Activation Protein 1–Dependent Transcriptional Activation of Interleukin 2 Gene by Ca^{2+}/Calmodulin Kinase Type IV/Gr

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Summary

The Ca^{2+}/calmodulin–dependent protein kinase (CaMK) type IV/Gr is selectively expressed in T lymphocytes and is activated after signaling via the T cell antigen receptor (TCR), indicating that it mediates some of the Ca^{2+}-dependent transcriptional events that follow TCR engagement. Here we show that CaMKIV/Gr induces the transcription factor activation protein 1 (AP-1) alone or in synergy with T cell mitogens and with the $p21^{ras}$ oncoprotein. CaMKIV/Gr signaling is associated with transcriptional activation of *c-fos* but is independent of $p21^{ras}$ or calcineurin. AP-1 is an integral component of the nuclear factor of activated T cells (NFAT) transcriptional complex, which is required for interleukin 2 gene expression in T cells. We demonstrate that CaMKIV/Gr reconstitutes the capacity of the cytosolic component of NFAT to direct transcription from NFAT sites in non–T cells. These results reveal a central role for CaMKIV/Gr as a Ca^{2+}-regulated activator of gene transcription in T lymphocytes.

Ca^{2+} signaling pathways play a particularly important role in lymphocyte biology. Elevation of free intracellular Ca^{2+} concentrations after engagement of lymphocyte antigen receptors is required for a broad array of events in the course of lymphocyte development and activation. These include positive and negative selection of developing lymphocytes, induction of lymphocyte proliferation and lymphokine gene expression, and activation-induced cell death. Previous studies have identified the Ca^{2+}/calmodulin–dependent phosphatase calcineurin as an important mediator of Ca^{2+} signaling in lymphocytes (1, 2). Calcineurin is critical for the induction of lymphokine gene expression in response to T cell mitogens, and its inhibition by the immunosuppressive agents cyclosporin and FK506 leads to the suppression of lymphokine gene transcription (3).

In addition to calcineurin, lymphocytes express other Ca^{2+}/calmodulin–binding proteins, prominent among which are the multifunctional Ca^{2+}/calmodulin–dependent protein kinases (CaMKs)[1]. These serine/threonine–specific protein kinases, which include CaMKI, II, and IV/Gr, have broad substrate specificity and serve to regulate a wide array of cellular processes (4). Of these enzymes, CaMKIV/Gr is of special interest in that its expression is largely restricted to T lymphocytes and neurons (5, 6). CaMKIV/Gr is the product of a single gene and is expressed in tissues as two monomeric isoforms, both of which share a domain structure common to other CaMKs and that includes a catalytic domain, a calmodulin-binding domain, and an associative domain (7–10). Previous studies have documented the presence of CaMKIV/Gr in the nucleus (11, 12), and the kinase has been shown to phosphorylate and transactivate the transcription factor cAMP response element binding protein (12–15), establishing a role for CaMKIV/Gr as a Ca^{2+}-dependent transcriptional regulator.

In T lymphocytes, CaMKIV/Gr is selectively enriched in the $CD4^+$ subpopulation. When isolated from resting T cells it exhibits minimal catalytic activity even in the presence of Ca^{2+} and calmodulin. Its activity is strongly upregulated upon antigen receptor stimulation (6, 16, 17) by a mechanism involving a protein kinase cascade (17, 18). This indicated that CaMKIV/Gr may play a role in Ca^{2+}-dependent transcriptional events triggered by antigen receptor signaling that are critical to the helper functions of T cells, namely the production of lymphokines. In this study, we examined the capacity of CaMKIV/Gr to activate lymphokine gene transcription using the IL-2 promoter as a model. Our results demonstrate that CaMKIV/Gr plays an important role in promoting IL-2 gene transcription, in part

[1] *Abbreviations used in this paper:* AP-1, activation protein 1; CaMK, Ca^{2+}/calmodulin–dependent protein kinase; mutants: CaMKII(c), constitutively active CaMKII-γ_B; CaMKIV/Gr(i), inactive CaMKIV/Gr; CaMKIV/Gr(wt), wild-type CaMKIV/Gr; ΔCaMKIV/Gr(c), constitutively active CaMKIV/Gr; ΔCaMKIV/Gr(i), double mutant constitutive/inactive; CAT, chloramphenicol acetyl transferase; NFATc, cytosolic component of NFAT; NFAT, nuclear factor of activated T cells.

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by mediating a novel, Ca^{+2}-dependent pathway for the induction of the transcription factor activation protein 1 (AP-1).

Materials and Methods

Cells. Jurkat Tag cells are derived from the Jurkat human leukemia T cell line and stably express the SV40 large T antigen (2). They were maintained in RPMI 1640 medium supplemented with 10% FCS. BJAB, an EBV-negative Burkitt lymphoma B cell line, and HeLa, a cervical adenocarcinoma cell line, were maintained in DMEM medium supplemented with 10% FCS.

Plasmids. FLAG epitope–tagged wild-type human CaMKIV/Gr (CaMKIV/Gr[wt]) was derived and subcloned into pSG5 vector as described (11). CaMKIV/Gr(wt) contains an eight–amino acid FLAG epitope at its NH_2 terminus to allow detection by immunoblotting. Inactive CaMKIV/Gr (CaMKIV/Gr[i]) was prepared by substituting the conserved Lys75 in the ATP-binding site of CaMKIV/Gr(wt) with a Glu residue by site-directed mutagenesis using the unique site elimination method (19). The constitutively active CaMKIV/Gr mutant ΔCaMKIV/Gr(c) was prepared by replacing the Gln318 codon of CaMKIV/Gr(wt) with a stop codon. ΔCaMKIV/Gr(c) does not bind to and is not activated by Ca^{2+}/calmodulin. The double mutant constitutive/inactive ΔCaMKIV/Gr(i) was prepared by mutating Lys75 of ΔCaMKIV/Gr(c) into a Glu. cDNA of all CaMKIV/Gr mutants were sequenced and ascertained to be free of errors. The constitutively active human CaMKII-γ_B mutant (CaMKII[c]) was a kind gift of Dr. Howard Schulman (Stanford University, Stanford, CA) and was generated by substituting Thr287 in the autoinhibitory domain with an Asp residue (20). CaMKII(c) was subcloned into the EcoRI site of pSG5 for use in our studies.

The plasmid IL-2 luciferase contains bp −326 to +45 of the human IL-2 enhancer/promoter directing the transcription of a luciferase reporter gene (21). Nuclear factor of activated T cells (NFAT) luciferase contains three copies of the NFAT site in the human IL-2 gene (21), and AP-1 luciferase contains two copies of the AP-1 site in the rodent α chorionic gonadotropin gene (22). The plasmid CMV-luciferase contains a luciferase gene under the control of the immediate early promoter region of CMV (23). The plasmid c-*fos*-chloramphenicol acetyl transferase (CAT) contains bp −356 to +109 of the c-*fos* promoter coupled to a CAT gene (24). The plasmids pEF-v-Ha-*ras* and pEF-Ha-*ras*-N17 ras encode a constitutively active and a catalytically inactive mutant of p21Ha-ras, respectively (25). cDNA encoding the cytoplasmic component of NFAT (NFATc) was fitted with EcoRI linkers and subcloned into the EcoRI site of the vector pBJ5 (26).

In Vitro Transcription/Translation Studies. In these studies, 2 μg of cDNA encoding either wild-type or mutant CaMKIV/Gr proteins were transcribed and translated in vitro using the TNT transcription and translation system (Promega Corp., Madison, WI) according to the instructions of the manufacturer. When indicated, the proteins were metabolically labeled during the translation process by including 4 μCi of [^{35}S]methionine in the transcription/translation reaction mixture. The labeled products were resolved on a 10% denaturing acrylamide gel and visualized by autoradiography.

