A Unique Phosphorylation-dependent Mechanism for the Activation of \( \text{Ca}^{2+} / \text{calmodulin-dependent Protein Kinase Type IV/GR} \)*

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The activity of the \( \text{Ca}^{2+} / \text{calmodulin-dependent protein kinase IV/Gr (CaMKIV/Gr)} \) is shown to be strictly regulated by phosphorylation of three residues both in vitro and in response to antigen receptor-mediated signaling in lymphocytes. One residue, Thr-200, is indispensable for enhancement of \( \text{Ca}^{2+} / \text{calmodulin-dependent basal activity by CaMKIV/Gr kinase. This event requires Ca}^{2+} / \text{calmodulin in the full-length CaMKIV/Gr but is Ca}^{2+} / \text{calmodulin-independent when a truncated version of CaMKIV/Gr is used as a substrate (ΔCaMKIV/Gr1–317, (Δ-317)). The other two residues, Ser12 and Ser13, are apparently autophosphorylated by the Ca}^{2+} / \text{calmodulin-bound CaMKIV/Gr. Phosphorylation of neither Ser12-Ser13 nor Thr312 (the residue in a homologous position to Thr312 of CaMKIα) influences the development of Ca}^{2+} / \text{calmodulin-independent activity or any other property of CaMKIV/Gr examined. Similarly, removal of the NH2-terminal 20 amino acids has no effect on the activation or function of CaMKIV/Gr. However, mutation of both Ser12 and Ser13 residues to Ala in Δ1-317 completely abrogates activity, while individual substitutions have no effect. These results indicate that the NH2-terminal Ser cluster mediates a novel type of intrasteric inhibition and suggest that three events are required for CaMKIV/Gr activation: 1) Ca\(^{2+}\)/calmodulin binding; 2) phosphorylation of the Ca\(^{2+}\)/calmodulin-bound enzyme on Thr200 by a Ca\(^{2+}\)/calmodulin-dependent protein kinase; and 3) autophosphorylation of Ser12-Ser13. This three-step requirement is unique among the multifunctional Ca\(^{2+}\)/calmodulin-dependent kinases.

The Ca\(^{2+}\)/calmodulin-dependent protein kinase type IV/Gr (CaMKIV/Gr) is a developmentally regulated enzyme that is expressed predominantly in a subset of neurons, developing T lymphocytes and postmeiotic male germ cells (1–3). This enzyme has been implicated as a transducer of receptor-mediated Ca\(^{2+}\) signaling in T lymphocytes (4, 5). It is not found in primary B lymphocytes, but its expression is induced by transformation with the Epstein-Barr virus (6). Whereas the physiologically relevant substrates have yet to be determined, CaMKIV/Gr is expressed in the nucleus and has been determined to phosphorylate and regulate the transactivation function of several transcription factors including cAMP-responsive element-binding protein (CREB) and cAMP-responsive element modulator t (CREMt) (7–12) and serum response factor (SRF) (8, 13). Although CaMKIV/Gr appears to be the product of a single gene in rat and human (14, 15), the protein exists in tissues as two monomeric forms, each of which binds to and is activated by Ca\(^{2+}\)/calmodulin. It has been suggested that the larger \( \beta \) monomer differs from the smaller \( \alpha \) monomer only by the presence of a unique 28-amino acid NH2-terminal extension (16), a postulate that is compatible with the structure of the gene (17). However, expression in transformed cell lines of cDNA encoding the \( \alpha \) form of CaMKIV/Gr gives rise to two recombinant CaMKIV/Gr monomers that co-migrate with tissue-derived \( \alpha / \beta \) monomers, indicating that the \( \beta \) monomer may also result in part from post-translational modification of the \( \alpha \) form (6). The catalytic region of the kinase is in the NH2-terminal half of the protein and is followed by an autoinhibitory calmodulin binding domain in a manner analogous to other members of the calmodulin-regulated kinase family (8, 18).

