Inhibition of antigen processing by the internal repeat region of the Epstein–Barr virus nuclear antigen-1

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The Epstein–Barr virus (EBV)-encoded nuclear antigen (EBNA1) is expressed in latently EBV-infected B lymphocytes that persist for life in healthy virus carriers[1,2], and is the only viral protein regularly detected in all malignancies associated with EBV[3,4]. Major histocompatibility complex (MHC) class I-restricted, EBNA1-specific cytotoxic T lymphocyte (CTL) responses have not been demonstrated[3,5]. Using recombinant vaccinia viruses encoding chimaeric proteins containing an immunodominant human leukocyte antigen A11-restricted CTL epitope, amino acids 416–424 of the EBNA4 protein[6], inserted within the intact EBNA1, or within an EBNA1 deletion mutant devoid of the internal Gly–Ala repetitive sequence, we demonstrate that the Gly–Ala repeats generate a *cis*-acting inhibitory signal that interferes with antigen processing and MHC class I-restricted presentation. Insertion of the Gly–Ala repeats downstream of the 416–424 epitope inhibited CTL recognition of a chimaeric EBNA4 protein. The results highlight a previously unknown mechanism of viral escape from CTL surveillance, and support the view that the resistance of cells expressing EBNA1 to rejection mediated by CTL is a critical requirement for EBV persistence and pathogenesis.

EBNA1 is a phosphoprotein[7] composed of unique amino- and carboxy-terminal domains (amino acids 1–89 and 327–641, respectively, in the prototype B95.8 EBV strain) joined by a repetitive sequence of Arg–Gly-containing motifs surrounding an internal Gly–Ala repeat[8]. Gly–Ala repeats of different length are present in all EBV isolates and represent the major target of EBNA-specific antibody responses[9] but their function is unknown. We reasoned that presence of repetitive sequences covering over one-third of the molecule could influence the recognition of EBNA1 by the cellular immune system. Taking advantage of the observation that EBV induced CTL responses in caucasian human leukocyte antigen (HLA) A11-positive individuals are often dominated by A11-restricted reactivities to peptide epitopes corresponding to residues 399–408 and 416–424 of the EBNA4 protein[6,10], we constructed recombinant vaccinia viruses expressing chimaeric genes that contained the EBNA4 416–424 epitope inserted in-frame within the intact EBNA1 sequence, or within EBNA1 deletion mutants that did not contain the Gly–Ala repeats (Fig. 1*a*). Because of toxicity of the Gly–Ala repeats for vaccinia virus replication[11], only recombinants containing the Gly–Ala-positive chimaeras inserted in negative orientation relative to the vaccinia P7.5 promoter were selected. Expression of the inserts in negative orientation is likely to originate from a cryptic poxvirus promoter[12] resulting in delayed kinetics and relatively lower levels of expression. This allows vaccinia virus assembly, probably by counteracting the capacity of the repeats to compete for viral proteases that are required for maturation of virion proteins by cleavage at Ala–Gly-X motifs[13].

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Expression of the chimaeric proteins in human fibroblasts was confirmed by immunofluorescence (Fig. 1*b*, *c*) and immunoblotting (not shown). EBNA1 deletion mutants containing the EBNA4 416–424 epitope inserted at the His 39, Pro 446 or Lys 520 positions, relative to the B95.8 sequence (Vacc-E1ΔGAN-E4, Vacc-E1ΔGAP-E4 and Vacc-E1ΔGAB-E4, respectively) sensitized HLA A11-positive fibroblasts to lysis by EBV-specific CTLs (Fig. 2*a*). The level of killing was in each case comparable to that observed after infection with a vaccinia recombinant expressing EBNA4. In contrast, fibroblasts expressing a chimaeric full-size EBNA1 with the EBNA4 416–424 epitope inserted at His 39 (Vacc-E1N-E4) were not recognized by the CTLs (Fig. 2*b*). This was true even after infection for up to 24 h although the recombinant protein was detected in most infected cells starting from 12 h (Fig. 1*c*). Fibroblasts infected with Vacc-E1ΔGAN-E4 were lysed already after 6 h, and remained sensitive to lysis until 24 h when the experiment had to be terminated because of extensive cytopathic effects. The different recognition of cells infected with Vacc-E1ΔGAN-E4 and Vacc-E1N-E4 supports the view that presence of the Gly–Ala repeats may be directly involved in the failure of the E1N-E4 chimaera to sensitize target cells to lysis.