Assay of Recombinant Kinase Activity. Recombinant CaMKIV/Gr and CaMKII(c) proteins were expressed by in vitro transcription translation, as described above. Kinase assays were carried out for 10 min at 23°C in a final volume of 50 μl containing 25 μl of the transcription/translation reaction and 25 μl of a kinase reaction mixture consisting of 20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 50 μM ATP, 4 μCi γ-[^{32}P]ATP, 20 μM of the substrate peptide syntide-2 (Bachem, Torrance, CA), and either 5 mM $CaCl_2$ and 600 nM calmodulin (Pharmacia, Piscataway, NJ) or 5 mM EGTA. The reaction was terminated by spotting 25 μl of the mixture onto P81 phosphocellulose filters (Whatman, Maidstone, UK). The filters were washed three times in 0.5% phosphoric acid, allowed to air dry, and counted.

Detection of Recombinant Kinase Proteins in Cells. Expression of recombinant CaMKIV/Gr in cells was analyzed by immunoblotting using an anti-FLAG epitope antibody (Eastman Kodak Co., Rochester, NY). Cells transfected with CaMKIV/Gr constructs were lysed on ice for 15 min in a buffer containing 25 mM Hepes, pH 7.5, 0.5% NP40, 50 mM NaCl, 25 mM NaH_2PO_4, 25 mM NaF, 12.5 mM $Na_4P_2O_7$, 2 mM EDTA, 2 mM EGTA, 1 mM Na_3VO_4, 100 mg/ml PMSF, and 10 mg/ml of the protease inhibitor leupeptin. The lysates were cleared of nuclei and other insoluble material by centrifugation at 16,000 *g* for 30 min at 4°C. 100 μg of the lysate proteins was resolved by SDS-PAGE and then electroblotted onto nitrocellullose membranes. The filters were incubated for 1 h at room temperature in a Tris-buffered saline solution (TBST; 20 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween 20 detergent) containing 5% nonfat milk; they were then washed and incubated for 1 h more with M2 anti-FLAG epitope antibody at 1 μg/ml in TBST solution. The filters were then washed and incubated with horseradish peroxidase–conjugated goat anti–mouse IgG (Amersham Corp., Arlington Heights, IL). The blots were subsequently washed and developed using the enhanced chemiluminescence system for peroxidase-based detection (Amersham Corp.).

Endogenous CaMKIV/Gr in cells was also detected by immunoblotting as detailed above but using as a primary antibody 1:1,000 dilution of a rabbit antiserum that is directed against the terminal 17 amino acids of the human enzyme (27).

Expression of recombinant CaMKII(c) was visualized by calmodulin overlay blotting (28). Filters were incubated with biotinylated calmodulin at 1 μg/ml in TBST solution containing $CaCl_2$ at 0.5 mM and $MgCl_2$ at 50 mM, and the two salts were also included in the entire immunoblotting process. After washing, the blots were incubated with horseradish peroxidase–conjugated streptavidin (Amersham Corp.), then washed again and developed using the enhanced chemiluminescence system.

Transfections and Reporter Assays. Jurkat Tag cells were suspended at 1.5×10^7 cells in 10% FCS RPMI medium, mixed with the indicated plasmids, then transfected by electroporation at 250 V and 960 μF. BJAB cells were suspended at 10^7 cells in 10% FCS DMEM medium and transfected by electroporation at 220 V and 960 μF. HeLa cells were transfected by calcium phosphate precipitation. After transfection, cells were handled as detailed in the figure legends. Luciferase and CAT activities were assayed as previously described (29, 30). Results were normalized for protein content and are expressed as fold induction over values obtained in cells transfected with reporter gene and empty vectors.

Electrophoretic Mobility Shift Assays. Nuclear extracts were prepared and electrophoretic mobility shift assays were conducted as previously described, with modifications (30). Cells were harvested at 48 h after transfection. For PMA induction of AP-1, cells were stimulated with 25 ng/ml of PMA for 1 h before harvesting. The cells were washed in PBS and then lysed in a buffer containing 10 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithioerythritol, 0.2 mM PMSF, 10 μg/ml leupeptin, and 0.025% NP40. The nuclei were pelleted and then extracted in a buffer containing 20 mM Hepes, pH 7.4, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM dithioerythritol, 0.2 mM EDTA, 0.2 mM

PMSF, 10 μg/ml leupeptin, and 5% glycerol. For electrophoretic mobility shift assays, 5 μg of nuclear extracts was incubated together with 0.1 ng of ^{32}P-labeled double stranded monomers of the indicated response elements in a buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM dithioerythritol, 0.5 mM EDTA, 5% glycerol, and 0.5–1 μg/ml poly(dI-dC). Gel shift probes used in these studies included: the AP-1–binding site found in the human metalothionein promoter, 5′-GATCCGTGACTCAGCGCGG-3′ (31); a mutant oligonucleotide derived from the aforementioned AP-1 site (mAP-1) that displays poor affinity for AP-1, 5′-GATCCAAGACTCTGCGCGG-3′ (32); and the murine IL-2 distal NFAT-binding site, 5′-GATCGCCCAAAGAGGAAAATTTGTTTCATACAG-3′ (33). For competition experiments, 100-fold molar excess of unlabeled oligonucleotide was included in the reaction mixture. After 30 min of incubation at 23°C, the reaction mixture was resolved on a prerun 5% nondenaturing polyacrylamide gel, and the protein–DNA complexes were visualized by autoradiography.

Results

Characterization of CaMKIV/Gr Constructs. To examine the role of CaMKIV/Gr in T cell activation, we generated cDNA constructs encoding recombinant wild-type and mutant CaMKIV/Gr proteins and examined the capacity of these proteins to recapitulate some of the activation events associated with Ca^{2+} signaling in T cells. Proteins encoded by these constructs include CaMKIV/Gr(wt) and its inactive counterpart CaMKIV/Gr(i), ΔCaMKIV/Gr(c) and the constitutively active mutant and its inactive counterpart ΔCaMKIV/Gr(i). We have also utilized in our studies a construct generated by Nghiem et al. (20), CaMKII(c). It is an isoform of CaMKII that is expressed in several tissues, including human T lymphocytes. CaMKII(c) binds to and its constitutive activity is upregulated by Ca^{2+}/calmodulin, and it was used in our studies as a kinase specificity control. The characteristics of recombinant CaMKIV/Gr and CaMKII(c) proteins are illustrated in Fig. 1. Fig. 1 *A* shows the migration profile on SDS/PAGE of recombinant CaMKIV/Gr and CaMKII(c) proteins that have been expressed in vitro and labeled with [^{35}S]methionine using a rabbit reticulocyte lysate transcription translation system. CaMKIV/Gr(wt) and CaMKIV/Gr(i) resolved as a 58-kD band on SDS/PAGE. CaMKII(c) resolved as a single 60-kD band, whereas both ΔCaMKIV/Gr(c) and ΔCaMKIV/Gr(i) resolved as a 40-kD band. Fig. 1 *B* demonstrates the activity profile of the respective recombinant kinase proteins. Specifically, CaMKIV/Gr(wt) mediated Ca^{2+}/calmodulin–dependent phosphorylation of substrate peptide whereas ΔCaMKIV/Gr(c) exhibited constitutive activity independent of Ca^{2+}/calmodulin. In contrast, CaMKIV/Gr(i) and ΔCaMKIV/Gr(i) were both inactive. CaMKII(c) exhibited constitutive activity that was further upregulated by Ca^{2+}/calmodulin.

