

Identification of N-acetylglucosaminyltransferase-IV as a modifier of Epstein-Barr virus BZLF1 activity

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ABSTRACT

Epstein-Barr virus is a prevalent human herpesvirus, with about 95% of the world's adult population positive for anti-EBV antigen antibodies. After the initial infection and production of new virus particles, the virus may enter a latent state within a subset of cells, and therefore can remain within the host indefinitely. Epstein-Barr virus contributes to a variety of diseases, including many types of cancers. We have created a model system in *Drosophila melanogaster* to study the effect of expression of the Epstein-Barr virus protein BZLF1, and to identify cellular proteins that mediate BZLF1 activity. Here we present the results of a genetic screen that determined that the *Drosophila melanogaster* CG9384 gene (an N-acetylglucosaminyltransferase) is a significant modulator of BZLF1 activity and EBV early lytic replication.

Keywords: Epstein-Barr Virus; BZLF1; *Drosophila*; CG9384; Gnt-IVb

1. INTRODUCTION

Epstein-Barr virus (EBV) is a widespread human herpesvirus, as nearly 95% of the world's human adult population are positive for anti-EBV antigen antibodies [1]. EBV was discovered in 1957 by Denis Burkitt and Anthony Epstein in tumor samples from Burkitt's lymphoma [2]. Since then, EBV has been found in association with many cancers, including nasopharyngeal carcinoma, breast cancer, gastric carcinoma, as well as many types of lymphomas, including post-transplant lymphoproliferative disease, Hodgkin's lymphoma, and T-cell lymphomas [1]. As EBV is so prevalent, and is associated with such a variety of cancers, it is important to understand how the virus contributes to pathogenesis and carcinogenesis.

EBV replication is controlled by two different types of life cycles: lytic, which occurs upon infection of cells and leads to the production of infectious viral particles, and latent, which is the dormant phase in which the virus produces minimal viral proteins to maintain the viral

genomes within immortalized B cells. Lytic replication, which occurs in epithelial cells as well as in B cells (before latency ensues) is broken down into three distinct phases of viral protein production: immediate-early, early and late. The immediate-early (IE) genes, BZLF1 and BRLF1, act like switches to activate lytic replication. The IE proteins are transcription factors that turn on the EBV early genes (which act to replicate the viral genome). The late genes encode virion structural elements [2].

BZLF1 (Z) and BRLF1 (R) not only bind to and transactivate early gene promoters, but they also modulate the host's intracellular environment by interacting with and altering the activities of many cellular proteins. For example, Z has been shown to interact with the histone acetylase CBP, the transcription factors CREB and Pax5, and the promyelocytic leukemia protein PML [3-5]. Such interactions benefit viral replication, but can be detrimental to the cell.

We previously created a model genetic system to screen for and identify host proteins that interact with Z. We expressed Z in *Drosophila* eyes, via the *GMR* system (*Glass-mediated response*; *Glass* is an eye-specific transcription factor in *Drosophila*) [6], which produced a significant mutant phenotype [5]. We performed candidate gene genetic screens, which involved crossing our *GMR-Z* flies to flies with known mutations in specific genes (such as tumor suppressors), and found some very interesting interactions [5,7]. These interactions have proven to impact EBV lytic replication in human cells [5] (and unpublished observations).

Here, we have performed a random genetic screen by mutagenizing flies and crossing them to *GMR-Z* flies. We isolated several mutant fly lines that modified the *GMR-Z* mutant phenotype. One of these modifiers, *AJ101*, which enhanced the *GMR-Z* phenotype, was characterized and determined to be a homolog of human Gnt-IVb.

Gnt-IVb is an N-acetylglucosaminyltransferase, which participates in sugar-chain branch formation on proteins that move through the secretory pathway [8,9]. Interes-

tingly, human Gnt-IVb has been found to be misexpressed in several types of cancers, including pancreatic cancer, colorectal carcinoma, and renal cancer [8,10-13]. We found that overexpression of human Gnt-IVb in a human gastric carcinoma cell line enhanced EBV early lytic replication. Therefore, expression levels of Gnt-IVb impact EBV replication and this may indirectly promote carcinogenesis.

