

The activation-induced expression of DHX32 in Jurkat T cells is specific and involves calcium and nuclear factor of activated T cells

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Abstract

In this report, we studied DHX32 expression in human Jurkat T cells. Co-stimulation of CD3 and CD28 resulted in upregulation of DHX32. No significant changes in the expression of the closely related RNA helicases were seen. Ionomycin treatment alone was sufficient to upregulate the expression of DHX32 mRNA isoform transcribed from the proximal promoter. We cloned DHX32 proximal promoter and identified a 218 bp fragment containing two potential binding sites for the transcription factor nuclear factor of activated T cells (NF-AT). Mutation of core sequence of NF-AT resulted in reduced transcriptional activity, with more reduction observed in the second NF-AT site. Electrophoretic mobility shift assay results were consistent with a specific binding of NF-AT from ionomycin stimulated nuclear extract of Jurkat cells to oligonucleotide probes from DHX32 proximal promoter. These results suggest that the DHX32 expression is modulated in Jurkat T cells via a pathway that involves NF-AT.

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1. Introduction

T cells are major components of the immune system with important regulatory [1] and effector functions [2]. Two signals are required for optimal T cell activation during the immune response, ligation of T cell receptor (TCR) and ligation of co-stimulatory molecules such as CD28 [3]. Activation of T cells leads to signal transduction via two pathways mediated by protein kinase C (PKC) [4] and increases in intracellular Ca^{2+} [5]. These signals can be mimicked through antibody (Ab)-mediated ligation of the CD3 chain of the TCR and CD28 or through treatment of T cells with phorbol myristate acetate (PMA) to activate PKC and calcium ionophores such as ionomycin. Upon T cell activation, nuclear factors of activated T cells (NF-AT) are dephosphorylated by the calcium-dependent protein phosphatase calcineurin, leading to their translocation to the

nucleus where they pair with AP1 and bind to consensus sequences in the promoters of several genes [6].

Several T cell response genes undergo processing at the RNA level including alternative splicing [7]. However, the factors involved in RNA processing in T cells are not fully characterized. Since RNA molecules tend to form secondary structures and interact with other RNA molecules, certain enzymes, called RNA helicases, are required to modulate RNA structure and interactions [8]. RNA helicases utilize energy derived from adenosine triphosphate (ATP) hydrolysis to unwind RNA–RNA, and in some cases disrupt RNA–protein, interactions [9,10]. To achieve this, RNA helicases have the “helicase domain,” consisting of several motifs which contain the amino acids required for ATP binding and hydrolysis and RNA binding and unwinding. Sequence variations within the motifs constituting the helicase domain are used to classify RNA helicases [11]. We identified a novel putative RNA helicase gene DDX32 based on its down regulation in acute lymphoblastic leukemia [12]. The gene designation was changed to DHX32 according to the revised nomenclature of human

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RNA helicases to reflect homology to the DHX family [13]. DHX32 expression in normal lymphoid tissues is variable and appears to correlate with their differentiation and activation status [14]. We have shown that the expression of DHX32 in T cell precursors is related to their differentiation [15]. We carried out this study to determine the activation signals and the pathways involved in regulating the expression of *DHX32* in Jurkat T cells.

2. Materials and methods

2.1. Cell lines, reagents, and culture conditions

Jurkat T cells were obtained from ATCC and maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Antibodies against CD3 (clone HIT3a), CD28 (clone CD28.2), and NF-ATc1 (clone 7A6) were obtained from Pharmingen/BD Bioscience (Oakville, Ontario, Canada). PMA and ionomycin were obtained from Sigma (Oakville, Ontario, Canada). Jurkat cells were grown at a concentration of $0.5\text{--}1 \times 10^6/\text{ml}$ in complete RPMI in tissue culture plates pre-coated with either anti-CD3 alone or a combination of anti-CD3 and anti-CD28 or control Ab ($10\text{ }\mu\text{g}/\text{ml}$ of each Ab in PBS for 5 h). PMA and ionomycin were used at a concentration of $50\text{ ng}/\text{ml}$ and 100 nM , respectively, for the specified times, unless otherwise indicated.

