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A Ca^{2+}/Calmodulin-Dependent Protein Kinase, CaM Kinase-Gr, Expressed after Transformation of Primary Human B Lymphocytes by Epstein-Barr Virus (EBV) Is Induced by the EBV Oncogene LMP1

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CaM kinase-Gr is a multifunctional Ca^{2+}/calmodulin-dependent protein kinase which is enriched in neurons and T lymphocytes. The kinase is absent from primary human B lymphocytes but is expressed in Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines, suggesting that expression of the kinase can be upregulated by an EBV gene product(s). We investigated the basis of CaM kinase-Gr expression in EBV-transformed cells and the mechanisms that regulate its activity therein by using an EBV-negative Burkitt lymphoma cell line, BJAB, and BJAB cells converted to expression of individual EBV proteins by single-gene transfer. CaM kinase-Gr expression was upregulated in BJAB cells by EBV latent-infection membrane protein 1 (LMP1) but not by LMP2A or by nuclear proteins EBNA1, EBNA2, EBNA3A, and EBNA3C. In LMP1-converted BJAB cells, the kinase was functional and was dramatically activated upon cross-linking of surface immunoglobulin M. Overlapping cDNA clones that encode human CaM kinase-Gr were sequenced, revealing 81% amino acid identity between the rat and human proteins. Transfection of BJAB cells with an expression construct for the human enzyme resulted in a functional kinase which was shown by epitope tagging to localize primarily to cytoplasmic and perinuclear structures. Induction of CaM kinase-Gr expression by LMP1 provides the first example of a Ca^{2+}/calmodulin-dependent protein kinase upregulated by a viral protein. In view of the key role played by LMP1 in B-lymphocyte immortalization by EBV, these findings implicate CaM kinase-Gr as a potential mediator of B-lymphocyte growth transformation.

Ca^{2+} is a key second messenger that mediates a broad array of cellular responses induced by diverse extracellular stimuli, such as growth factors, hormones, neurotransmitters, etc. Many of the effects of elevated free intracellular Ca^{2+} are transmitted by the Ca^{2+} receptor calmodulin which, upon binding Ca^{2+}, activates a diverse family of effector molecules. An important component of the Ca^{2+} signalling cascade in mamalian cells is a family of serine-threonine-specific, Ca^{2+}/calmodulin-dependent protein kinases, whose members have been implicated in a broad array of cellular processes, including neuronal development and plasticity, cellular contractility and motility, carbohydrate metabolism, cell cycle progression, and gene expression (reviewed in reference 11). Ca^{2+}/calmodulin-dependent protein kinases are classified into two groups. Members of the first group, which include myosin light-chain kinase, phosphorylase kinase, and the Ca^{2+}/calmodulin-dependent protein kinases type III, are dedicated to the phosphorylation of one specific substrate and hence serve to regulate single important cellular processes. Members of the second group, which include the Ca^{2+}/calmodulin-dependent protein kinase types Ia, Ib, and II and Ca^{2+}/calmodulin-dependent protein kinase type Gr (CaM kinase-Gr), are multifunctional protein kinases that have broad substrate specificities and serve to regulate a wide array of cellular processes (35).

CaM kinase-Gr is unique among the multifunctional Ca^{2+}/calmodulin-dependent protein kinases in that its expression is largely restricted to neurons and T lymphocytes. The kinase was purified and cloned from rat brain tissue, where it exists in two monomeric isoforms, a 65,000-M_r α polypeptide and a 67,000-M_r β polypeptide. These two isoforms share a domain structure common to other Ca^{2+}/calmodulin-dependent protein kinases that includes a catalytic domain, a calmodulin-binding domain, and an associative domain which may serve to mediate interactions with other proteins (14, 23, 28, 30). The two isoforms of CaM kinase-Gr are encoded by a single gene, which also codes for calspermin, a testis-specific calmodulin-binding protein which comprises the calmodulin-binding and associative domains but lacks the catalytic domain. Calspermin is generated by alternate transcription initiation and alternative splicing of internal exons (14, 23, 28, 30).

CaM kinase-Gr is widely distributed in the rat brain and is particularly enriched in cerebellar granule cells, from which the enzyme derives its name (6, 29). In peripheral tissues, CaM kinase-Gr is highly expressed in developing T lymphocytes in the thymus, where its levels are comparable to those in the cerebellum (5). The kinase is also expressed at low levels in the spleen and testes but is undetectable in several other tissues (5, 23).

In human lymphoid tissues, CaM kinase-Gr is exclusively found in T lymphocytes; it is most abundant in immature thymocytes, and its levels drop by severalfold in the course of T-cell maturation (9). The human enzyme has two monomeric isoforms with sizes of 57 and 60 kDa. The catalytic activity of the human kinase was dramatically upregulated upon engagement of T-cell antigen receptor molecules by ligands, indicating that CaM kinase-Gr is a component of the Ca^{2+} signalling pathway in T lymphocytes.

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While the kinase was not detected in primary human B lymphocytes, it is expressed in Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines (9), raising the possibility that this kinase is important for EBV-mediated B-lymphocyte growth transformation. In transforming primary B lymphocytes, EBV expresses six nucleoproteins (EBNAs), two integral membrane proteins (LMPs), and two small RNA species (EBERs) (16, 19). EBNA LP, EBNA2, EBNA3A, EBNA3C, and LMP1 are essential for primary B-lymphocyte growth transformation, while EBNA3B, LMP2, and the EBERs are not (16, 19). In this study, we examined the capacity of these EBV genes to induce CaM kinase-Gr expression in a kinase-negative, non-EBV-infected Burkitt's lymphoma (BL) cell line, BJAB. Induction of CaM kinase-Gr expression was shown to be mediated by EBV LMP1. The LMP1-induced kinase was found to be activated by the increase in the intracellular Ca^{2+} concentration which follows cross-linking of surface immunoglobulins. A cDNA which encodes both isoforms of the kinase was derived from EBV-infected BL cells, sequenced, and expressed in BJAB cells. The recombinant kinases localized to the cyoplasmic and perinuclear areas and were also activated upon signalling via surface immunoglobulin molecules.

MATERIALS AND METHODS

Cells. BJAB is an EBV-negative BL cell line (24). BJAB clones stably expressing LMP1, LMP2A, both LMP1 and LMP2A, EBNA1, EBNA2, EBNA3A, or EBNA3C were generated as previously described (21, 25, 35). Jurkat human leukemic T-cell clone JE6.1 was obtained from the American Type Culture Collection (Bethesda, Md.). Human EBV-transformed B-lymphoblastoid cell lines were prepared and maintained as previously described (37).

