

necks in the antigen-processing pathway (1–4). In addition, the translation initiation mechanism for generating these peptides can use a leucine rather than a methionine residue. Understanding this mechanism should illuminate not only the expression of otherwise hidden antigenic peptides but also a growing list of non-AUG-initiated genes in the sequenced genomes.

References and Notes

- E. Pamer, P. Cresswell, *Annu. Rev. Immunol.* **16**, 323 (1998).
- K. Rock, A. Goldberg, *Annu. Rev. Immunol.* **17**, 739 (1999).
- N. Shastri, S. Schwab, T. Serwold, *Annu. Rev. Immunol.* **20**, 463 (2002).
- J. Yewdell, *Mol. Immunol.* **39**, 139 (2002).
- E. A. J. Reits, J. Vos, M. Gromme, J. Neefjes, *Nature* **404**, 774 (2000).
- U. Schubert *et al.*, *Nature* **404**, 770 (2000).
- T. Boon, A. Van Pel, *Immunogenetics* **29**, 75 (1989).
- A. Greenfield *et al.*, *Nature Genet.* **14**, 474 (1996).
- S. Malarkannan *et al.*, *J. Immunol.* **161**, 3501 (1998).
- Materials and methods and supporting material are available on Science Online.
- H. Pircher *et al.*, *EMBO J.* **8**, 719 (1989).
- J. Banachereau, R. Steinman, *Nature* **392**, 245 (1998).
- S. St-Jacques, U. Cymerman, N. Pece, M. Letarte, *Endocrinology* **134**, 2645 (1994).
- D. Scott *et al.*, *Immunity* **12**, 711 (2000).
- S. Malarkannan *et al.*, *Immunity* **13**, 333 (2000).
- E. Choi *et al.*, *Immunity* **17**, 593 (2002).
- U. Rajbhandary, C. Chow, in *tRNA: Structure, Biosynthesis, and Function*, D. Soll, U. Rajbhandary, Eds. (American Society for Microbiology, Washington, DC, 1995), p. 511.
- D. Peabody, *J. Biol. Chem.* **264**, 5031 (1989).
- M. Kozak, *Crit. Rev. Biochem. Mol. Biol.* **27**, 385 (1992).
- S. Malarkannan, T. Hornig, P. Shih, S. Schwab, N. Shastri, *Immunity* **10**, 681 (1999).
- We thank M. Botchan for virus-producing cells and T. Serwold for comments on the manuscript. Supported by grants to N.S. from the NIH.

Supporting Online Material

www.sciencemag.org/cgi/content/full/301/5638/1367/DC1

Materials and Methods
Figs. S1 to S3

14 April 2003; accepted 21 July 2003

Self-Inhibition of Synthesis and Antigen Presentation by Epstein-Barr Virus-Encoded EBNA1

Yili Yin,¹ Bénédicte Manoury,² Robin Fåhræus^{1*}

The glycine-alanine repeat domain (GAR) of Epstein-Barr virus-encoded nuclear antigen 1 (EBNA1) prevents major histocompatibility complex (MHC) class I-restricted presentation of EBNA1 epitopes to cytotoxic T cells. This effect has previously been attributed to the ability of GAR to inhibit its own proteasomal degradation. Here we show, both *in vitro* and *in vivo*, that GAR also inhibits messenger RNA translation of EBNA1 *in cis* and that this effect can be distinguished from its effect on proteasomal degradation. Hence, inhibition of messenger RNA translation, but not protein degradation, is essential to prevent antigen presentation on MHC class I molecules. Thus, by minimizing translation of the EBNA1 transcript, cells expressing EBNA1 avoid cytotoxic T cell recognition. At the same time, blocking degradation maintains the EBNA1 expression level.

