# Phosphorylation of Calmodulin Fragments by Protein Kinase CK2. Mechanistic Aspects and Structural Consequences†

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ABSTRACT: Calmodulin is phosphorylated in vivo and in vitro by protein kinase CK2 in a manner that is unique among CK2 substrates for being inhibited by the regulatory $\beta$-subunit of the kinase and dramatically enhanced by polybasic peptides. Using synthetic fragments of calmodulin variably encompassing the CK2 phosphorylation sites here we show that individual phosphorylation of Thr79, Ser81, Ser101, and Thr117 is critically influenced by the size and composition of the peptides and that the C-terminal domain of calmodulin is implicated both in down-regulation of calmodulin phosphorylation by the $\beta$-subunit and in its abnormal responsiveness to polylysine. A far-Western blot analysis discloses polylysine-dependent interaction between calmodulin and the N-terminal domain of the $\beta$-subunit. We also show that phosphorylation of Ser81 hampers subsequent phosphorylation of Thr79 and by itself promotes the unfolding of the central helix, whose flexibility is instrumental to the interaction with calmodulin-dependent enzymes. Collectively taken, our data are consistent with a multifaceted regulation of calmodulin phosphorylation through the concerted action of distinct CaM domains, the catalytic and regulatory subunits of CK2, and polycationic effectors mimicking in vivo the effect of polylysine.

Calmodulin (CaM[1]) is a ubiquitous, highly conserved Ca-binding protein playing a paramount role in calcium signaling (*1*−*4*). It has a $Ca^{2+}$ affinity of $10^{-6}$ M and thus acts as a switch whenever intracellular $Ca^{2+}$ concentration rises transiently to $10^{-5}$ from its resting value of approximately $10^{-7}$ M. The modus operandi of CaM consists of the binding of $Ca^{2+}$ at its four Ca binding loops forming two globular clusters at its N- and C-terminal domains, which are connected by a flexible central α-helix (*5*). When calcium is sequestered, the α-helix content of CaM increases with a concomitant exposure of two hydrophobic patches, which bind specifically to the CaM binding domains of a variety of proteins the activity of which is either dependent on or anyway affected by CaM (*6*). A number of $Ca^{2+}$-regulated enzymes, moreover, notably phosphorylase kinase and calcineurin (protein phosphatase 2B), incorporate CaM in their holoenzymes as a bona fide regulatory subunit.

The biological function of CaM, however, may be not confined to its universal role as primary $Ca^{2+}$-signaling "decoder" in the cell, since it interacts with several proteins in its $Ca^{2+}$-free form as well (*2*, *7*).

Given the crucial implication of CaM in signal transduction and the central role protein phosphorylation plays in this process, one would expect CaM to be a target for protein kinases and protein phosphatases, by analogy with the majority of signaling molecules. This would also provide a means to integrate signals generated by diverse stimuli but all mediated by CaM.

CaM includes 18 phosphorylatable residues altogether, 4 serines, 12 threonines, and 2 tyrosines. In vitro phosphorylation of both tyrosines, of four threonines and of two serines by a variety of tyrosine and serine/threonine kinases has been reported (reviewed in ref *8*) and shown in some cases to alter the properties of CaM. In particular, phosphorylation of calmodulin by CK2 has been reported to variably affect the interaction with several CaM-dependent enzymes, notably CaM-dependent cyclic nucleotide phosphodiesterase, $Ca^{2+}$-ATPase, $Ca^{2+}$/CaM-dependent protein kinase II, myosin light chain kinase, and NO synthase (*8*−*14*). Regarding the in vivo occurrence of these phosphorylation events, while tyrosine phosphorylation in nontransfected cells has been a matter of debate (*15*−*17*), incontrovertible evidence has been provided that Thr79, Ser81, and Ser101 are phosphorylated in a fraction of phosphocalmodulin (accounting for about 15% of the whole cell CaM) isolated from rat liver (*18*). Indeed a serine/threonine phosphorylated form of CaM had been previously isolated also from chicken brain and muscle (*19*). Thr79 and Ser81 are located in the central helix, while Ser101 is in the Ca-binding loop III, and all conform to the consensus sequence of protein kinase CK2 (S/T-X-X-E/D). The same residues, plus Thr117, are also phosphorylated in vitro by CK2 (*20*−*23*), a constitutively active, highly pleiotropic serine/threonine kinase of which the growing list of substrates already includes, besides CaM, >300 proteins, mostly implicated in signal transduction and gene expression (*24*). Endogenous phosphorylation of CaM by CK2 has been recently reported to occur in bovine aortic endothelial cells

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[1] Abbreviations: CaM, calmodulin; CK2, casein kinase 2; FWB, far-Western blotting.

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(*9*). Thus CK2 appears to be the main, if not the only, serine/threonine-phosphorylating agent of CaM in living cells. It was early realized, however, that phosphorylation of CaM by CK2 is unique in at least two respects: first it is suppressed, instead of being stimulated as it is with most CK2 substrates, by the regulatory $\beta$-subunit of CK2 to such an extent that the holoenzyme is virtually inactive on CaM, which instead is appreciably phosphorylated by the isolated catalytic subunits, either $\alpha$ (*25*) or $\alpha'$ (*26*). Second phosphorylation of CaM by CK2 holoenzyme can be triggered by polybasic peptides, such as polylysine, some histones, and protamines the general efficacy of which as stimulators of CK2 holenzyme becomes especially dramatic if CaM is the substrate (*21*, *22*, *25*). Such a behavior raises the question as to how the in vivo phosphorylation of CK2 takes place, whether catalyzed by a pool of free catalytic subunits of CK2 (the existence of which, suggested by a number of coincidental arguments, has never been unambiguously proven) or rather mediated by the concerted action of CK2 holenzyme and as yet unidentified polybasic proteins mimicking the in vitro effect of polylysine.