Immunoblotting. For Western blot (immunoblot) analysis, cells were lysed on ice for 15 min in a buffer (cell lysis buffer) containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5), 0.5% Nonidet P-40, 50 mM NaCl, 25 mM NaH_2PO_4, 25 mM NaF, 12.5 mM $Na_4P_2O_7$, 2 mM EDTA, 2 mM ethylene glycol-bis(β-aminoethyl ether-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM Na_3VO_4, 100 μg of phenylmethylsulfonyl fluoride per ml, and 1 μg each of the protease inhibitors leupeptin, pepstatin, chymostatin, and antipain per ml. The lysates were then cleared of nuclei and other insoluble material by centrifugation at 16,000 × *g* for 30 min at 4°C. Supernatant samples containing 200 μg of protein were boiled in sodium dodecyl sulfate (SDS)-containing sample buffer, subjected to denaturing polyacrylamide gel electrophoresis, and then electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, Mass.). The filters were then incubated for 1 h at 23°C in blotting buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 0.05% Tween 20) containing 5% nonfat milk and then rinsed with blotting buffer and incubated for 3 h at 23°C with either CaM kinase-Gr-specific polyclonal rabbit antiserum at a 1:200 dilution in blotting buffer (9) or with anti-LMP1 monoclonal antibody S12 at a 1:2 dilution in blotting buffer (21). The filters were washed, and the LMP1 immunoblots were additionally incubated for 1 h at 23°C with affinity-purified rabbit anti-mouse immunoglobulin G (IgG) (Cappel) at 1 μg/ml in blotting buffer. The filters were subjected to a final incubation with ^{125}I-labeled protein G (New England Nuclear) at 1 to 2 μCi per membrane for 1 h at 23°C and then washed free of ^{125}I-labeled protein G and dried. Radiolabeled bands were visualized by autoradiography. For some CaM kinase-Gr immunoblots, alkaline phosphatase-conjugated goat anti-rabbit IgG (Cappel) was used as a secondary antibody. These blots were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Immunoprecipitation and gel electrophoresis. Cells were washed and then suspended at 2.5 × 10^7 cells per ml in RPMI 1640 medium supplemented with 10% fetal calf serum, incubated at 37°C for 10 min, and treated as indicated in the figure legends. The treatment was terminated by pelleting the cells for 5 s in a microcentrifuge and lysing them for 15 min on ice in lysis buffer. Nuclei and insoluble material were removed by centrifugation, and the supernatants were precleared overnight at 4°C with 25 μl of packed protein G-Sepharose (Boehringer Mannheim Biochemicals) precoated with normal rabbit serum. CaM kinase-Gr was then precipitated by incubating the precleared lysates for 4 h at 4°C with 25 μl of protein G-Sepharose precoated with anti-CaM kinase-Gr serum. The beads were washed four times with lysis buffer, and the immunoprecipitates were solubilized and subjected to denaturing acrylamide gel electrophoresis as previously described (9).

Assay of CaM kinase-Gr activity in immunoprecipitates. CaM kinase-Gr immunoprecipitates were prepared as described above, except that the kinase-containing beads were washed twice in a buffer containing 25 mM HEPES (pH 7.5), 0.5 mM EDTA, 0.5 M NaCl, 100 μg of phenylmethylsulfonyl fluoride per ml, and 1 μg each of the protease inhibitors leupeptin, pepstatin, chymostatin, and antipain per ml and then washed two additional times in this buffer but without NaCl. The beads were finally resuspended in 100 μl of the last buffer and used for enzyme assays. Assays of purified CaM kinase-Gr activity were carried out for 10 min at 23°C in a final volume of 100 μl containing 50 μl of the bead-wash buffer suspension and 50 μl of a reaction mixture containing 20 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 50 μM ATP, 1 μCi of [γ-^{32}P]ATP, a 20 μM concentration of the substrate peptide syntide-2 (Bachem), and either 5 mM $CaCl_2$ and 600 nM calmodulin (Pharmacia) or 5 mM EGTA. The reaction was terminated by spotting 25 μl of the mixture onto P81 phosphocellulose filters (Whatman). The filters were washed three times in 0.5% phosphoric acid, allowed to air dry, and counted in vials containing scintillation fluid.

cDNA library screening. A lambda gt10 cDNA library prepared from EBV-positive BL cell line BL41/B95-8 (1) was screened for isolation of a human lymphoid cDNA for CaM kinase-Gr. Approximately 150,000 phage plaques were screened. The primary screening was performed with a probe made from a 454-base-pair (bp) fragment of a human CaM kinase-Gr cDNA corresponding to bp +727 to +1171 of rat CaM kinase-Gr (23). The probe was generated by PCR amplification (9) and was labeled with a random-hexanucleotide DNA-labeling kit (Boehringer Mannheim Biochemicals). The phage plaques were lifted with colony-plaque screen filters (NEN), and the filters were hybridized at 42°C for 18 to 24 h in a buffer containing 50% formamide, 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 1% SDS, 1× Denhardt's solution, and 100 μg of sonicated salmon sperm DNA per ml. The filters were washed twice under high-stringency conditions for 30 min each time at 65°C in a buffer containing 0.2× SSPE, 1% SDS, and 0.05% sodium PP_i and then subjected to autoradiography. Positive clones were plaque purified. Certain positive clones were also screened with a probe spanning bp +1 to +752 of rat CaM kinase-Gr cDNA (23). Lambda DNA was prepared from the positive clones. Inserts were excised with *Eco*RI digestion and subcloned into plasmid pSG5 (Stratagene) for sequencing. Overlapping clones were sequenced with primers synthesized on the basis of a newly generated sequence (Sequenase kit; United

States Biochemical). Alignment of the amino acid sequences of the human, rat, and mouse enzymes was done with the CLUSTAL program (PCGene; InteliGenetics, Mountain View, Calif.).

Amplification and subcloning of human CaM kinase-Gr cDNA. The coding region of human CaM kinase-Gr was PCR amplified from lambda clone 4.1 by using primers GR-1 (5′-CGCAGATCTGCGAAGATGCTCAAAGTCAC-3′) and GR-2 (5′-GCCAGATCTGAAGGAAGCTGTTTAG-3′). The PCR product was purified by extraction with phenol and chloroform and then ethanol precipitated. Following purification, the PCR product was digested with *Bgl*II, purified by gel electrophoresis, and subcloned into the *Bgl*II site of plasmid pSG5 to generate pHGR. To place a FLAG epitope tag (IBI) on the amino terminus of CaM kinase-Gr, the coding region of human CaM kinase-Gr was PCR amplified from lambda clone 4.1 by using FLAG-encoding primer GR-FLAG (5′-GAAG ATCTCTGAGATGGACTACAAGGACGACGATGACAA ACTCAAAGTCACGGTGCCCTCCTGCTC) and primer GR-2. The PCR product was subcloned into the *Bgl*II site of pSG5 as described above to generate plasmid pHGR-FLAG.