2. MATERIALS AND METHODS

2.1. Genetic Screen

iso e males (of a stock with isogenized third chromosomes marked with *ebony*) were placed in vials containing Whatman paper saturated with 25 mM ethylmethanesulfonate (EMS) in 1% dextrose for 12 hours. These males were crossed to virgin *weak GMR-Z* females. Male progeny were scored for modification of the *weak GMR-Z* phenotype.

2.2. Mapping

Recombination mapping: *AJ101* was mapped against the third chromosome P element stocks #13230, 14984, 13372, 14258, 13115, 13114, 15252, 16532, 12803, 13126, 12824, 12868, 12629, 12694, 12719 from the Bloomington Stock Center. The lowest level of recombination was found between *AJ101* and stock 13115 (at position 70A8). Deficiency mapping: *AJ101* was crossed to 18 overlapping deficiencies spanning the region 69A to 77B. All stocks were acquired from the Bloomington Stock Center. *AJ101* failed to complement the stock #3126 (with breakpoints 70D2-3; 71E4-5), as well as the smaller deficiency 8074 (70F4-71E1); all other deficiencies in the area complemented *AJ101*, narrowing the region of *AJ101* to 70F. The available mutant alleles for the genes within this region were tested, including *Trl* (stocks 12088, 13334), *CG9384* (stock 22582), *mop* (stock 15817), *bmm* (stocks 25926, 15828), *CG13472* (stocks 12790, 15978), *gnu* (stock 3321), and *CG17839* (stocks 19525, 22869). *AJ101* failed to complement the *CG9384* mutant allele only.

2.3. Determination of Heterozygous versus Homozygous *AJ101* Larvae

The *AJ101* chromosome was balanced over a GFP-marked balancer chromosome, creating the stock *AJ101/TM3 GFP Ser*. Adults of this stock were allowed to lay embryos on molasses plates, and the plates examined under a fluorescent dissecting microscope to separate GFP-expressing embryos (heterozygotes) and non-GFP embryos (homozygotes). Separated embryos were allowed to develop into third instar larvae.

2.4. Sequencing

Genomic DNA was isolated from homozygous *AJ101* larvae. Briefly, larvae were squished in 50 μ L of buffer SB (10 mM Tris pH 8.2, 1 mM EDTA pH 8.0, 25 mM NaCl, 200 μ g/ml proteinase K), incubated at 25°C for 30 min, then incubated at 95°C for 2 min 5 μ L of the lysate was added to PCR reactions (GoTaq, Promega) with three sets of primers from the *CG9384* gene sequence (set 1: 5' CAGCGCCTGCATTAGTCATA3', 5' GTTT-CGTTCT GTCCTCGTC3'; set 2: 5' GTAAGGGTGG-CAGTGCA AAT3', 5' TTTCTTTTCAGCGACGAACT-3'; set 3: 5' A GTCCAAGGTTTGTGCGACG3', 5' GC-TACCACGAC TGCATCTCA3'). DNA was isolated from an agarose gel with the GeneClean III kit (Q-BIO-gene), according to manufacturer's directions. The primers used for PCR were also used for sequencing the three overlapping gene segments.

2.5. Cell Lines and Transfection

AGS-BDneo (gift of Lindsey Hutt-Fletcher) is an EBV-positive gastric carcinoma cell line and was maintained in Ham's F12 medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, fungicide, and 500 μ g/ml G418. Cells were transfected with either vector alone or a Gnt-IVb-expressing vector (OriGene SC110257) with FuGene HD transfection reagent (Promega), as per the manufacturer's instructions. 24 hr. post-transfection, cells were either left uninduced, or were induced into lytic replication by the addition of 20 ng/ml TPA and 3 mM sodium butyrate.