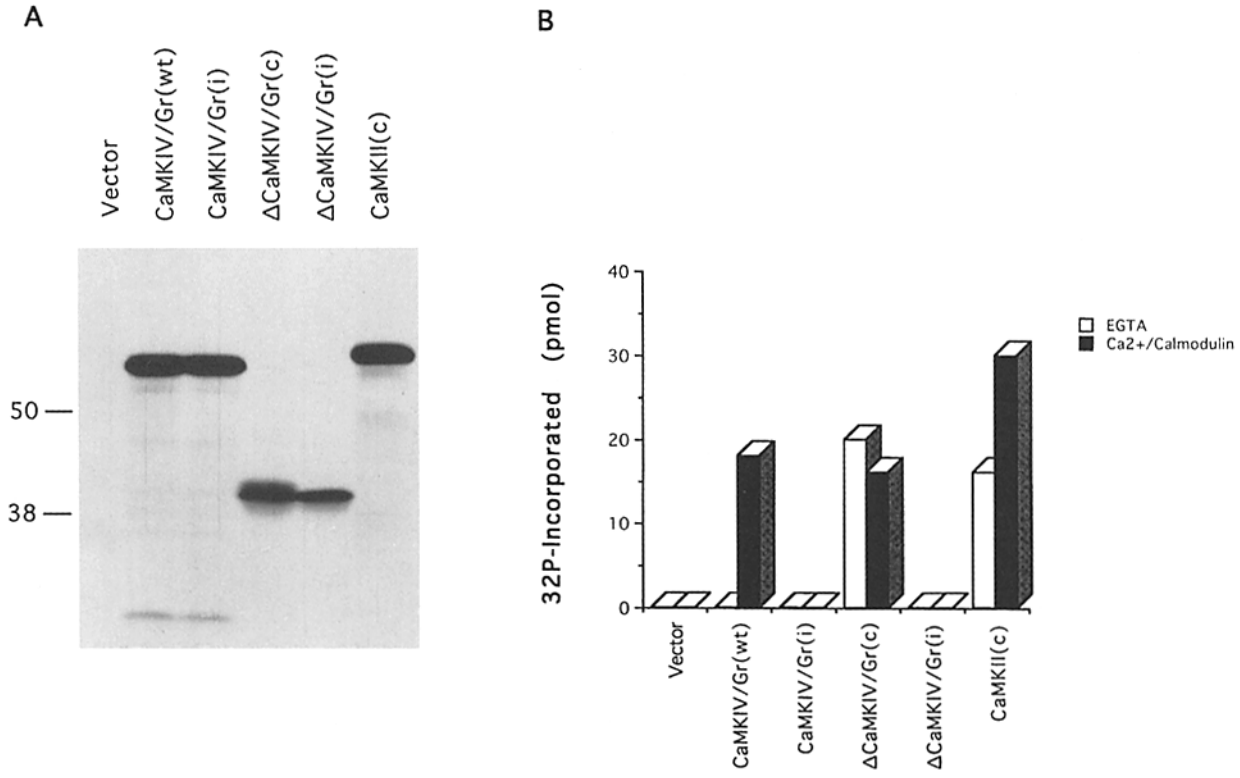

Figure 1. Characterization of recombinant CaMK proteins. (*A*) SDS/PAGE analysis of ^{35}S-labeled, in vitro transcribed and translated recombinant CaMKIV/Gr and CaMKII(c) proteins. The numbers on the left represent mol wt markers. (*B*) Phosphorylation of the substrate peptide syntide-2 by in vitro transcribed and translated recombinant CaMK proteins. Results are expressed as picomoles of phosphate incorporated into peptide substrate over the course of the reaction and represent means of triplicate determinations; similar results were found in two other experiments.

We next examined the expression of CaMK constructs in transfected Jurkat cells by immunoblotting and/or calmodulin overlay assays. Fig. 2 *A* demonstrates that both CaMKIV/Gr(wt) and CaMKIV/Gr(i) were expressed as two monomeric α/β bands with molecular masses of 58 and 60 kD, respectively. ΔCaMKIV/Gr(c) was expressed as two bands migrating with molecular masses of 40 and 42 kD. The latter band represents an autophosphorylated form of the 40-kD species (Chatila, T., unpublished observations). ΔCaMKIV/Gr(i) was expressed at lower levels as a single 40-kD band. In separate studies, we established that three independent inactivating mutations all resulted in decreased expression of ΔCaMKIV/Gr(c), indicating that the low level of expression of ΔCaMKIV/Gr(i) in Jurkat cells reflects a requirement for an autophosphorylation event to stabilize this mutant (Chatila, T., and A.R. Means, manuscript in preparation). We have also determined that when expressed at levels comparable with those of ΔCaMKIV/Gr(i), the constitutively active kinase mutant exhibited activity equivalent to ~15% of that found with unrestricted ΔCaMKIV/Gr(c) expression. In some experiments (e.g., Fig. 2 *A*), low levels of a split product of CaMKIV/Gr(wt) could be detected that comigrated with ΔCaMKIV/Gr(c). Fig. 2 *B* demonstrates that CaMKII(c) was well expressed in Jurkat cells as detected by calmodulin overlay assays. In separate studies, it could be demonstrated by calmodulin overlay assays that the levels of CaMKII(c) achieved in lymphoid and nonlymphoid cells closely approximated those of recombinant CaMKIV/Gr(wt) (data not shown).

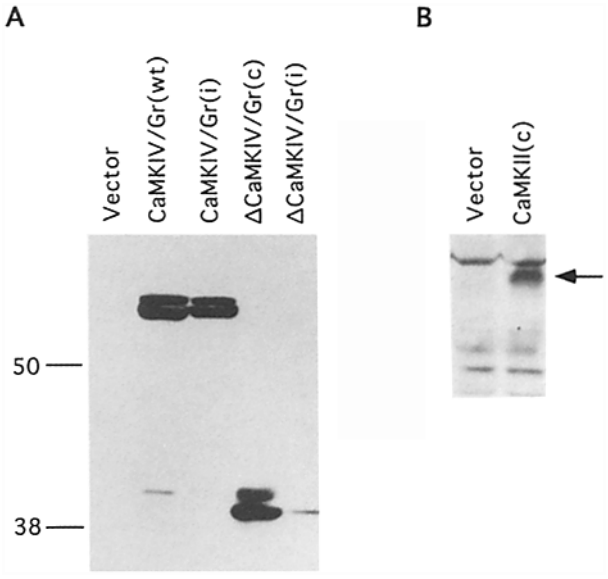

Figure 2. Expression of wild-type and mutant CaMKIV/Gr and CaMKII(c) in Jurkat cells. (*A*) Expression of CaMKIV/Gr derivatives. 100 μg of protein lysates derived from cells that had been transiently transfected with the indicated CaMKIV/Gr construct were resolved by SDS/PAGE and probed for kinase expression using an anti-FLAG epitope antibody, as described in Materials and Methods. (*B*) Expression of CaMKII(c) in Jurkat cells. Lysates derived from cells transfected with pSG5 vector alone or with CaMKII(c) in pSG5 were resolved by SDS/PAGE and probed for calmodulin-binding proteins as described in Materials and Methods. (*Arrow*) Position of the CaMKII(c) band.

Activation of AP-1–dependent Transcription by CaMKIV/Gr. Ca^{2+} signaling pathways have previously been implicated in the activation of AP-1 in T lymphocytes by mechanisms that include transcriptional activation of *c-fos* (34) and posttranscriptional activation of a c-Jun kinase termed JNK or stress-activated protein kinase (35, 36). To determine the capacity of CaMKIV/Gr to activate AP-1–dependent transcription, Jurkat cells were transiently cotransfected with the respective CaMKIV/Gr construct together with an AP-1–responsive luciferase reporter gene, and the cells were subsequently examined for luciferase activity. Fig. 3 *A* demonstrates that transfection of Jurkat cells with a construct encoding CaMKIV/Gr(wt) resulted in an eightfold upregulation of luciferase activity. Furthermore, transfection of a cDNA construct encoding a constitutively active form of the kinase resulted in 50-fold induction of luciferase activity. Significantly, both wild-type and the constitutively active kinase mutant acted in synergy with the phorbol ester PMA and with the TCR-stimulating lectin PHA to upregulate the induction of AP-1 reporter gene expression (Fig. 3 *B*). In contrast, inactive CaMKIV/Gr mutants derived from the wild-type and constitutively active kinase species failed to induce the transcription of the AP-1 reporter or to enhance PMA or PHA-induced reporter gene expression.