Viruses have evolved numerous mechanisms to evade the host immune system (1, 2). A well-documented example is provided by EBNA1, which is expressed in all Epstein-Barr virus (EBV)-carrying cells and is essential for maintaining viral replication (3). In certain types of cells, such as Burkitt's lymphoma, expression of EBV antigens is restricted to EBNA1 only (4, 5). CD8⁺ cytotoxic T lymphocytes (CTLs) specific for EBNA1-derived peptides are generated via cross-priming and can readily be isolated from EBV-infected individuals (6–8). Although these CTLs can kill human leukocyte antigen-compatible cells loaded with exogenous EBNA1-derived peptides, they fail to recognize cells expressing endogenous full-length EBNA1 (8). This is a result of a failure to present antigens and can be corrected after deletion of the Gly-Ala repeat domain (GAR) of EBNA1 (8). Furthermore, fusion of GAR to an unrelated protein can

confer inhibition of presentation to CTLs (9). Previous studies have also shown that GAR increases the half-life of EBNA1 (and other proteins to which it is fused *in cis*) by inhibiting degradation via the proteasomal pathway. This self-inhibition of degradation has been proposed as the mechanism by which MHC class I presentation of EBNA1-derived peptides is inhibited (10, 11).

However, this model leaves questions concerning the mechanisms by which EBNA1-expressing cells avoid CD8⁺ T cell recognition unanswered. For example, an increase in the half-life conferred by GAR should result in a higher steady-state level of full-length EBNA1 than of EBNA1 with a deleted GAR. In fact, this is not observed and EBNA1 does not accumulate to a high level in EBV-infected B cells, nor in cells expressing exogenous EBNA1 (8, 10). Furthermore, by depending solely on inhibition of proteasomal degradation of full-length EBNA1 as a means of avoiding CTL recognition, GAR would not be capable of preventing presentation of peptides from the EBNA1 transcript that are derived through rapid degradation of so-called defective ribosomal products (DRiPs) (12, 13). Such DRiPs have been

estimated to make up at least 30% of newly synthesized mRNA translation products, and are thus considered a potentially important source of peptides for presentation by MHC class I molecules (14, 15). These questions prompted us to further investigate the role of the GAR sequence in preventing EBNA1 from producing antigenic peptides.

Contrary to expectations, the EBNA1 protein steady-state level was increased in cells that expressed an EBNA1 with a deleted GAR (EBΔGA) relative to cells expressing the wild type (EBNA1wt) (Fig. 1, A and B). Because GAR inhibits EBNA1 degradation but does not affect the level of EBNA1 mRNA (Fig. 1C), this result indicated that additional mechanisms might be regulating EBNA1 expression levels. Examination of *in vitro* transcribed EBNA1wt and EBΔGA mRNAs by *in vitro* translation (IVT) assays revealed that translation of EBNA1wt transcript was less efficient than that of EBΔGA by a factor of about 11 (Fig. 2A). Because the stability of GAR-carrying EBNA1wt mRNA was similar to that of EBΔGA both *in vitro* (Fig. 2B) and *in vivo* (Fig. 1C), we concluded that the presence of GAR inhibited translation of the EBNA1wt transcript. We then determined whether GAR could interfere with the translation of other transcripts (i.e., *in trans*) or if its effect is restricted to its own mRNA. Increasing amounts of EBNA1wt mRNA that were cotranslated in the presence of a fixed amount of EBΔGA mRNA did not influence EBΔGA translation; hence, the inhibitory effect of GAR is restricted to its own transcript by an effect mediated *in cis* (Fig. 2C).

The double-stranded RNA-activated kinase (PKR) phosphorylates the translation initiation factor eIF2α at Ser⁵¹; this is a well-known mechanism of regulating protein synthesis *in vitro* and *in vivo* (16). It was thus possible that addition of GAR RNA to the IVT lysates might activate PKR under these conditions. However, the presence of GAR RNA in rabbit reticulocyte lysates did not lead to a specific increase in phosphorylation of eIF2α, and treatment with the PKR inhibitor 2-aminopurine (2-

¹Division of Molecular Physiology, ²Division of Cell Biology and Immunology, Faculty of Life Sciences, University of Dundee, Dundee DD1 5EH, UK.

