Peptide Specificity Determinants at P–7 and P–6 Enhance the Catalytic Efficiency of Ca$^{2+}$/Calmodulin-dependent Protein Kinase I in the Absence of Activation Loop Phosphorylation*

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Phosphorylation of Ca$^{2+}$/calmodulin-dependent protein kinase I (CaM KI) at Thr-177 by recombinant rat Ca$^{2+}$/calmodulin-dependent kinase kinase B (CaM KKB) modulates the kinetics of synapsin-(4–13) peptide phosphorylation by reducing the $K_m$ 4-fold and decreasing the $K_{CaM}$ 4-fold. There is also a slight decrease in $K_m$ for ATP and increase in enzyme $V_{max}$. A synthetic peptide substrate from the yeast transcription factor, ADR1-(222–234)G233 is a 15-fold better substrate for the Thr-177 dephospho-form of CaM KI than synapsin-(4–13). The Thr-177 dephospho-enzyme has a $K_m$ and $V_{max}$ for ADR1-(222-234)G233 similar to the values with synapsin-(4–13) using the Thr-177 phosphorylated enzyme. Likewise, with ADR1-(222-234)G233 as substrate, phosphorylation of Thr-177 or substitution of T177A had very little effect on the kinetic values. Using chimeric peptides between synapsin-(4–13) and ADR1-(222–234)G233 we found that N-terminal basic residues at P–7 and P–6 positions were sufficient to allow efficient phosphorylation by the Thr-177 dephospho-form of CaM KI. Phosphorylation of Thr-177 expands the substrate specificity of CaM KI and is not merely an “on-off” switch for kinase activity.

Protein phosphorylation plays a regulatory role in signal transduction for many physiological processes in eukaryotic cells. Frequently, both receptor and nonreceptor kinases not only phosphorylate cellular proteins to elicit biological responses, but are also phosphoproteins themselves. Phosphorylation of kinases by either autophosphorylation in response to ligand binding, in the case of receptor kinases, or by an upstream kinase, in the case of nonreceptor kinases, is an important step in the signal transduction cascade. The regulatory phosphorylation event(s) usually occur on a single (or multiple) residue(s) located in a region termed the activation loop between kinase subdomains VII and VIII.

Virtually every kinase family, including the mitogen-activated protein kinases (1–4), CDKs² (5), protein kinase Cs (6–10), Janus kinases (11–13), nonreceptor tyrosine kinases such as the Src kinases (14), and receptor tyrosine kinases such as the insulin (15, 16) and Trk (17–20) receptors, contains members regulated by activation loop phosphorylation. In all of these cases, activation loop phosphorylation markedly activates the protein kinase, and the phosphorylated form of the kinase is thought to be physiologically relevant. For example, mutation of Tyr-1007 to Ala in the Janus 2 kinase eliminated activity, and the mutant could not restore erythropoietin signaling in cells lacking Janus 2 kinase (12). A second example of the biological significance of activation loop phosphorylation is the phosphorylation of Ig-B kinase α by the NF-κB-inducing kinase. A mutated Ig-B kinase α in which the activating Ser-176 was changed to Ala acts as a dominant negative inhibitor of interleukin-1 and tumor necrosis factor-induced NF-κB activation (21). These data are illustrative of the biological importance of phosphorylatable residues that reside in the activation loop of kinases.

The most extensively characterized kinase that is regulated by activation loop phosphorylation is the CaM-dependent protein kinase, PKA. In the crystal structure of PKA, Thr-197 is phosphorylated, and the phosphate makes electrostatic contacts with the small and large lobes of the kinase. These contacts were proposed to facilitate both ATP and peptide substrate binding (22–24). Biochemical analysis of PKA confirmed that the phosphate of Thr-197 is required for both ATP binding as well as efficient phosphate transfer to the peptide substrate (25, 26). When PKA is expressed in bacteria, Thr-197 phosphorylation occurs by autophosphorylation, but in mammalian cells Thr-179 may be phosphorylated by another protein kinase. Phosphoinositide-dependent protein kinase 1 can phosphorylate and activate PKA in vitro (27) favoring the idea that PKA is a downstream target of a protein kinase cascade.

Two members of the Ca$^{2+}$/calmodulin (CaM)-dependent family of protein kinases, CaM KI and IV, are activated by upstream kinases (28, 29). Activation loop phosphorylation of CaM KI and CaM KIV by CaM kinase kinase A and CaM KKB, both of which are also members of the CaM kinase family, increases enzyme activity 10–50-fold (30). Both CaM KKS contain phosphorylatable residues within their activation loops and also may be targets of upstream kinases, although to date, no kinases have been identified. Activation loop phosphorylation of CaM KI and IV has been shown to occur in intact cells.