2.6. Flow Cytometry

Cells were removed from their culture dishes, washed with PBS, and fixed with 60% acetone in PBS for 10 min. at 4°C. Cells were washed with PBS/0.5% BSA, and incubated in anti-BMRF1 antibody (Capricorn), diluted 1:200 in Incubation mix (0.3% BSA, 5% goat serum, 0.1% Triton X in PBS) for 1 hr. at room temperature. Cells were washed with PBS/0.5% BSA, and incubated in donkey-anti-mouse-DyLight 488 secondary antibody (Jackson ImmunoResearch), diluted 1:400 in Incubation mix, for 1 hr. at room temperature. Cells were washed with PBS/0.5% BSA and resuspended in PBS. Flow cytometry was performed with a Guava easyCyte flow cytometer.

3. RESULTS

3.1. A Random Genetic Screen in *Drosophila* Identified an Enhancer of the *weak GMR-Z* Phenotype

To find genetic modifiers of Z, *Drosophila iso e* male flies were mutagenized with 25 mM ethylmethanesul-

fonate (EMS) and crossed to *weak GMR-Z* females, as detailed in Material and Methods. Over 10,000 male progeny were examined for modification of the *weak GMR-Z* phenotype. One of the modifiers identified, which we named *AJ101*, yielded a significant enhancement of the *weak GMR-Z* phenotype (**Figure 1**). This enhancement included a flattening of the ommatidia, yielding a smoother eye cuticle (compare **Figures 1(D)** to **1(F)**), and a complete loss of eye pigment.

While *AJ101* heterozygotes had no mutant phenotype on their own, the *AJ101* mutation was found to be homozygous lethal (data not shown). *AJ101* homozygotes died at the third instar larval stage.

3.2. *AJ101* Maps to the Cytological Region 70F4 and to the *Drosophila* Gene *CG9384*

To identify the *Drosophila* gene which corresponded to the *AJ101* modifier, we performed recombination mapping. We initially determined that *AJ101* resided on the third chromosome, thus we mapped the location of *AJ101* against fifteen P element insertions along the third chromosome. This mapping indicated that *AJ101* mapped to a location near chromosomal region 70A8 (data not shown). We then crossed *AJ101* to eighteen overlapping deficiencies, spanning chromosomal regions 69A to 77B. The lethal *AJ101* mutation failed to complement one of these deficiencies, a deficiency with

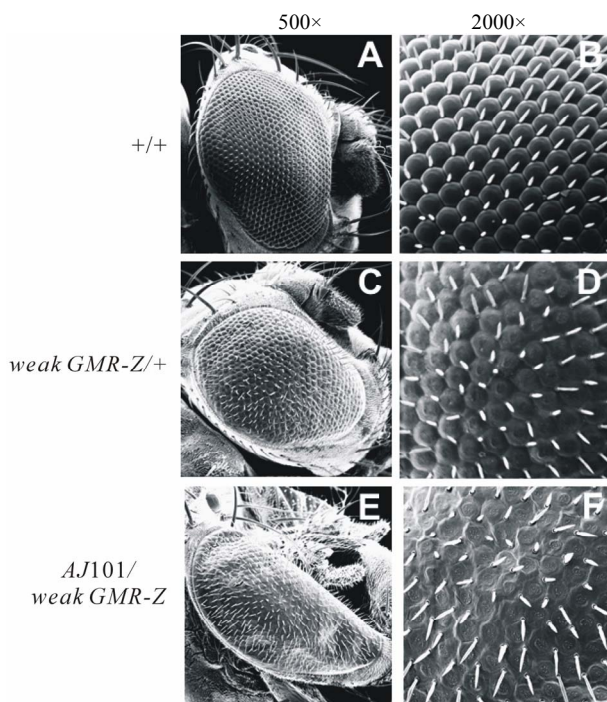


Figure 1. *AJ101* is a genetic modifier of *weak GMR-Z*. A, B: Wild-type. C, D: *weak GMR-Z* heterozygote. E, F: *AJ101/weak GMR-Z* trans-heterozygote. SEMs are presented at 500 \times (A), (C), (E) and 2000 \times (B), (D), (F).