*To whom correspondence should be addressed. E-mail: r.fahræus@dundee.ac.uk

REPORTS

AP) did not stimulate EBNA1wt translation (Fig. 2D). This indicates that GAR RNA does not block translation via signal transduction pathways. This finding is further supported by the observation that fusion of the GAR sequence to the 3' untranslated region (3'UTR) of the EBΔGA transcript (EB-GAC3') failed to confer the inhibitory effect of the GAR sequence on translation (Fig. 2E). Taken together, these results indicate that the GAR RNA sequence per se is unlikely to interfere with translation, either through a third party or through direct interaction with the translation machinery. Instead, the data revealed that the GAR sequence is required to be within the coding sequence to affect translation. However, when the GAR sequence was expressed in the C terminus of the EBΔGA transcript (EB-GAC), it had significantly less inhibitory effect on translation than it did in its N-terminal position in EBNA1wt (Fig. 2E).

To determine whether other parts of the EBNA1 transcript might contribute to the inhibition of translation, which could help to explain the location-dependent effect of GAR, we fused GAR to the C terminus or to the N terminus of an ovalbumin (Ova) sequence that lacks the N-terminal 50-amino acid signal sequence (Ova-GAC and Ova-GAN, respectively) (Fig. 2E). These Ova transcripts were translated at levels similar to the corresponding EBNA1 transcripts; hence, no other parts of the EBNA1 mRNA are required for GAR to block translation. We also fused a shortened GAR sequence (corresponding to 20 amino acids) to the C terminus or to the N terminus of the Ova transcript (Ova-Cpep and Ova-Npep, respectively) (Fig. 2E). This short sequence had a reduced

effect on translation relative to full-length GAR when located at the N terminus and displayed no effect on translation when fused to the C terminus of the Ova mRNA.

The observation that the effect of GAR is dependent on its position within the coding region led us to consider the possibility that the GAR peptide sequence was capable of inhibiting its own synthesis. We reasoned that if this assumption were correct, the GAR peptide might inhibit mRNA translation by interfering with the activity of a ribosomal factor, and translation of the GAR transcript might be stimulated if the

GAR peptide were hindered from such an interaction. To test this idea, we purified GAR sequence-specific antibodies derived from EBV-positive human sera and from rabbits immunized with GAR peptides (17), confirming GAR specificity by immunoblotting (Fig. 2F, upper panel) and enzyme-linked immunosorbent assay (18). When these antibodies were added to IVT assays, they increased the translation of the EBNA1wt transcript but had no effect on translation of the EBΔGA transcripts (Fig. 2F, lower panel). A monoclonal antibody (mAb) to the C terminus of EBNA1 had no effect on mRNA

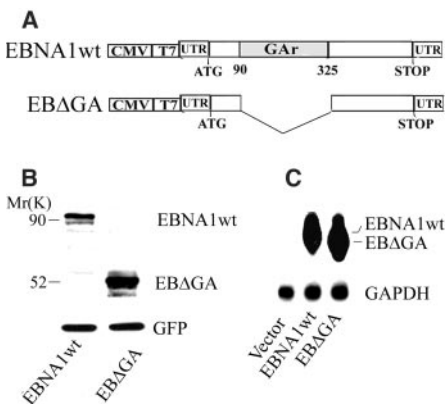


Fig. 1. Deletion of the GAR sequence increases EBNA1 steady-state level. (A) The EBNA1 constructs have identical 5' and 3' UTRs. The shaded area shows the location of GAR in EBNA1. (B) Western blot of EBV-negative H1299 cells expressing EBNA1wt or an EBNA1 protein with a deleted GAR (EBΔGA) and a green fluorescent protein transfection control. (C) Northern blot of total RNA shows that cells transfected under the same conditions as in (B) express similar levels of EBNA1wt and EBΔGA mRNAs (10% more EBΔGA RNA).