To explore this possibility further, we compared the CTL sensitivity of cells infected with vaccinia recombinants expressing EBNA4, or a chimaeric EBNA4 protein with the EBNA1 Gly–Ala repeats region inserted in-frame between Trp 624 and Pro 625, that is, downstream of the 399–408 and 416–424 epitopes (E4IR, Fig. 1*a*). Judging from the percentage of positive cells and intensity of fluorescence detected in experiments performed over a wide range of multiplicity of infection (m.o.i.), the recombinant proteins were expressed with identical kinetics and at similar levels (Fig. 1*b*, *c*). HLA A11-positive fibroblasts infected with Vacc-EBNA4 were sensitive to CTL clones specific for the 399–408 or 416–424 epitopes with maximal lysis after infection for 6 h (Fig. 3*a*) at a m.o.i. of 5 (Fig. 3*c*). In contrast, cells infected with Vacc-E4IR were lysed only weakly, even after infection at sixfold higher m.o.i. or for prolonged periods of time. Infection of the spontaneous lymphoblastoid cell line from donor QJZ (QJZsp LCL) that carries a Chinese EBV isolate with mutations abrogating recognition of the endogeneous EBNA4 399–408 and 416–424 epitopes[10] gave essentially similar results (Fig. 3*b*, *d*). Thus, the EBNA1 Gly–Ala repeats seem to affect processing and MHC class I-restricted presentation independently of the target cell lineage.

In the final set of experiments we asked whether overexpression of EBNA1 could by itself influence antigen processing. High levels of EBNA1 can be expressed in cells infected with vaccinia virus through the use of an inducible T7 RNA polymerase system[14]. HLA A11-positive fibroblasts infected for 12 h with Vacc-T7 and Vacc-EBNA1 or, as controls, Vacc-T7 and Vacc-EBNA3 were superinfected for 6 h with Vacc-EBNA4 (Fig. 4*a*) and then tested for sensitivity to lysis by CTLs specific for the 399–408 or 416–424 epitopes. Overexpression of EBNA1 or EBNA3 did not prevent recognition of the Vacc-EBNA4-infected fibroblasts although a 30 to 50% reduction of lysis was observed, probably due to competition of the viruses for the cellular transcription/translation machinery (Fig. 4*b*). Thus, presence of the repeats within the context of the target protein seems to generate a signal which either prevents processing or, alternatively, sequesters the processing products to a cellular compartment which is inaccessible to MHC class I-restricted presentation.

Additional studies are needed to clarify the mechanism by which the EBNA1 Gly–Ala repeats influence CTL recognition. Recent evidence suggests that processing of antigens for MHC class I-restricted presentation requires both ubiquitination[15] and subsequent degradation by the proteasome[16]. The ubiquitin system has been implicated in the processing or complete destruction of many cellular proteins including proteins that are mutated, misfolded or otherwise damaged[17]. Our observation