It had been reported that following Ca\(^{2+}\)/calmodulin binding, CaMKIV/Gr isolated from rat brain underwent a rapid auto-phosphorylation that resulted in the generation of Ca\(^{2+}\)/calmodulin-independent activity (19, 20). Phosphorylation of the activated kinase was detected on multiple Ser residues within the NH2-terminal 20 amino acids as well as on Thr196 and Thr200 in subdomain VIII of the catalytic region of the kinase (21). Subsequent experiments by a number of laboratories using enzyme produced from recombinant cDNA expression vectors demonstrated that a CaMK kinase(s) was responsible for the initial phosphorylation of CaMKIV/Gr following Ca\(^{2+}\)/calmodulin binding (18, 22–24). Selbert et al. (24) found that a CaMK kinase exclusively phosphorylated Thr196 of the rat enzyme, which markedly enhanced activity coincident with additional phosphorylation on Ser residues and the appearance of some degree of autonomous activity. When Thr196 was changed to Ala, neither phosphorylation nor activation of the enzyme occurred. It was concluded that the binding of Ca\(^{2+}\)/calmodulin exposed Thr196 to the activating CaMK kinase and that, when phosphorylated on this residue, the enzyme underwent auto-phosphorylation on Ser residues that led to generation of autonomous activity.

The present study was undertaken to address the role of enzyme phosphorylation in regulating the activity of CaMKIV/Gr. We have examined the process in vitro using recombinant forms of the human enzyme as well as in a cellular model of CaMKIV/Gr activation. The results show that Thr200, the res-
Regulation of CaMKIV/Gr by Phosphorylation

idie in the human enzyme homologous to Thr196 of the rodent kinase, is absolutely required for the activation of and signal cascades generated by CaMKIV/Gr. The subsequent autophosphorylation of the NH2-terminal Ser residues 12 and 13 may be involved in relief of autoinhibition by a novel mechanism but is not involved in generation of autonomous activity.

MATERIALS AND METHODS

cDNA Constructs—Human CaMKIV/Gr cDNA was derived and sub- 
doned into the pSG5 vector, as described (6). Δ1–317 is a constitutively active deletion mutant of CaMKIV/Gr fitted with a FLAG epitope tag at the NH2terminus and subcloned into the BglII site of pSG5. Substitutions at Ser6, Ser10, Ser12, Ser13, Ser15, Ser16, Lys75, and Thr200 were introduced into the full-length kinase or into the Δ1–317 fragment by site-directed mutagenesis using the unique site elimination method (25). The NH2-terminal 25 amino acids of the wild-type kinase and was subcloned by blunt end ligation into the BamHI site of pSG5. The CaMKIV/Gr mutant T312A and the Δ1–317 mutants S12A and S13A were generated by site-directed mutagenesis as described (26). The cDNAs of all kinase mutants were sequenced and were confirmed to be free of errors. The plasmid AP-1 luciferase contains two copies of the AP-1 site of the rodent α chorionic gonadotropin gene and was a kind gift of Dr. Kevin Chien (University of California, San Diego) (27).

Bacterial Expression and in Vitro Assay of CaMKIV/Gr Fusion Proteins—Recombinant proteins were expressed from pGEX2T plasmid (Pharmacia Biotech Inc.) in Escherichia coli strain BL21 (DE3) as glutathione S-transferase fusion proteins and purified in a single step with glutathione-Sepharose 4B. Kinase activity was assayed by incubating fusion protein for 5 min at 30°C, and 32P incorporation was determined following binding of the peptide to p81 phosphocellulose paper, as described previously (28). The CaMK kinase used to activate CaMKIV/Gr was purified pig brain CaMKI kinase (29), generously provided by Dr. Arthur Edelman (State University of New York, Buffalo, New York).

In Vitro Transcription/Translation Studies—Two μg of cDNA encoding either wild-type or mutant CaMK kinase IV/Gr proteins were transcribed and translated in vitro using the Promega T3 system (Promega Corp.) as described by the manufacturer. The proteins were labeled during translation by including 1.5 μCi of [35S]methionine in the reaction mixture, and the labeled products were resolved on a 5–16% denaturing acrylamide gradient gel. The gel was then dried, and the bands were visualized by autoradiography.

Transfections and Reporter Gene Assays—B(A)J is an Epstein-Barr virus-negative Burkitt lymphoma B cell line (30) that is responsive to a bacterial expression vector pSG5, and the encoded protein is constitutively active. Mutations of Thr200 to Ala (T200A) does not affect the Ca2+/calmodulin-dependent form of CaMKIV/Gr by truncating the protein at Leu-317 to generate Δ1–317. The following mutations, all Ala substitutions except Glu for Lys75, were introduced into Δ1–317: Ser8, Ser10, Ser12, Ser13, Ser15, Ser16, Lys75, and Thr200. Each of the mutant cDNAs was incorporated into bacterial (pGEX2T) and eukaryotic cell (pSG5) expression vector.