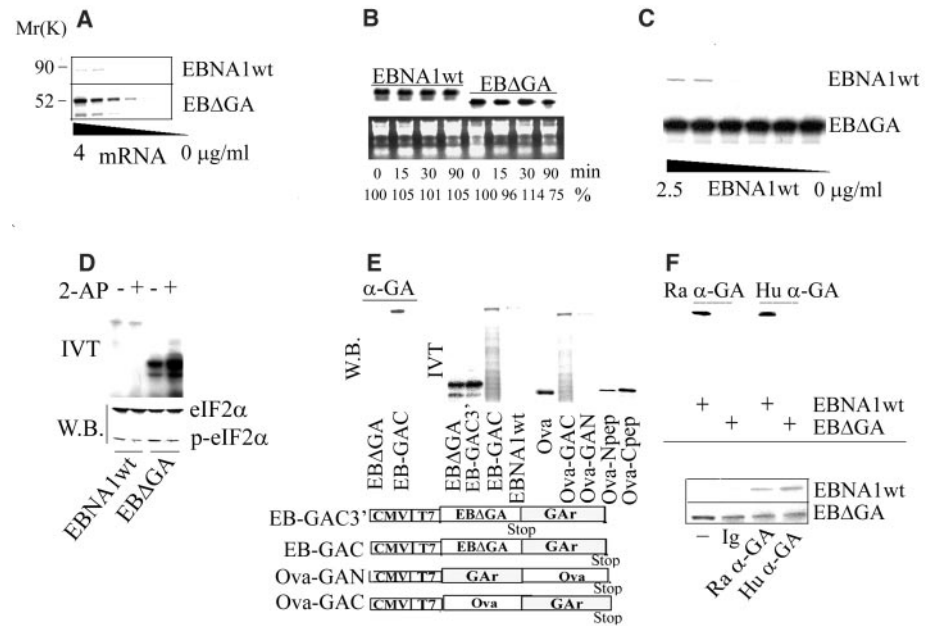


Fig. 2. GAR inhibits its own mRNA translation. (A) Capped mRNAs expressing EBNA1wt and EBΔGA were transcribed in vitro and purified before addition (twofold dilution series) to a rabbit reticulocyte lysate IVT system. [³⁵S]Methionine-labeled proteins were visualized by autoradiography. Phosphoimager analysis revealed that deletion of GAR results in a factor of ~11 increase in EBNA1 translation at 4 μg/ml mRNA. (B) EBNA1wt and EBΔGA mRNAs were trace-labeled with α[³²P]uridine triphosphate in vitro and added to rabbit reticulocyte lysate for the indicated lengths of time. Total RNA was isolated and visualized by ultraviolet light (lower panel), and the stability of EBNA1 mRNAs was determined by phosphoimager measurement of EBNA1wt and EBΔGA mRNA levels (upper panel). This result shows that the steady-state levels of EBNA1wt and EBΔGA mRNAs in rabbit reticulocyte lysates are similar, with EBΔGA having the shorter half-life. (C) Autoradiograph of an IVT assay where an increasing amount of EBNA1wt was cotranslated with a fixed amount (5 μg/ml) of the non-GAR carrying EBΔGA mRNA. The result shows that the presence of GAR mRNA has no effect on translation on EBΔGA, hence GAR inhibits translation in cis. (D) Autoradiograph from IVT of lysates incubated with indicated mRNA (2.5 μg/ml) in the presence or absence of 1 mM 2-AP. Western blot of the same lysates using the eIF2α Ser⁵¹ phospho-specific polyclonal antibody or a non-phospho-specific eIF2α mAb shows that the GAR RNA sequence does not activate PKR. (E) The GAR cDNA sequence was cloned into the 3'UTR and in the C terminus of the EBΔGA construct (EB-GAC3' and EB-GAC, respectively) and the expression of GAR from the EB-GAC construct was confirmed by Western blot with rabbit antisera to GAR (left panel). The translation efficiencies of these mRNAs were compared with those of EBΔGA and EBNA1wt in an IVT assay (right panel). The GAR-encoding DNA sequence was also fused to the N terminus of an Ova cDNA lacking the first 50-amino acid secretory signal sequence (Ova-GAN) and to its C terminus (Ova-GAC), and mRNA translations of these constructs were compared with Ova itself in vitro. A sequence corresponding to a 20-amino acid GAR was also fused to the N terminus or the C terminus of Ova (Ova-Cpep and Ova-Npep, respectively). This short GAR peptide sequence affected Ova translation only when fused to the N terminus of the transcript. (F) Autoradiograph (top panel) of cell lysates from H1299 cells transfected with either EBNA1wt or EBΔGA cDNA and blotted against GAR-specific antibodies purified from EBV-positive human sera (Hu α-GA) or sera from rabbits that were immunized against a 24-amino acid synthetic GAR peptide (Ra α-GA) (17). Purified antibodies to GAR (lower panel) were added at a final concentration of 10 μg/ml (human) and 25 μg/ml (rabbit) to IVT containing EBNA1wt and EBΔGA mRNAs (1.5 μg/ml each). The GAR-specific antibodies stimulate translation of the EBNA1wt mRNA only.