RNA isolation and hybridization. Total cellular RNA was prepared from cell lines by guanidinium isothiocyanate lysis, and poly(A)$^+$ RNA was selected over poly(dT) (34). A 5-μg sample of each poly(A)$^+$ RNA was size fractionated by electrophoresis in a 1% agarose–formaldehyde gel, transferred to a nitrocelluose filter, and hybridized with a randomly primed, ^{32}P-labeled cDNA probe spanning the entire coding region of human CaM kinase-Gr. The filters were washed under stringent conditions, dried, and subjected to autoradiography.

PCR analysis of CaM kinase-Gr transcripts. PCR analysis of CaM kinase-Gr transcripts was carried out as previously described, with some modifications (9). Total cellular RNA was prepared by lysis of cells with guanidinium isothiocyanate, followed by centrifugation of the lysate over a CsCl cushion. A 20-μg sample of total RNA from each cell type was primed with random hexamers and reverse transcribed. The reverse transcription reaction products were resuspended in a final volume of 20 μl. Aliquots (2 μl) of the reverse transcription reaction products were used for PCR amplification with the Perkin-Elmer-Cetus RNA PCR kit (catalog no. N808-0017) in accordance with the manufacturer's instructions. CaM kinase-Gr amplifications used primers 5′-GCGATCAGTTCATGT TCAGG-3′ (sense primer), corresponding to bp 836–855, and 5′-ACCTTCATCAGCTCAGCCTGTG-3′ (antisense primer), complementary to bp 1268 to 1290 of the human CaM kinase-Gr sequence. Control amplification reactions used the human β-actin primers (Stratagene catalog no. 302010). Both CaM kinase-Gr and β-actin antisense primers were end labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; New England Nuclear) by using T4 polynucleotide kinase, and 1.0×10^6 cpm was used for each PCR. PCR conditions included 30 cycles of 1 min of denaturation at 95°C, 2 min of annealing at 50°C, and 3 min of extension at 72°C. Following amplification, 5-μl aliquots of the CaM kinase-Gr and β-actin PCR products were combined and then resolved by electrophoresis on a 12% acrylamide gel. The gel was dried under vacuum and exposed to Kodak X-Omat film.

Transfections. BJAB cells (1.5×10^7) were electroporated with 30 μg of plasmid DNA in 0.4 ml of RPMI containing 10% fetal calf serum by using a Bio-Rad Gene Pulser at 200 V and 960 μF. CaM kinase-Gr expression was analyzed by indirect immunofluorescence or immunoblotting 18 to 72 h later.

Immunofluorescence. Transfected cells were washed in phosphate-buffered saline (PBS) and dried quickly onto slides at 37°C. The cells were then blocked with PBS containing 20% goat serum for 30 min. The cells were then incubated at 37°C for 1 h with 10 μg of monoclonal antibody M2 (IBI), which recognizes the FLAG epitope, per ml. Following incubation, the cells were washed five times for 4 min each time with PBS containing 20% goat serum and then incubated as described above, with a fluorescein isothiocyanate-conjugated goat anti-mouse antibody. The cells were subsequently washed five times for 4 min each time in PBS, mounted, and visualized with a Zeiss Axioskop.

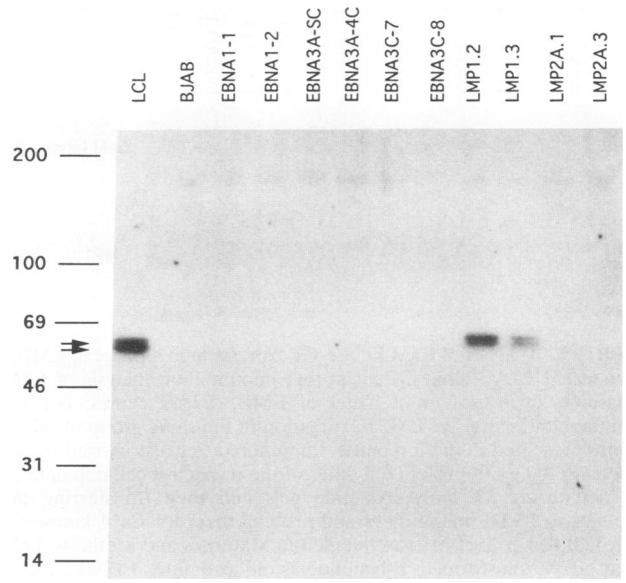

FIG. 1. CaM kinase-Gr expression in BJAB BL cells is specifically induced by LMP1. Protein (200 μg) of lysates of the respective cell populations was resolved on 5 to 16% denaturing polyacrylamide gels, transferred to PVDF membranes, and probed for CaM kinase-Gr polypeptides as detailed in Materials and Methods. LCL is an EBV-transformed B-lymphoblastoid cell line. BJAB is an EBV-negative human lymphoma cell line. EBNA1-1 and EBNA1-2 are BJAB cell clones that stably express EBNA1. EBNA3A-SC and EBNA3A-4C are BJAB clones that stably express EBNA3A. EBNA3C-7 and EBNA3C-8 are BJAB clones that stably express EBNA3C. LMP1.2 and LMP1.3 are BJAB clones that stably express LMP1. LMP2A.1 and LMP2A.3 are BJAB clones that stably express LMP2A. CaM kinase-Gr isoforms are indicated by the arrows. The numbers on the left are molecular size markers in kilodaltons.

Nucleotide sequence accession number. The human CaM kinase-Gr cDNA sequence has been deposited in the GenBank data base and assigned accession no. L24959.

RESULTS

Induction of CaM kinase-Gr expression by EBV is mediated by LMP1. CaM kinase-Gr was not detectable by immunoblotting in EBV-negative BL cell line BJAB (Fig. 1). However, the kinase was readily detectable in an EBV-transformed B-lymphoblastoid cell line, used as a positive control, and in BJAB clones expressing LMP-1. Both the 57- and 60-kDa isoforms of CaM kinase-Gr were abundant in EBV-transformed B-lymphoblastoid cell lines and in LMP1-transfected BJAB clones. Significantly, the kinase was absent from other clones expressing LMP2A, EBNA1, EBNA3A, or EBNA3C. The kinase was also absent from two EBNA2-expressing BJAB clones and was readily detected in nine LMP1-expressing

