Molecular Immunology 48 (2011) 539–545

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Studies of the *H60a* locus in C57BL/6 and 129/Sv mouse strains identify the *H60a* 3′UTR as a regulator of H60a expression

Hui Zhang, Chanae Hardamon, Bright Sagoe, Jennifer Ngolab, Jack D. Bui*

Department of Pathology, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093, United States

ARTICLE INFO

Article history:
Received 26 June 2010
Received in revised form 1 October 2010
Accepted 22 October 2010
Available online 19 November 2010

Keywords:
NKG2D
H60
Natural killer cells
Interferon
microRNA

ABSTRACT

The minor histocompatibility antigen 60 (H60a) is expressed in BALB/C and 129/Sv but not in C57BL/6 strains of mice. We recently found that IFNγ down-regulates H60a, but the mechanism of regulation is not known. To better understand the regulation of H60a, we examined the genomic locus of *H60a* in 129/Sv and C57BL/6 strains. We found that the upstream regulatory region of *H60a* was present and functional in both strains. Interestingly, IFNγ can down-regulate *H60a* transcripts in cell lines from 129/Sv but not C57BL/6 strains of mice, suggesting that IFNγ-dependent regulation of H60a proceeds through cis elements other than the conserved promoter region. We determined that the regulation of H60a by IFNγ proceeds through the 3′UTR of *H60a*, which is present in 129/Sv, but not C57BL/6 cells. We also found that the *H60a* 3′UTR and microRNAs can contribute to the level of constitutive expression of H60a in tumor cell lines. We conclude that in 129/Sv strain mice, H60a can be regulated by its 3′UTR through IFNγ and unknown microRNAs. Since H60a mediates NK cell target recognition, our studies identify a cis element that can regulate virus and tumor surveillance.

1. Introduction

Natural Killer Group 2D (NKG2D) is a receptor expressed on NK cells that mediates the detection of stressed cells that are infected by viruses or undergoing transformation (Cerwenka and Lanier, 2001; Raulet, 2003). It recognizes a ligand family that is heterogeneous and generally not expressed at functional levels in normal tissues, but can be up-regulated by certain stimuli, including DNA damage and viral infection (Gasser et al., 2005; Yokoyama, 2000). H60a is an NKG2D ligand that was originally identified as a minor histocompatibility antigen between BALB/b and C57BL/6 mice (Malarkannan et al., 1998). It is known to be expressed by hematopoietic cells and tumor cell lines from various strains, including BALB/C and 129/Sv (Bui et al., 2006a; Cerwenka et al., 2000; Diefenbach et al., 2000; Malarkannan et al., 1998), but is a pseudogene in C57BL/6 mice. The NKG2D ligands H60b and H60c were recently identified (Takada et al., 2008; Whang et al., 2009) and share 73% and 44% amino acid identity with H60a. H60b was shown to be expressed in BALB/C and C57BL/6 tissues, but its expression in the 129/Sv-strain has not been studied.

The expression of NKG2D ligands is regulated via complex pathways involving transcriptional, post-transcriptional, and post-translational mechanisms (Mistry and O'Callaghan, 2007; Nausch and Cerwenka, 2008; Yadav et al., 2009). Although these mechanisms have been described for many of the NKG2D ligands, surprisingly little is known about the signals that regulate H60a. For example, increased *MULT1*, *RAE*, and *MICA/B* transcripts can be found in cells undergoing DNA damage (Gasser et al., 2005), but it is not known whether H60a is induced by similar signals. Furthermore, virus infection seems to induce *RAE* (Lodoen et al., 2003) and *H60b* (Takada et al., 2008), but not *H60a*. Interestingly, the expression pattern of *H60a, b*, and *c* in normal cells is different, even though these molecules all can function as NKG2D ligands and activate NK cell recognition. *H60c* is expressed exclusively in keratinocytes (Whang et al., 2009), while *H60b* and *H60a* seem to be more broadly expressed (Takada et al., 2008).