The multifunctional CaMKII is similar to CaMKIV/Gr in that both are homologous, Ca^{2+}/calmodulin–dependent protein kinases that exhibit broad substrate specificity and that phosphorylate the same minimal consensus substrate sequence (RXXS/T) (4). We therefore examined the capacity of CaMKII(c) for its capacity to induce AP-1. We found that CaMKII(c) failed to induce AP-1 activation in transfected Jurkat cells (Fig. 3 *A*) or to upregulate induction by phorbol esters and PHA of AP-1 reporter expression (Fig. 3 *B*). Other reports have documented moderate inhibition of AP-1 activation by CaMKII(c) (37, 38). The specificity of CaMKIV/Gr induction of AP-1 reporter gene expression was also ascertained by the failure of ΔCaMKIV/Gr(c) to upregulate the transcription of a luciferase reporter gene driven by the CMV immediate early gene promoter or to enhance the upregulation of the CMV promoter activity by PMA and PHA (Fig. 3 *C*).

Induction of AP-1–dependent transcription by CaMKIV/Gr was associated with increased AP-1 DNA-binding activity. Electrophoretic mobility shift assays performed using a radiolabeled AP-1–binding oligonucleotide probe revealed enhanced AP-1 DNA-binding activity in nuclear extracts of ΔCaMKIV/Gr(c)-transfected Jurkat cells as compared with those extracts isolated from cells transfected with vector alone or with ΔCaMKIV/Gr(i) (Fig. 4). The increase in AP-1 DNA-binding activity by ΔCaMKIV/Gr(c) was less than that observed upon treatment of Jurkat cells with PMA. This can be explained in part by the low number of Jurkat cells successfully transfected with ΔCaMKIV/Gr(c) (typically 20–30%) as compared with 100% of cells being amenable to PMA treatment.

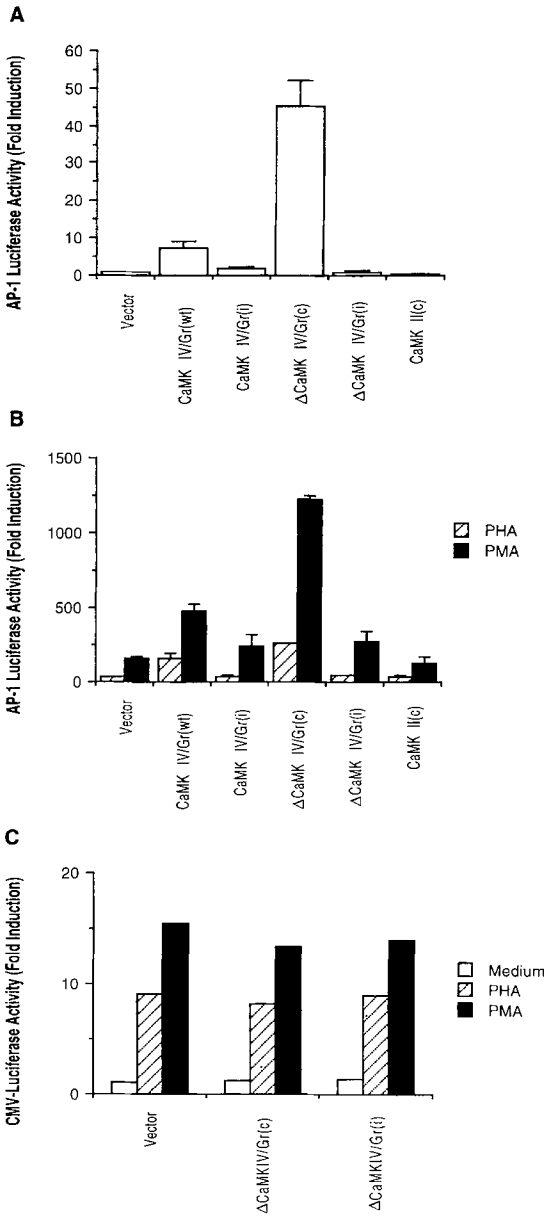

Figure 3. Activation of AP-1 by CaMKIV/Gr. (*A*) CaMKIV/Gr independently activates AP-1–dependent gene transcription in T cells. Jurkat Tag cells were transiently cotransfected with 5 μg of AP-1-luciferase reporter gene together with 30 μg of empty pSG5 vector (*Vector*) or pSG5 encoding wild-type CaMKIV/Gr (*CaMKIV/Gr[wt]*), inactive CaMKIV/Gr (*CaMKIV/Gr[i]*), constitutively active CaMKIV/Gr (*ΔCaMKIV/Gr[c]*), double mutant constitutive/inactive (*ΔCaMKIV/Gr[i]*), or a constitutively active CaMKII-γ_B mutant (*CaMKII[c]*). Transfected cells were incubated for 48 h, then harvested and assayed for luciferase activity. (*B*) CaMKIV/Gr upregulates AP-1 activation by T cell mitogens. Jurkat Tag cells cotransfected with the AP-1 reporter gene and the indicated constructs were stimulated for 6 h with PHA-P (10 μg/ml) or PMA (25 ng/ml). (*C*) CaMKIV/Gr does not activate transcription from the CMV immediate early gene promoter. Jurkat Tag cells were cotransfected with 0.5 μg of CMV-luciferase reporter gene (23); 30 μg of the indicated constructs were then stimulated for 6 h with PHA-P or with PMA. Data in *A* and *B* represent means ± SEM of three to five independent experiments. Data in *C* are representative of two independent experiments.

Previous studies have demonstrated that p21^{ras}-regulated signaling pathway(s) are involved in TCR coupling to AP-1 activation (39). To examine the potential role of p21^{ras} in CaMKIV/Gr-dependent induction of AP-1, we monitored AP-1 activity in Jurkat cells cotransfected with CaMKIV/Gr and either a mutationally activated or a dominant negative p21^{ras} mutant. Fig. 5 *A* demonstrates that CaMKIV/Gr acted in synergy with activated p21^{ras}, encoded by v-Ha-*ras*, in inducing AP-1–dependent transcription. This result agrees with the data shown in Fig. 3 *B* since both PHA and PMA have been shown to convert p21^{ras} to its active, GTP-bound state and thereby activate p21^{ras}-coupled pathways. However, Fig. 5 *B* demonstrates that CaMKIV/Gr induced AP-1 activation by mechanism(s) that were largely independent of p21^{ras} since cotransfection of the dominant negative p21^{ras} mutant Ha-*ras*-N17 resulted in only modest inhibition (<20% in three independent experiments). In contrast, and in agreement with previous studies, the Ha-*ras*-N17 mutant inhibited the induction of AP-1 activity by PMA by ~85%.