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² The abbreviations used are: CDK, cyclin-dependent kinase; CaM KI, Ca$^{2+}$/calmodulin-dependent protein kinase I; CaM, calmodulin; KKB, kinase kinase B; CaM KKB, Ca$^{2+}$/calmodulin-dependent protein kinase kinase B; PKA, cAMP-dependent protein kinase; CaM KIV, Ca$^{2+}$/calmodulin-dependent protein kinase IV; PCR, polymerase chain reaction; GST, glutathione S-transferase; ADR1, alcohol dehydrogenase repressor protein 1; MBP, maltose-binding protein; SKMLCK, skeletal muscle myosin light chain kinase; PKI, protein kinase inhibitor; LKK-synapsin-(4–13), LKK LRRRLSDANF; LRR-synapsin-(4–13), LRRRLRRRLSDANF.
Aletta et al. (31) have shown that elevated intracellular Ca\(^{2+}\) concentrations, resulting from either ionomycin or KCl treatment of PC12 cells, lead to phosphorylation of CaM KI on Thr-177. CaM KI immunoprecipitated from cells treated with either agent was significantly more active than CaM KI immunoprecipitated from unstimulated cells and showed decreased responsiveness to purified CaM KK in vitro. In addition, anti-IgM treatment of the B cell line, BJAB (32), as well as T cell receptor stimulation of Jurkat cells (33) have been shown to activate CaM KIV. CaM KIV induction of AP-1 (34) and cAMP response element-binding protein-mediated transcription (35) is inhibited by a T200A mutation in human CaM KIV. These data provide compelling evidence that phosphorylation of CaM KI and IV is a physiological response to signals that increase intracellular Ca\(^{2+}\) with these kinases serving as intermediates in signal transduction cascades.

Recent data from Chin et al. (36) revealed that Thr-177-dephosphorylated CaM KI has one of the highest specific activities reported for any protein kinase, and peptide substrates could show greater than 400-fold differences in specificities. These surprising observations raised the possibility that activation loop phosphorylation of CaM KI may not be essential for kinase activity but could modulate substrate specificity. Here we demonstrate that phosphorylation is not an absolute requirement for CaM KI activity but, indeed, serves to expand the peptide substrate specificity of the enzyme. We have also found that CaM KIV is regulated in a similar manner. CaM KI and IV, therefore, represent the first kinases whose peptide substrate specificity has been shown to be regulated by activation loop phosphorylation and raises the possibility that such control may be extended to members of other protein kinase families.

Experimental Procedures

Reagents—\(\gamma\)-\(^{32}\)P\textsubscript{ATP} was from Amersham Pharmacia Biotech. TWEEN 20, isopropyl-1-thio-\(\beta\)-galactopyranoside, glutathione, dithiothreitol, bovine serum albumin, and Tris were all obtained from Sigma. Glutathione-Sepharose 4B was from Amersham Pharmacia Biotech. Calcium chloride and magnesium chloride were purchased from Fisher. P81 phosphocellulose filter paper was from Whatman. CaM KIV was purified from SF-9 cells as described previously (37).

Protein Expression and Puriﬁcation—the human CaM KI clone was isolated from a differentiated HL-60 cDNA library as described previously (28). The T177A mutant was generated by PCR using a 5'-oligodinucleotide primer containing the appropriate base change. The PCR product was digested with SmaI and EcoRI, gel purified, and used to replace the wild-type sequence in the CaM KI clone. The entire PCR fragment was sequenced to ensure no secondary mutations had occurred (28).

BL2 (DE3) Escherichia coli strains were transformed with the appropriate CaM KI clones. Overnight starter cultures were used to inoculate 1-liter large scale cultures. These were grown to an A\textsubscript{600} of 0.7 at 37 °C, isopropyl-1-thio-\(\beta\)-galactopyranoside, glutathione, dithiothreitol, bovine serum albumin, and Tris were all obtained from Sigma. Glutathione-Sepharose 4B was from Amersham Pharmacia Biotech. CaM KI purified from Sf-9 cells as described previously (37). Glutathione-Sepharose 4B was from Amersham Pharmacia Biotech. Tween 20, isopropyl-1-thio-\(\beta\)-galactopyranoside, glutathione, dithiothreitol, bovine serum albumin, and Tris were all obtained from Sigma. Glutathione-Sepharose 4B was from Amersham Pharmacia Biotech.

The radioactive bands were carefully excised and counted by liquid scintillation spectrometry.