The function of H60a as a recognition determinant for NK cells during tumor formation and viral infection is supported by multiple studies. *H60a* is induced during carcinogenesis (Girardi et al., 2001), and overexpression of H60a on tumor cells is sufficient to mediate tumor rejection by NK cells and prime adaptive immunity (Diefenbach et al., 2001). Nevertheless, little is known about other signals that induce its expression during carcinogenesis and maintains it on tumor cell lines. We have found that the cytokines IFNα/β and IFNγ potently down-regulate H60a on tumor cells, rendering them resistant to NK cell lysis (Bui et al., 2006a), but the mechanism by which IFNs regulate H60a in tumor cells is not known. In this study, we defined the *H60a* locus in the 129/Sv and C57BL/6 strains

Abbreviations: NKG2D, natural killer group 2D; NK, natural killer; H60a, histocompatibility antigen 60a; MULT1, murine ULBP-like transcript; RAE, retinoic acid early transcript; MICA/B, MHC class I-like chain A/B; IFN, interferon; UTR, untranslated region; qRT-PCR, quantitative real-time polymerase chain reaction; miRNA, microRNA.

* Corresponding author. Tel.: +1 858 534 3890; fax: +1 858 822 5580.
E-mail address: jbui@ucsd.edu (J.D. Bui).

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doi:10.1016/j.molimm.2010.10.015

and found that the promoter region of *H60a* is identical and functional in both strains. We further show that IFNγ regulates *H60a* transcripts via its 3′UTR. Inhibition of DICER led to enhanced 3′UTR activity and H60a protein expression, thus suggesting that a mouse NKG2D ligand is regulated by microRNAs (miRNAs).

2. Materials and methods

2.1. *Shot-gun sequencing of the H60a locus in 129S6/SvEv Tac strain*

We screened a bacterial artificial chromosome (BAC) library constructed from cells from a 129S6/SvEvTac mouse (http://bacpac.chori.org) for *H60a* sequences. Shot-gun sequencing (http://genome.wustl.edu/) of one clone generated a 44 kilobase (kB) contiguous sequence (contig) (accession number: HM590820). This sequence was aligned with publicly available sequence using a BLAST program in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2. Cell lines and stimulations

MCA-induced sarcomas were isolated and passaged *in vitro* as described (Bui et al., 2006b; Shankaran et al., 2001). Cell lines were maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, NEAA, sodium pyruvate, sodium bicarbonate, pen/strep, and β-mercaptoethanol. Recombinant murine IFNγ was obtained from eBioscience (San Diego, CA) and used at 100 U/ml.

2.3. *Real-time PCR*

RNA was generated using Trizol Reagent (Invitrogen, San Diego). cDNA was made using the Applied Biosystems (Foster City, CA) protocol. Real-time Taqman PCR reactions were performed using the following primers: H60a for, 5′GAG CCA CCA GCA AGA GCA A; H60a rev, 5′CCA GTA TGG TCC CCA GAT AGC T; H60a probe VIC-5′TTG CCT GAT TCT GAG CCT TTT CAT TCT GCT-TAMRA (Bui et al., 2006a); GAPDH for, 5′CTT AGC ACC CCT GGC CAA G; GAPDH rev, 5′TGG TCA TGA GTC CTT CCA CG; GAPDH probe, VIC-5′CAT CCA TGA CCA CCC CTG GCC AAG-MGB (Buchau et al., 2010). Sybr green reactions were performed with the following primers: RAE1 for, 5′ATC AAC TTC CCC GCT TCC A; RAE1 rev, 5′AGA TAT GAA GAT GAG TCC CAC AGA GAT A (Bui et al., 2006a); H60b for, 5′AGC CTT TTG GTC CTG CTG AAT; H60b rev, 5′ATG TTT TTT ATC ACC AAA ATC AAG GAG T (Takada et al., 2008); H60a exon 2–4 for, 5′CTG AGC TAT CTG GGG ACC AT; H60a exon 2–4 rev, 5′AGA TTG TGT TGT GAC ATT CAA GG. The RAE1 primer recognizes all *RAE1* family members. H60a exon 2–4 sybr primers detect *H60a* transcripts from 129/Sv but not C57BL/6 mice. H60a taqman primers detect *H60a* transcripts from both strains. All experiments use at least duplicate conditions and duplicate real-time wells and are done at least twice.