translation of EBNA1 *in vitro* (19), which indicates that the GAR peptide is involved in blocking its own synthesis *in cis*.

We next looked at the effect of GAR on intracellular protein synthesis. Cells expressing Ova or Ova-derived fusion proteins carrying the GAR sequence in the C terminus or N terminus were pulse-labeled with [³⁵S]methionine for 10, 20, and 40 min. Figure 3A shows that, as *in vitro*, GAR-mediated inhibition of synthesis *in vivo* depended on the location of the GAR sequence within the protein.

Taken together, these results may explain the paradox of why removal of the GAR sequence from EBNA1 has only a limited effect on EBNA1 expression in cells. Apparently, the increase in synthesis of EBNA1 after removal of GAR is almost completely compensated for by the subsequent increase in the rate of degradation.

The idea that GAR-mediated inhibition of proteasomal degradation of full-length EBNA1 is sufficient to avoid EBNA1-derived peptides from being processed and loaded onto MHC class I molecules (9, 11) is not in accordance with the DRiP hypothesis (13, 14, 20). We therefore tested directly whether the inhibition of proteasomal degradation was sufficient to protect GAR chimeric proteins from being processed and presented on MHC class I molecules. To do this, we took advantage of the fact that GAR-mediated self-inhibition of mRNA translation is dependent on the location of GAR within the protein, whereas inhibition of proteasomal degradation is not (Fig. 3, A and B) (11). Thus, by changing the location of the GAR sequence, the rate of translation could be altered while maintaining the rate of degradation of the full-length protein.

We used the Ova-GAR fusion constructs to compare the effects of inhibition of protein degradation (Ova-GAC) with those of the combined inhibition of degradation and synthesis (Ova-GAN) on generating antigenic peptides. K^b-positive EL4 cells expressing Ova-GAN, Ova-GAC, or Ova were incubated with the B3Z CD8⁺ T cell clone specific for the Ova-derived SIINFEKL peptide (Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu, residues 257 to 264) for 20 hours (21). Expression of Ova and Ova-GAC led to T cell stimulation, whereas expression of Ova-GAN did not (Fig. 3C, left panel). To ensure that GAR did not affect the MHC class I antigen presentation pathway, we exposed cells that expressed the different Ova transcripts to exogenous SIINFEKL peptide before incubating them with the B3Z cells. Under these conditions, there was no difference in the capacity of GAR-expressing EL4 cells to present SIINFEKL peptides (Fig. 3C, right panel). We also compared the rate of production of SIINFEKL peptide from the different Ova constructs by treating Ova-expressing EL4 cells with brefeldin A (BFA) for 12 hours, followed by the addition of the reversible proteasome inhibitor MG132. An increase in the presentation of SIINFEKL peptide was observed over a 3-hour time period after BFA and MG132 were removed from cells expressing Ova or Ova-GAC but not Ova-GAN (Fig. 3D). A similar increase in SIINFEKL presentation was observed in cells given fresh medium after being treated only with MG132 for 4 hours (18). The rate of SIINFEKL production from the Ova-GAC construct is about one-third that of Ova, which correlates with the difference in the rate of mRNA translation of these two transcripts. Hence, we conclude that inhibition of proteasomal degradation by GAR is not sufficient to prevent presentation of epitopes from GAR chimeras to the MHC class I pathway, and that the increase in antigenic peptide production observed from the Ova-GAC transcript (relative to that of Ova-GAN) reflects its higher rate of mRNA translation.