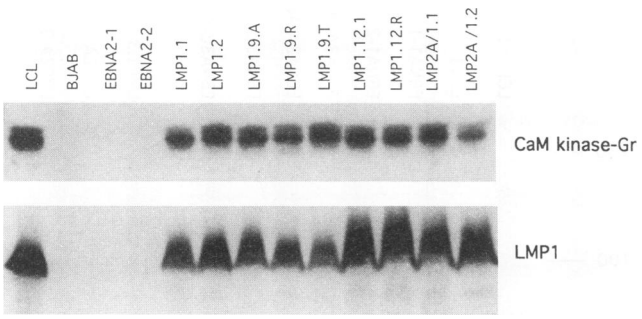

FIG. 2. Analysis of CaM kinase-Gr expression in a panel of LMP1-transfected BJAB clones. Panels: top, immunoblot analysis of CaM kinase-Gr expression in a panel of LMP1 BJAB clones; bottom, immunoblot analysis of LMP1 expression in the same group of BJAB clones presented in the top panel. Immunoblotting was carried out by resolving 200 μg of protein of lysates of the respective cell populations on denaturing 8% polyacrylamide gels and then transferring the proteins to PVDF membranes and probing them for CaM kinase-Gr polypeptides or for LMP1 as detailed in Materials and Methods. LCL is an EBV-transformed B-lymphoblastoid cell line. EBNA2-1 and EBNA2-2 are BJAB clones that stably express EBNA-2. LMP1.1, LMP1.2, LMP1.9.A, LMP1.9.R, LMP1.9.T, LMP12.1, and LMP12.R are BJAB clones that stably express LMP1. LMP2A/1.1 and LMP2A/1.3 are BJAB clones that stably express both LMP1 and LMP2A.

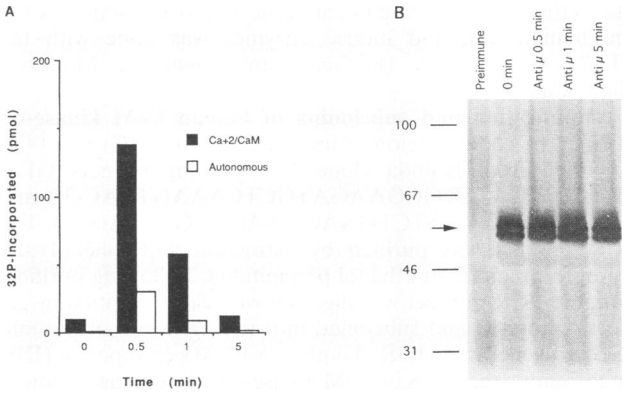

FIG. 3. Upregulation of CaM kinase-Gr catalytic activity in LMP1-transfected BJAB clones by surface IgM signalling. (A) Activation of CaM kinase-Gr by signalling via surface IgM. Aliquots of LMP1 clone 9-2 cells (2×10^7 cells per time point) were treated with goat anti-human IgM antibodies at 20 μg/ml for the indicated time periods. At the end of the incubation periods, the cells were lysed in 0.5% Nonidet P-40 lysis buffer and CaM kinase-Gr immunoprecipitates were prepared and assayed for Ca^{2+}/calmodulin-dependent activity or autonomous (Ca^{2+}/calmodulin-independent) kinase activity. The assays were run as described in Materials and Methods, and the data shown represent the means of three replicates run for each time point. Standard deviations were less than 10% of the means. Similar results were found in six other independent experiments. (B) Immunoblot analysis of CaM kinase-Gr immunoprecipitates isolated from LMP1 clone 9-2 cells either left unstimulated or stimulated with goat anti-human IgM antibodies (Anti μ) for the indicated periods. CaM kinase-Gr immunoprecipitates were resolved on a denaturing 8% acrylamide gel, transferred to PVDF membranes, and immunobloted with anti-CaM kinase-Gr sera as described in Materials and Methods. The prominent doublet represents CaM kinase-Gr isoforms (arrow), while the faint broad band is the rabbit IgG heavy chain. The numbers on the left are molecular size markers in kilodaltons.

BJAB clones (Fig. 2). These included seven clones which expressed LMP1 alone and two clones which coexpressed LMP1 and LMP2A (Fig. 2). The CaM kinase-Gr level in the LMP1/LMP2A doubly converted clones was comparable to that in LMP1 converted clones, indicating that LMP2A does not influence kinase induction by LMP-1.

Activation of CaM kinase-Gr by cross-linking of surface IgM in LMP1-converted BJAB cells. To determine whether the CaM kinase-Gr expressed in LMP1-converted BJAB clones is functional, the enzyme was immunoprecipitated from LMP1-expressing BJAB clones that had been left untreated or had been stimulated with goat anti-human IgM for various times, and the kinase immunoprecipitates were examined for autonomous (i.e., Ca^{2+}/calmodulin-independent) and Ca^{2+}/calmodulin-dependent kinase activities. CaM kinase-Gr immunoprecipitates from untreated LMP1-converted BJAB cells exhibited minimal autonomous activity and modest Ca^{2+}/calmodulin-stimulated activity (Fig. 3A). Treatment with anti-IgM antibodies resulted in dramatic upregulation of both autonomous and Ca^{2+}/calmodulin-dependent activities (Fig. 3A). Enzyme activation by surface IgM signalling was highest at 30 s after receptor engagement and rapidly declined thereafter. The increased CaM kinase-Gr activity was not due to increased immunoprecipitation of CaM kinase-Gr following anti-IgM treatment, since equal amounts of the kinase were detected in immunoprecipitates of cells before or after cross-linking (Fig. 3B). The initial increase and subsequent decline in CaM kinase-Gr activity following ligation of surface IgM molecules correlated with the previously reported time course for the rise in free intracellular Ca^{2+} concentrations in LMP1-converted BJAB clones (25). Activation of CaM kinase-Gr following surface IgM cross-linking was partially blocked by chelation of extracellular Ca^{2+} with EGTA, consistent with a role for extracellular Ca^{2+} in kinase activation following surface IgM cross-linking (data not shown).

LMP2A has been reported to block Ca^{2+} mobilization following ligation of surface immunoglobulins, CD19, or major histocompatibility complex class II molecules in B lymphocytes. LMP1 partially reverses the inhibitory effect of LMP2A on Ca^{2+} signalling. LMP2A does not appear to influence the capacity of LMP1 to induce the expression of CaM kinase-Gr (Fig. 2). However, it could be demonstrated in preliminary experiments that the magnitude of CaM kinase-Gr activation following surface IgM cross-linking is reduced by more than 50% in LMP2A-superconverted LMP1 BJAB clones compared with LMP1 clones. This would be consistent with the previously proposed view that LMP2A may act to downmodulate LMP1 effects on cell growth or to antagonize induction of lytic EBV infection following receptor ligation (25).