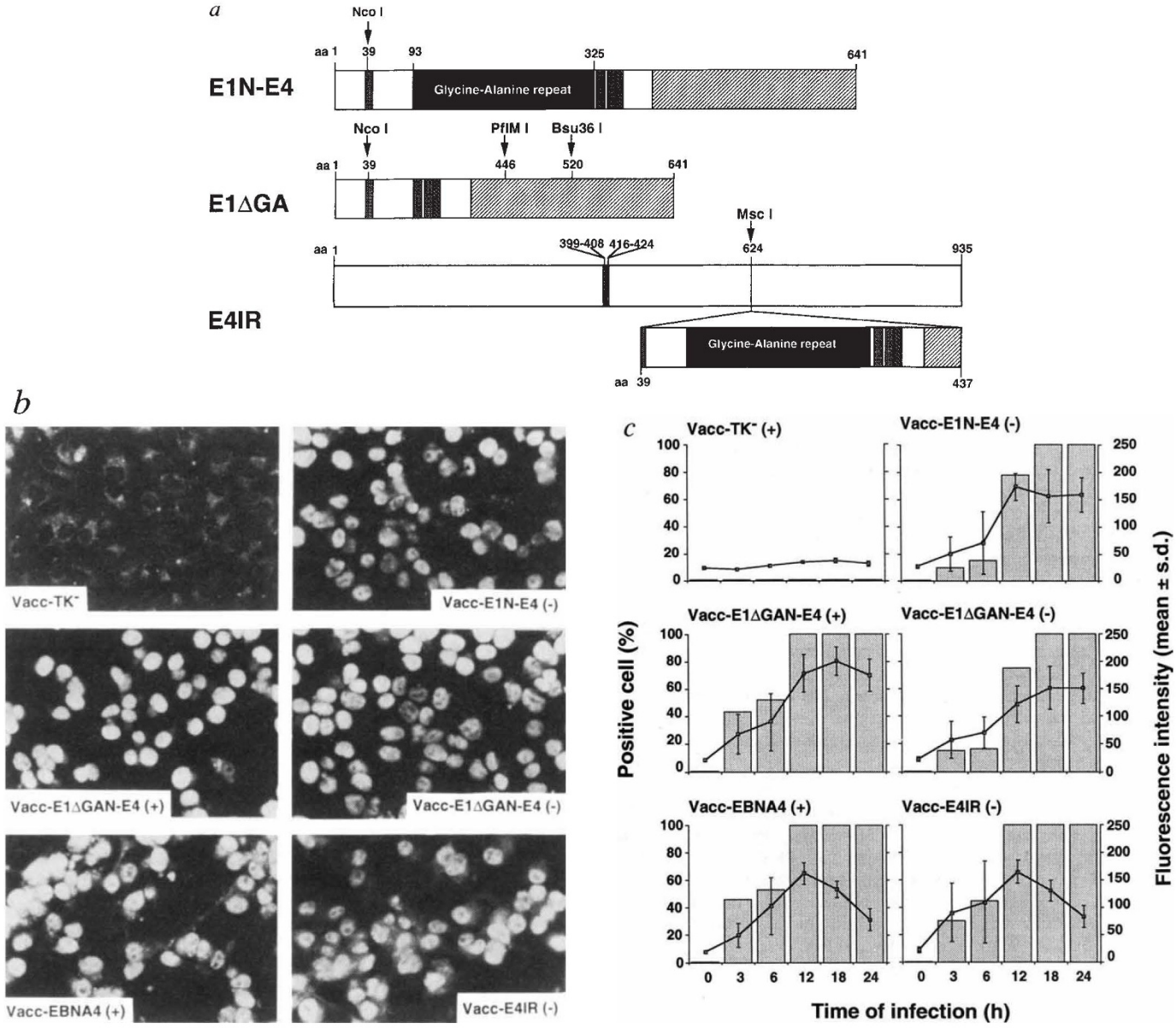
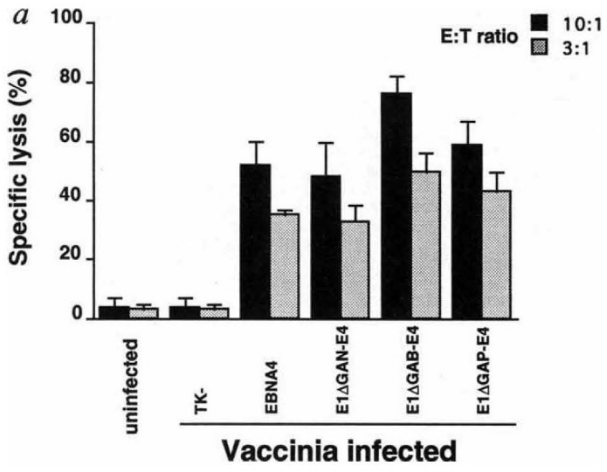