There are many examples of how viruses evade immune recognition of host cells, but EBV appears unique in that its latent form of existence in cell types that express EBNA1 only (such as Burkitt's lymphoma cells) is reliant on the GAR peptide motif to protect the host cell from immune recognition. Even though it is clear that the ability to block its own degradation is an important part of the viral strategy to control the expression level of EBNA1, these results show that protection of the full-length protein from proteasomal processing is not sufficient to block antigen presentation. Instead, our results show how EBV has evolved a mechanism to avoid immune surveillance of EBNA1-expressing latent host cells. This is a beautiful example of an adaptation to the rules of the DRiP model, in that the most effective way to prevent the production of potentially antigenic

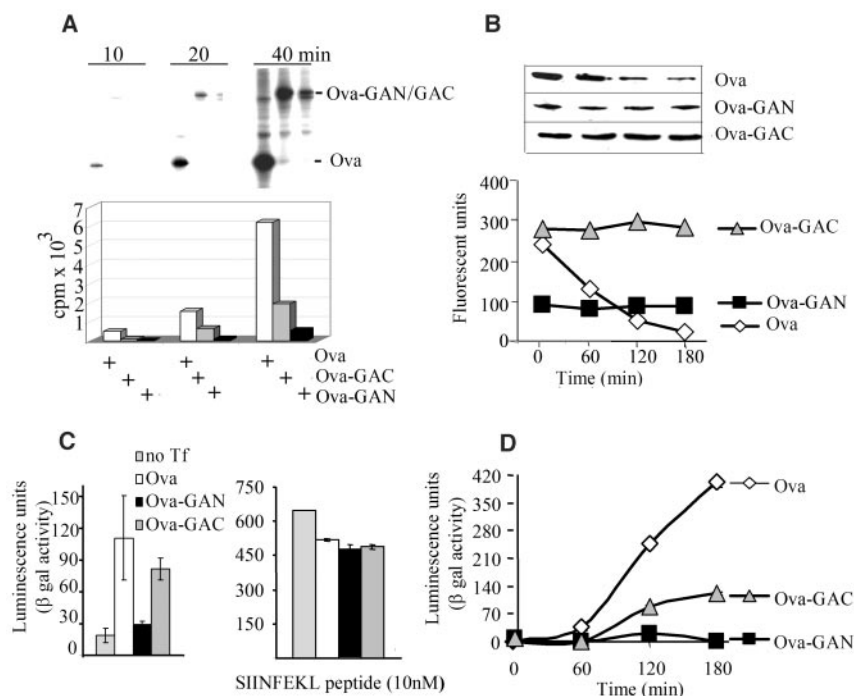


Fig. 3. Inhibition of proteasomal degradation is not sufficient to prevent GAR-carrying chimeras from presenting peptides to MHC class I molecules. (A) Cells transiently transfected with different Ova constructs for 48 hours were labeled with [³⁵S]methionine for indicated time in the presence of the proteasome inhibitor MG132. Top panel shows autoradiograph of the anti-Ova immunoprecipitates; the corresponding phosphorimager data are shown in the lower panel. The results show that GAR-dependent inhibition of synthesis is most effective when GAR is located at the N terminus of the chimeric protein. (B) Western blot of lysates from cells expressing the different Ova constructs under the same conditions as in (A) and treated with cycloheximide for the indicated times shows that GAR-mediated inhibition of proteasomal degradation is independent of the location of GAR within the protein. The values in the graph were determined with Alexa 680-labeled secondary antibodies to rabbit immunoglobulin G and the Odyssey (Light Core) fluorescent reader. (C) K^b-positive EL4 cells transiently transfected with Ova, Ova-GAC, or Ova-GAN cDNA constructs (3 μg per 10⁶ cells) for 48 hours were incubated with the B3Z CD8⁺ T cell clone specific for the Ova SIINFEKL peptide (amino acids 257 to 264) (21). B3Z cells express the *LacZ* gene under the control of the interleukin-2 promoter; B3Z activation was determined by measuring *LacZ* activity after 20 hours of incubation with EL4 cells. The right graph shows B3Z activation after EL4 cells expressing the different Ova transcripts had been exposed to 10 nM exogenous SIINFEKL peptide. (D) Cells transfected as in (C) were treated with BFA (5 μg/ml) for 12 hours to block the MHC class I export pathway before adding 10 μM MG132 for 4 hours. Cells were washed and chased in fresh warm medium for the indicated lengths of time before being fixed and incubated with B3Z cells as in (C).