The activity of the bacterially expressed full-length CaMKIV/Gr is compared with that of the full-length mutants in Fig. 1B. Purified CaMKIV/Gr was preincubated in the absence or in the presence of CaMK kinase, as indicated, under conditions previously shown to result in maximal activation of CaMKIV/Gr by CaMK kinase. CaMKIV/Gr activity was then assayed in the presence of Ca2+/calmodulin or EGTA. The addition of Ca2+/calmodulin produces a small increase in activity, which is further enhanced 10-fold by CaMK kinase to a specific activity of 1.8 μmol min−1 mg−1. Addition of Ca2+/calmodulin kinase is accompanied by the generation of autonomous activity amounting to 15% of maximal activity. Neither removal of the first 20 amino acids nor conversion of Thr312 to Ala (T312A) altered the activity generated under the four conditions relative to the wild type enzyme. Mutations of Thr200 to Ala (T200A) does not affect the Ca2+/calmodulin dependent increase in CaMKIV/Gr activity but completely abrogates the up-regulation of CaMKIV/Gr activity by the CaMK kinase. Conversion of Lys75 to Glu (K75E) inactivates the enzyme regardless of the experimental conditions of the assay.

The same constructs analyzed in Fig. 1B were subcloned into the mammalian expression vector pSG5, and the encoded proteins were analyzed by in vitro transcription/translation and by transfection into the human Burkitt lymphoma B cell line B(A)J, which lacks endogenous CaMKIV/Gr (6). Fig. 2A demonstrates that both wild type and mutant proteins were expressed equally well when their respective cDNAs (in pSG5 vector) were subjected to in vitro transcription/translation. SDS-PAGE analysis revealed the wild type kinase, the T200A mutant and the K75E mutant to co-migrate at around 58 kDa while the Δ21–473 mutant migrated at around 56 kDa. Fig. 2B reveals the expression of wild type and mutant CaMKIV/Gr

Immunoprecipitation—This was performed using either CaMKIV/Gr-specific polyclonal rabbit antiserum to detect full-length kinase proteins or using the M2 anti-FLAG monoclonal antibody (Eastman Kodak Co.) at 1 μg/ml to detect Δ1–317 fragments. Following incubation with the appropriate secondary reagents, the blots were developed using an enhanced chemiluminescence system for peroxidase-based detection (Amerham Corp.).

RESULTS

The structure of CaMKIV/Gr is depicted schematically in Fig. 1A to illustrate the location and the nature of the mutations introduced (3, 6, 34–36). Lying at the extreme NH2 terminus are multiple Ser residues (Ser6–Ser16), some of which have been previously shown to become autophosphorylated (21, 37). The Lys residue involved in ATP binding is Lys75 (K75), and the Thr residue in the activation loop of the catalytic domain (subdomain VIII) which is phosphorylated by CaMKIV/Gr kinase is Thr200 (T200). Thr312 (T312) exists in an analogous position relative to the CaM binding site, as does Thr286 in CaMKII. It is the intersubunit phosphorylation of Thr200 that is responsible for the generation of Ca2+/calmodulin-independent activity of CaMKIV/Gr. Leu317 is proximal to the CaM binding domain, and truncation at this residue has been shown to produce an enzyme that is independent of Ca2+/calmodulin (8). This truncated fragment still requires phosphorylation of Thr200 for maximal activity (24). In the full-length kinase, mutations were made by deleting the first 20 amino acids to generate Δ21–473 and also by individual conversion of Lys75 into Glu (K75E) and Thr200 or Thr312 into Ala (T200A and T312A, respectively). In addition we made a Ca2+/calmodulin-independent form of CaMKIV/Gr by truncating the protein at Leu-317 to generate Δ1–317. The following mutations, all Ala substitutions except Glu for Lys75, were introduced into Δ1–317: Ser8, Ser10, Ser12, Ser13, Ser15, Ser16, Lys75, and Thr200. Each of the mutant cDNAs was incorporated into bacterial (pGEX2T) and eukaryotic cell (pSG5) expression vector.