2.2. Reverse transcription-PCR

The expression of DHX32 was determined by reverse transcription (RT) of total RNA as follows: primers for exon 2 transcript: forward: 5'-CGG AAC CCA CCA AAC TTT AAA CAC CAG CCT-3' (position 57–86 of NM_018180) and reverse: 5'-CCA TCG CTG GAA TCC AGG GAT TCA GGA AAA-3' (position 542–571 of NM_018180) 514 bp product. Primers for exon 1 transcript: forward primer 5'-GTG CCC CGA CGT CGC CGA C-3' (position 111–129 of BC068471), reverse primer 5'-GCT GGT AGT GGA TGG AAA G-3' (position 798–816 of BC06471), product is 687 bp. Primers for DHX8: forward 5'-TCG CAA GCA GAT GTT AGG CAT-3', reverse: 5'-ACG GAA GAA CCC ACT GCA GAT-3', DHX15: forward 5'-GCG AAA CAG AAG GTC TAC-3' and reverse 5'-CTC ATC TGC GGC TTT CTT-3', DHX16: forward 5'-CTG CAC ATC AGA GCG AAC TGT C-3', reverse: 5'-ATC CAC CAT CAC CAC GCT GTA-3', DHX34 forward 5'-GAC CTT CTT TGA ACG CCT-5', reverse 5'-CGA ACG TGT GCT CTC AAA-3', DHX35 forward: 5'-TCT GGC TGA AAA CTC TGG-3', reverse 5'-TGC AGT CAT CAA AGC GGA-3'.

Primers for actin: forward 5'-TGG ACA TCC GCA AAG ACC TGT ACG C-3', and reverse 5'-TGT CAC CTT CAC CGT TCC AGTT-3'. The PCR conditions were as follows: an initial 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 48°C for 30 s, and 72°C for 3 min, and a final 72°C for 10 min.

2.3. DHX32 proximal promoter constructs

Two primers were designed to clone 1325 bp segment of the genomic DNA sequence of DHX32 proximal promoter from -1321 to $+4$ with $+1$ as the translational start site based on GenBank entries GI:15391100 and GI:20336299 with following sequence: forward (-1321) 5'-ATG ATG GGT ACC CCC TTC ACC TGA CAT GTG-3' and reverse ($+4$) 5'-CAT CAT GCT AGC CAT CTT GTC TGA CAG TG-3'. The following primers were designed to clone various regions of this segment: Forward (-816) 5'-ATG ATG GGT ACC GCC AAC CAT AAC TAC AT-3', Forward (-410) 5'-ATG ATG GGT ACC AGC CTA AAT TAT TCC TG-3', forward (-197) 5'-ATG ATG GGT ACC AGC ACA GTG GGT AAC AC-3', reverse (-599) 5'-CAT CAT GCT AGC GTG AAA GAG GAG CTG GAC CT-3'. All forward primers have one *KpnI* restriction site and all reverse primers have one *NheI* restriction site (underlined). In addition, primers were designed to clone segment containing either the wild type or a mutated NF-AT sites with a two-nucleotide mutation in the GGAAA core sequence of NF-AT by the overlap extension procedure as previously described by Vallejo et al. [16] (TC instead of GG, shown in bold face) as follows: NF-AT1 (-805) ATG ATG GGT ACC CTA CAT ATG GAA **GGA** AAT AAT AAA TTC and Mutated NF-AT1 forward (-805) 5'-ATG ATG GGT ACC CTA CAT ATG GAA **TCA** AAT AAT AAA TTC-3'. NF-AT2 5'-ATG ATG GGT ACC ACA TAA GCA AAA **GGA** AAA TGA AAA GTT-3' and mutated NF-AT2 forward (-751) 5'-ATG ATG GGT ACC ACA TAA GCA AAA **TCA** AAA TGA AAA GTT-3'. The PCR products were cloned into the pGL3-basic vector at the *KpnI* and *NheI* sites (Promega, Madison, WI). The integrity of all constructs was confirmed by sequencing.

2.4. Transient transfections and luciferase assay

Transfection was achieved by using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Briefly, 2×10^5 Jurkat cells in triplicate were resuspended in RPMI 1640 with $0.8\text{ }\mu\text{g}$ of various pGL3 constructs. Transfection efficiencies were normalized by co-transfecting 80 ng of a pCMV- β reporter construct followed by quantification of the β -galactosidase expression using the expression kit as per manufacturer's instructions (Promega, Maddison, WI). Twenty-four to forty-eight hours after transfection, cells were harvested, washed three times with PBS, lysed, and centrifuged to remove cellular debris. The protein concentrations were determined by Bradford and equal amounts of the supernatant proteins were analyzed for luciferase activity using assay kits available from Promega according to manufacturer's instructions. Measurements of luciferase relative light units were performed by using Berthold LB9507 Lumat Single Tube Luminometer (Fisher Scientific, Ottawa, Canada). The inhibitory effect was not due to nonspecific cellular toxicity since all cells were equally viable (as determined by trypan blue exclusion).

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