Axion Exchange Chromatography—The procedure was similar to Kemp et al. (40). Bio-Rad anion exchange resin AG1 \(\times 8\) (acetate form) was equilibrated in water, and the buffer was then changed to 30% acetic acid. Prior to the experiments, the ATP binding capacity of the resin was monitored by absorbance at 290 nm so >95% of the ATP bound. Chimeric peptides were phosphorylated by 5 ng of CaM KI for 5 min in the presence of 500 \(\mu\)M ATP and 5 \(\mu\)Ci of (\(\gamma\)-\(^{32}\)P)ATP in a volume of 40 \(\mu\)l. Reactions were stopped by adding 360 \(\mu\)l of 30% acetic acid. The reactions were loaded onto individual AG1 \(\times 8\) columns at 4 °C. The flow through and 6-ml 30% acetic acid washes were counted by a Beckman LS 6000 scintillation spectrometer. For kinetic analyses the 5 or 6 ATP concentrations ranged from 400 to 5000 \(\mu\)M. ADR1-(222–234)G233 peptides contain the peptide activites reported for any protein kinase, and peptide substrates could show greater than 400-fold differences in specificities. These surprising observations raised the possibility that activation loop phosphorylation of CaM KI may not be essential for kinase activity but could modulate substrate specificity. Here we demonstrate that phosphorylation is not an absolute requirement for CaM KI activity but, indeed, serves to expand the peptide substrate specificity of the enzyme. We have also found that CaM KIV is regulated in a similar manner. CaM KI and IV, therefore, represent the first kinases whose peptide substrate specificity has been shown to be regulated by activation loop phosphorylation and raises the possibility that such control may be extended to members of other protein kinase families.
Thr-177 dephospho-CaM KI has a KI and 563 ng of MBP-KKB fusion protein were incubated alone or together for the indicated time periods at 30 °C in the standard assay buffer (see “Experimental Procedures”) in the presence of 1 μM CaM and 500 μM ATP. Reactions were stopped by spotting the mixture on P81 filter paper and precipitation with 20% trichloroacetic acid. Filters were washed 3 times for 1 h or more in 20% trichloroacetic acid, rinsed briefly in 100% acetone, and air-dried. KKB autophosphorylation was subtracted to determine stoichiometry of CaM KI phosphorylation. CaM KI autophosphorylation was not above background. Open circles denote phosphorylation of CaM KI by KKB. Open squares denote KKB auto-phosphorylation. Wt, wild type.

ing CaM KKB from both human and rat brain libraries, expressed the rat kinase as a maltose-binding fusion protein, and examined its properties (35). Here we asked whether recombinant CaM KKB can phosphorylate CaM KI as does its tissue-purified counterpart (28, 30). Recombinant CaM KKB catalyzes a time-dependent phosphorylation of CaM KI to approximately 0.8 mol of phosphate/mol of enzyme (Fig. 1). The extent of phosphate incorporation at Thr-177 directly correlates with the degree of CaM KI kinase activity. In the presence of Ca2+/CaM, KKB also autophosphorylates to a stoichiometry of 0.2 mol of phosphate/mol of enzyme (Fig. 1), but this autophosphorylation does not alter KKB activity (35).

To determine the effect of Thr-177 phosphorylation on the kinetics of peptide phosphorylation, we stoichiometrically phosphorylated CaM KI using recombinant KKB and examined changes in its ability to phosphorylate the most extensively studied CaM KI peptide substrate, synapsin-(4–13). This peptide, LRRRLSEDANF, is derived from the sequence surrounding site 1, LRRRLSDSNF, of synapsin. We determined that Thr-177 dephospho-CaM KI has a KM for the synapsin-(4–13) peptide of 209 μM and a Vmax of 26.1 μmol/min/mg (Table 1). After CaM KI phosphorylation by recombinant KKB, the KMB for synapsin-(4–13) decreases 44-fold (209 to 4.7 μM) and the Vmax increases to 65.5 μmol/min/mg. After Thr-177 phosphorylation, the catalytic efficiency of the enzyme, as indicated by Vmax/Km, increases 107-fold.

Phosphorylation within the autoinhibitory and CaM binding domains of Ca2+/calmodulin-dependent protein kinase II (43) and myosin light chain kinase (MLCK) (44) is known to affect enzyme activation by CaM. To address whether activation loop phosphorylation of CaM KI affects its sensitivity to CaM, we determined the concentration of CaM required to produce 50% maximal enzyme activity (KCaM) with Thr-177 dephospho- and phospho-CaM KI. CaM KI (10 ng) was phosphorylated for 20 min by 400 ng of 1-487 KKB in the presence of 20 nM CaM. The 1-487 KKB is a constitutively active truncation mutant from which the C-terminal autoinhibitory and CaM binding domains were removed. Thus, the CaM included in the preincubation mixture served only to bind CaM KI and expose Thr-177 to KKB (28). After the 20 min preincubation, the reaction mixture was diluted 200-fold in the presence of EGTA. This effectively decreased the concentration of CaM. The CaM activation curve was then generated by assaying CaM KI activity toward synapsin-(4–13) in the presence of 2 mM CaCl2 and CaM concentrations ranging from 100 pM to 1 μM (Fig. 2). In this experiment, representative of five, the KCaM for Thr-177 dephospho-CaM KI was 14.0 ± 0.95 nM. The CaM curve for Thr-177 phospho-CaM KI was left-shifted from that of the Thr-177 dephospho-CaM KI and the KCaM 4-fold lower or 3.7 ± 0.39 nM. Thus, Thr-177 phosphorylation causes a decrease not only in the peptide km but also in the KCaM of CaM KI.