An important corollary to this question is the understanding of the structural features underlying the anomalous mode of CaM phosphorylation with respect to its inhibition by the $\beta$-subunit and its exceptional stimulation by polybasic peptides (note that polyamines fail to surrogate polybasic peptides in this respect (*25*)). We have previously shown that both properties are not conferred by any peculiar feature of the sequence encompassing the two main phosphoacceptor sites, Thr79/Ser81 and Ser101, since discrete peptides reproducing the two sequences are readily phosphorylated by CK2 in a "canonical" manner, that is, stimulated by the $\beta$-subunit and not dependent on polylysine whenever the phosphorylating agent is the heterotetrameric holoenzyme (*25*, *27*). Here, by exploiting large synthetic peptides and phosphopeptides encompassing in some cases almost half of the CaM molecule, we show that the C-terminal domain of CaM is critical to make CaM phosphorylation susceptible to inhibition by the $\beta$-subunit and dependent on polylysine. We also show that in the presence of polylysine (but not in its absence) CaM binds to the N-terminal domain of CK2 $\beta$-subunit and that phosphorylation of Ser81 unfolds the central helix of CaM.

## MATERIALS AND METHODS

*Materials.* Native protein kinase CK2 was isolated and purified from rat liver cytosol as previously described (*28*). Recombinant calmodulin was expressed in *Escherichia coli* and purified as previously described (*29*). Recombinant $\alpha$ and $\beta$ subunits of human protein kinase CK2 were purified according to Sarno (*30*) after expression in *E. coli*.

*Peptide Synthesis.* The calmodulin fragments were synthesized as previously described (*27*). The phosphopeptide CaM[54−106]pS81 was obtained by direct incorporation of phosphoserine as the Fmoc-Ser(PO(Obzl))-OH derivative (Novabiochem).

The crude peptides were purified by ion-exchange chromatography on DEAE Ceramic HyperD F resin (Biosepra) and by HPLC on a Prep-NovaPak HR C18, 6 $\mu$m, 25 mm × 100 mm (Waters), using a linear gradient of 0%−50% acetonitrile/0.1% TFA with a flow rate of 12 mL/min. The resulting purity of peptides was >95% by analytical HPLC on a column C18 Symmetry300, 5 $\mu$m, 4.6 mm × 250 mm (Waters), using a linear gradient of 5%−60% acetonitrile/0.1% TFA at 1 mL/min flow rate. The molecular masses of the peptides were confirmed by mass spectrometry using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectrometer (Micromass). All peptides were soluble in water up to at least 5 mM. The water solutions were stored at −20 °C for several weeks without appreciable alterations in physical chemical properties.

*Phosphorylation Assay.* Phosphorylation reactions were performed by incubating for 10 min at 37 °C the phosphorylatable substrate at concentrations detailed in tables and figures in 25 $\mu$L of a medium containing 50 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, 0.1 M NaCl, 40 $\mu$M [$\gamma$-$^{32}$P]ATP (specific radioactivity 500−1000 cpm/pmol), and 10−20 mU of CK2 holoenzyme (one unit being the amount of kinase transferring 1 nmol of $P_i$ per minute to the specific peptide substrate). All CK2 activity assays were linear with respect to time and enzyme concentration in each incubation. The phosphate incorporated into protein substrates was evaluated by subjecting samples to SDS−PAGE, staining, and autoradiography or direct scanning on Instant Imager (Canberra-Packard) apparatus. The radiolabeled peptides were isolated and quantified by phosphocellulose filters procedure (*31*). Recovery was always >90%.

*Phosphoamino Acid Analysis.* Aliquots of radiolabeled protein or peptide substrates were subjected to partial acid hydrolysis in 6 N HCl at 105 °C for 4 h, followed by high-voltage paper electrophoresis and corrections for hydrolytic loss of SerP (48%) and ThrP (14%) as previously detailed (*32*). The hydrolytic loss was not significantly influenced by the structure of the peptide substrates.

*Trypsin Digestion and 2D Separation of Tryptic Peptides.* Radiolabeled protein or peptide samples were eluted from polyacrylamide gels with 50 mM ammonium hydrogen carbonate, lyophylized, and subjected to tryptic digestion in freshly prepared 50 mM ammonium hydrogen carbonate (4 h at 37 °C) with a 1:50 w/w L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone (TPCK)-treated trypsin/substrate ratio. The tryptic peptides were separated on 2D thin layer cellulose plates (20 cm × 20 cm) by combining electrophoresis in the first dimension (30 min at 1000 V in 1% ammonium carbonate buffer, pH 8.9) with ascending chromatography in organic solvent buffer (39.2% *n*-butanol, 30.3% pyridine, 6.1% acetic acid) in the second dimension according to Boyle (*33*). Cellulose plates were then dried and scanned on Instant Imager apparatus (Canberra-Packard) for the detection of the radiolabeled spots.

*Far-Western Blot Analysis.* Samples of CK2 $\beta$-subunit and of its shorter fragments were subjected to SDS−PAGE and transblotted to nitrocellulose membranes (Bio Rad) in a Hoefer apparatus at 250 mA for 2.30 h. Membranes were rapidly stained with 0.2% Ponceau Red in 3% TCA, blocked for 1 h at room temperature with 3% bovine serum albumin in 10 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl and then treated as previously described (*34*). In particular, membranes were first equilibrated for 1 h with binding buffer (20 mM Tris-HCl, pH 8.0, containing 300 mM KCl and 0.1% Tween 20) and then gently stirred for 2 h at 4 °C in the same buffer in the presence of 0.06 mg/mL calmodulin (CaM) as a ligand. Unbound CaM was removed by washing

the membranes three times with binding buffer and the bound ligand was immunodetected by using polyclonal anti-CaM antibodies (Santa Cruz Biotechnologies).