Cloning and expression of a human cDNA that encodes CaM kinase-Gr. Since human CaM kinase-Gr cDNA had not been previously cloned, we isolated and sequenced cDNA clones for the LMP1-induced kinase mRNA. A lambda gt10 cDNA library from EBV-positive BL cell line BL41/B95-8 was screened with a 454-bp fragment of human CaM kinase-Gr cDNA (see Materials and Methods). Six positive clones were identified (clones 3.1, 4.1, 4.3, 5.1, 5.2, and 5.3). All six hybridized to probes from the middle or the 3′ *Eco*RI fragments of rat CaM kinase-Gr cDNA. Clones 4.1 and 5.2 also hybridized to probes from the 5′ *Eco*RI fragment of rat kinase cDNA (bp +1 to +752) (23). *Eco*RI digestion of phage DNA, followed by sequencing of the inserts, revealed that clone 3.1 contained a 203-bp insert which corresponds to the middle *Eco*RI fragment of the rat cDNA and a 594-bp *Eco*RI insert which corresponds to the 3′ *Eco*RI fragment of the rat cDNA. Clone 4.1 contained 869-, 203-, and 529-bp inserts that, by

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CCCGGACCCAGGTGCTGCCAGGGCGGCGGCTGGCGGCCGGCTTCTCGCTCGGGCAGCGGCGGCGGCGGCGGC   72
GGCGGCTTCCGGAGTCCCGCTGCGAAGATGCTCAAAGTCACGGTGCCCTCCTGCTCCGCCTCGTCCTGCTCT  144
                    METLeuLysValThrValProSerCysSerAlaSerSerCysSer           15

TCGGTCACCGCCAGTGCGGCCCCGGGGACCGCGAGCCTCGTCCCGGATTACTGGATCGACGGCTCCAACAGG  216
SerValThrAlaSerAlaAlaProGlyThrAlaSerLeuValProAspTyrTrpIleAspGlySerAsnArg   39

GATGCGCTGAGCGATTTCTTCGAGGTGGAGTCGGAGCTGGGACGGGGTGCTACATCCATTGTGTACAGATGC  288
AspAlaLeuSerAspPhePheGluValGluSerGluLeuGlyArgGlyAlaThrSerIleValTyrArgCys   63
                                  *

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LysGlnLysGlyThrGlnLysProTyrAlaLeuLysValLeuLysLysThrValAspLysLysIleValArg   87

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IleValHisArgAspLeuLysProGluAsnLeuLeuTyrAlaThrProAlaProAspAlaProLeuLysIle  183

GCTGATTTTGGACTCTCTAAAATTGTGGAACATCAAGTGCTCATGAAGACAGTATGTGGAACCCCAGGGTAC  720
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LeuGluLysValLysGlyAlaAspIleAsnAlaGluGluAlaProLysMetValProLysAlaValGluAsp  423

GGGATAAAGGTGGCTGACCTGGAACTAGAGGAGGGCCTAGCAGAGGAGAAGCTGAAGACTGTGGAGGAGGCA 1440
GlyIleLysValAlaAspLeuGluLeuGluGluGlyLeuAlaGluGluLysLeuLysThrValGluGluAla  447

GCAGCTCCCAGAGAAGGGCAAGGAAGCTCTGCTGTGGGTTTTGAAGTTCCACAGCAAGATGTGATCCTGCCA 1512
AlaAlaProArgGluGlyGlnGlySerSerAlaValGlyPheGluValProGlnGlnAspValIleLeuPro  471

GAGTACTAAACAGCTTCCTTCAGATCTGGAAGCCAAACACCGGCATTTTATGTACTTTGTCCTTCAGCAAGA 1584
GluTyr***                                                                  473
AAGGTGTGGAAGCATGATATGTACTATAGTGATTCTGTTTTTGAGGTGCAAAAAAC
```

FIG. 4. Sequence of human CaM kinase-Gr cDNA. The DNA and deduced amino acid sequences of human CaM kinase-Gr are shown. The nucleotide sequence is shown on the upper line, and the deduced amino acid sequence is shown on the lower line with the three-letter nomenclature for amino acids. Glycine 53, the first residue of the consensus ATP-binding site, is indicated by the underlying asterisk. The putative calmodulin-binding site (amino acids 322 to 335) is double underlined. The latter was identified on the basis of its homology with the calmodulin-binding site of rat Ca^{2+}/calmodulin-dependent protein kinase type IIα (amino acids 296 to 309) (31). Both peptides share structural features deemed important for calmodulin binding, including an N-terminal stretch of basic amino acids followed by another stretch of hydrophobic residues and an overall α-helical secondary structure (100% for CaM kinase-Gr peptide amino acids 322 to 335), as determined by the algorithims of Garnier et al. (8) and Chou and Fasman (2).

```
HGRPROT     MLKVTVPSCSASSCSSVTASAAPGTASLVPDYWIDGSNRDALSDFFEVES   50
MGRPROT     MLKVTVPSCPSSPCSSVTAS----TENLVPDYWIDGSNRDPLGDFFEVES   46
RGRPROT     MLKVTVPSCPSSPCSSVTSS----TENLVPDYWIDGSKRDPLSDFFEVES   46
            *********..*.*****.*    *..**********.**.*.*******

HGRPROT     ELGRGATSIVYRCKQKGTQKPYALKVLKKTVDKKIVRTEIGVLLRLSHPN   100
MGRPROT     ELGRGATSIVYRCKQKGTQKPYALKVLKKTVDKKIVRTEIGVLLRLSHPN    96
RGRPROT     ELGRGATSIVYRCKQKGTQKPYALKVLKKTVDKKIVRTEIGVLLRLSHPN    96
            **************************************************

HGRPROT     IIKLKEIFETPTEISLVLELVTGGELFDRIVEKGYYSERDAADAVKQILE   150
MGRPROT     IIKLKEIFETPTEISLVLELVTGGELFDRIVEKGYYSERDARDAVKQILE   146
RGRPROT     IIKLKEIFETPTEISLVLELVTGGELFDRIVEKGYYSERDAADAVKQILE   146
            ********************************************* ********

HGRPROT     AVAYLHENGIVHRDLKPENLLYATPAPDAPLKIADFGLSKIVEHQVLMKT   200
MGRPROT     AVAYLHENGIVHRDLKPENLLYATPAPDAPLKIADFGLSKIVEHQVLMKT   196
RGRPROT     AVAYLHENGIVHRDLKPENLLYATPAPDAPLKIADFGLSKIVEHQVLMKT   196
            **************************************************

HGRPROT     VCGTPGYCAPEILRGCAYGPEVDMWSVGIITYILLCGFEPFYDERGDQFM   250
MGRPROT     VCGTPGYCAPEILRGCAYGPEVDMWSVGIITYILLCGFEPFYDERGDQFM   246
RGRPROT     VCGTPGYCAPEILRGCAYGPEVDMWSVGIITYILLCGFEPFYDERGDQFM   246
            **************************************************

HGRPROT     FRRILNCEYYFISPWWDEVSLNAKDLVRKLIVLDPKKRLTTFQALQHPWV   300
MGRPROT     FRRILNCEYYFISPWWDEVSLNAKDLVKKLIVLDPKKRLTTFQALQHPWV   296
RGRPROT     FRRILNCEYYFISPWWDEVSLNAKDLVKKLIVLDPKKRLTTFQALQHPWV   296
            **************************.**************************

HGRPROT     TGKAANFVHMDTAQKKLQEFNARRKLKAAVKAVVASSRLGSASSSHGSIQ   350
MGRPROT     TGKAANFVHMDTAQKKLQEFNARRKLKAAVKAVVASSRLGSASSSHTSIQ   346
RGRPROT     TGKAANFVHMDTAQKKLQEFNARRKLKAAVKAVVASSRLGSASSSHTNIQ   346
            **************************************************..**

HGRPROT     ESHKASRDPSPIQDGNEDMKAIPEGEKIQG---DGAQAAVKGAQAELMKV   397
MGRPROT     ENHKASSDPPSTQDAKDSTDLL--GKKMQEEDQEEDQVEAEASADEMRKL   394
RGRPROT     ESNKASSEAQPAQDGKDKTDPL--ENKIQAGDHEA----AKAAADETMKL   390
            *..***... . **.... . .  ..*.*.   ..     ......* .*.