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Fig. 1. Characterization of wild type and mutant CaMKIV/Gr-GST fusion proteins. A, schematic representation of CaMKIV/Gr structure. The general domain structure of CaMKIV/Gr is illustrated in the upper diagram. In the lower diagram the sites where point mutations (S8, S10, S12, S13, S15, S16, K75, T200, and T312) and truncations (A21, L317) were made are indicated, along with the primary amino acid sequence at these sites. The sequence His^{309}--Ala^{329} is shown aligned with the similar sequence from CaM kinase II. S10, S12, S13, S15, S16, K75, T200, and T312) and truncations (A21, L317) were made are indicated, along with the primary amino acid sequence at these sites. The sequence His^{309}--Ala^{329} is shown aligned with the similar sequence from CaM kinase II.

molecules at 48 h post-transfection in BJAB cells. Expression of the Δ21-473 mutant was comparable with that of the wild type kinase, while the levels of the T200A mutant achieved following transfection were markedly decreased at 30-50% of wild type. Significantly, decreased expression was also observed in another mutant in which the conserved Lys^{75} residue in the ATP binding pocket was replaced with a Glu. These results indicated that mutations which compromise kinase activity including T200A and K75E are associated with decreased expression and/or stability in BJAB cells.

Fig. 3A demonstrates that wild type kinase immunoprecipitates isolated from unstimulated BJAB cells exhibited modest Ca^{2+}/calmodulin-dependent catalytic activity toward the peptide substrate syntide-2. Stimulation with anti-IgM antibodies induced strong Ca^{2+}/calmodulin-dependent activity in CaMKIV/Gr immunoprecipitates. Maximal kinase activation was achieved within 30 s and 1 min, but activity swiftly declined thereafter. Fig. 3B demonstrates that CaMKIV/Gr wild type also exhibited appreciable autonomous activity in response to anti-IgM treatment, which followed a time course identical to that of the Ca^{2+}/calmodulin-dependent activity. The Ca^{2+}/calmodulin and autonomous activities of the Δ21-473 mutant were also up-regulated by anti-IgM treatment, and the magnitude and time course of this activation closely approximated that of the wild type kinase. In contrast, treatment of BJAB cells expressing the T200A mutant with anti-IgM antibodies did not induce appreciable Ca^{2+}/calmodulin-dependent or autonomous activity. The K75E mutant was devoid of inducible activity. For each kinase species equal amounts of protein were present in the immunoprecipitates as detected by immunoblotting with an anti-CaMKIV/Gr antiserum (data not shown), indicating that the up-regulation by anti-IgM of the activities of wild type kinase and Δ21-473 mutant was not due to the enhanced retrieval by immunoprecipitation of the respective kinase species following anti-IgM treatment. These results reveal that Thr^{200} is absolutely required for both Ca^{2+}/calmodulin-dependent and autonomous activation of CaMKIV/Gr in a cellular context and that the first 20 amino acids that contain the Ser residues known to be phosphorylated in vitro are not required for this activation.

To determine whether phosphorylation of the Thr^{200} or the NH_{2}-terminal Ser residues influence a downstream effect of CaMKIV/Gr, we examined the ability of the T200A and Δ21-473 mutants to activate an AP-1 reporter gene relative to the wild type kinase (38). Fig. 4 demonstrates that overexpression of wild type kinase or the Δ21-473 mutant in BJAB cells results in the transcriptional activation of a luciferase reporter gene driven by an AP-1 promoter. Moreover, induction of AP-1 activity by both wild type kinase and the Δ21-473 mutant was increased about 2-fold by anti-IgM treatment. In contrast, the T200A mutant failed to induce expression of the AP-1 reporter in the absence or in the presence of anti-IgM antibodies. This result is in full agreement with the lack of inducible Ca^{2+}/calmodulin activity of the T200A mutant both in vitro (Fig. 1B) or in BJAB cells (Fig. 3).
by mutating to Ala individual Ser residues or pairs of Ser residues that occur at positions 8, 10, 12, 13, 15, and 16. These mutations were introduced into Δ1–317 (see Fig. 1A) in order to separate the regulatory effects of the NH2-terminal Ser residues from the autoinhibitory sequences present in the calmodulin binding domain of CaMKIV/Gr (8, 18). The resulting mutants were called S8A, S10A, S12A/S13A, and S15A/S16A, and the activity of this series of proteins produced in bacteria is shown in Fig. 1C. The truncated version of the wild type enzyme, Δ1–317, was completely independent of Ca2+/calmodulin (compare bars 1 and 2) but was stimulated 10-fold by the CaMKIV/Gr kinase. All of the increased activity was independent of Ca2+/calmodulin (compare bars 3 and 4). The mutant proteins containing Ala substitutions for Ser6, Ser10, or Ser15, Ser16 behaved identically to Δ1–317. However, the Δ1–317 S12A/S13A mutant showed no protein kinase activity under any of the four experimental conditions. The lack of kinase activity was similar to the results obtained with the inactive Δ1-317K75E mutant. Whereas the Δ1–317T200A mutant was resistant to activation by CaMK kinase, it did exhibit Ca2+/ calmodulin-independent activity that was greater than that of either Δ1-317S12/13A or Δ1–317K75E mutant enzymes.