FIG. 1 *a*, Schematic outline of the full-length EBNA1 chimaera (E1N–E4), the Gly–Ala-deleted EBNA1 chimaeras (E1ΔGA) and the Gly–Ala-containing EBNA4 chimaera (E4IR). The position of insertion of the EBNA4 416–424 epitope (E4) and the EBNA1 internal repeat region (IR) are indicated by arrows pointing to the amino-acid number in the B95.8 sequence. The EBNA1 Gly–Ala repeats (solid); Arg–Gly repeats (light shaded); nuclear localization signal (dark shaded); DNA binding and dimerization domains (cross-hatched) are indicated. *b*, ACIF stainings illustrating the nuclear localization of the recombinant E1N-E4, E1ΔGAN-E4 inserted in positive (+) or negative (−) orientation relative to the P7.5 promoter, EBNA4 and E4IR in vaccinia virus-infected fibroblasts. The E1ΔGAP-E4 and E1ΔGAB-E4 exhibited similar fluorescence patterns (not shown). *c*, Quantification of expression of the recombinant proteins detected by ACIF. E1N-E4 and E1ΔGAN-E4 reached a plateau of expression between 12 to 18 h whereas the expression of EBNA4 and E4IR reached a maximum at 12 h and decreased thereafter. The negative orientations E1N-E4 and E1ΔGAN-E4 were expressed with slower kinetics and at lower levels compared to the positive orientation E1ΔGAN-E4 whereas the orientation of insertion did not affect the expression of E4IR. It should be noted that the expression of E1N-E4 and E4IR is probably overestimated because of the presence of Gly–Ala-specific antibodies in the human serum.

METHODS. Vaccinia recombinants carrying the coding sequences of EBNA1 and EBNA4 (also known as EBNA-3B) from B95–8 were described previously[14]. Chimaeric proteins containing the EBNA4 416–424 epitope (IVTDFSVIK)[6] within EBNA1 (E1), or an EBNA1 deletion mutant that lacks the Gly–Ala repeat region (E1ΔGA)[11] were produced by inserting an oligonucleotide corresponding to the E4 epitope in the *Nco*I, *Pfl*MI or *Bsu*36I site of the EBNA1 coding sequence (genomic positions: 108,067, 109,291 and 109,510, respectively). The oligonucleotide pairs E1N-E4F/E1N-E4R (5′-CATGCCATAGTAACTGACTTTAGTGTAATCAAG-3′/5′-CATGCTTGATTACACTTAAGTCAGTTACTAT-3′); E1P-E4F/E1P-E4R (5′-CGATCGTAACTGACTTTAGTGTAATCAGG-3′/5′-CCTTGATTACACTAAAGTCAGTTACGATCGTGC-3′) and E1B-E4F/E1B-E4R (5′-TAACGATCGTAACTGACTTTAGTGTAATCAAGG-3′/5′-TTACCTTGATTACACTAAGTCAGTTACGATCG-3′) were annealed and ligated to appropriately digested pBS-E1 or pBS-E1ΔGA at 100:1 ratio. The chimaeric E1 and E1ΔGA open reading frames were excised by *Bst*YI and *Hin*cII digestion and inserted at the *Sma*I site of pSC11. The pBS-E4IR plasmic was constructed by inserting an *Nco*I–*Apa*I EBNA1 fragment (IR, gp: 108,067–109,261) into the *Msc*I site of EBNA4 (gp: 97,302). The E4IR open reading frame was excised by *Eco*RI and *Sst*I digestion and ligated into pSC11. Insert containing plasmids were sequenced to determine correct orientation and reading frame alignment. Recombinant viruses were generated by transfection into TK-143 cells infected with WR strain wild-type vaccinia and viral stocks were prepared and titrated in CV-1 cells[14]. Western blot analysis using a previously characterized human serum containing high antibody titres to all EBNAs (HR) confirmed that the E1N-E4 chimaera runs as a major band of 80K, corresponding to the full-length protein, and a 70K band, probably arising from recombination within the Gly–Ala sequence, whereas the three E1ΔGA chimaeras have a rough size of 52K (not shown). Attempts to detect the E4IR chimaera by immunoblotting gave inconsistent results, probably because of poor transfer of the 1,334 amino-acid-long product. The HR ▶

