGATGCCAAAAG -3' (position 746-765). The segments of mRNA amplified from primers Forward –exon 1a and Reverse-exon3 were predicted to be 319bp, 296bp and 182 bp, respectively. This is primer set A. The segment amplified from Forward-exon1b to Reverse-exon1b is 214bp, and is designated as primer set B. Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as an internal loading control. The segment produced from the GAPDH forward and reverse primers was expected to be 225bp.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR): cDNA was generated from 20 ηg of RNA isolated from HeLa cell line by RT-PCR using the primers described

above and the Qiagen 1-Step RT-PCR kit. The reaction mixture contained 3µl 5X buffer, 0.6 µl DNTPs, 0.6µl of enzyme mix, 1µl of RNA template (with a concentration of 20 ηg/µl) and ddH_2O was added until the final reaction volume was 15 µl. In order to prevent evaporation, 12µl of mineral oil was added to the RT-PCR tubes. The amplifications for WaRS primers' were performed by the following method; initial reverse trancriptase reaction (50°C for 30 mins and 95°C for 15 mins), 49 cycles of denaturation, annealing and extension (94°C for 20 s, 60°C for 30 s and 72°C for 30 s), and final extension 72°C for 2 mins and cooled to 4°C. The GAPDH primers had the same denaturation, annealing and extension times, except they were run for 25 cycles. The resulting cDNA was visualized by UV light on a 1% agarose gel containing ethidium bromide.

PCR Purification: The Rapid PCR Purification kit (Marligen Biosciences, Inc.) was used to purify the RT-PCR product as per the manufacture's instructions. Briefly, 400µl of Binding Solution H1 was added to the RT-PCR products, the mixture was loaded onto a spin column, centrifuged at 12,000g for a minute, and the flow through was discarded. 700µl of Wash Buffer H2 containing ethanol was added, and centrifuged at 12,000g for a minute, and the flow through was discarded. In order to remove all residual wash buffers, this process was repeated. To elute the cDNA, 26µl of 65°C dH_20 was added to the center of the spin column, incubated at room temperature for one minute, and then centrifuged at 12,000g for two minutes.

PCR Purification from gel slices: The Rapid Gel Extraction kit (Marligen Biosciences, Inc.) was used to purify the DNA bands that were excised from an agarose gel as per the manufacture's instructions. However, to elute the cDNA, 23µl of 65°C dH_20, not TE

buffer, was added to the center of the spin column, incubated at room temperature for one minute, and then centrifuged at 12,000g for two minutes.

Sanger Sequencing: cDNA sequencing was achieved using the Sanger dideoxy method using radiolabeled ATP. Briefly, a master mix containing multiplicities of 4ml of 10X cycling buffer, .5µl of ^{33}P-α ATP, 50fm of cDNA were added to a tube and the reaction was brought up to 28µl with 33.13µl ddH_2O. 28µl of this master mix was allocated out into tubes, and 2µl of 10pmol primer was added to each tube. 6µl of this reaction was pipetted out to 4 tubes, and 2µl of either dideoxy GTP, ATP, CTP or TTP was added. In order to prevent evaporation, 12µl of mineral oil was added to the reactions. The amplifications were performed by initial denaturation (94°C for 3 min), 25 cycles of denaturation, annealing and extension (94°C for 30 s, 58°C for 30 s and 72°C for 1 min), and cooled to 4°C. Following the amplification, 4µl of stop solution was added to the tubes, and the reaction was heated to 94°C for three minutes to denature the products. This product was analyzed on a polyacrylamide gel, and the subsequent nucleotide sequence was visualized by autoradiography.

Sequence alignment: The partial sequence of WaRS obtained from the sequencing gel was aligned using BLAST with the NCBI sequence BC095453.

Results

Alternative splice sites for the WaRS gene

The WaRS gene has 3 alternative products for 5' splicing events. Figure 1 demonstates the three permutations and their relative sizes.

A A

B B

Fig 1. Primer set A produces three different transcripts; with all the exons included, the transcript would be 319bp, with exon 1b missing it would be 296bp and with exon 2 sliced out the transcript would be182 bp. Primer set B produces a 215bp product.