We also evaluated the expression and function of wild type and mutant Δ1–317 proteins in eukaryotic cells. Fig. 5A shows the expression of the Ser mutants in a rabbit reticulocyte lysate system compared with the T200A and kinase-lacking (K75E) versions of Δ1–317. It can be seen that each protein was effectively expressed in this coupled transcription/translation system. Wild type Δ1–317 as well as the S8A, S10A, and S15A/S16A mutants migrated as two closely spaced bands on SDS-PAGE. The Δ1–317 mutants S12A/S13A, K75E, and T200A exclusively migrated as the faster moving (lower) band. Since these mutants lack kinase activity (Fig. 1C), the upper band may represent an autophosphorylated form of the constitutively active kinase fragment.

We next examined the expression of Δ1–317 and the set of mutants shown in Fig. 5A in BJ AB cells and also assessed their ability to stimulate the activity of the AP-1 luciferase gene. Fig. 5B demonstrates that substitutions that disrupt kinase activity including S12A/S13A, T200A, and K75E all resulted in poor expression as compared with the wild type Δ1–317 control. In contrast, the S15A/S16A mutant was expressed at levels comparable with the wild type control. The S8A and S10A mutants were expressed at intermediate levels. Fig. 5C demonstrates that the S8A, S10A, and S15A/S16A mutants induced luciferase reporter gene transcription from an AP-1 promoter at levels approximating 50–70% that of the wild type Δ1–317 protein. In contrast, cells transfected with the Δ1–317 mutants S12/13A, T200A, and K75E showed no evidence of inducible, AP-1-driven gene transcription. Overall, these results indicate that Ser12

![Figure 2](http://www.jbc.org/)

**Fig. 2. Expression of wild type and mutant CaMKIV/Gr molecules in BJ AB cells.** A, in vitro transcription/translation of the various CaMKIV/Gr derivatives. In vitro transcribed and translated CaMKIV/Gr proteins were labeled during the translation process by including [35S]methionine in the transcription/translation reaction mixture and then resolved by SDS-PAGE and visualized by autoradiography. The wild type kinase, the T200A mutant, and the K75E mutant all co-migrated at about 58 kDa, while the Δ21–473 mutant migrated at about 56 kDa. Results are representative of three independent experiments. B, expression of CaMKIV/Gr derivatives in BJ AB cells. BJ AB cells were transfected with the indicated kinase construct. The cells were lysed 48 h later, and 100 μg of protein present in the respective lysates was resolved by SDS-PAGE and probed for kinase expression using a rabbit anti-CaMKIV/Gr antiserum. Immunoreactive proteins were visualized by enhanced chemiluminescence and autoradiography, as described under “Materials and Methods.” Results are representative of seven independent experiments.

![Figure 3](http://www.jbc.org/)

**Fig. 3. Response of recombinant wild type and mutant CaMKIV/Gr molecules expressed in BJ AB cells to activation via surface IgM molecules.** A, Ca2+/calmodulin-dependent activity of the respective kinase species following treatment of transfected BJ AB cells with anti-surface IgM antibodies (anti μ) B, autonomous activity. BJ AB cells (total 3 × 107/kinase species) were transfected with the indicated kinase species and cultured for 48 h. The cells were then harvested, divided into equal aliquots, and treated with goat anti-human IgM antibodies (anti μ) at 20 μg/ml for the indicated time periods. The cells were then lysed, and wild type and mutant CaMKIV/Gr proteins were immunoprecipitated using a rabbit anti-CaMKIV/Gr antiserum. The immunoprecipitates were assayed for Ca2+/calmodulin-dependent kinase activity (panel A) or for autonomous activity (panel B) as described under “Materials and Methods.” Results are expressed as pmol of [32P] incorporated into substrate peptide (sytide-2) over the 10-min period of the assay. Results represent the mean of three replicates done for each time point; S.E. values were less than 10% of the means. Similar results were found in two other independent experiments.
and Ser13 are indispensable to the expression (or stability) and function of Δ1–317. The other NH2-terminal Ser residues may additionally contribute to the expression (Ser8 and Ser10) and/or to the function of Δ1–317 in cells (Ser8, Ser10, and Ser12-Ser13).