Substrate Specificity of Dephospho-CaM KI—ADR1 is phosphorylated by PKA in Saccharomyces cerevisiae (45). A peptide surrounding the PKA phosphorylation site has also been shown to be a good substrate for Ca2+/calmodulin-dependent protein kinase II, CaM KIV (45), and CaM KI (36). We have tested 10 variants of ADR1 and found that substitutions at positions within the synapsin-(4–13) peptide, which were shown to be critical or dispensable for phosphorylated CaM KI by Lee et al. (46), showed similar importance in the context of ADR1 using unphosphorylated CaM KI.2 For example, changing Arg at −3 to Leu completely abolished the ability of CaM KI to phosphorylate the ADR1-(222–234) peptide, a result consistent with the observation of Lee et al. (46) that substitution of Arg at −3 in synapsin-(4–13) caused a 240-fold decrease in Vmax/Km. On the other hand, changes in P−2 and P−1 had only very modest effects on CaM KI activity. The best variant of ADR1-(222–234), the ADR1-(222–234)G233 (substitution of Ala with Gly at the P+3 position) was an approximately 14-fold better substrate at a concentration of 100 μM than was the synapsin-(4–13) peptide. Amino acid requirements at the P+3 position have not been analyzed for CaM KI previously, and because substitutions at this position can change CaM KI activity at least 17-fold, it appears to be very important in substrate recognition.2 The best peptide, ADR1-(222–234)G233, was chosen for further studies.

Efficient Peptide Phosphorylation in the Absence of Activation Loop Phosphorylation—Because ADR1 was such an effective substrate for Thr-177 dephospho-CaM KI, we were interested in determining whether phosphorylation of CaM KI on Thr-177 would further increase enzyme activity. Our control substrate for these studies was the synapsin-(4–13) peptide. Phosphorylation of CaM KI by KKB causes a large increase in specific activity toward synapsin-(4–13) (Fig. 3A). The activity of CaM KI T177A (a mutant with the activation loop Thr changed to Ala) is not enhanced by KKB. Thus, the activity of CaM KI toward the synapsin-(4–13) peptide is dependent on Thr-177 phosphorylation. When using the ADR1-(222–234)G233 peptide as the substrate, however, phosphorylation of wild type CaM KI by KKB results in only a 25% increase in specific activity (Fig. 3A). In addition, the T177A mutant, in the presence or absence of KKB, shows similar specific activities to the native CaM KI. Hence, KKB causes only a small increase in CaM KI activity, which is independent of Thr-177 phosphorylation. This was not due to phosphorylation of ADR1-(222–234)G233 by KKB because no peptide tested with recombinant KKB was appreciably phosphorylated. Thus, these data suggest that the activity of CaM KI toward synapsin-(4–13) depends on Thr-177 phosphorylation, whereas the activity toward ADR1-(222–234)G233 is largely Thr-177 phosphorylation-independent.

Because CaM KIV is also regulated by activation loop phosphorylation, we tested if this kinase, like CaM KI, could efficiently phosphorylate ADR1-(222–234)G233 without Thr-196 being phosphorylated. In Fig. 3B, we have used as our positive control the GS-(1–10)A9,10 peptide, a variation of the glycogen

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2 S. S. Hook and A. R. Means, unpublished observations.
The synthase sequence, which has previously been used to assay CaM KIV activation (29). CaM KIV has very low activity toward GS-(1–10)A9,10 that is enhanced 7-fold by KKB. When using ADR1-(222–234)G233, however, the specific activity was 13 times that of GS-(1–10)A9,10, and phosphorylation of CaM KIV by KKB causes only a 50% increase in activity. Thus, CaM KIV is a second example where activation loop phosphorylation serves to expand its substrate specificity range.

We synthesized chimeric peptides between synapsin-(4–13) and ADR1-(222–234)G233 to determine which residues in ADR1-(222–234)G233 are responsible for the Thr-177 dephospho- and Thr-177 phospho-CaM KI activity. The results shown here are representative of five experiments. Plus symbols denote dephospho-CaM KI. Open circles denote phospho-CaM KI.

FIG. 2. CaM activation curve for the Thr-177 dephospho- and Thr-177 phospho-CaM KI. 10 ng of CaM KI was pre-incubated for 20 min with 400 ng of MBP-KKB 1–487, a Ca2+/CaM-independent form of the kinase kinase. The preincubation mixture contained 20 nM CaM because CaM binding to the full-length CaM KI is required in order for KKB to phosphorylate CaM KI (28). The activated CaM KI and CaM were then diluted in the presence of EGTA and assayed for activity (3 min) using the synapsin-(4–13) peptide (200 μM) in an excess of 2 mM CaCl2 and CaM concentrations ranging from 100 pM to 1 μM. Thr-177 dephospho-CaM KI was assayed for 5 min with CaM concentrations ranging from 250 pM to 1 μM. The results shown here are representative of five experiments. Plus symbols denote dephospho-CaM KI. Open circles denote phospho-CaM KI.

FIG. 3. Specific activities of CaM KI and CaM KIV in the absence and presence of KKB. A, specific activities of Thr-177 dephospho, Thr-177 phospho, and T177A CaM KI using synapsin-(4–13) and ADR1-(222–234)G233 peptide substrates. CaM KI (10 ng) was preincubated with 563 ng of KKB for 20 min and assayed as described under “Experimental Procedures” for 3 min. CaM KI without KKB was not preincubated alone because the enzyme lost activity during the preincubation. Instead, the kinase was assayed for 3 min without prior preincubation. The final concentration of peptide was 200 μM and ATP was 500 μM. The results here are a single representative experiment repeated three times in duplicate. Gray bars denote CaM KI activity in the absence of KKB. Black bars denote CaM KI activity in the presence of KKB. Wt, wild type. B, specific activities of Thr-196 dephospho and phospho-CaM KIV using GS-(1–10)A9,10 and ADR1-(222–234)G233 peptides. CaM KIV (250 ng) was preincubated with 563 ng of KKB for 20 min and assayed as in A. The final concentration of the peptides was 75 μM and ATP was 500 μM. The results here are a single representative experiment repeated four times in duplicate. Gray bars denote CaM KIV activity in the absence of KKB. Black bars denote CaM KIV activity in the presence of KKB.