that presentation of the E1N-E4 and E4IR chimaeras is inhibited rather than enhanced, as may be expected following modification of protein structure by insertion of foreign sequences, supports the notion that the Gly–Ala repeats may directly influence protein degradation.

MHC class I-restricted CTLs have potent antiviral activity both *in vitro* and *in vivo*[18 20]. It is therefore not surprising that viruses have evolved sophisticated mechanisms to escape CTL recognition. Previously identified routes of escape include suppression of target cell antigenicity by downregulation of MHC class I antigens[21], or selective mutation of immunodominant CTL target structures[10,22–25]; and modulation of the host immune response by clonal exhaustion or induction of immunosuppression[26,27]. The results presented here highlight a



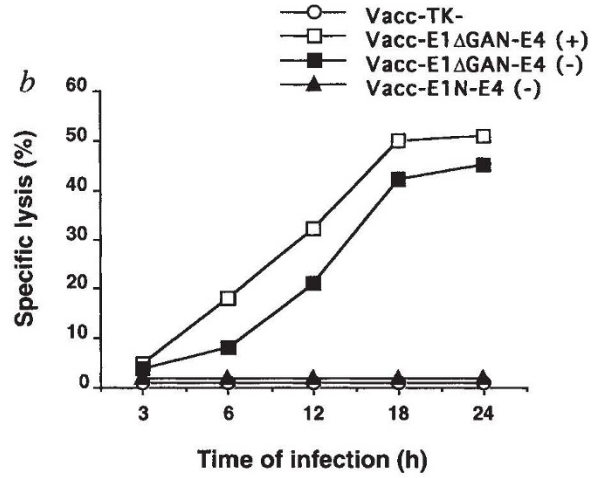


FIG. 2 Lysis of fibroblasts expressing the full-size and Gly–Ala-deleted EBNA1 chimaeras by CTLs specific for the EBNA4 416–424 epitope. Fibroblasts expressing the Gly–Ala-deleted chimaeras were lysed as efficiently as fibroblasts expressing EBNA4, whereas fibroblasts expressing the full-size EBNA1 chimaera were not killed. *a*, Lysis of fibroblasts expressing EBNA4, E1ΔGAN-E4, E1ΔGAP-E4 or E1ΔGAB-E4. Mean and s.e. of four experiments. *b*, Lysis of fibroblasts infected for different lengths of time with the Vacc-E1ΔGAN-E4 recombinant containing the Gly–Ala-deleted chimaera insert in positive (+) or negative (−) orientation Vacc-E1N-E4. Specific lysis at 10:1 effector:target ratio in one representative experiment out of three.

METHODS. Semiconfluent monolayers of fibroblasts from donor QJZ. (HLA A11 B13,B51) were grown in 96-well plates. Infection was done in the assay wells in the presence of 3 μCi $^{51}NaCrO_4$ per well either for 12 h at a m.o.i. of 10 (*a*), or for the indicated times starting from 24 h before addition of the effectors (*b*). CTL clones specific for the EBNA4 416–424 epitope were obtained by stimulation of lymphocytes from the EBV seropositive donors BK (HLA A2,A11 B7,B35) with the autologous B95.8 virus transformed LCL as previously described[6]. The cytotoxic activity was assayed in triplicate in standard 4 h ^{51}Cr-release assays.