*MS Analysis: Identification of Phosphorylation Sites.* Mass analysis was carried out using an Ultima Q-Tof (Micromass). After phosphorylation by CK2, the solutions containing the different phosphopeptides were desalted with $C_{18}$-E SPE STRATA cartridges (Phenomenex), concentrated under vacuum, and then loaded into the instrument.

Collision-induced dissociation (CID) spectra of the phosphorylated peptides were acquired to determine the position of the phosphoresidues. Due to the loss of phosphoric acid dominating the CID spectra of phosphopeptides, the solutions containing the peptides phosphorylated by CK2 were treated with a saturated solution of $Ba(OH)_2$ for 30 min, the pH was lowered by adding formic acid, and the mixture was desalted using C18 Zip-Tip (Millipore) and analyzed again. The reaction with $Ba(OH)_2$ implies a $\beta$-elimination of $H_3PO_4$ and the generation of dehydrated serine or threonine at the site of phosphorylation, allowing one to obtain more informative MS/MS spectra (*35*−*37*). The fragmentation spectra were then manually interpreted according to Hunt (*38*), and the phosphorylation sites were identified.

*MS Analysis: Identification of Tryptic Peptides by HPLC and MALDI Analysis.* The peptides CaM[54−106] and CaM-[54−106]pS81 were digested with trypsin in 100 mM $NH_4HCO_3$, pH 7.8, using an enzyme/substrate ratio of 1/50 (w/w) for 4 h at 37 °C. The tryptic peptides were then separated by HPLC using a reverse-phase C18 Symmetry300 column (Waters), 5 $\mu$m, 3.9 mm × 150 mm, with a linear gradient from 0%−50% of acetonitrile in 40 min and with a flow rate of 1 mL/min. The fractions corresponding to the isolated peaks were then concentrated under vacuum and analyzed by MALDI mass spectrometry using a MALDI-TOF (Micromass). α-Cyano-4-hydroxycinnamic acid (Aldrich) in 50% acetonitrile/0.1% formic acid was used as matrix.

*Circular Dichroism Analysis.* CD spectra were recorded with a Jasco (Easton, MD) J-710 spectropolarimeter. The instrument was calibrated with *d*-(+)-camphorsulfonic acid. Far-UV CD spectra of peptides CaM[54−106] and CaM-[54−106]pS81 were recorded at 25 °C using 0.1 cm path length quartz cells in 10 mM Tris/HCl, 50 mM NaCl, 5 mM $CaCl_2$, pH 7.5. The spectra were then normalized as mean residue ellipticity [Θ] (deg·cm$^2$·dmol$^{-1}$) according to the formula $[\Theta]_{MRW} = (\Theta_{obs}/10)MRW/(lc)$, where $\Theta_{obs}$ is the observed ellipticity at a given wavelength, MRW is the mean residue weight of the peptide, *l* is the path length in centimeters, and *c* is the peptide concentration in grams per milliliter.

## RESULTS AND DISCUSSION

*1. Synthetic Fragments of Calmodulin.* Figure 1 reports a schematic representation of the calmodulin molecule aligned with the synthetic peptides used in this study the sequences of which are on display in Table 1. The peptides variably encompass the three CK2 phosphorylation sites, namely, Thr79/Ser81, Ser101, and Thr117, with the largest peptide, 74−142, including all of them and spanning the whole C-terminal half of the protein from the central helix to the C-terminus (except for the last five amino acids). The other peptides include one or two phosphoacceptor sites, and in few cases, they have also undergone modifications to assess the relevance of individual structural elements. Note that peptide 54−106, encompassing the central part of calmodulin with the central helix flanked by Ca-binding loops II and III has been synthesized either as such or with serine 81, a residue that is phosphorylated both in vivo and in vitro by CK2, replaced by phosphoserine.

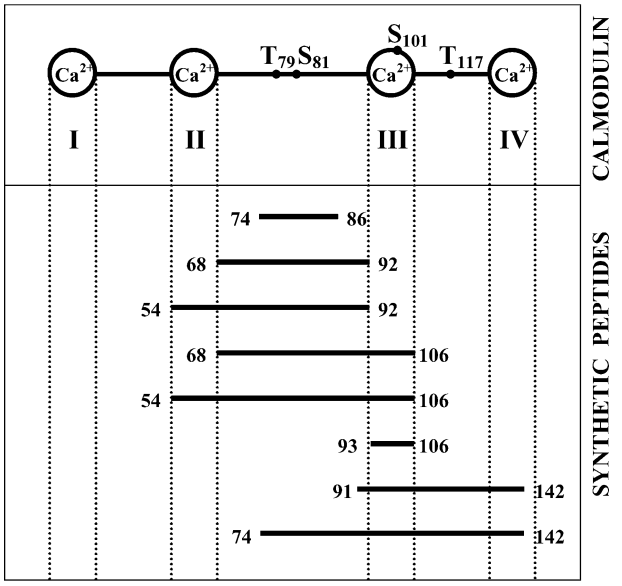


FIGURE 1: Schematic representation of calmodulin and its synthetic fragments. I−IV denote the four calcium binding loops of full-length calmodulin. The four main sites phosphorylated by CK2 ($T_{79}$, $S_{81}$, $S_{101}$, and $T_{117}$) are also indicated.