products from the EBNA1 mRNA is to suppress its translation. A slower rate of translation might ensure that peptides that are translated out of frame (or that carry nonfunctional GAR sequences for other reasons) will not be produced from the EBNA1 transcript. Such a reassurance could not be achieved by simply lowering the rate of transcription of the gene encoding EBNA1. And because EBNA1 has no other known function for interfering with antigen presentation, inhibition of mRNA translation is clearly an effective strategy. The identification of a virus-encoded protein such as EBNA1 that minimizes MHC class I antigen presentation by inhibiting its own synthesis adds further support to the importance of rapidly degraded translation products as the main source for antigenic peptides.

This example of mRNA translation regulation by a peptide sequence within the encoded polypeptide is unusual, and there are to our knowledge few, if any, similar examples (22–24). This unusual self-regulating mechanism of protein synthesis most likely reflects the fact that GAR carries out a unique dual function, as an inhibitor of both ribosomal and proteasomal

activity. Thus, to minimize translation while maintaining a functional expression level of EBNA1, GAR appears to adapt to and explore as yet unidentified common mechanisms that control both proteasomal and ribosomal activity.

References and Notes

1. J. W. Yewdell, A. B. Hill, *Nature Immunol.* **3**, 1019 (2002).
2. D. Tortorella, B. E. Gewurz, M. H. Furman, D. J. Schust, H. L. Ploegh, *Annu. Rev. Immunol.* **18**, 861 (2000).
3. H. Wu, P. Kapoor, L. Frappier, *J. Virol.* **76**, 2480 (2002).
4. F. Chen *et al.*, *J. Virol.* **69**, 3752 (1995).
5. R. J. Tierney, N. Steven, L. S. Young, A. B. Rickinson, *J. Virol.* **68**, 7374 (1994).
6. N. Blake, T. Haigh, G. Shaka'a, D. Croom-Carter, A. Rickinson, *J. Immunol.* **165**, 7078 (2000).
7. R. Khanna *et al.*, *Virology* **214**, 633 (1995).
8. N. Blake *et al.*, *Immunity* **7**, 791 (1997).
9. J. Levitskaya *et al.*, *Nature* **375**, 685 (1995).
10. J. Levitskaya *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12616 (1997).
11. A. Sharipo, M. Imreh, A. Leonchiks, S. Imreh, M. G. Masucci, *Nature Med.* **8**, 939 (1998).
12. J. W. Yewdell, L. C. Anton, J. R. Bennink, *J. Immunol.* **157**, 1823 (1996).
13. J. W. Yewdell, U. Schubert, J. R. Bennink, *J. Cell Sci.* **114**, 845 (2001).
14. U. Schubert, L. C. Anton, J. Gibbs, C. C. Norbury, J. Yewdell, J. R. Bennink, *Nature* **404**, 770 (2000).

15. M. F. Princiotto *et al.*, *Immunity* **18**, 343 (2003).
16. T. Pe'ery, M. B. Mathews, in *Translational Control of Gene Expression*, N. Sonenberg, J. W. B. Hershey, M. B. Mathews, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2000).
17. J. Dillner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4652 (1984).
18. Y. Yin, B. Manoury, R. Fähræus, unpublished data.
19. See supporting data on Science Online.
20. E. A. Reits, J. C. Vos, M. Grommé, J. Neefjes, *Nature* **404**, 774 (2000).
21. J. Karttunen, S. Sanderson, N. Shasrri, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6021 (1992).
22. A. P. Geballe, D. R. Morris, *Trends Biochem. Sci.* **19**, 159 (1994).
23. N. K. Gray, M. Wickens, *Annu. Rev. Cell Dev. Biol.* **14**, 399 (1998).
24. P. S. Lovett, E. J. Rogers, *Microbiol. Rev.* **60**, 366 (1996).
25. Supported by the Cancer Research UK (CRUK). R.F. is a CRUK Senior Fellow. We thank C. Stephen for help with the initial work on the effect of GAR on its own translation and for cloning the EBNA1wt and EBΔGA constructs into the pCDNA3 vector. Both EBNA1wt and EBΔGA cDNA were a kind gift from N. Blake and A. Rickinson.