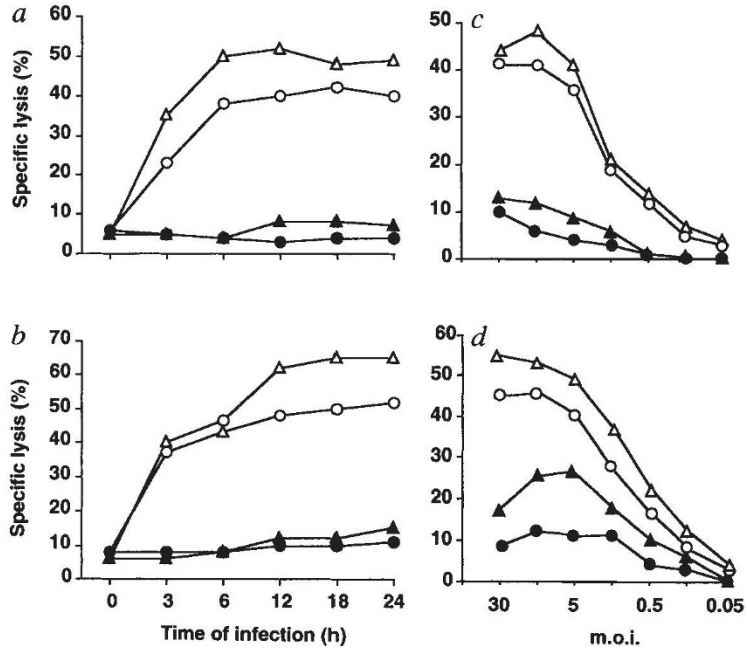


FIG. 3 Lysis of cells expressing EBNA4 or the E4IR chimaera by CTLs specific for the EBNA4 399–408 and 416–424 epitopes. QJZ fibroblasts (*a* and *c*) or the QJZsp LCL that carries a Chinese EBV isolate with mutations abrogating HLA A11-restricted recognition of the endogenous EBNA4 protein[10] (*b* and *d*), were infected with Vacc-EBNA4 (△, ○) or Vacc-E4IR (▲, ●) at a m.o.i. of 10 for the indicated times (*a* and *b*) or at the indicated m.o.i. for 12 h (*c* and *d*) before use as targets for CTLs specific for the EBNA4 399–408 (○, ●) or 416–424 (△, ▲) epitopes. Specific lysis at 10:1 effector: target ratio in one representative experiment out of three done with each effector:target combination.

METHODS. Semiconfluent monolayers of QJZ fibroblasts were infected and labelled as described in Fig. 2. Aliquots of 5×10^6 QJZsp LCL cells were placed in 5-ml tubes and infected for 1 h at 37 °C with 1 ml concentrated virus before addition of 2 ml complete medium and further incubation for the indicated times. 100 μCi $^{51}NaCrO_4$ were added to each tube 2 h before the initiation of the assay.

▶ serum was routinely used in complement enhanced immunofluorescence (ACIF) staining[28] to detect expression of the recombinant proteins in vaccina virus-infected human fibroblasts. Parallel slides were stained with affinity-purified anti-Gly–Ala human antibodies[9] and with the EBNA1-specific monoclonal antibody EBNA.OTx[29]. For quantification experiments the fibroblasts were grown in 8-chamber slides. Separate chambers were infected at a m.o.i. of 10 starting from 24 h before the termination of the assay. Pairs of slides were stained with the HR serum and with an EBV antibody-negative human serum as control. The percentage of positive cells was estimated visually using an epifluorescence microscope (LEICA, DMRB). Digital image recording was done with a cooled CCD camera (Hamamatsu C4880-01) controlled by the HiPic software. The images were analysed with the NIH Image1.56 software where fluorescence is proportional to the pixel value on a grey scale from 0 to 255. The mean fluorescence intensity of each sample was calculated from the pixel value of at least 50 nuclei.