A possible mechanism by which Ca^{2+} signaling pathways may activate AP-1 is through induction of c-*fos* transcription (34). The capacity of CaMKIV/Gr to activate c-*fos* transcription was examined by cotransfecting kinase derivatives and a reporter gene regulated by the c-*fos* enhancer/

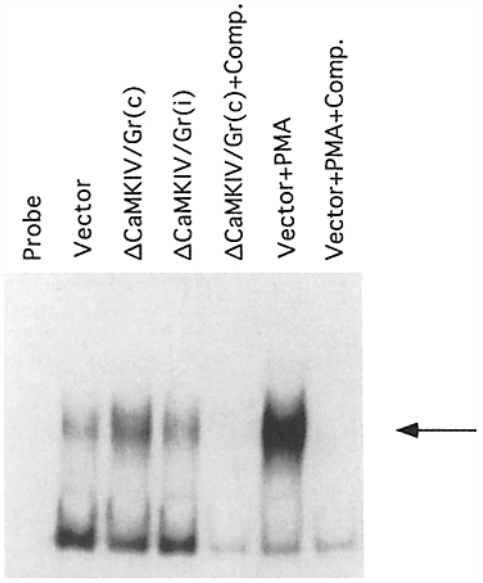

Figure 4. Upregulation of nuclear AP-1 DNA-binding activity by CaMKIV/Gr. Nuclear extracts were prepared from Jurkat Tag cells that were transiently transfected with either pSG5 vector (*Vector*) or with the indicated kinase cDNA. 5 μg of the respective extract was incubated with ^{32}P-labeled AP-1 oligonucleotide in the absence or presence of excess unlabeled competitor AP-1 oligonucleotide (*Comp.*), and the bound complexes were resolved on 5% nondenaturing acrylamide gel. (*Vector + PMA*) Nuclear extracts derived from Jurkat Tag cells that were transfected with pSG5 vector and that were treated with PMA at 25 ng/ml for 1 h before cell lysis. (*Probe* lane) Radiolabeled reaction mixture incubated in the absence of nuclear proteins. (*Arrow*) Specific AP-1/DNA complexes. The lower band represents a nonspecific DNA–protein complex, while unbound radiolabeled probe was allowed to run out of the gel. Results are representative of two independent experiments.

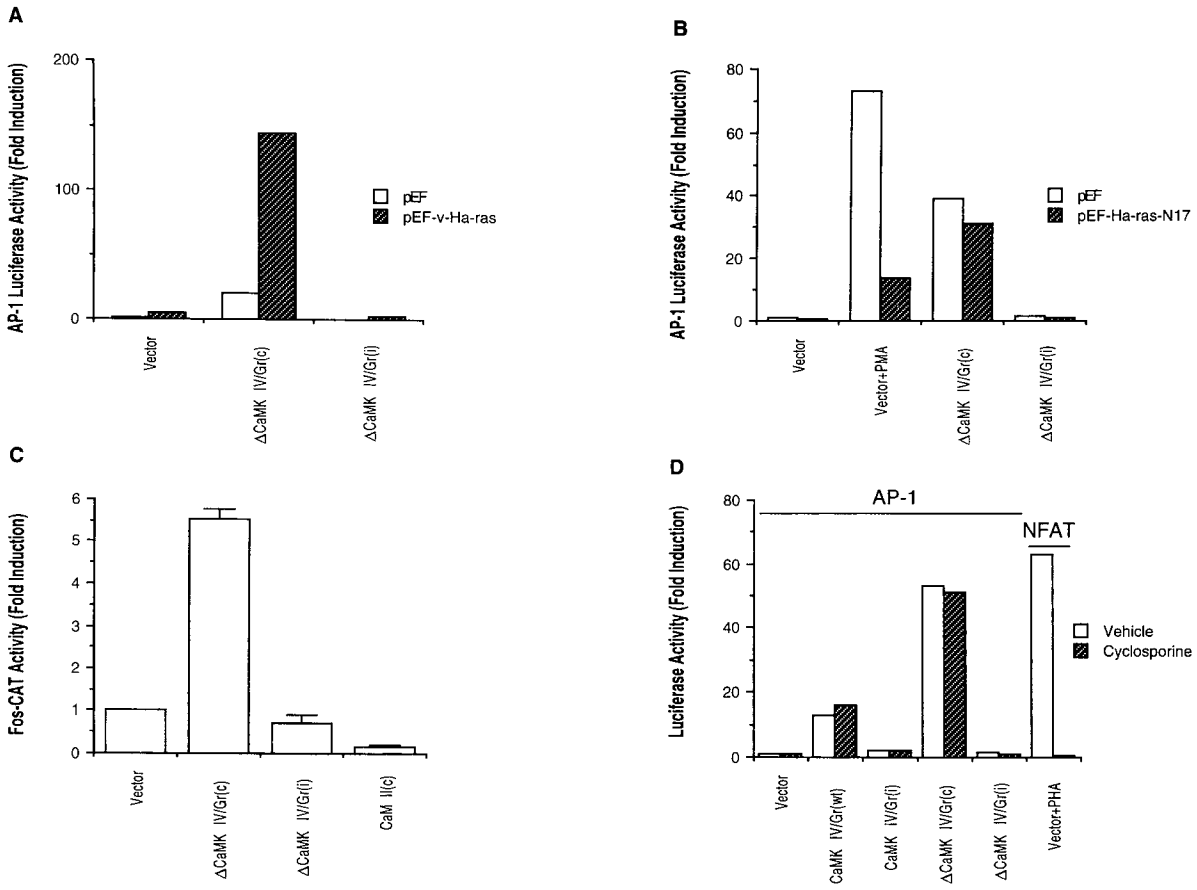

Figure 5. Characterization of AP-1 activation by CaMKIV/Gr. (*A*) Synergistic activation of AP-1 by CaMKIV/Gr and $p21^{v\text{-}Ha\text{-}ras}$. Jurkat Tag cells were cotransfected with 5 μg of the AP-1 reporter gene and 30 μg of the respective kinase construct together with 30 μg of empty pEF vector or pEF containing the constitutively active $p21^{ras}$ mutant v-Ha-*ras* (pEF-vHa-*ras*) (25). The cells were incubated for 48 h in the absence of any added stimulus; they were then harvested and assayed for luciferase activity. Data are expressed as fold induction over levels achieved upon cotransfection of AP-1 reporter gene together with empty pSG5 and pEF vectors. (*B*) Resistance of AP-1 activation by CaMKIV/Gr to inhibition by Ha-*ras*-N17. Jurkat Tag cells were cotransfected as per *A* with AP-1 reporter and the indicated kinase constructs together with empty pEF vector or pEF containing the dominant negative $p21^{ras}$ mutant Ha-*ras* N17 (pEF-Ha-*ras*-N17). PMA induction of AP-1 was performed by treatment with 25 ng/ml of PMA during the last 6 h of the incubation period. (*C*) Transcriptional activation of c-*fos* enhancer/promoter by CaMKIV/Gr. Jurkat Tag cells were cotransfected with 5 μg of c-*fos* (−356 to +109)–CAT reporter gene (24), and 30 μg of the indicated kinase construct was then harvested and assayed for CAT activity. (*D*) Resistance of AP-1 activation by CaMK IV/Gr to inhibition by cyclosporin A. Jurkat Tag cells were cotransfected with either the AP-1 or NFAT reporter gene (21) together with the indicated kinase construct and were thereafter treated for 48 h in culture with cyclosporin A at 100 ng/ml or with solvent alone. Cells transfected with the NFAT luciferase reporter gene were treated with PHA-P at 10 μg/ml during the last 6 h of the incubation period to induce reporter expression. Data presented in *A*, *B*, and *D* are representative of at least three independent experiments for each panel, and data in *C* are means values ± SEM of three experiments.

promoter. Fig. 5 *C* demonstrates that ΔCaMKIV/Gr(c) is a potent inducer of c-*fos* reporter gene transcription in Jurkat cells. In contrast, CaMKII(c) inhibited (>90%) basal transcription of the c-*fos* reporter.

Ca^{2+} may also act in synergy with phorbol esters to activate AP-1 in T cells by a calcineurin-dependent mechanism that involves JNK/SAPK, a member of the mitogen-activated protein kinase family that phosphorylates c-Jun and upregulates its transcriptional activity (35, 36). If calcineurin were to be involved in CaMKIV/Gr–dependent activation, then it follows that the response would be sensitive to the immunosuppressive drug cyclosporin A. Fig. 5 *D* demonstrates that this was not the case, since AP-1 activation by CaMKIV/Gr is cyclosporin A resistant, whereas the drug, as expected, blocks PHA induction of a NFAT-driven reporter gene. This result agrees with our observation that TCR-dependent activation of CaMKIV/Gr is cyclosporin A resistant (data not shown).