*2. Phosphorylation of Peptides Encompassing the Thr79/Ser81 Site.* Peptide 68−92 reproduces the central helix of calmodulin, spanning between Ca-binding loops II and III; it contains two of the four residues phosphorylated by CK2, Thr79 and Ser81, forming together the main phosphoacceptor site. This site alone is also contained in a shorter peptide, 74−86, and in a longer one, 54−92, this latter extended on the N-terminal side to include also Ca-binding loop II. The kinetic constants for the phosphorylation of these peptides by CK2 holoenzyme and $^{32}P$ incorporated into either serine or threonine are reported in Table 2. Note that although the peptides 68−92 and 54−92 include one and two additional threonines, respectively, besides Thr79, the phosphorylation of these residues was ruled out by tryptic digestion and HPLC separation of the phosphorylated products (not shown). Therefore the phosphoamino acid analysis reflects the phosphorylation of just Thr79 and Ser81. While the phosphorylation efficiency is affected, albeit not dramatically, by the length of the peptide, the SerP to ThrP ratio is invariably low with all these peptides (Table 2, peptides 1−3). These figures are not affected by the incubation time (not shown), a circumstance arguing against an ordered or hierarchical mode of phosphorylation of Thr79 and Ser81 or both. Preferential phosphorylation of Thr79 over Ser81 came as a surprise considering that CK2 by far prefers serine over threonine under comparable conditions (*24*, *39*); it has to be assumed therefore that for some reason position 79 is better suited than position 81 for CK2 phosphorylation. To assess this point, a derivative of the 74−86 peptide has been synthesized in which Thr79 and Ser81 have been interchanged: this was phosphorylated much more efficiently

Table 1: Amino Acid Sequence of the Synthetic Fragments of Calmodulin[a]

| SYNTHETIC PEPTIDE | SEQUENCE |
|---|---|
| CaM[74-86] | RKMKD**T**D**S**EEEIR |
| CaM[74-86]A81 | RKMKD**T**D**A**EEEIR |
| CaM[74-86]S79T81 | RKMKD**S**D**T**EEEIR |
| CaM[68-92] | FLTMMARKMKD**T**D**S**EEEIREAFRVF |
| CaM[54-92] | EVDADGNGTIDFPEFLTMMARKMKD**T**D**S**EEEIREAFRVF |
| CaM[68-106] | FLTMMARKMKD**T**D**S**EEEIREAFRVFDKDGNGYI**S**AAELR |
| CaM[68-106]A82,83,84,87 | FLTMMARKMKD**T**D**S**AAAIRAAFRVFDKDGNGYI**S**AAELR |
| CaM[[68-106]A93,95 | FLTMMARKMKD**T**D**S**EEEIREAFRVFAKAGNGYI**S**AAELR |
| CaM[54-106] | EVDADGNGTIDFPEFLTMMARKMKD**T**D**S**EEEIREAFRVFDKDGNGYI**S**AAELR |
| CaM[54-106]pS81 | EVDADGNGTIDFPEFLTMMARKMKD**T**D**pS**EEEIREAFRVFDKDGNGYI**S**AAELR |
| CaM[93-106] | DKDGNGYI**S**AAELR |
| CaM[93-106]A93,95 | AKAGNGYI**S**AAELR |
| CaM[93-106]A104 | DKDGNGYI**S**AAALR |
| CaM[91-142] | VFDKDGNGYI**S**AAELRHVMTNLGEKL**T**DEEVDEMIREANIDGDGQVNYEEFV |
| CaM[74-142] | RKMKD**T**D**S**EEEIREAFRVFDKDGNGYI**S**AAELRHVMTNLGEKL**T**DEEVDEMIREANIDGDGQVNYEEFV |

[a] The residues phosphorylated by CK2 in position 79, 81, 101, and 117 are highlighted in bold. The residues mutated with respect to the wild-type sequence are underlined.

Table 2: Kinetic Constants and Phosphoamino Acid Analysis of Central Helix Derived Calmodulin Fragments Phosphorylated by CK2 Holoenzyme[a]

| | peptide | $V_{max}$ (nmol·min$^{-1}$· mg$^{-1}$) | $K_m$ ($\mu$M) | P-Ser (%) | P-Thr (%) |
|---|---|---|---|---|---|
| 1 | CaM[54−92] | 134 | 212 | 33 | 67 |
| 2 | CaM[68−92] | 126 | 270 | 28 | 72 |
| 3 | CaM[74−86] | 125 | 254 | 30 | 70 |
| 4 | CaM[74−86]S79T81 | 324 | 236 | 94 | 6 |
| 5 | CaM[74−86]A81 | 41 | 250 | | 100 |
| 6 | CaM[91−142] | 32 | 138 | 76 | 24 |
| 7 | CaM[93−106] | 47 | 135 | 100 | |
| 8 | CaM[93−106]A93,95 | 26 | 131 | 100 | |
| 9 | CaM[93−106]A104 | 12 | 166 | 100 | |
| 10 | CaM[68−106] | 314 | 160 | 82 | 18 |
| 11 | CaM[68−106]A82,83,84,87 | 255 | 158 | 97 | 3 |
| 12 | CaM[68−106]A93,95 | 283 | 135 | 60 | 40 |
| 13 | CaM[54−106] | 353 | 153 | 79 | 21 |
| 14 | CaM[54−106]pS81 | *b* | *b* | 99 | 1 |
| 15 | CaM[74−142] | 61 | 137 | 46 | 54 |