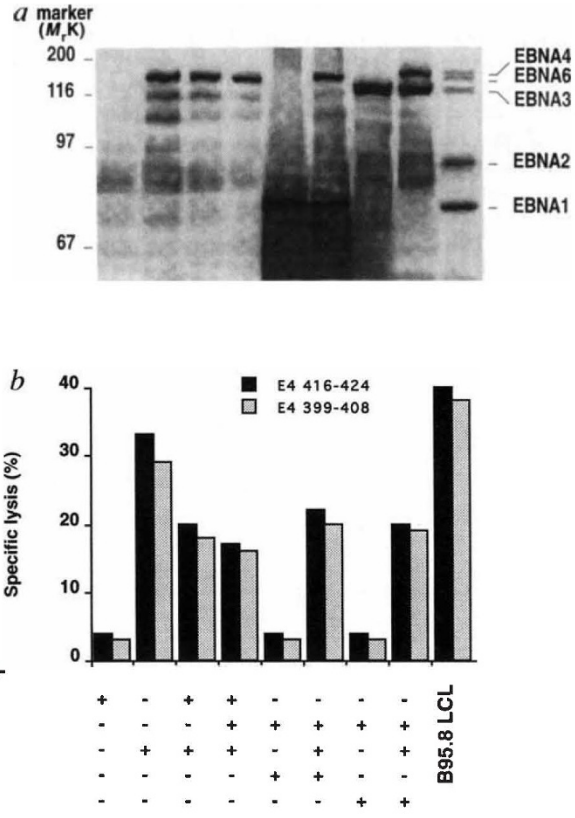


FIG. 4 Overexpression of EBNA1 does not inhibit presentation of the EBNA4 399–408 and 416–424 epitopes. *a*, Western blot illustrating the expression of EBNA4, EBNA1 and EBNA3 and coexpression of EBNA1 + EBNA4 and EBNA3 + EBNA4 in QJZ fibroblasts. The vaccinia-expressed EBNA3 and EBNA4 run as major bands of about 145K and 160K with minor degradation bands, whereas the T7 RNA polymerase-induced EBNA1 is seen as a major band of about 80K and a broad smear of immunoreactive material. *b*, Lysis of the same cells by CTLs specific for the EBNA4 399–408 (shaded) and 416–424 (solid) epitopes. Specific lysis at 10:1 effector:target ratio in one representative experiment out of three.

METHODS. The vaccinia recombinant carrying the coding sequence of EBNA3 (also known as EBNA-3A) from B95.8, and the T7 RNA polymerase-inducible EBNA1 expression system have been described previously[14]. QJZ fibroblasts were infected for 12 h with Vacc-EBNA1 + Vacc-T7, Vacc-EBNA3 + Vacc-T7 or Vacc-TK$^-$ + Vacc-T7 before superinfection for 6 h with Vacc-EBNA4. Control samples were infected with Vacc-TK$^-$ or Vacc-EBNA4 for 6 h. Each recombinant was used at a m.o.i. of 10. Western blots of 10^6 infected cells were probed with the HR serum. A B95.8 virus-transformed LCL from donor QJZ was used as control.

new mechanism of immune evasion which is targeted to a particular stage of the virus–host interaction. Failure to undergo processing and MHC class I-restricted presentation of a constitutively expressed viral protein may afford protection independently of the class I type of the host. The high selectivity of this viral strategy may favour the long-term persistence of virus-infected cells without involving immunosuppression, thus maintaining the strong rejection-geared responses that prevent the uncontrolled proliferation of EBV-transformed immunoblasts. □