breakpoints of 70D2-3; 71E4-5 (#3126; see **Figure 2**). Crosses with smaller deficiencies within these breakpoints narrowed the location of *AJ101* to 70F (including #8073, 6551, 8074, 8075, and 24945; **Figure 2**). Within this area were ten genes for consideration [14]. *AJ101* was crossed to available mutant alleles for these genes (**Figure 2**), and failed to complement a mutation in *CG9384*. Specifically, we crossed *AJ101* to the *CG9384* mutant fly line *CG9384^{EY22766}/TM3 Sb¹ Ser¹* (Bloomington stock number 22582). The mutation in this line is due to a P element insertion inserted within the gene, which disrupts the upstream untranslated region at the 5' end of the mRNA [14]. The resulting *AJ101/CG9384^{EY22766}* trans-heterozygote was lethal at third instar, just as the *AJ101* homozygotes were. As *AJ101* failed to complement this specific mutation, we confirmed the identity of *AJ101* as *CG9384*.

To identify the genomic alteration that caused the *AJ101* mutation, the genomic DNA corresponding to *CG9384* was PCR-amplified from *AJ101* homozygous larvae and sequenced. A nucleotide mutation was found that would cause a conversion of amino acid 512 from a serine to an asparagine within the *CG9384* protein.

3.3. *CG9384* Is a Homolog of the Human MGAT4B

CG9384 appears to have one spliced transcript, 1764 nucleotides in length, encoding a 587 amino acid long protein. The transcript contains a 105 nucleotide region that is 70% identical to the human mannosyl (alpha-1,3-) glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme B (also called MGAT4B) [14]. The *CG9384* protein is 46% identical (65% with conservative substitutions) across amino acids 120 - 567 to the MGAT4B protein, and contains an N-acetylglucosaminyltransferase-IV (GnT-IV) conserved region from amino acids 99 - 412.

N-acetylglucosaminyltransferases (GnTs) participate

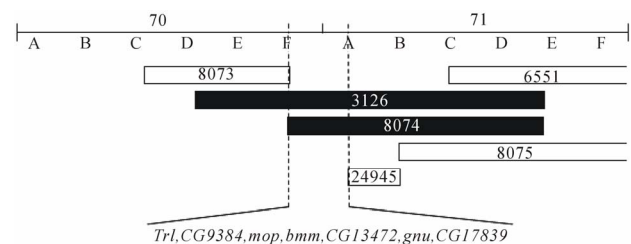


Figure 2. The *AJ101* mutation maps to the *CG9384* gene. Deletion mapping indicated that the *AJ101* mutation was located in the cytogenetic region of 70F. Of the genes in this region (between dotted lines) with available mutant alleles, *AJ101* failed to complement *CG9384*. White boxes represent deletions that complemented *AJ101*, black boxes represent deletions that were lethal in combination with *AJ101*. Regions shown may not be to scale.

in sugar-chain branch formation within the Golgi apparatus [8,9]. In vertebrates there are six GnTs, which are designated as GnT-I to -VI; these catalyze the transfer of GlcNAc to the core mannose residues of asparagine-linked sugar chains. GnT-IV specifically catalyzes the transfer of GlcNAc from UDP-GlcNAc to the GlcNAc 1 - 2 Man 1 - 3 arm of core oligosaccharide, and forms a GlcNAc 1 - 4 (GlcNAc 1 - 2) Man 1 - 3 structure on the core oligosaccharide [8].

3.4. Overexpression of Gnt-IVb Enhances EBV Early Lytic Replication in EBV-Positive Gastric Carcinoma Cells

To determine whether altered levels of human Gnt-IVb in a human cell line would impact EBV lytic replication, we overexpressed the human Gnt-IVb gene in AGS-BDneo cells (gastric carcinoma cells latently infected with EBV), and assessed the impact upon EBV early lytic replication. The EBV early protein BMRF1 is commonly used as an indicator of EBV early lytic replication (for example [15-17]). **Figure 3** shows that when cells overexpressed Gnt-IVb, and were subsequently induced into lytic replication, the cells displayed significantly higher levels of the EBV early lytic protein BMRF1, in a dose-dependent manner.