[a] All peptides were subjected to phosphorylation by 10 min incubation with CK2 holoenzyme as described in the Materials and Methods section, and kinetic parameters were calculated from double-reciprocal plots according to the Lineweaver−Burk equation as the mean of at least three independent experiments. Phosphorylation never exceeded 5% of the peptide substrates. SE never exceeded 10%. Aliquots of the peptides radiolabeled at 200 $\mu$M concentration were subjected to partial acid hydrolysis and high-voltage paper electrophoresis as described in the Materials and Methods section. The radioactivity corresponding to the spots of phosphoserine and phosphothreonine, quantitated and corrected for the hydrolytic loss (48% and 14%, respectively) (*32*), is expressed as percentage of the total incorporation. [b] Not determined.

than the parent peptide with nearly all the phosphate being now incorporated into serine rather than into threonine (peptide 4 in Table 2). This corroborates the concept that indeed the 79 position is preferred over the 81 one by CK2 and that, under comparable conditions, serine is phosphorylated more readily than threonine. The additional finding that Thr79 is almost unaffected if Ser81 is replaced by alanine in the parent peptide 74−86 (Table 2, peptide 5) would indicate that Ser81 by itself is a better determinant than alanine of Thr79 phosphorylation regardless of its phosphorylation, which is less pronounced than that of Thr79. Interestingly the same substitution of Ser81 by alanine is also detrimental to the phosphorylation of CaM transfected into aortic endothelial cells (*9*).

A thin layer 2D separation of the phosphopeptides obtained by tryptic digestion of phosphoradiolabeled peptide 68−92 is shown in Figure 2, panel a. A row of four radioactive spots is detectable with two spots (B and C) slightly differing for chromatographic mobility ($R_f$ = 0.34 and 0.32, respectively) and two spots of which the electrophoretic mobility is higher (A) and lower (D) than that of the spots B and C. In accordance with previous reports (*40*) such a heterogeneity is mainly due to incomplete tryptic digestion, partially due to the phosphate incorporated, as also shown by HPLC and MS analysis of the tryptic fragments generated from the peptide 54−106 either as such or phosphorylated at Ser81 (Table 3). Tryptic cleavage of the K77−D78 bond is partial, giving rise to two peptides 78−86 and 75−86, both including the phosphorylation site. A third peptide including the 79/81 site is generated by trypsin if Ser81 is phosphorylated due to partial cleavage at Arg86 as well (phosphopeptide 75−90). Thus phosphorylation of Ser81 alone is sufficient to account for three radioactive spots differing in electrophoretic mobility detected in Figure 2, panel a. Accordingly, although traces of a peptide doubly phosphorylated at both Thr79 and Ser81 have been actually detected in the tryptic digest of calmodulin phosphorylated in vivo (*18*), no

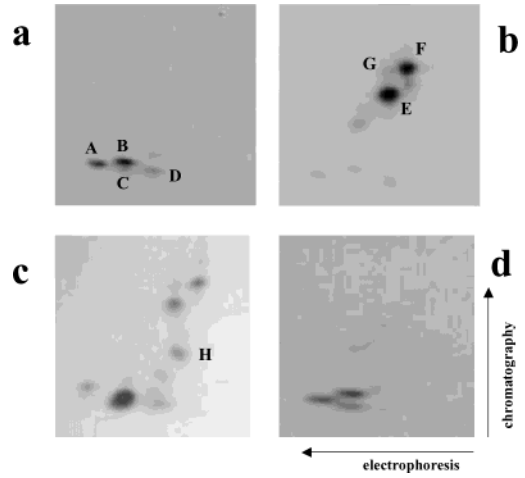


FIGURE 2: 2D thin layer separation of tryptic peptides obtained from calmodulin fragments phosphorylated by CK2 holoenzyme. Phosphorylation of the peptides (200 $\mu$M), digestion with trypsin, and 2D separation were performed as described in the Materials and Methods section. In the case of calmodulin (10 $\mu$M), 330 nM polylysine was also present in the phosphorylation medium. After the radioactive spots were scraped and the phosphoamino acid analysis was performed (see Materials and Methods), the radiolabeled spots E−G of panel b and spot H of panel c gave rise only to phosphoserine and phosphothreonine, respectively. Spots A and B (panel a) gave rise only to phosphoserine and to phosphothreonine, respectively, while both phosphoamino acids could be detected in spots C and D. Panel a presents results for peptide CaM[68−92]; panel b presents results for peptide CaM[54−106]; panel c presents results for peptide CaM[74−142]; panel d presents results for calmodulin. A−H labels indicate the main spots discussed in the text. The directions of electrophoresis at pH 8.9 and of chromatography are also indicated.

Table 3: MS Characterization of Peptides Obtained by Tryptic Digestion of CaM[54−106] and CaM[54−106]pS81[a]

| tryptic peptide | theoretical mass (Da) | experimental mass (Da) |
|---|---|---|
| | CaM[54−106] | |
| CaM[78−86] | 1092.46 | 1092.45 |
| CaM[75−86] | 1479.70 | 1479.70 |
| CaM[91−106] | 1753.87 | 1753.88 |
| CaM[54−74] | 2328.05 | 2328.06 |
| | CaM[54−106]pS81 | |
| CaM[78−86]pS81 | 1092.46 | 1092.45 |
| CaM[75−86]pS81 | 1479.70 | 1479.70 |
| CaM[75−90]pS81 | 2062.91 | 2062.92 |
| CaM[91−106] | 1753.87 | 1753.88 |
| CaM[54−74] | 2328.05 | 2328.06 |