Substrate Specificity of CaM KI

| Peptide | K<sub>m</sub> (μM) | V<sub>max</sub> (μmol/min/mg) | V<sub>max</sub>/K<sub>m</sub> (mol/min/μM) |
|---------|------------------|-----------------------------|---------------------------------|
| Synapsin-(4–13) | 209 | 26.1 | 0.13 |
| ADR1-(222–234)G233 | 17.4 | 39.4 | 1.98 |
| MYNLRRRLSDANF | 32.0 | 33.6 | 1.05 |
| LKKLTRRASFSGQ | 59.8 | 38.9 | 0.65 |
| LKKLTRRASDANF | 6.7 | 40.4 | 0.63 |
| LKKLRRLSDANF | 18.7 | 36.7 | 1.96 |

The underlined type represents the sequences from synapsin, and the bold type represents the sequences from ADR1.

* ND, not determined.
KI were assayed for 5 min in the presence of 200 μM ATP. The results here are an average of three separate experiments performed in duplicate.

234)G233 sequence did not result in high CaM KI activity in the absence of Thr-177 phosphorylation. Based on these results, neither peptide length nor the residues at positions P+1 through P+4 dictate Thr-177 dephospho-CaM KI activity.

Previously, Lee et al. (46) had shown for phospho-CaM KI that substitutions at P−2 and P−4 had minimal effects on CaM KI activity. Accordingly, we chose to focus on the N terminus of the peptides and added LKK at positions P−8 through P−6 onto synapsin-(4–13). As seen in Fig. 4, either LKK or LRR at these positions transforms synapsin-(4–13) into a substrate as good as ADR1-(222–234)/G233. Moreover, Thr-177 phosphorylation no longer has a dramatic effect on CaM KI kinetics if the LKK or LRR-synapsin-(4–13) peptides are used (Table I).

These data indicate that a peptide containing an aliphatic hydrophobic amino acid at P−8 with two basic residues at P−7 and P−6 is an excellent substrate for Thr-177 dephospho-CaM KI.

Kinetic analysis with the synapsin-(4–13) peptide revealed that activation involves primarily a decrease in the peptide Km. We determined that the Km peptide values for ADR1-(222–234)/G233 and the LKK and LRR-synapsin-(4–13) chimeric peptides using dephospho-CaM KI were similar to values seen with Thr-177 phospho-CaM KI using the synapsin-(4–13) substrate (Table I). Thr-177 dephospho-CaM KI has a Km for ADR1-(222–234)/G233 of 17.4 μM and it decreases to 7.1 μM after activation. As seen with synapsin-(4–13), the Vmax for ADR1-(222–234)/G233 increases a similar magnitude from 39.4 μmol/min/mg for dephospho-kinase to 87 μmol/min/mg for the Thr-177 phospho-CaM KI. The VmATP for ATP when using either synapsin-(4–13) or ADR1-(222–234)/G233 decreases approximately 40% after activation (110 to 68 μM). Thus, the Vmax and Km ATP is increased or decreased, respectively, independent of the peptide substrate. These results indicate that the primary effect of CaM KI activation is to lower the Km peptide substrate. ADR1-(222–234)/G233 is a much better substrate for Thr-177 dephospho-CaM KI than synapsin-(4–13) because of its low Km. The kinetic data obtained using the line underlined chimeric peptides also support this conclusion. The addition of LKK at P−8 through P−6 in synapsin-(4–13) creates a substrate with a Km of 6.7 μM and Vmax of 40.4 μmol/min/mg, a Km lower and Vmax even higher than ADR1-(222–234)/G233. Remarkably, the catalytic efficiency (Vmax/Km) of dephospho-CaM KI for synapsin-(4–13) increases over 45-fold by the addition of LKK at P−8 through P−6. Phosphorylation of CaM KI results in an increase in Vmax/Km, using synapsin-(4–13) of 107-fold, whereas the Vmax/Km of LKK-synapsin-(4–13) increases a mere 3.6-fold. Thus, the addition of LKK onto the synapsin-(4–13) peptide creates a substrate that is largely independent of activation loop phosphorylation in that it is 30-fold less responsive to CaM KI Thr-177 phosphorylation.

Our data also suggest that CaM KI phosphorylation serves to broaden its substrate specificity. The relative specificity (Vmax/Km) for ADR1-(222–234)/G233 over synapsin-(4–13) using dephospho-CaM KI is 15.2-fold (Table I). Thus, ADR1-(222–234)/G233 is strongly preferred by dephospho-CaM KI. However, with phospho-CaM KI, the specificity for ADR1-(222–234)/G233 relative to synapsin-(4–13) is very close to unity (0.88). So, whereas phospho-CaM KI can phosphorylate both ADR1-(222–234)/G233 and synapsin-(4–13), synapsin-(4–13) is slightly preferred as a peptide substrate.