2.4. Luciferase assay

The *H60a* promoter region containing 527 bp of sequence upstream of the transcriptional start site and 501 bp of 5′UTR was amplified from a 129/SvEv BAC clone and ligated into a luciferase reporter plasmid (PGL3-basic, Promega) using pGLOW TOPO cloning protocol (Invitrogen) and the following primers: 5′AGA AGA AAA CCT GAG GGT GGG3′, 5′GGT CTT CCC TCA GAC CCT GCT3′. The *H60a* 3′UTR was amplified from a 129/SvEv BAC clone into a TOPO shuttle vector (Invitrogen) and subcloned into a reporter construct that contained both the firefly and renilla luciferase genes downstream of independent promoters (OmicsLink, Genecopoeia, Rockville, MD) using the following primers: 5′ TCA TGT GAC TCT TCA AC3′, 5′TAT GCA AGC TTA GTT C. The *H60a* 3′UTR was inserted downstream of the firefly luciferase gene. Reporter activity was measured by dividing firefly luciferase light values by *Renilla* luciferase light values using a dual luciferase assay (Promega). Transfection was done through Lipofectamine 2000 (Invitrogen) in triplicate wells in a 96-well plate using four different DNA to cell ratios. Cells were left unstimulated or stimulated with IFNγ the next day, and activity was measured after 24 h of IFNγ stimulation. In some experiments, siRNA control or siRNA specific for DICER were used (Dharmacon). All experiments were done at least twice.

2.5. Antibodies and FACS analysis

All cell stainings were done with 50–80% confluent cells. Cells were harvest with dPBS or HBSS supplemented with 2.5 mM EDTA. Trypsin was not used since it decreased H60a staining (unpublished observations, J.D.B.). Monoclonal antibodies to H60a were obtained from R&D (Minneapolis, MN). Secondary antibodies were obtained from Biolegend (San Diego, CA). Staining was conducted for 15–30 min at 4 °C in FACS tubes containing 0.5–2 million total cells, 0.5–1 μl of antibody, and 100 μl of FACS buffer (PBS + 1% FCS + 0.09% NaN_3, Sigma). All analyses were done on live cells identified by forward and side scatter properties. All experiments were done at least twice.

3. Results

3.1. Alignment of H60a and H60b transcripts with 129/Sv genomic sequence

Most mouse genomic data is derived from the C57BL/6 strain, which does not have the intact *H60a* gene (Malarkannan et al., 1998; Samarakoon et al., 2009). To examine the *H60a* genomic locus, we screened a 129/Sv-strain BAC library and sequenced a putative clone that contains the *H60a* gene. We generated a 44 kb contig of the *H60a* genomic locus via this approach (accession number: HM590820). This sequence was aligned with transcripts of *H60a* (NM_010400.2) and *H60b* (NM_001177775) (Fig. 1). We found that when the *H60a* transcript was aligned to the 129/Sv genomic sequence, there were regions of 100% identity that corresponded to the six exons of *H60a* (Takada et al., 2008), thus confirming the accuracy of our sequence. In contrast, the *H60b* transcript did not align with 100% identity to our contig. There was 92–95% sequence identity between exons 3, 4, and 5 of *H60a* and *H60b*, but not between exons 1, 2, or 6. These results are concordant with previous studies showing high homology in the alpha 1, alpha 2, and transmembrane regions of the H60a and H60b proteins (Samarakoon et al., 2009; Takada et al., 2008) and further substantiates our approach. We conclude that our 129/Sv contig contains the entire *H60a* gene, including 30 kb of upstream sequence and 5 kb of downstream sequence, but does not contain any region of the *H60b* coding sequence.

3.2. Alignment of H60a and H60b transcripts with C57BL/6 genomic sequence

Next, we wished to elucidate the structure of the *H60a* locus in the C57BL/6 genome, where it is a pseudogene. Fig. 2 shows that when the *H60a* transcript was aligned to C57BL/6 genomic sequences from chromosome 10 (NCBI Build 37.1), there was partial alignment of the first 2 exons and part of the 3′UTR; however, the C57BL/6 genome did not contain exons 3, 4, and 5 of *H60a*. In fact, when these exons were used to BLAST the C57BL/6 genome, they corresponded to exons 3, 4, and 5 of *H60b* with >90% identity. Interestingly, we found that the *H60b* gene locus lies approximately 20 kb upstream of the *H60a* putative transcriptional start site in the C57BL/6 strain (Fig. 2), while the corresponding region in the