[a] The peptides were digested with trypsin and the products separated by HPLC and analyzed by mass spectrometry as described in the Materials and Methods section. Note that three peptides including the phosphoacceptor site were identified in the tryptic products of CaM[54−106]pS81 instead of two found in the tryptic products of its unphosphorylated counterpart. The largest of these peptides (CaM[75−90]) was not detected in the tryptic products of CaM[54−106], suggesting that the phosphate at position 81 is responsible for incomplete cleavage at Arg86.

evidence of bisphosphorylated tryptic products could be obtained either by HPLC analysis (not shown) or by a MS approach (see Figure 3). Both strategies have yielded monophosphorylated products containing either pSer81 or pThr79 but failed to detect bisphosphorylated peptides. The fourth spot in Figure 2a can be due to phosphorylation of Thr79. In fact, while spot A upon acid hydrolysis gives rise only to SerP, ThrP alone could be recovered from spot B. Both SerP and ThrP were found in the acid hydrolysates of the faint spots C and D. Their electrophoretic mobility argues against the possibility that they are bisphosphorylated peptides, however. It seems more likely that they are a mixture of products of partial tryptic cleavage monophosphorylated at either Thr79 or Ser81.

Collectively taken the experiments with peptides encompassing the 79/81 phosphoacceptor site support the conclusion that position 79 is featured in such a way that it is preferred by CK2 over position 81 even though threonine is per se not as good as serine as a CK2 target. Moreover, individual phosphorylation of either Ser81 or Thr79 seemingly stabilizes local conformations that hamper the subsequent phosphorylation of the other residue, thus accounting for the failure to detect bisphosphorylated products. Consistent with this, a peptide encompassing residues 54−106 in which Ser81 has been replaced by phosphoserine has lost its ability to be phosphorylated at Thr79 (see below), despite the fact that a phosphorylated side chain at position $n + 2$ is a strong positive determinant for CK2 phosphorylation of short peptides (*32*).

*3. Phosphorylation of Peptides Encompassing Ser101 and Thr117.* Besides Thr79 and Ser81, also Ser101 in Ca-binding loop III and Thr117 between Ca-binding loops III and IV are phosphorylated by CK2. Ser101 has been found phosphorylated also in vivo (*18*), whereas evidence for Thr117 phosphorylation is grounded only on in vitro evidence (*41*). Accordingly, a peptide encompassing both Ser101 and Thr117 (91−142) is readily phosphorylated at both serine and threonine residues (Table 2, peptide 6).

While Thr117 displays the stigmata of an optimal CK2 site with acidic determinants at all the important positions, $n + 3$, $n + 1$, and $n + 2$, Ser101 conforms to the minimun consensus (S-X-X-E) lacking additional acidic residues downstream as they are found in the majority of CK2 sites (*24*), and it also has an arginyl residue at position $n + 5$, which is expected to act as a negative determinant (*42*). Therefore efficient phosphorylation of peptides encompassing Ser101 by CK2 was unexpected. On the basis of the crystal structure of calmodulin (*43*), it was suggested that phosphorylation of Ser101 could be assisted by a "3D consensus" generated by aspartates at positions 93 and 95, which are brought close to Ser101 by the folding of the molecule (*27*). To provide experimental support to this hypothesis, three peptides have been synthesized reproducing the 93−106 sequence of calmodulin either as such or with acidic residues variably replaced by alanine. As shown in Table 2, not only the substitution of Glu104 (i.e., the crucial element of the S-X-X-E consensus) but also that of Asp93/Asp95 are detrimental to phosphorylation. This corroborates the view that phosphorylation of calmodulin Ser101 is grounded on an atypical consensus generated not only by the canonical determinant at position $n + 3$, but also by a couple of acidic residues at remote upstream positions ($n - 6$ and $n - 8$), which are generally unimportant as determinants of peptide phosphorylation by CK2.

*4. Phosphorylation of Peptides Encompassing Phosphoacceptor Sites 79/81, 101, and 117.* The somewhat unexpected good performance of Ser101 as a phosphoacceptor residue (see above) is further improved within peptides that include also the central helix (and therefore the Thr79/Ser81 site), as shown by comparing the phosphorylation of peptides