Activation of NFAT-dependent Transcription in T Cells by CaMKIV/Gr. The transcriptional complexes NFAT and NFIL2-A regulate lymphokine gene transcription in T cells and contain AP-1 components that are essential for the function of these complexes (40, 41). In the case of NFAT, NFATc (26) translocates to the nucleus to combine with an AP-1 (nuclear) component (21, 33, 42) and activates transcription from specific response elements. Assembly and activation of NFATc in the nucleus is thought to reflect the action of two signaling pathways: *(a) a* Ca^{2+}-dependent pathway involving the Ca^{2+}/calmodulin–dependent phosphatase calcineurin and mediating NFATc translocation to

the nucleus (43); and (*b*) a PKC- and $p21^{ras}$-dependent pathway mediating activation of AP-1 (44). To determine if AP-1 activation by CaMKIV/Gr is associated with increased NFAT activity, we examined if CaMKIV/Gr modulates basal and mitogen-activated transcription from a NFAT-driven repoter gene transfected into Jurkat cells. Fig. 6 *A* demonstrates that CaMKIV/Gr upregulated the activation of NFAT-dependent transcription by PMA and the Ca^{2+} ionophore ionomycin. In contrast, and in agreement with previous studies (37, 38), the constitutively active CaMKII(c) moderately inhibited NFAT activation under the same conditions. The observed synergy between CaMKIV/Gr and ionomycin in inducing NFAT-dependent transcription is of interest since it suggests synergistic interaction between calcinuerin, which mediates NFATc translocation to the nucleus, and CaMKIV/Gr, which activates AP-1. This interpretation is in agreement with the previously reported capacity of transfected AP-1 components, especially *c-fos*, to promote NFAT activation by ionomycin in Jurkat cells (21).

In line with the activation of NFAT-driven transcription, CaMKIV/Gr also enhanced the transcriptional activation by PMA and ionomycin of a reporter gene driven by the complete IL-2 enhancer/promoter and transfected into Jurkat cells. This effect was more pronounced in the presence of suboptimal concentrations of PMA, in agreement with the synergistic induction of AP-1 by PMA and CaMKIV/Gr (Fig. 6 *B*).

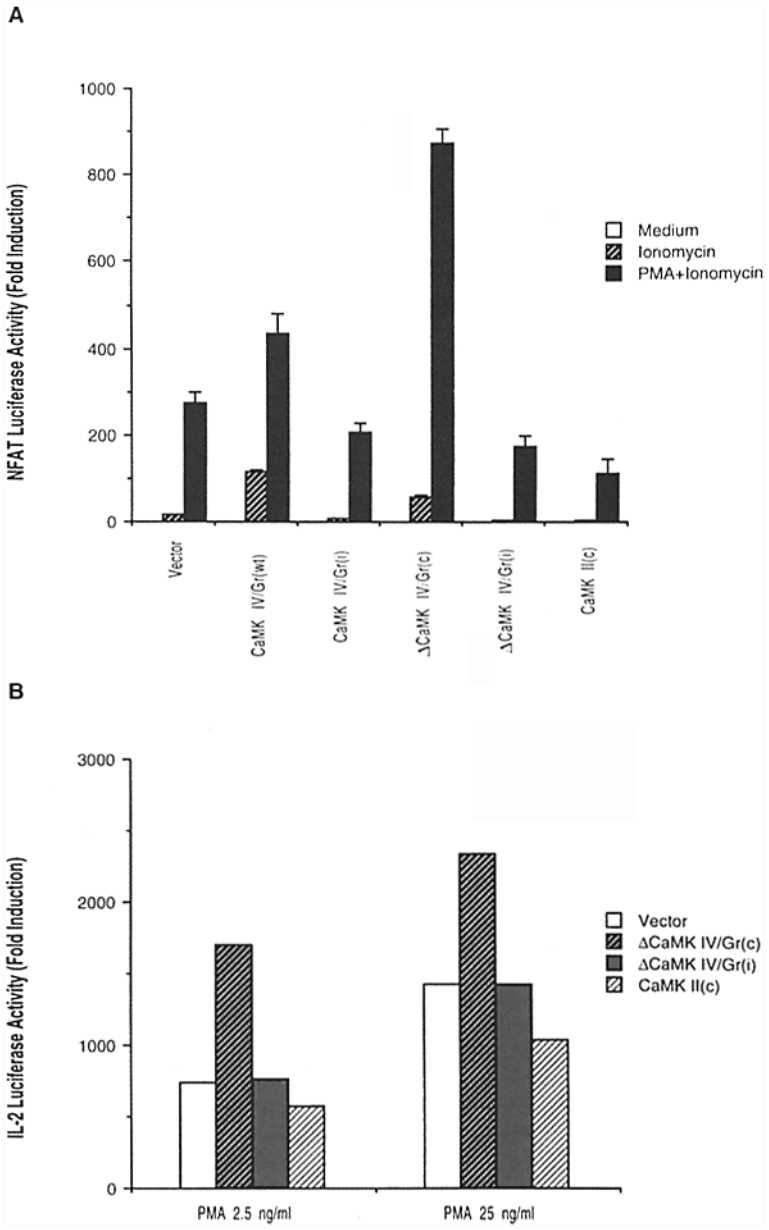

Figure 6. Upregulation of NFAT and IL-2 enhancer/promoter activities by CaMKIV/Gr in Jurkat cells. (*A*) Jurkat Tag cells were cotransfected with 5 μg of NFAT luciferase reporter together with 30 μg of pSG5 vector or pSG5 vector containing the indicated kinase construct. The cells were incubated for 48 h and thereafter treated for 6 h with either medium alone, ionomycin at 1 μM, or with PMA at 25 ng/ml and ionomycin at 1 μM. Results are means of three experiments ± SEM and are expressed as fold induction over values obtained with empty pSG5 vector alone. (*B*) Jurkat Tag cells were cotransfected with an IL-2 enhancer/promoter–driven luciferase reporter gene (21) and the indicated kinase construct and incubated as above; they were then stimulated with ionomycin at 1 μM and PMA at 2.5 or 25 ng/ml, as indicated. Data are representative of three independent experiments.

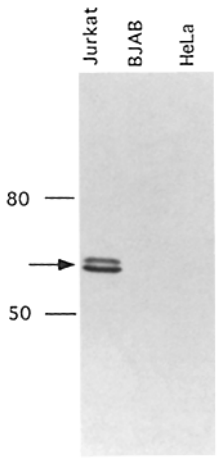

Figure 7. Immunoblot analysis of CaMKIV/Gr expression in Jurkat, BJAB, and HeLa cells. 100 μg of protein lysates derived from the respective cell populations was resolved by SDS/PAGE and probed for endogenous CaMKIV/Gr using a polyclonal rabbit anti–human CaMKIV/Gr antiserum, as described in Materials and Methods. (*Arrow*) Position of the two CaMKIV/Gr isoforms.