Increased Expression of WaRS transcripts in HeLa cells treated with IFN-γ

cDNA was generated from both treated and untreated HeLa cells, and subsequently analyzed by RT-PCR using a combination of primers as described in the Methods and Materials section. As noted by others (Jorgensen et al. 2000, Shaw et al. 1994 and Wakasugi et al. 2002), treatment with IFN-γ lead to an increase in the amount of WaRS mRNA produced (Fig2). However, similar to Liu et al (2004), even though there was an induction in the transcript produced with treated cells, the relative amounts of each transcript increased uniformly. Alternative splicing due to IFN-γ induction was not observed. The similar levels of GAPDH indicate that the result is due to augmented amounts of WaRS RNA and not due to a loading error.

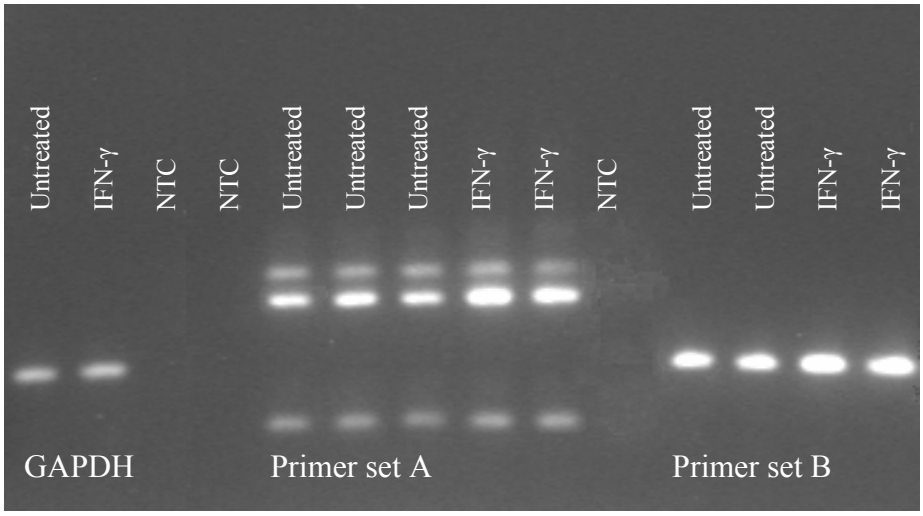

Fig 2. RT–PCR analysis of IFN-γ up-regulated WaRS in HeLa cells. Cells were treated with or without IFN-γ (500 U/ml) for 8 hours. The reaction was done in duplicate or triplicate to avoid errors, to increase product for sequencing and to ensure reproducibility. Total RNAs were isolated and converted to cDNA using RT-PCR as described in Materials and Methods and analyzed by 1% agarose gel electrophoresis. Under these conditions, the differences between the levels of WaRS mRNA could be seen. GAPDH is an internal control.

Sequencing Data:

The sequences of the products obtained from RT-PCR analysis were used to confirm that the data and the primers were specific. Of the three products generated using primers 1a and 3, all showed strong homology to the Genbank sequence. Figure 4a shows the 319bp product, figure 4b shows the 296bp product and figure 4c shows the 182bp product. Each sequence contained data that was specific for its respective exon combinations, with a homology of 96%, 97% and 98%, respectively. PCR product from primers for exon 1b (primer set B) shows 92% homology to the WaRS gene in the location it was meant to amplify (fig4d).

Figure 3a
Identities = 96%

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CCTCATCCCAATACAATACCCAGTTTCTTTAAGTGCCTTATTATGCTCCTCCACCGCAGT
||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||  |
CCTCATCCCAATACAATACCCAGTTTCTTTAAGTGCCTGATTATGCTCCTCCACCGCGAT

GGAAACTTTCAGAATGAGCACAAGATTCAAGTTCTTCAA
|||||||||||||| |||||||||||||||||||||| ||
GGAAACTTTCAGAAAGAGCACAAGATTCAAGTTCTTGAA
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Figure 3b
Identities = 97%