Because the substitution of LKK at positions P−8 through P−6 in the synapsin-(4–13) peptide markedly increased catalytic efficiency in the absence of Thr-177 phosphorylation, we tested the effect of Ala substitutions at these positions. We found that the AAA and LAA variants did not bind to P81 phosphocellulose filters (Fig. 5A). This result was surprising because previous work had demonstrated that two basic residues with a free amino group within a peptide were sufficient for P81 phosphocellulose filter binding (48, 49). CaM KI activity toward the peptides was quantified following electrophoresis in 10% Laemmli gels, which were immediately dried and visualized by autoradiography. The bands were excised and counted by liquid scintillation spectrometry. The results are shown in Fig. 5B. The AAALTRRASFSGQ peptide phosphorylation is 50% greater than the synapsin-(4–13) peptide. The LAALTRRASFSGQ peptide is only a slightly better substrate than the AAALTRRASFSGQ peptide, and the AKKLTRRASFSGQ peptide is phosphorylated as well as the ADR1-(222–234)/G233 peptide. These results indicate that Leu at P−8 is

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**Fig. 4.** Specific activities of chimeric peptides (P+1 through P+4 and P−8 through P−6) between synapsin-(4–13) and ADR1-(222–234)/G233. Sequences from ADR1-(222–234)/G233 are in bold. Sequences from synapsin-(4–13) are underlined. 5 ng of dephospho-CaM KI were assayed for 5 min in the presence of 200 μM peptide and 500 μM ATP. The results here are an average of three separate experiments performed in duplicate.

**Fig. 5.** Dephospho-CaM KI activity toward ADR1-(222–234)/G233 peptides with substitutions at P−8 through P−6. A, P81 filter assays. ADR1-(222–234)/G233 sequences are shown in bold. Synapsin-(4–13) sequences are underlined. Changes within the ADR1-(222–234)/G233 sequence at P−8 through P−6 are shown with an asterisk. Assay conditions are as described in Fig. 4. The results here are an average of three separate experiments performed in duplicate. B, gel quantitation. Assay conditions are as described in A. The results shown here are from a single representative experiment repeated three times in triplicate.
not a critical determinant for dephospho-CaM KI activity. Individual mutation of each Lys indicates that both P–7 and P–6 contribute to the efficiency of phosphorylation by dephospho-CaM KI, with Lys at P–7 having the dominant effect. The results obtained in these experiments were also confirmed by anion exchange chromatography (40). In this assay, the peptides are passed over a positively charged resin and the free ATP binds, whereas the phosphorylated peptides are not retained. The results of this assay are consistent with those of the gel assay. Thus, the most important determinants for efficient peptide phosphorylation by dephospho-CaM KI are the Lys residues at P–7 and P–6, with the Lys at P–7 being most critical.

DISCUSSION

A large number of protein kinases have Ser/Thr or Tyr within their activation loops, and many of these kinases are phosphorylated on these residues. In all cases examined thus far, phosphorylation is thought to be required for appreciable kinase activity (24). It was previously thought that CaM KI also required phosphorylation on Thr-177 in order for the enzyme to be active (28, 30, 39, 50–52). However, in analyzing the substrate specificity of Thr-177 dephospho-CaM KI, data that agree with studies done with the phosphorylated kinase (46), we were surprised to find that ADR1-(222–234)/G233 was phosphorylated 15-fold better than the best CaM KI substrate, synapsin-(4–13). We then asked whether the specific activity toward ADR1-(222–234)/G233 could further be enhanced by Thr-177 phosphorylation. Because phosphorylation had only modest effects on CaM KI activity toward ADR1-(222–234)/G233, kinetic analysis was undertaken to determine the mechanism of activation and why certain substrates could apparently diminish the need for Thr-177 phosphorylation. Using the synapsin-(4–13) substrate, we found that Thr-177 phosphorylation modulates virtually every enzymatic parameter, V_max, K_m for substrate, K_m for ATP, and K_m CaM. The largest effect is a 44-fold decrease in K_m for substrate. These data are in contrast to Inoue et al. (51) who used an inefficient substrate for CaM KI, syntide-2, to show that K_m for peptide decreases 7-fold and K_m ATP decreases 3-fold after CaM KI phosphorylation.