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1. Tierney, R. J., Steven, N., Young, L. S. & Rickinson, A. B. *J. Virol.* **68,** 7374–7385 (1994).
2. Chen, F. *et al. J. Virol* (in the press).
3. Masucci, M. G. & Ernberg, I. *Trends Microbiol.* **2,** 125–130 (1994).
4. Klein, G. *Cell* **77,** 791–793 (1994).
5. Rickinson, A. *et al.* in *A New Look at Tumor Immunology* (eds McMichael, A. & Franks, L.) 53–80 (Cold Spring Harbor Laboratory Press, New York, 1992).
6. Gavioli, R. *et al. J. Virol.* **67,** 1572–1578 (1993).
7. Rawlins, D. R., Milman, G., Hayward, D. S. & Haywards, G. S. *Cell* **42,** 859–868 (1985).
8. Baer, R. *et al. Nature* **310,** 207–211 (1984).
9. Dillner, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **81,** 4652–4656 (1984).
10. de Campos-Lima, P. O. *et al. J. exp. Med.* **179,** 1297–1305 (1994).
11. Steigewald-Mullen, P. M. & Kurilla, M. G. *J. gen. Virol.* (in the press).
12. Bangham, C. R. M. *et al. J. Immun.* **137,** 3973–3977 (1986).
13. Lee, P. & Hruby, D. E. *J. Virol.* **67,** 4252–4263 (1993).
14. Murray, R. *et al. J. exp. Med.* **176,** 157–168 (1992).
15. Michalek, M. T., Grant, E. P., Gramm, C., Goldberg, A. L. & Rock, K. L. *Nature* **363,** 552–554 (1993).
16. Rock, K. L. *et al. Cell* **78,** 761–771 (1994).
17. Ciechanover, A. *Cell* **79,** 13–21 (1994).
18. Tsubota, H., Lord, C. I., Watkins, D. I., Morimoto, C. & Letvin, N. L. *J. exp. Med.* **169,** 1421–1434 (1989).
19. Lukacher, A. E., Braciale, V. L. & Braciale, T. J. *J. exp. Med.* **160,** 814–826 (1984).
20. Byrne, J. A. & Oldstone, M. B. A. *J. Virol.* **51,** 682–686 (1984).
21. Burgert, H. G., Maryansky, J. L. & Kvist, S. *Proc. natn. Acad. Sci. U.S.A.* **84,** 1356–1359 (1987).
22. Phillips, R. *et al. Nature* **354,** 453–459 (1991).
23. Pircher, H. *et al. Nature* **346,** 629–633 (1990).
24. Klenerman, P. *et al. Nature* **369,** 403–407 (1994).
25. Bertoletti, A. *et al. Nature* **369,** 407–410 (1994).
26. Tishon, A., Borrow, P., Evans, C. & A., O. M. B. *Virology* **195,** 397–405 (1993).
27. Moskophidis, D., Lechner, F., Pircher, H. & Zinkernagei, R. M. *Nature* **362,** 758–761 (1993).
28. Reedman, B. M. & Klein, G. *Int. J. Cancer* **11,** 499–520 (1973).
29. Chen, M.-R., Middletorp, J. M. & Hayward, D. *J. Virol.* **67,** 4875–4885 (1993).

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Tilting of the light-chain region of myosin during step length changes and active force generation in skeletal muscle

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FORCE generation and relative sliding between the myosin and actin filaments in muscle are thought to be caused by tilting of the head region of the myosin crossbridges between the filaments[1–3]. Structural and spectroscopic experiments have demonstrated segmental flexibility of myosin in muscle[4–6], but have not shown a direct linkage between tilting of the myosin heads and either force generation or filament sliding. Here we use fluorescence polarization to detect changes in the orientation of the light-chain region of the head, the part most likely to tilt[5,7,8], and synchronized head movements by imposing rapid length steps[9–11]. We found that the light-chain region of the myosin head tilts both during the imposed filament sliding and during the subsequent quick force recovery that is thought to signal the elementary force-generating event.

During muscle contraction, myosin crossbridges cycle through a series of biochemical, structural and mechanical states. We obtained partial synchronization of the transitions between these