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GGATCCAGAAACACTCCTGGGCCACATTC-CTTCTCAGCTGCTCCAGAATTGGC-TGTCT
|||||||||||||||||||||||||||||| ||||||||||||||||||||||| |||||
GGATCCAGAAACACTCCTGGGCCACATTCTCTTCTCAGCTGCTCCAGAATTGGCCTGTCT

TGATGCCACCCTTCCCCAGATTGCAGAATCA-GCGTGTCCTGATCTCTGTGAGATGATTT
| ||||||||    ||||||| ||||| ||| | ||||||||||||||||||||||||||
T-ATGCCACCT---CCCAGAT-GCAGA-TCACGTGTGTCCTGATCTCTGTGAGATGATTT

TGATGCAACCCAAGTCTTTATCA
|||||||||||||||||||||||
GATGCAACCCAAGTCTTTATCAT
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Figure 3c
Identities = 98%

Figure 3d
Identities = 92%

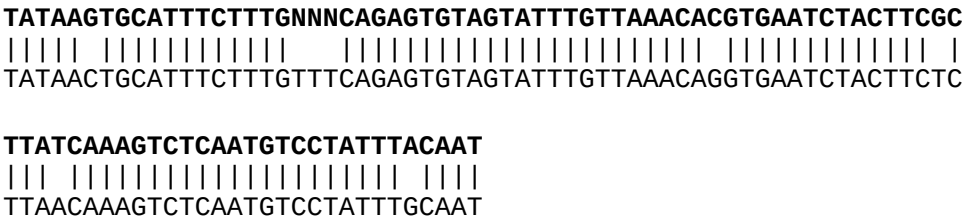

Fig 3. Alignment of sequencing data using BLAST. 319bp (fig 3a), 296bp (fig 3b), and 182 (fig 3c) product produced from primer set A, Forward –exon 1a and Reverse-exon3. Product produced from primer set B (fig 3d), Forward-exon1b to Reverse-exon1b. The data I generated is in bold in comparison to the GenBank accession sequence (number BC095453) that is underneath it.

Discussion

Alternative splicing enables the cell to use the same gene to target different proteins, have greater stability, or signal different pathways (Kim et al. 2000). The full length mRNA for WaRS is critical in charging tryptophans and adding them onto tRNAs (Liu et al 2004). My results show that IFN-γ is a strong stimulator of tryptophanyl-tRNA synthetase. I expected to see an increase in the specific type of alternative splice variant produced, however my results show that after treatment, the proportion of mRNA variants increase uniformly. Instead of a specific variant being up regulated, there is a twofold increase in all the mRNA variants. Contrary to this study, others (Jorgensen et al.

2000, Shaw et al. 1994 and Wakasugi et al. 2002) have shown that the relative amount of each variant is capricious, and it was problematic to quantify the splice variants produced. The quantification may have been difficult if the splice variants for the mRNA was only slightly up regulated, or if the fidelity of Taq polymerase was not uniform across all samples. The differences between the variants was difficult to quantify, however, further work using Real Time PCR should help determine if there is any diversity in the splice variants produced.

The truncated variants produced are cell and situation specific, and have other functions; the shortest transcript has been shown to be essential for activation of angiogenesis activity (Wakasugi et al. 2002), allowing increased blood flow. Due to IFN-γ ability to stimulate angiogenic cytokines, cleavage of WaRS transcripts might be a type of signaling mechanism. Others (Shaw et al. 1994, Wakasugi et al. 2002) have shown that the shortest 182bp mRNA is specifically responsible for the production of angiogenic cytokines. There is no known role for the 296bp size transcript produced, and further work focused on determining its own function should prove valuable for understanding the role of alternative splicing of WaRS. Further work is needed to determine whether the 182bp truncated transcript with exon 2 missing acts in the same signaling mechanism as other factors responsible for angiogenesis.

Acknowledgments

I would like to thank Jinsong Qiu for his long hours, infinite amounts of wisdom and patience. I would also like to thank Lisa Sarran for her help, Dr. Rubin for his advice, Dr. Henriksen for her assistance through a few sticky situations and my classmates for all their entertaining virtues.

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