HGRPROT     QALEKVKGADINAEE--------APKMVPKAVEDGIKVADLELEEGLAEE   439
MGRPROT     QSEEVEKDAGVKEEE--------TSSMVPQDPEDELETDDPEMKRD-SEE   435
RGRPROT     QSEEVEEEEGVKEEEEEEEEEEETSRMVPQEPEDRLETDDQEMKRN-SEE   439
            *. *  .......**          ...***.. ** ....* *.. . .**

HGRPROT     KLKTVEEAAAPREGQGSSAVGFEVP-QQDVILPEY   473
MGRPROT     KLKSVEEEMDPMTEEEAPDAGLGVP-QQDAIQPEY   469
RGRPROT     TLKSVEEEMDPKAEEEAAAVGLGVPPQQDAILPEY   474
            .**.***. .*.........*..** ***.* ***
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FIG. 5. Amino acid alignment of mouse (MGRPROT), rat (RGRPROT), and human (HGRPROT) CaM kinase-Gr sequences. The mouse and rat CaM kinase-Gr sequences have been previously published (14, 23, 28). Amino acids are represented by the single-letter code. Identical amino acids are indicated by stars, and conserved amino acid substitutions are indicated by dotes. Gaps inserted to optimize the alignment are indicated by dashes.

sequence analysis, correspond to the 5′, middle, and 3′ *Eco*RI fragments of the rat cDNA. The middle and 3′ *Eco*RI fragments of the human CaM kinase-Gr cDNA were fully sequenced from clone 3.1, and the 5′ *Eco*RI fragment was fully sequenced from clone 4.1. Clone 4.1 was used for PCR amplification of the entire open reading frame of CaM kinase-Gr (see Materials and Methods). The PCR product of this amplification was subcloned into pSG5 and sequenced.

The nucleotide and deduced amino acid sequences of the human CaM kinase-Gr obtained from clones 3.1 and 4.1 are shown in Fig. 4, and alignment of the amino acid sequences of the human, mouse, and rat CaM kinase-Gr enzymes are shown in Fig. 5. The human enzyme is predicted to consist of 473 amino acids, with a calculated M_r of 51,925, and contains many of the amino acid residues found to be conserved among serine-threonine-specific protein kinases (10). In common with other members of the family of Ca^{2+}/calmodulin-dependent protein kinases (36), human CaM kinase-Gr was found to contain three major domains: an amino-terminal catalytic domain, starting at the first residue of the consensus ATP-binding site (glycine 53; subdomain I of Hanks et al. [10]) and ending at the conserved residue arginine 288 (subdomain XI of Hanks et al. [10]), a regulatory domain containing the putative calmodulin-binding site (alanine 322 to alanine 335), as well as pseudosubstrate sequences, and a carboxyl-terminal associative domain that starts at alanine 342. Additionally, human CaM kinase-Gr possesses a fourth amino-terminal domain (methionine 1 to leucine 52) that is also found in rodent CaM kinase-Gr enzymes and is particularly rich in serine residues (22). This domain has been determined, in the case of the rat enzyme, to contain the major site of enzyme autophosphorylation and may thus function as a second regulatory domain that upregulates the activity of the enzyme upon autophosphorylation.

As seen in Fig. 5, sequence comparison of the human, mouse (14), and rat (23, 28) enzymes reveals virtual identity between the catalytic and regulatory domains of the human enzyme and their rodent counterparts. In contrast, significant sequence divergence between the human associative domain and its rodent counterparts was found. The carboxyl-terminal region of the human enzyme is significantly less acidic (the net negative charge is 13) than the corresponding region of the mouse (net negative charge of 31) and rat (net negative charge of 32) enzymes.

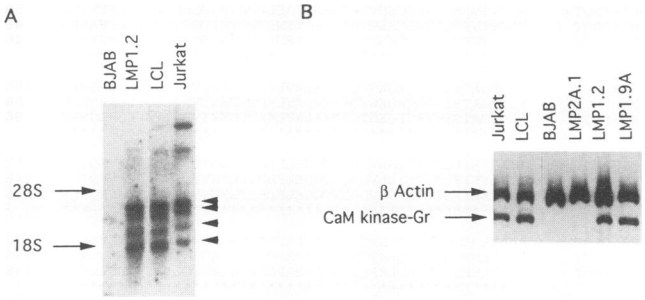

FIG. 6. Analysis of CaM kinase-Gr transcripts in lymphoid cell populations. (A) Northern blot analysis of CaM kinase-Gr transcripts in selected B- and T-lymphoid cell lines. Samples (5 μg) of poly(A)$^+$ mRNAs derived from BJAB, LMP1-expressing BJAB clone LMP1.2, lymphoblastoid cell line LCL, and the Jurkat T-cell line were applied per lane and probed with a randomly labeled cDNA probe spanning the entire coding region of human CaM kinase-Gr. Prominent hybridizing mRNA species are indicated by arrowheads, while the locations of the 18S and 28S rRNA bands are indicated by arrows. (B) Analysis of CaM kinase-Gr transcripts in lymphocyte cell lines by a radiolabeled PCR technique. Samples (2 μg) of total RNA from Jurkat, LCL, and BJAB cells and LMP2A-expressing BJAB clone LMP2A.1 and LMP1-expressing BJAB clones LMP1.2 and LMP1.9A were primed with random hexamers, reverse transcribed, and amplified by using CaM kinase-Gr-specific or β-actin-specific primers. PCR was carried out as detailed in Materials and Methods, and the amplified products were separated by electrophoresis on a 12% acrylamide gel.