Supporting Online Material

www.sciencemag.org/cgi/content/full/301/5638/1371/DC1

Materials and Methods

8 July 2003; accepted 6 August 2003

Predominant Autoantibody Production by Early Human B Cell Precursors

Hedda Wardemann,¹ Sergey Yurasov,^{1,3} Anne Schaefer,¹ James W. Young,⁴ Eric Meffre,^{1,5*} Michel C. Nussenzweig^{1,2*}

During B lymphocyte development, antibodies are assembled by random gene segment reassortment to produce a vast number of specificities. A potential disadvantage of this process is that some of the antibodies produced are self-reactive. We determined the prevalence of self-reactive antibody formation and its regulation in human B cells. A majority (55 to 75%) of all antibodies expressed by early immature B cells displayed self-reactivity, including polyreactive and anti-nuclear specificities. Most of these autoantibodies were removed from the population at two discrete checkpoints during B cell development. Inefficient checkpoint regulation would lead to substantial increases in circulating autoantibodies.

Self-reactivity has concerned immunologists since the time of Ehrlich, who referred to this potential problem as leading to “horror autotoxicus” (1). Landsteiner’s finding that the immune repertoire is highly diverse focused the problem on how such diversity could be generated while avoiding self-reactivity (2).

We now know that antibody diversity is produced by V(D)J recombination, and experiments using transgenic mice have shown that at least three mechanisms account for silencing of self-reactive antibodies during B cell development: receptor editing, deletion, and anergy (3–6). However, the actual number of self-reactive antibodies that arise during B cell development has not been accurately determined, nor is it known precisely when such antibodies are removed from the repertoire under physiologic circumstances.

To examine the development and silencing of autoreactive B cells, we cloned antibodies from single B cells derived from the bone marrow and blood of three healthy human donors and tested them for reactivity against cell lysates and a panel of defined

antigens (fig. S1 and tables S1 to S10) (7, 8). Precursor B cells with the surface phenotype of pre-B cells that expressed functional Igκ or Igλ chain transcripts were designated as early immature B cells. These were distinguished from pre-B cells that did not express functional light chains and from immature B cells that expressed cell surface immunoglobulin M (IgM) (fig. S1 and tables S1 to S6).

Although amino acid sequence alone cannot predict whether an antibody will be self-reactive, long Ig heavy chain complementarity-determining regions 3 (IgH CDR3) have been associated with self-reactive or polyreactive antibodies (9–12). Analysis of human antibodies cloned from pools of developing B cells showed that IgH CDR3s from progenitor B cells are significantly longer on average than those from peripheral B cells (13, 14). A second feature associated with self-reactivity is the presence of positively charged amino acids within IgH CDR3 (15, 16). We found that antibodies with long and/or highly positively charged IgH CDR3s were enriched in pre-B cells and early immature B cells (Fig. 1). These features were selectively lost from the repertoire as B cells progressed through development (Fig. 1). We found no significant differences in Ig light chain (IgL) CDR3 length or charge, nor in IgH or IgL V/J usage, between B cell fractions (fig. S1). Thus, IgH CDR3 features associated with self-reactive antibodies appear to be removed from the B cell repertoire as B cells progress through development.

To determine whether individual antibodies cloned from different B cell subsets were self-reactive, we expressed them in 293A

¹Laboratory of Molecular Immunology, ²Howard Hughes Medical Institute, Rockefeller University, New York, NY 10021, USA. ³Department of Pediatrics, ⁴Allogeneic Bone Marrow Transplant and Clinical Immunology Services, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA. ⁵Hospital for Special Surgery, Weill Medical College of Cornell University, New York, NY 10021, USA.

*To whom correspondence should be addressed. E-mail: meffre@hss.edu, nussen@mail.rockefeller.edu