DISCUSSION

Rat CaMKIV/Gr must be phosphorylated on Thr196 in the activation loop by a CaMK kinase for maximal activity in vitro (24). This site is masked in the full-length enzyme and is only exposed upon binding of Ca2+/calmodulin. Even when the enzyme is rendered independent of Ca2+/calmodulin by removal of the Ca2+/calmodulin binding region, phosphorylation of Thr196 is still required for maximal activity. Our studies reveal that the highly homologous human form of CaMKIV/Gr is similarly regulated in vitro by Ca2+/calmodulin binding followed by phosphorylation of Thr200 (the identical residue to rat Thr196). In addition, we have extended the relevance of a CaMKIV/Gr kinase and the importance of a Thr200 phosphorylation to a cellular context. When transfected into BJ AB cells, Thr200 is absolutely required for kinase activity measured in immunoprecipitates or for stimulation of a downstream target of the CaMKIV/Gr signaling pathway, namely AP-1-mediated transcription (38). A similar requirement for the homologous Thr196 residue of the rat enzyme has also been recently demonstrated in a cellular model of CaMKIV/Gr activation by Ca2+ ionophore treatment (39).

The presence of an activating kinase in BJ AB cells raises the question of whether (or how) this enzyme participates in signal transduction cascades leading to CaMKIV/Gr activation. Two observations suggest that the activating kinase might be fully active in unstimulated BJ AB cells. First, the capacity of Δ1–317 to induce AP-1-dependent transcription in unstimulated BJ AB is independent of IgM treatment and is at least as great as that of the full-length kinase in IgM-stimulated cells. Second, transfection of the full-length kinase is sufficient to induce...
activity of the AP-1 reporter gene in the absence of receptor-mediated activation (although IgM treatment does result in a 2-fold increase in reporter activity). A possible explanation for this observation is that transfection of the full-length enzyme results in the production of a small amount of a catalytically active but Ca\(^{2+}\)/calmodulin-independent fragment due to proteolytic cleavage of recombinant CaMKIV/Gr. This is supported by our demonstration elsewhere that transfection of full-length CaMKIV/Gr into cells gives rise to small amounts of a recombinant kinase fragment that co-migrates with Δ1-317 (38).

The original characterization of CaMKIV/Gr isolated from rat brain and subjected to Ca\(^{2+}\)/calmodulin dependence in vitro identified several phosphoserine residues at the extreme NH\(_2\) terminus (see Fig. 1A) and a small number of phosphothreonine residues at positions 196 and 200 (21). Subsequent experiments with enzymes produced from recombinant cDNAs indicated that the initial activating phosphorylation occurred on Thr\(^{196}\) (24) and was catalyzed by a separate protein kinase that also exhibited Ca\(^{2+}\)/calmodulin dependence in vitro (18, 23, 24). Phosphorylation of Thr\(^{196}\) resulted in further Ser phosphorylation and generation of autonomous activity. It seemed likely that the Ser phosphorylation occurred at the NH\(_2\) terminus and that this phosphorylation was responsible for development of Ca\(^{2+}\)/calmodulin-independent activity. Our results reveal that the mechanisms governing the activity of CaMKIV/Gr are considerably more complex than originally anticipated. First, the NH\(_2\)-terminal Ser cluster is not involved with the development of autonomous activity. Δ21-473 behaves in an identical manner to the wild type kinase both in vitro and in response to receptor stimulation. Both forms require Ca\(^{2+}\)/calmodulin and phosphorylation of Thr\(^{200}\) for maximal activity. Second, whereas the NH\(_2\)-terminal Ser cluster was not required for Ca\(^{2+}\)/calmodulin-dependent activation, phosphorylation of Thr\(^{200}\) or generation of autonomous activity, Ser\(^{12}\) and Ser\(^{13}\) were indispensable for kinase activity under all conditions tested. Particularly striking was the behavior of the constitutively active truncated CaMKIV/Gr fragment Δ1-317. Even when this Ca\(^{2+}\)/calmodulin-independent fragment was phosphorylated on Thr\(^{200}\) by the activating kinase, no activity of CaMKIV/Gr was observed when both Ser\(^{12}\) and Ser\(^{13}\) were mutated into Ala. In contrast, single substitutions at Ser\(^{12}\) or Ser\(^{13}\) were indistinguishable from wild type Δ1-317. These results suggest the presence of an additional autoinhibitory mechanism of the intrasteric type mediated by Ser\(^{12}\)-Ser\(^{13}\) that is relieved by phosphorylation of one or both residues. The α, β, and γ isoforms of protein kinase C and the related Raf kinases represent precedents for the location of an autoinhibitory domain at the NH\(_2\) terminus of a protein kinase (40). Like CaMKIV/Gr, protein kinase C autophosphorylates on multiple amino acids, including residues at the NH\(_2\) terminus (Ser\(^{16}\) and Thr\(^{17}\)), although the functional significance of these events has not been determined (41).