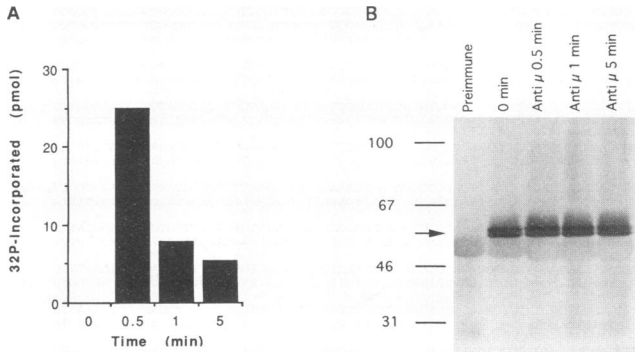

FIG. 7. Expression and activation of recombinant CaM kinase-Gr transcripts in BJAB cells. BJAB cells were transfected with plasmid pHGR, containing the coding sequence of human CaM kinase-Gr. At 72 h later, aliquots of transfected BJAB cells (2.5×10^7 cells per time point) were treated with goat anti-human IgM antibodies at 20 μg/ml for the periods indicated. The cells were then lysed, and CaM kinase-Gr immunoprecipitates were prepared and immunoblotted or assayed for Ca^{2+}/calmodulin-dependent kinase activity as described in Materials and Methods. (A) Activation of wild-type recombinant CaM kinase-Gr molecules following treatment of transfected BJAB cells with anti-surface IgM antibodies (Anti μ). The enzymatic activity data in panel B are means of three replicates done for each time point. The standard deviation was less than 10% of the mean. Results similar to those reproduced here were found in two other independent experiments. (B) Immunoblots of recombinant CaM kinase-Gr molecules immunoprecipitated from unstimulated and anti-IgM antibody-treated, transfected BJAB cells. CaM kinase-Gr immunoprecipitates were resolved on a denaturing 5 to 16% acrylamide gel, transferred to PVDF membranes, and immunoblotted with anti-CaM kinase-Gr sera as described in Materials and Methods. The prominent doublet represents CaM kinase-Gr isoforms (arrow), while the faint broad band is the rabbit IgG heavy chain. The numbers on the left are molecular size markers in kilodaltons.

An interesting feature of the enzyme's cDNA is the presence in the 5′-untranslated region of a stretch of seven CGG triplet repeats (bp 58 to 78). Size amplification of trinucleotide repeats in disease genes has been determined to be the underlying genetic defect in myotonic dystrophy, Kennedy disease, Huntington disease, and fragile X syndrome (17). Instability of CGG repeats has been specifically associated with the fragile X syndrome, a disease in which the affected gene, *FMR1*, exhibits dramatic expansion of these repeats (7). It remains to be established whether the CGG repeats in the CaM kinase-Gr 5′ region are stable or whether they vary in size between individuals and, if so, whether this is associated with a disease state(s).

Expression of CaM kinase-Gr mRNA was analyzed in human B and T lymphocytes by hybridization of poly(A)$^+$ RNA under stringent conditions with a cDNA probe encompassing the coding region of human CaM kinase-Gr. No hybridizing RNA species was detected in BJAB cells, whereas 2.3-, 3.5-, 4.6-, and 4.8-kb hybridizing RNA species were clearly and reproducibly detected in an LMP1-converted BJAB clone, in an EBV-transformed B-lymphoblastoid cell line, and in the Jurkat human leukemic T-cell line. Additionally, larger hybridizing RNA species were also apparent in all CaM kinase-Gr-expressing cell lines; however, their significance remains unknown. The multiplicity of mRNA transcripts that hybridized with a CaM kinase-Gr probe may be due to alternative splicing, the presence of several transcription initiation sites, and/or the presence of several polyadenylation sites.

Expression of CaM kinase-Gr transcripts in lymphoid cell populations was also analyzed by using a sensitive radiolabeled PCR method. Even by this technique, no CaM kinase-Gr transcripts could be detected in BJAB cells or in LMP2A-converted BJAB cells (Fig. 6B). CaM kinase-Gr transcripts were readily detected in LMP1-converted BJAB clones, in EBV-transformed lymphoblastoid cell lines, and in the human T-cell line Jurkat. These results indicated that LMP1 induces CaM kinase-Gr expression at the mRNA level.

Transfection of BJAB cells with the full-length CaM kinase-Gr cDNA under control of the simian virus 40 promoter and enhancer in pSG5 resulted in the expression of two closely migrating proteins which cross-reacted with anti-human CaM kinase-Gr serum and were identical in size to the enzyme proteins expressed in the Jurkat and the EBV-transformed human lymphoblastoid cell lines (Fig. 7 and data not shown). Since the cDNA has only one ATG codon near the beginning of the open reading frame and since plasmid expression of the cDNA in BJAB cells gives rise to both isoforms of the human kinase, the cDNA is likely to comprise the complete coding sequence and the isoforms are probably the product of post-translational processing. The recombinant kinase was functional, as evidenced by its enhanced activity following cross-linking of surface IgM molecules in transfected BJAB cells (Fig. 7). The time course of recombinant kinase activation in BJAB cells mirrored that noted for kinase molecules in LMP1-expressing BJAB clones: enzyme activity was highest at 30 s poststimulation and declined thereafter (compare Fig. 7A and 3A).

Since the antipeptide serum used for immunoblotting and immunoprecipitation of the human enzyme did not work in immunofluorescence experiments, we examined the intracellular localization in transfected BJAB cells of a recombinant human CaM kinase-Gr which had been tagged at its N terminus with the FLAG epitope (see Materials and Methods). The FLAG epitope-tagged kinase was similar to its wild-type counterpart in that it was also expressed as two isoforms (slightly shifted in molecular weight because of the additional

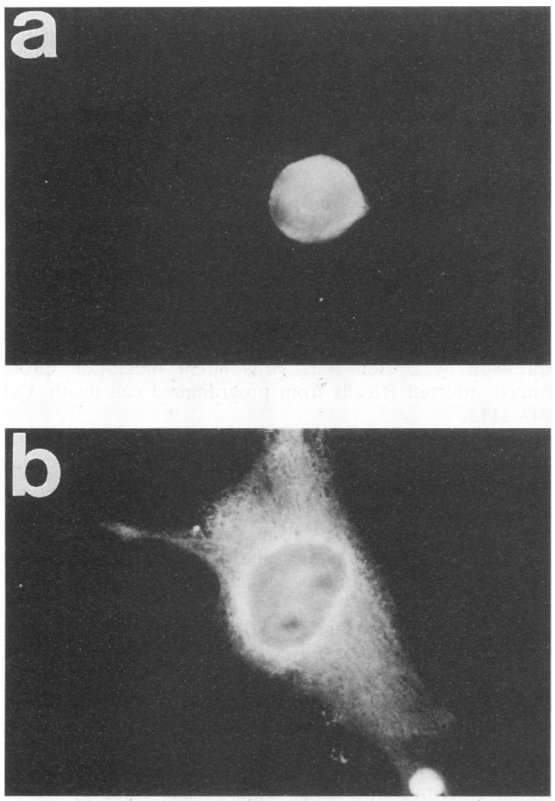

FIG. 8. Recombinant CaM kinase-Gr exhibits both nuclear and cytoplasmic localizations in transfected BJAB and COS cells. (a) Immunofluoresence analysis of BJAB transfectants. BJAB cells (1.5×10^7) were transfected with plasmid pHGR-FLAG, which codes for FLAG epitope-tagged human CaM kinase-Gr. At 18 h later, the cells were analyzed for CaM kinase-Gr expression with an anti-FLAG monoclonal antibody (Kodak) and fluorescein isothiocyanate-conjugated goat $F(ab')_2$ anti-mouse immunoglobulin, as detailed in Materials and Methods. (b) Immunofluorescence of COS cell transfectants. COS cells (1.5×10^7) were transfected with plasmid pHGR-FLAG, and CaM kinase-Gr expression was analyzed 2 days later, as described for panel a.