Reconstitution of NFAT-dependent Transcription in Non–T Cells by CaMKIV/Gr and NFATc. Endogenous CaMKIV/Gr in Jurkat cells is activated by mitogens and is thus likely to mask the full magnitude of NFAT activation by recombinant CaMKIV/Gr transfectants (6, 16). We therefore examined the capacity of CaMKIV/Gr transfectants to activate NFAT-dependent transcription in non–T cell systems that lack endogenous CaMKIV/Gr expression. Fig. 7 demonstrates that the two cell lines used in these studies, the human B cell Burkitt lymphoma cell line BJAB (11) and the human cervical adenocarcinoma cell line HeLa, were lacking in endogenous CaMKIV/Gr expression, as detected by immunoblotting with a specific anti–human CaMKIV/Gr antiserum. Cells were transfected with cDNA encoding NFATc and CaMKIV/Gr, either alone or in combination, and transcription from reporter gene constructs driven by the NFAT response element or by the complete IL-2 enhancer/promoter was analyzed. Fig. 8 *A* demonstrates that a construct encoding NFATc was ineffective in activating transcription of the NFAT reporter gene in BJAB in the absence of a costimulus. Stimulation with PMA resulted in a modest level of reporter transcription. Transfection of BJAB cells with a construct encoding ΔCaMKIV/Gr(c) resulted in modest activation of NFAT-

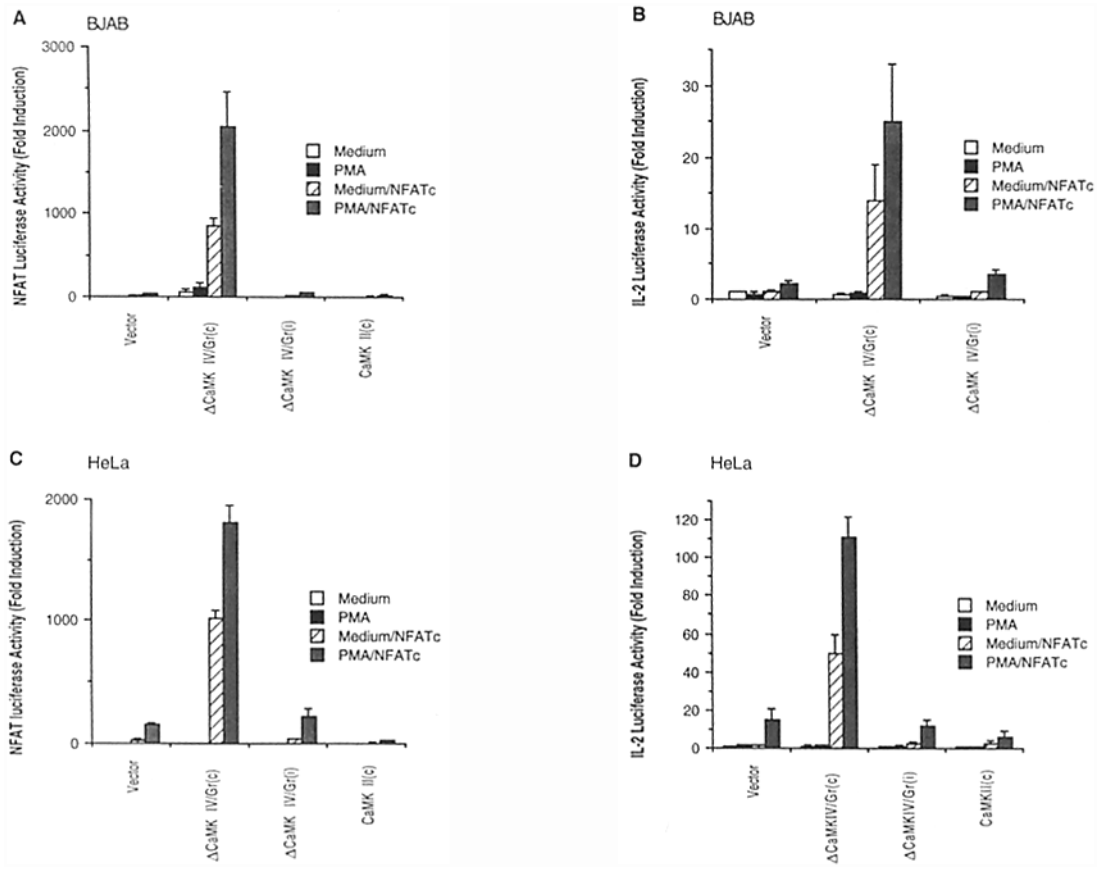

Figure 8. Reconstitution of NFAT and IL-2 promoter activities by CaMKIV/Gr and NFATc in kinase-negative cells. (*A* and *B*) 10^7 BJAB cells were cotransfected by electroporation with 5 μg of either NFAT or IL-2 luciferase reporters and 30 μg of the indicated kinase construct with or without an additional 5 μg of NFATc cDNA in pBJ5 vector (26). The cells were incubated for 48 h and then treated as follows and left incubating for an additional 6 h before harvesting. (*Medium*) Cells receiving kinase construct alone and otherwise left untreated; (*PMA*) cells receiving kinase construct alone and treated with PMA at 25 ng/ml; (*Medium/NFATc*) cells cotransfected with NFATc and kinase constructs but otherwise left untreated; (*PMA/NFATc*) cells cotransfected with NFATc and kinase constructs and treated with PMA at 25 ng/ml. Results represent mean values of three independent experiments + SEM and are expressed as fold induction over values obtained in cells transfected with reporter gene and empty pSG5 and pBJ5 vectors and incubated in medium alone. (*C* and *D*) HeLa cells were cotransfected by the Ca^{2+} phosphate method with 2.5 μg of either a NFAT or a IL-2 enhancer/promoter–driven reporter gene and 15 μg of the indicated kinase construct with or without an additional 2.5 μg of NFATc cDNA in pBJ5. The cells were incubated for 48 h and thereafter treated for 6 h as per *A* and *B*. Results represent mean values of three independent experiments ± SEM.

driven reporter gene expression. This induction was most likely mediated by endogenous NFAT proteins normally found in B cells and was abrogated upon treatment with cyclosporin A (data not shown). In contrast, cotransfection of of BJAB cells with constructs encoding ΔCaMKIV/Gr(c) and NFATc resulted in the activation of NFAT-driven reporter gene expression to levels 25-fold higher than those obtained with NFATc alone in the presence of PMA and 10-fold higher than those induced upon transfection of BJAB cells with CaMKIV/Gr(c) alone. Induction of the NFAT reporter gene expression by NFATc and ΔCaMKIV/Gr(c) was further upregulated by PMA by about twofold, which is consistent with the observed synergy between PMA/Ras and CaMKIV/Gr in upregulating AP-1 function (Figs. 3 and 5).

The results obtained in BJAB cells were reproduced in the human adenocarcinoma cell line HeLa. Fig. 8 *C* demonstrates that a construct encoding NFATc was ineffective in activating transcription of the NFAT reporter gene in HeLa cells. Similarly, transfection of a construct encoding ΔCaMKIV/Gr(c) failed to activate NFAT-driven reporter gene expression. In contrast, cotransfection of HeLa cells with constructs encoding ΔCaMKIV/Gr(c) and NFATc resulted in vigorous activation of NFAT-driven reporter gene expression. Induction of the NFAT reporter gene expression by NFATc and ΔCaMKIV/Gr(c) was further upregulated by PMA, similar to what was observed with BJAB cells. The validity of these results was extended to an IL-2 enhancer/promoter–driven luciferase reporter gene, where ΔCaMKIV/Gr(c) was found to synergize with NFATc to drive the transcription of this reporter in both BJAB and HeLa cells (Fig. 8, *B* and *D*). The specificity of CaMKIV/Gr stimulation of NFATc activity in BJAB and HeLa cells was suggested by the failure of CaMKII(c) to activate transcription of the analyzed reporter genes (Fig. 8).