4. DISCUSSION

We performed a random genetic screen in *Drosophila* to find cellular modifiers of EBV Z activity, with the hope of finding novel interactors that would perhaps be unexpected (as opposed to, say, nuclear transcription factors).

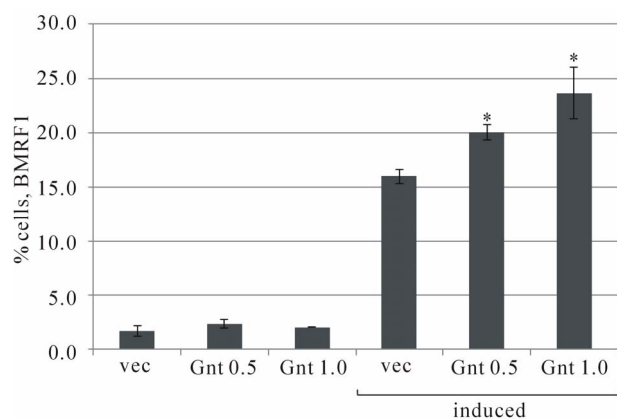


Figure 3. Overexpression of Gnt-IVb enhances EBV early lytic replication. Flow cytometry was performed on cells that overexpressed vector alone (vec), 0.5 μ g Gnt-IVb expression vector (Gnt 0.5), or 1.0 μ g Gnt-IVb expression vector (Gnt 1.0), either without or with induction into lytic replication (24 hr. post-transfection). Cells were immunostained for the EBV early protein BMRF1. The percent of BMRF1-positive cells is presented. The average of three separate experiments are presented. * = $p \leq 0.05$ relative to vec induced.

We identified a mutation, which we called *AJ101*, that enhanced the *GMR-Z* mutant phenotype, thus enhanced Z activity within epithelial cells. We successfully mapped the *AJ101* mutation to the third *Drosophila* chromosome at position 70F, and more specifically to the *Drosophila CG9384* gene.

The human homolog of *CG9384* is MGAT4B, also called Gnt-IVb. Gnt-IVb is a glycosyltransferase that participates in the formation of N-glycan branching structures on glycosylated proteins. Gnt-IV, localized to the Golgi, specifically contributes the 1-4GlcNAc branch to a mannose on the core structure [8]. Alteration of Gnt levels, either reduced or increased, have serious consequences upon a cell, as cell surface and secreted proteins, among others, are glycosylated. This glycosylation is important for proper localization (or secretion) and function of proteins. The alteration of Gnt-IV levels (both the a and b forms) has been linked to cancer and diabetes. Increased Gnt-IVb levels have been found in association with pancreatic and colorectal cancers [10-12], and decreased levels have been found in association with renal cell cancer [13]. Interestingly, Takamatsu *et al.* found that Gnt-IVb-deficient mice had an increase in Gnt-IVa expression (an apparent compensation mechanism), but still showed abnormalities to hemostasis, including a decreased number of neutrophils and increased lymphocyte cellularity [18].

In relation to EBV Z, which is a nuclear transcription factor that would not pass through the Golgi and therefore would not likely be exposed to Gnt-IVb, the effect of misexpression of Gnt-IVb must be an indirect one. However it is logical to assume that alterations to glycosylation of proteins (namely cell surface and secreted proteins) would alter those proteins' functions, and affect numerous signal transduction processes. Z has previously been shown to interact with signal transduction pathways, as well as transcription factors that are activated via signal transduction pathways [3,17] (and unpublished observations). Furthermore, EBV biology in general is dependent upon signal transduction pathways [19-22]. Therefore, alterations to the levels and/or functions of signaling molecules and cell surface receptors would likely affect Z activity and EBV replication in general, as well as affect host cell immune responses. Misexpression of glycosyltransferases, such as Gnt-IVb, has been associated with various cancers, and as an alteration of the *Drosophila* Gnt-IVb in Z-expressing tissues enhanced Z activity, it follows that misexpression of glycosyltransferases in tissues that are infected with EBV may lead to increases in EBV replication and perhaps to increases in EBV's contribution to carcinogenesis.

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