tag) and was similarly activated upon signalling via surface IgM molecules (data not shown). The FLAG epitope-tagged enzyme appeared to associate, in part, with cytoplasmic structures when expressed in BJAB cells (Fig. 8A). To better visualize the intracellular distribution of CaM kinase-Gr, we expressed the FLAG epitope-tagged kinase in COS cells to take advantage of the superior resolution allowed by the larger cytoplasmic compartment of these cells. The FLAG epitope-tagged kinase was associated with a fine cytoplasmic fibrillar network which was accentuated around the nucleus. Nuclei also stained, consistent with possible nuclear localization of the enzyme. However, the apparent nuclear staining could alternatively be due to the intensity of the perinuclear staining. Previous intracellular localization studies with rat neurons have demonstrated the localization of the rat enzyme to both cytoplasmic and nuclear compartments (13).

DISCUSSION

The present study demonstrates that CaM kinase-Gr, a Ca^{2+}/calmodulin-dependent protein, is inducible in human B lymphocytes by EBV transforming protein LMP1 and that the induced protein is fully functional, as evidenced by its responsiveness to Ca^{2+} signalling via surface immunoglobulin molecules. This protein kinase is not normally expressed in primary B lymphocytes; thus, its presence in EBV-transformed primary B lymphocytes suggests specific association with latent infection or the concomitant cell growth and transformation. The specific induction of the kinase mRNA in BJAB cell by LMP1 ties the kinase to the LMP1 pathway and strengthens the hypothesis that the kinase plays a role in the downstream mediation of LMP1 activating and/or transforming effects.

The CaM kinase-Gr from EBV-transformed B cells is nearly identical to the rodent enzymes in the first 351 amino acids of the enzyme, which include the amino-terminal regulatory domain, the catalytic domain, and in the calmodulin-binding domain. The last 122 amino acids of the human, rat, and mouse enzymes, which comprise most of the carboxyl-terminal associative domain, are significantly divergent. The carboxyl termini of the rodent enzymes are particularly rich in glutamate residues, a structural feature common to several nuclear proteins which may allow these proteins to associate with chromatin (4). Indeed, the rodent enzyme has been shown to localize, in part, to the nuclei of neurons (13). The C-terminal region of the human enzyme is less endowed with glutamate residues and is consequently less acidic. Nevertheless, the human enzyme also localizes in part to nuclear structures of BJAB and COS cells.

LMP1 is an integral membrane phosphoprotein which bears no direct homology to other signal-transducing membrane proteins. LMP1 is indespensible for viral transformation and is one of a restricted number of viral gene products that are expressed during viral latency (15, 38). LMP1 exerts profound effects on host cell growth. It transforms established rodent fibroblasts and renders B cells resistant to apoptosis. The latter effect may be mediated by bcl-2 and A20, whose expression is induced by LMP1 and which can protect B cells from apoptosis (12). LMP1 also upregulates the expression of several cell surface adhesion molecules in B cells, including LFA-1, LFA3, ICAM-1, and CD23 (39).

While the mechanisms by which this membrane-bound protein effects cellular activation and transformation are not well delineated, LMP1 may act in part by activating the function of nuclear transcription factors which, in turn, can initiate the transcription of LMP1-inducible genes. Evidence for such a mechanism has been provided in the case of the A20 zinc finger protein (18). This LMP1-inducible gene has been demonstrated to be transcriptionally activated in LMP1-expressing B cells through *cis*-acting sequences found within its promoter region which recognize nuclear transcription factor NF-κB. In the case of CaM kinase-Gr, it is likely that LMP1 upregulates the expression of this kinase by direct transcriptional activation rather than by a posttranscriptional mechanism, since no kinase RNA can be detected in BJAB cells by Northern (RNA) blot analysis or by a very sensitive PCR method. NF-κB could mediate the induction of CaM kinase-Gr expression by LMP1 as with the A20 gene.

CaM kinase-Gr is the first Ca^{2+}/calmodulin-dependent enzyme determined to be regulated by a viral gene product. Although the role CaM kinase-Gr plays in B-lymphocyte transformation by EBV is undefined, upregulated expression of CaM kinase-Gr may mediate some of the activating and/or growth-transforming effects of LMP1. The multifunctional Ca^{2+}/calmodulin-dependent protein kinase type II, whose substrate specificity overlaps that of CaM kinase-Gr (3, 26), has been recently reported to mediate cell cycle progression in fertilized *Xenopus* eggs by inactivating M-phase promoting and cytostatic factors (20). One can speculate that CaM kinase-Gr

plays a similar role in facilitating cell cycle progression in EBV-transformed B lymphocytes. The apparent cytoskeletal and perinuclear-nuclear localization of CaM kinase-Gr is compatible with its being a modulator of cell shape and/or motility and/or of the activity of a cytoskeleton-associated nuclear factor. Its capacity to influence cell growth and motility is also supported by the previously reported range of substrates of the rodent enzyme, which includes the $p21^{ras}$-related Rap1B GTP-binding protein (33), histone H1, myosin light chain- and microtubule-associated proteins MAP-2 and Tau (26), and transcriptional factors CREB (cyclic AMP response element-binding protein) and SRF (serum response factor) (3). Additional studies, including genetic approaches which target the expression and/or activity of the kinase, are required to delineate the role of CaM kinase-Gr in B-lymphocyte activation and transformation.

Finally, A role for CaM kinase-Gr in B-cell lymphomagenesis is further supported by the intriguing observation that while CaM kinase-Gr is absent from normal human B cells and from human pre-B-cell lines Reh and Nalm-6, it was found to be expressed in several of the EBV-negative BL cell lines examined (e.g., BL30, BL41, and Ramos), with the sole exception of BJAB (our unpublished observations). Unlike BJAB, which demonstrates no chromosomal translocations, all of the aforementioned BL cell lines bear the chromosome 8:14 translocations common to this malignancy (27, 32). Further studies are required to examine the link between chromosomal translocations in B-cell lymphomas, including BLs, and the expression of CaM kinase-Gr. It also remains to be established whether LMP1 induces expression of CaM kinase-Gr in epithelial cells, which are themselves a prime target of EBV transformation.

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