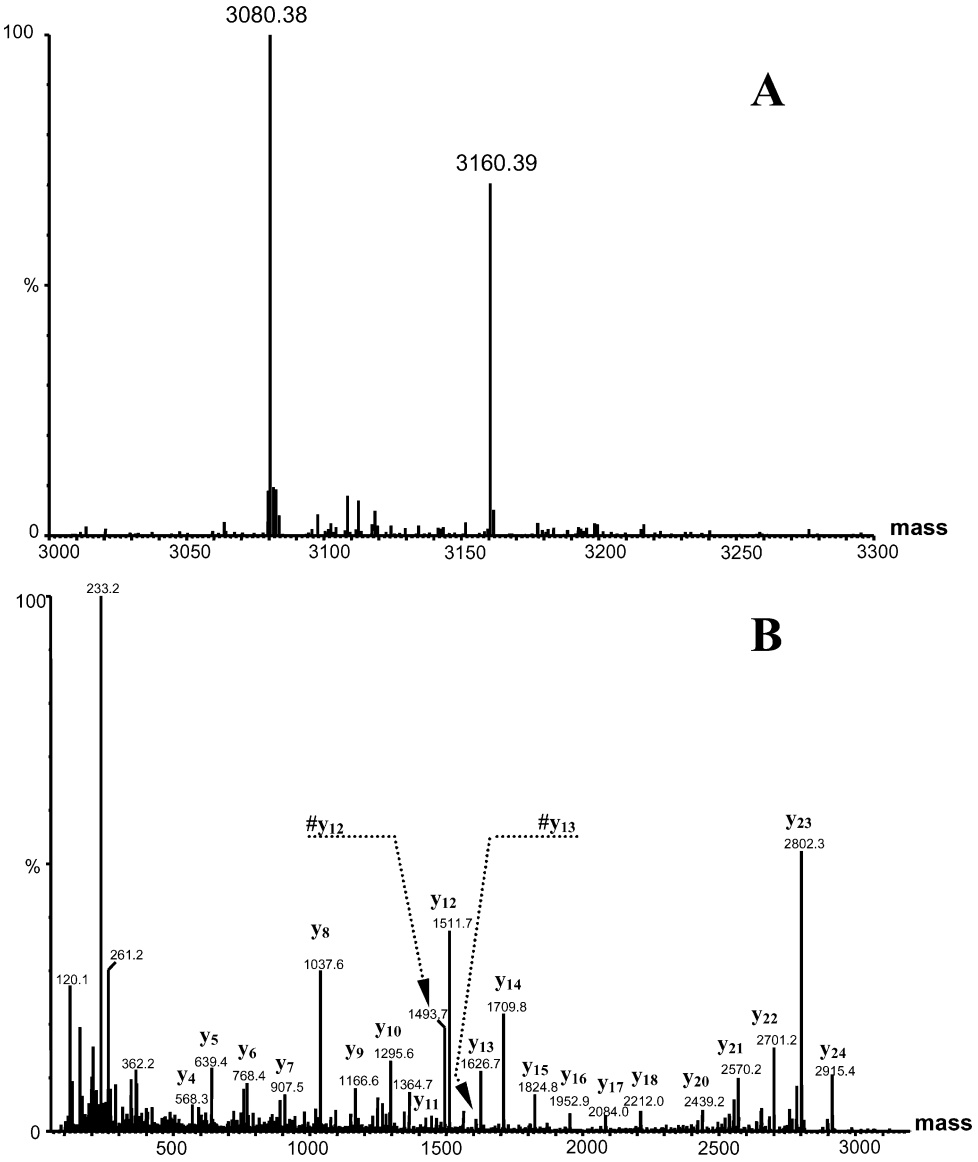


FIGURE 3: Mass analysis of CaM[68−92] phosphorylated by CK2. Panel A shows the mass spectrum of CaM[68−92] phosphorylated by CK2 determined as described in the Materials and Methods section. The peak at 3080.38 Da is due to the nonphosphorylated peptide, while the signal at 3160.39 Da is due to the monophosphorylated product. No bisphosphorylated peptide is detected. Phospho-CaM[68−92] was also chemically dephosphorylated with $Ba(OH)_2$ (see Materials and Methods section), and the corresponding CID spectrum is shown in panel B. An almost complete y series is detected, and the presence of the signals $\#y_{12}$ and $\#y_{13}$ shows that the phosphorylation took place at either Thr79 or Ser81.

68−106 and 54−106 on one side with that of 93−106 (and 68−92) on the other (Table 2). What is noteworthy is not just the increased phosphorylation efficiency of the longer peptides, which include both the phosphoacceptor sites (e.g., peptide 68−106 vs peptide 68−92 and 93−106) but the finding that phosphorylation is now mostly accounted for by Ser101, whereas phosphorylation of the Thr79/Ser81 site drops to negligible values. This is clearly demonstrated both by the decrease in $^{32}$P-threonine (see Table 2) and, even more conclusively, by the 2D TL pattern of the tryptic digest of the phosphorylated 54−106 peptide (Figure 2, panel b), where the spots accounted for by Thr79/Ser81 (A−D) are very faint as compared to two intense spots (E and F) (sometimes accompanied by a fainter spot, G), more mobile than spots A−D in the chromatographic direction. The confirmation that these spots are due to peptides including phospho-Ser101 was provided by phosphoamino acid analysis, revealing only phosphoserine and no phosphothreonine in spots E, F, and G (see legend of Figure 2). Note that, besides Ser81, which is also present in the 68−92 peptide and the phosphorylation of which contributed to the generation of the A−D cluster of spots (see panel a of Figure 2), the only other seryl residue in the 54−106 peptide is Ser101, which therefore accounts alone for the upper cluster of spots (E−G) in panel b of Figure 2. Their heterogeneity is probably due to an isoaspartyl bond between Asn97 and Gly98, as already observed by others (*40*), to a partial cleavage of the Asp93-Lys94-Asp95 site, which is quite resistant to trypsin (*44*), or to both.

If the same analysis is carried out with a peptide encompassing the whole C-terminal domain of calmodulin, 74−142, not only phosphorylation efficiency significantly decreases (Table 2, compare peptide 15 with peptide 10), but also the ratio between Ser101 and Thr79/Ser81 phosphorylation is deeply altered with the latter becoming predominant (Figure 2, compare panels b and c). Note that

Table 4: Summary of Phosphoresidues Generated by CK2 in Calmodulin Fragments[a]

| peptide | Thr79 | Ser81 | Ser101 | Thr117 |
|---|---|---|---|---|
| CaM[74−86] | 70 | 30 | | |
| CaM[74−86]S79T81 | 6 | 94 | | |
| CaM[74−86]A81 | 100 | 0 | | |
| CaM[68−92] | 72 | 28 | | |
| CaM[54−92] | 67 | 33 | | |
| CaM[68−106] | 18 | 4 | 78 | |
| CaM[68−106]A82,83,84,87 | 3 | 2 | 95 | |
| CaM[68−106]A93,95 | 40 | 25 | 35 | |
| CaM[54−106] | 21 | 4 | 75 | |
| CaM[54−106]pS81 | 1 | 0 | 99 | |
| CaM[93−106] | | | 100 | |
| CaM[93−106]A93,95 | | | 100 | |
| CaM[93−106]A104 | | | 100 | |
| CaM[91−142] | | | 76 | 24 |
| CaM[74−142] | 29 | 12 | 34 | 25 |
| calmodulin | 47 | 38 | 8 | 8 |