Our finding that phospho-CaM KI has an increased affinity for CaM as compared with dephospho-CaM KI may have important physiological implications. Previously, Meyer et al. (43) have shown that autophosphorylation of another CaM-dependent kinase, Ca2+/calmodulin-dependent protein kinase II, markedly decreases the K_m of the enzyme, resulting in a sensitization to repetitive Ca2+ spikes. Sensitization of Ca2+/calmodulin-dependent protein kinase II to changes in intracellular Ca2+ concentration is important in such a neuronal process as long term potentiation and long term depression. CaM KI is also expressed in neurons (53) and becomes phosphorylated on Thr-177 in response to KCl depolarization of PC12 cells (31). CaM KI phosphorylation and the accompanying left-shift in the CaM activation curve may also be important in regulation of the neuronal processes of long term potentiation and long term depression. Our kinetic analysis of dephospho- and phospho-CaM KI using both the synapsin-(4–13) and ADR1-(222–234)/G233 peptide substrates revealed that CaM KI has an extremely high activity in the absence of Thr-177 phosphorylation toward substrates with very low K_m values. Substrates that have high K_m values are phosphorylated quite poorly unless Thr-177 is phosphorylated. Poor substrates for Thr-177 dephospho-CaM KI, however, can be efficiently phosphorylated by this form of the enzyme by introducing Lys or Arg residues at P–7 and P–6 relative to the phosphoacceptor site. The literature contains several examples of protein kinases that have substrate specificity determinates at P–7 or P–6. Earlier studies on PKA showed that high affinity binding of the inhibitor peptide PKI depended on: 1) the presence of a basic residue at the P–6 position, 2) dibasic residues at P–2 and P–3, and 3) an aromatic residue at the P–11 position. The importance of the P–6 position is illustrated by the kinetics of PKA phosphorylation of the PKI(14–22)/S21 peptide, GRTGRRASAI, which has a K_m of 0.11 μm, 40-fold lower than Kemptide (K_m 4.7 μm), LRRASLG, a substrate that does not contain a basic residue at P–6 (54). Substrate specificity requirements seven residues away from the phosphoacceptor are also reported for smooth muscle MLCK (55, 56). Recent work by Millward et al. (57) demonstrated that basic residues at P–7 and P–6 might also be important substrate determinants for the S100-regulated kinase, Ndr. The peptide sequence AARNRTLSVA in which the Lys residues at P–7 and P–6 were changed to Ala was not phosphorylated by this kinase. These authors used the P81 phosphocellulose binding assay but did not report whether the Ala analog peptides bound to the P81 paper quantitatively. We have shown here that the ADR1 peptide, AAALTRRASFSGQ, containing substitutions at P–8 through P–6, although having two positively charged residues and a free N terminus, does not bind quantitatively to P81 phosphocellulose filters.

The kinetic mechanism of CaM KI activation is similar to that of PKA in that K_m for peptide is dramatically affected in both cases. Phosphorylation of PKA also significantly decreases its K_m ATP, whereas phosphorylation of CaM KI only slightly affects K_m ATP. Mutation of the equivalent Thr in PKA, Thr-197, to either Ala or Asp results in a 25–100-fold increase in K_m substrate and K_m ATP (26). In addition, Steinberg et al. (25) demonstrated that the unphosphorylated PKA also has elevated K_m values for substrate and ATP similar to those seen with the mutant catalytic subunits (26). The increased K_m for ATP was because of a decreased binding affinity, but the K_m substrate increases were not because of peptide binding affinities, but rather a reduced rate of phosphoryl transfer.

The catalytic subunit of PKA was crystallized as an “active” kinase with Thr-197 phosphorylated and Mg2+/ATP present. The structures of CaM KI (58), CDK2 (59), and extracellular signal-regulated kinase 2 (60) all reflect inactive kinases, where the activating residue(s) within the activation loop were not phosphorylated. It is thought that the conformation of the activation loops of both phosphorylated CDK2 and extracellular signal-regulated kinase 2 precludes substrate binding. However, mutagenesis data from PKA reveal that the loss of Thr-197 phosphate has no effect on peptide binding; the changes in K_m peptide are because of phosphate transfer. Based on our data, the nonphosphorylated activation loop of CaM KI, like the nonphosphorylated activation loop of PKA, does not preclude substrate binding, as was proposed for CDK2 and extracellular signal-regulated kinase 2. The crystal structure of the CaM KI 1–320 enzyme contains a disordered activation loop providing us with no information about the conformation of the unphosphorylated loop. Our studies suggest that the change in peptide K_m in response to Thr-177 phosphorylation, whether because of peptide binding or rate of phosphoryl transfer, can be overcome by the sequence of the substrate. It seems reasonable to suggest that the unphosphorylated loop of CaM KI does not adopt a conformation that prevents substrate binding because unphosphorylated CaM KI efficiently phosphorylates the ADR1-(222–234)/G233 substrate.