Our results suggest that activation of CaMKIV/Gr would consist of three possibly cooperative events: 1) Ca\(^{2+}\)/calmodulin binding, 2) phosphorylation of Thr\(^{200}\) by CaMKIV/Gr kinase, and 3) autophosphorylation on Ser\(^{12}\)-Ser\(^{13}\). The following observations suggest that the phosphorylation events triggered by CaM binding may not be strictly ordered. First, purified CaMK kinase can phosphorylate the inactive CaMKIV/Gr K75E mutant, indicating that phosphorylation of Thr\(^{200}\) can occur in the absence of phosphorylated Ser\(^{12}\)-Ser\(^{13}\). Second, as described above, mutagenesis of Ser\(^{12}\) and Ser\(^{13}\) to Ala results in an enzyme incapable of generating CaM-dependent basal activity, while the T200A mutant retains this ability. These results suggest that calmodulin binding, in the context of the wild type enzyme, is sufficient to trigger autophosphorylation of Ser\(^{12}\)-Ser\(^{13}\) in the absence of phosphorylated Thr\(^{200}\). This autophosphorylation relieves autoinhibition and generates basal enzymatic activity.

In the case of CaMKIIα, binding of Ca\(^{2+}\)/calmodulin to one subunit is sufficient to relieve autoinhibition without inducing autophosphorylation. However, binding of Ca\(^{2+}\)/calmodulin to two contiguous subunits results in the phosphorylation of Thr\(^{200}\) on one of the subunits. This phosphorylation is responsible for development of autonomous kinase activity (42–45) and a 1000-fold increase in the affinity of Ca\(^{2+}\)/calmodulin to the autophosphorylated subunit (46). CaMKIV/Gr does have a Thr residue (Thr\(^{312}\); Fig. 1A) at a position identical to Thr\(^{200}\) of CaMKIIα. Tokumitsu et al. (18) initially implicated this residue as important for development of autonomous activity in CaMKIV/Gr, since introduction of negatively charged residues surrounding Thr\(^{312}\) did result in the appearance of Ca\(^{2+}\)/calmodulin-independent activity. On the other hand, our results as well as those of Tokumitsu and Soderling (39) demonstrate that mutation of Thr\(^{312}\) to Ala (T312A; Fig. 1B) is not sufficient to generate autonomous activity either in the absence or presence of the CaMK kinase activator and, in fact, does not change the activity of the enzyme relative to those of the wild-type enzyme. While the mechanism for generation of autonomy in CaMKIV/Gr is currently unclear, one can speculate that a residue (or residues) that has yet to be identified becomes autophosphorylated in the activated enzyme and mediates autonomy. Alternatively, it is possible that phosphorylation of Thr\(^{200}\) is itself capable of generating autonomy. In such a scenario, a lowering of Ca\(^{2+}\) would release calmodulin, but as long as Thr\(^{200}\) remained phosphorylated the calmodulin binding domain could not reconfigure to completely inactivate the enzyme. Experiments are currently under way to evaluate these possibilities.

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28. Deleted in proof
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