To analyze the mechanism(s) by which CaMKIV/Gr(c) synergized with NFATc in inducing NFAT and IL-2 reporter gene expression in non–T cells, we examined nuclear extracts derived from transfected HeLa cells for the presence of NFAT DNA-binding activity using an oligonucleotide corresponding to the distal NFAT site in the murine IL-2 promoter. This oligonucleotide binds to NFAT proteins both in the absence and in the presence of associated AP-1 components to give rise to two distinct DNA–protein complexes that can be resolved on PAGE: a faster migrating complex containing NFAT and a slower complex containing NFAT/AP-1 (33). Fig. 9 demonstrates that nuclear extracts of HeLa cells that have been transfected with empty vectors alone exhibited no NFAT-specific DNA–protein complexes. Transfection with NFATc induced the appearance of two NFAT-specific complexes: a dominant, higher mobility complex (Fig. 9, *arrow*) and a minor slower moving complex (Fig. 9, *arrowhead*). The presence of these complexes was not inhibited by cyclosporin A, indicating that recombinant NFATc gained entry into nuclei of unstimulated HeLa cells by a calcineurin-independent mechanism (data not shown). Transfection of CaMKIV/Gr(c) induced no demonstrable NFAT-specific binding activity. In

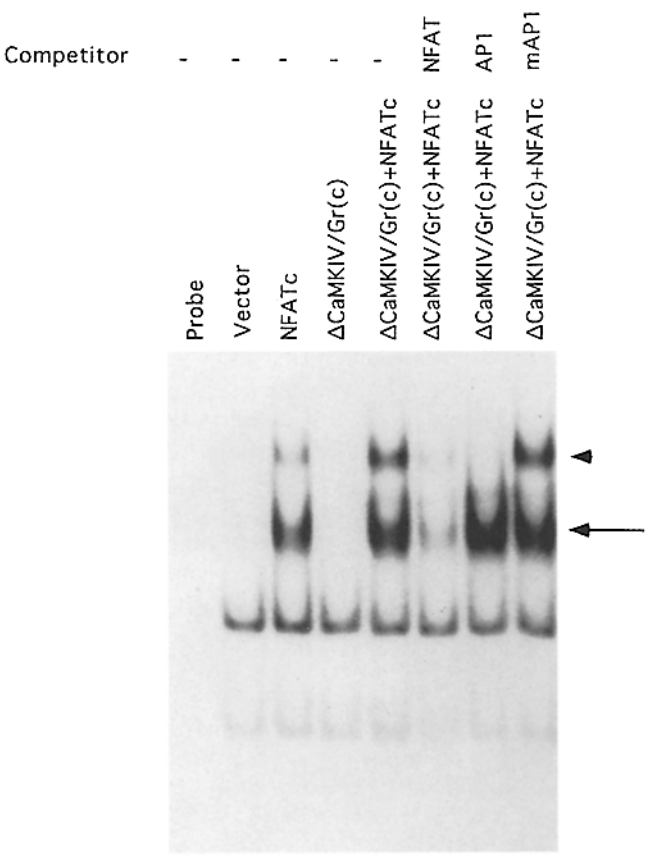

Figure 9. Upregulation of NFAT DNA-binding activity by CaMKIV/Gr. Nuclear extracts were prepared from HeLa cells that had been transiently transfected with the indicated construct(s) as detailed in the legend to Fig. 8. 5 μg of the respective extract was incubated with ^{32}P-labeled oligonucleotide probe corresponding to the murine IL-2 distal NFAT site in the absence or presence of 100-fold molar excess of unlabeled NFAT, AP-1, or mutant AP-1 (*mAP1*) competitor oligonucleotide, as indicated. The bound complexes were resolved on 5% nondenaturing acrylamide gel. (*Probe* lane) Radiolabeled reaction mixture incubated in the absence of nuclear proteins. (*Arrowhead*) DNA complexes containing NFAT proteins together with AP-1; (*arrow*) complexes containing NFAT proteins. The bottom two bands represents nonspecific DNA–protein complex, while unbound radiolabeled probe was allowed to run out of the gel. Results are representative of five independent experiments.

contrast, cotransfection of both NFATc and CaMKIV/Gr(c) resulted in marked increase in the upper complex, consistent with the induction of AP-1 activity by CaMKIV/Gr. It also resulted in some increase in the abundance of the lower complex, consistent with either enhanced transport of NFATc into the nucleus and/or increased retention. The specificity of NFAT–DNA complexes was ascertained by the capacity of excess unlabeled NFAT-binding oligonucleotide to competitively inhibit both NFAT–DNA complexes. Addition of excess unlabeled AP-1–binding oligonucleotide specifically inhibited the upper complex, consistent with the presence of AP-1 in that complex, whereas mutant AP-1–binding oligonucleotide, which exhibits poor affinity to AP-1, inhibited neither complex. These results indicated that the effects of CaMKIV/Gr(c) on NFAT-driven transcription could be accounted for, at

least in part, by the capacity of the kinase to activate AP-1 expression.

Discussion

The present study establishes the role of CaMKIV/Gr as a transducer of Ca^{2+}-dependent activation signals in T lymphocytes. CaMKIV/Gr upregulates NFAT and IL-2 enhancer/promoter–dependent transcription in T cells and, importantly, reconstitutes the capacity of NFATc to direct transcription from the respective regulatory sequence in non–T cells. The promotion by CaMKIV/Gr of NFAT and IL-2 enhancer/promoter–dependent transcription is due in part to the induction by the kinase of the transcription factor AP-1. This is the first example of AP-1 activation by a CaMK, and the results herein provide a novel mechanism by which Ca^{2+} signaling upregulates AP-1 function. CaMKIV/Gr induces AP-1 in part by activating the transcription of genes encoding AP-1 components. This is supported by the demonstration that CaMKIV/Gr upregulates the transcription in Jurkat cells of a reporter gene driven by the c-*fos* promoter/enhancer and by the demonstration that CaMKIV/Gr induces the transcription of c-*fos* in PC12 rat pheochromocytoma cells. CaMKIV/Gr induction of c-*fos* transcription proceeds by a serum response factor–dependent mechanism in contrast to induction by $p21^{ras}$, which is mediated by the serum response factor–associated protein Elk-1 (45). This illustrates how CaMKIV/Gr and $p21^{ras}$ can act independently yet synergistically to upregulate AP-1 function. It remains to be determined whether CaMKIV/Gr activates transcription of genes encoding other AP-1 components and whether it additionally upregulates AP-1 activity by a posttranscriptional mechanism.

Consistent with its induction of AP-1, CaMKIV/Gr upregulated the activation by mitogens of NFAT-dependent transcription in Jurkat cells. It also synergized with recombinant NFATc in promoting NFAT-dependent transcription in BJAB and HeLa cells. In the latter case, NFATc gained entry into the nucleus in the absence of a Ca^{2+}-mobilizing signal. This probably resulted from overexpression of NFATc in the cytosol leading to its transport into the nucleus in a calcineurin-independent manner. Whereas AP-1 induction provides an important mechanism underlying the activation of IL-2 transcription by CaMKIV/Gr, additional kinase-induced activation events may also be involved. This is indicated by the finding that CaMKIV/Gr greatly surpassed PMA in activating transcription of NFAT and IL-2 enhancer/promoter–driven reporter genes in non–T cells. An effect on NFATc itself is possible, suggested by the observation that CaMKIV/Gr increased NFAT DNA-binding activity in the nucleus. It should be noted that NFATc (26) and its related factors NFATc2/p (46), NFATc3 (47), and NFAT-X (48) contain numerous consensus phosphorylation sites for CaMKIV/Gr and CaMKII. The role of these phosphorylation sites in positive and negative regulation of NFAT protein function remains to be determined.

It is likely that activation of NFAT-dependent transcription in T cells is a cooperative process between several signaling pathways and that it involves calcineurin, CaMKIV/Gr, and PKC- and $p21^{ras}$-dependent pathways. However, unlike the aforementioned transducers of TCR signaling, CaMKIV/Gr is distinguished by its selective expression in T cells, particularly the $CD4^+$ subset (6). This and the capacity of CaMKIV/Gr to activate transcription from lymphokine response elements suggests that the kinase may be an important determinant of the type and level of expression of lymphokine genes present in T versus B cells and possibly among different T cell populations.

In contrast to the salutary effects of CaMKIV/Gr on transcription directed from lymphokine regulatory elements, the multifunctional CaMKII-γ_B inhibits transcription from these same elements. It has been suggested that CaMKII may play a role in mediating negative effects of Ca^{2+} signaling in lymphocytes, including anergy and negative selection (37, 38). However, the outcome of Ca^{2+} signaling in a particular T lymphocyte is more likely to be determined by a complex set of interactions between the various Ca^{2+}-responsive enzymes, including CaMKIV/Gr, CaMKII, and calcineurin. The contribution of each of these enzymes to the diverse effects of Ca^{2+} signaling in T lymphocytes remains to be fully mapped.

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