The crystal structures of CaM KI and PKA provide some insight as to how kinases recognize their substrate. CaM KI contains an autoinhibitory domain that, in the absence of Ca2+/CaM, lies within the substrate binding groove. When CaM KI is autoinhibited, its autoinhibitory domain is predicted to make
similar contacts with the catalytic domain as would a substrate. Ca\(^{2+}\)/CaM binding to the enzyme results in the removal of the autoinhibitory domain from the catalytic pocket and allows substrate binding. The CaM KI fragment (1–320) was crystallized in the absence of Ca\(^{2+}\)/CaM and Thr-177 phosphorylation. Hence, the structure reflects the autoinhibited kinase. The catalytic subunit of PKA does not contain an autoinhibitory domain but the kinase was crystallized in the presence of the inhibitory protein, PKI. PKI binds to PKA in a manner competitive with substrate. In addition, a variant of PKI that contains a PKA autophosphorylation site in its C terminus is no longer an inhibitor but a substrate (54). Thus, by inspecting the contacts of PKI and the autoinhibitory domain of CaM KI with the catalytic domain of PKA or CaM KI, respectively, we can predict how CaM KI recognizes individual residues surrounding the substrate phosphoacceptor site. CaM KI has a strict requirement for a basic residue at P\(_6\). CaM KI mimics Arg at P\(_6\) by dephospho-CaM KI, a residue that makes contacts with the basic Arg at P\(_6\) within the substrate (Fig. 6). Lys-300 within the autoinhibitory domain of CaM KI mimics Arg at P\(_3\) by interacting with Glu-221. Mutation of Lys-300 alters the enzyme specificity at P\(_3\) (58). Likewise, the preference of CaM KI for a hydrophobic residue at P\(_5\) within the substrate is illustrated by the autoinhibitory domain residue Phe-298 being buried within a deep hydrophobic pocket formed by Phe-104, Ile-210, and Pro-216. We can structurally rationalize how the addition of basic residues at P\(_{-7}\) and P\(_{-6}\) on a CaM KI substrate substantially decreases its K\(_{\text{m}}\). Our chimeric peptide studies indicated that the most crucial factor for efficient phosphorylation of ADR1-(222–234)G233 (a low K\(_{\text{m}}\) value) by dephospho-CaM KI was Lys at P\(_{-7}\). It seems reasonable that Lys at P\(_{-7}\) is interacting with Glu-221, a residue residing at the end of the loop preceding the G\(_{\alpha}\)-helix within the catalytic core. Alternatively, depending on how the Lys side chain at P\(_{-7}\) lies, it may be interacting with Val-108, a residue that interacts with P\(_{-8}\) within the autoinhibitory domain. Interestingly, CaM KIV, another enzyme that displays similar substrate phosphorylation properties to CaM KI in the absence and presence of activation loop phosphorylation, has conserved Glu and Val residues at these same positions. In PKA, the P\(_{-6}\) residue is represented by the positively charged Arg-15 in PKI, interacts with the negatively charged Glu-203 within the catalytic core (22). The equivalent residue to Glu-203 in CaM KI is Gly-183. It seems reasonable that if Gly-183 were a negatively charged Glu, as in PKA, the positively charged Lys at P\(_{-6}\) in ADR1-(222–234)G233 peptide may contribute more significantly to the “independence” of ADR1-(222–234)G233 for Thr-177 phosphorylation. Other enzymes shown to be regulated by activation loop phosphorylation such as protein kinase C, PKA, and CDK2 have a Glu (in the case of protein kinase C) or an Asp (in the cases of PKA and CDK2) at the equivalent position as Glu-221 in CaM KI, raising the possibility that they may also have peptide substrates that are efficiently phosphorylated in the absence of activation loop phosphorylation.

Our data reveal that Thr-177 phosphorylation expands the substrate specificity of CaM KI. What structural role does Thr-177 phosphorylation play with substrates, like synapsin-1 (4–13), whose V\(_{\text{max}}$/K\(_{\text{m}}\) is dramatically influenced by activation loop phosphorylation? The answer to this question may be partially explained by small angle x-ray and neutron scattering with another CaM-dependent enzyme, skeletal (sk) muscle MLCK. Krueger et al. (61) have reported that CaM binding to skMLCK causes the autoinhibitory sequences to swing away from the catalytic core. The addition of either the nonhydrolyzable ATP analogue, AMPPNP, or a peptide substrate from the myosin light chain to this CaM-MLCK complex causes a compaction of the catalytic domain (62). The closure between the small and large lobes of the kinase and the accompanying shift in the CaM and skMLCK centers of mass toward one another, however, require both Mg\(^{2+}\)/ATP and peptide substrate binding. Although the activity of skMLCK is not regulated by activation loop phosphorylation, its activity is stimulated by CaM in a manner similar to CaM KI. The crystal structure of CaM KI in the absence of CaM, Mg\(^{2+}\)/ATP, peptide substrate, and Thr-177 phosphorylation is in the open conformation. If we extend the findings with skMLCK to CaM KI, we predict that the ability of peptide substrate to induce conformational changes may be influenced by Thr-177 phosphorylation. Peptide substrates that are phosphorylated efficiently in the absence of Thr-177 bind to dephospho-CaM KI with high affinity and induce the requisite conformational change associated with substrate binding, whereas other “activation-dependent” substrates would require Thr-177 phosphorylation in order for them to induce the conformational change. Alternatively, Thr-177 phosphorylation may induce conformational changes in the kinase that can be mimicked by binding of a high affinity substrate.

Regardless of the molecular mechanism, our results identify the possibility that substrates directly regulate kinase activity. Previously, Walsh et al. (63) have pointed out that different substrate affinities may be important in regulating the order of substrate phosphorylation events within cells to coordinate different metabolic events. Our results highlight the possibility that phosphorylation of protein kinase activation loops may add a further regulatory dimension to the timing of phosphorylation events within cells.

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