[a] The percent phosphorylation of various calmodulin phosphoacceptor residues was calculated by combining phosphoamino acid analysis and 2D thin layer resolution of tryptic products obtained from the radiolabeled peptides. The data are the mean of three independent experiments with SE never exceeding 10%. All peptides were phosphorylated by CK2 holoenzyme. In the case of calmodulin, polylysine (330 nM) was also added. Similar results were obtained if calmodulin and CaM[74−142] were phosphorylated by CK2 α subunit and also if CaM[74−142] was phosphorylated by CK2 holoenzyme in the presence of polylysine.

in panel c a new spot appears (spot H), which is absent in panels a and b, the radiolabeling of which is entirely accounted for by $^{32}$P-threonine (see legend of Figure 2). This most likely corresponds to the predicted tryptic phosphopeptide 116−126, including phospho-Thr117, which is present in peptide 74−142 (panel c) but neither in peptide 54−106 (panel b) nor in peptide 68−92 (panel a).

In this respect, the 2D tryptic fingerprint of peptide 74−142 resembles that of full-size calmodulin (panel d) suggesting that the inclusion in it of the C-terminal region confers to this peptide the behavior of calmodulin as far as phosphorylation by CK2 is concerned.

A summary of $^{32}$P incorporation into the various phosphoacceptor sites in calmodulin and its synthetic fragments, inferred from the experiments illustrated in Figure 2 and others performed in the same way, is presented in Table 4. The data corroborate the concept that the C-terminal domain of calmodulin is required to depress the relative phosphorylation of Ser101 while increasing that of Thr79/Ser81. They also highlight the relevance of the acidic cluster 82−84 and 86 and of the acidic doublet D93/D95 for the phosphorylation of Thr79/Ser81 and of Ser101, respectively. In fact, a derivative of the 68−106 peptide in which the former have been replaced by alanines is no longer phosphorylated at Thr79/Ser81 but exclusively at Ser101; by contrast, the replacement of D93/D95 by alanines in the same peptide causes a drop in Ser101 phosphorylation paralleled by a relative increase in the phosphorylation of both Thr79 and Ser81. Note that a modelization study of the complex between calmodulin and the catalytic subunit of CK2 has highlighted the glutamyl residues 82, 84, and 87 among those that interact with basic residues of CK2 if Ser81 is positioned in the catalytic site (*45*). Also noteworthy is the finding that replacement of Ser81 by phosphoserine in peptide 54−106 instead of increasing phosphorylation of Thr79, as expected, nearly abolishes it, consistent with the failure to detect bisphosphorylated products of the 79/81 site in the peptides encompassing residues 68−92 (see Figure 3) and residues 74−86 (not shown). This also argues against the interchangeability of phosphoserine and aspartic acid, considering that the replacement of Ser81 by aspartate does not decrease the phosphorylation of Thr79 in transfected full-length calmodulin (*9*). Note that the same MS analysis performed on the peptide 74−142 revealed, besides a large excess (>90%) of tryptic peptides monophosphorylated at Thr79 (or Ser81), Ser101, and Thr117, also detectable traces of a tryptic peptide bisphosphorylated at both Thr79 and Ser81 (not shown). The structural features determining such a behavior are unclear. It may be worthy to note, however, that a minor occurrence of Thr79/Ser81 bisphosphorylation has been also reported in the case of full-size calmodulin isolated from rat liver (*18*). This provides additional support to the concept that peptide 74−142 resembles calmodulin more than its congeners lacking the C terminal end, as far as phosphorylation by CK2 is concerned. The data in Table 4 refer to 10 min phosphorylation experiments: by prolonging incubation, increasing the amount of CK2, or both, higher phosphorylation stoichiometries could be attained, not accompanied however by any significant changes in relative phosphorylation of the phosphoacceptor sites. This argues against a cooperative mode of phosphorylation.

*5. Variable Effects of CK2 β-Subunit and of Polylysine on the Phosphorylation of Calmodulin Peptides.* All the above experiments were performed with CK2 holoenzyme, either native (from rat liver) or recombinant composed of two catalytic subunits and two regulatory β-subunits with similar results in both cases. Calmodulin phosphorylation by CK2 is anomalous in that it is inhibited instead of being stimulated by the β-subunit (*25*, *46*). As a consequence CK2 holoenzyme is very poorly active on full-size calmodulin unless in the presence of polylysine or other polybasic peptides, which dramatically enhance calmodulin phosphorylation by CK2 holoenzyme. Such a behavior is not shared by short calmodulin peptides suggesting that structural elements outside the phosphoaccepor sites are responsible for both down-regulation by the β-subunit and hyperstimulation by polybasic effectors. To gain more information about this issue, the phosphorylation of full-size calmodulin and of its synthetic fragments either by the isolated catalytic subunit of CK2, α, or by the reconstituted holoenzyme ($\alpha_2\beta_2$) in the absence and presence of polylysine was determined under comparable conditions. The results are shown as histograms in Figure 4. While the 54−92 peptide encompassing the Ca-binding loop II and the central helix, similar to the shorter peptides 68−92 and 93−106, still behaves as a canonical substrate the phosphorylation of which is stimulated by the β-subunit ("class I" according to current nomenclature (*47*)), with other peptides, more extended on their C-terminal side, the β-subunit has lost its stimulatory potential and displays an inhibitory effect similar to, albeit less pronounced than, that observed with calmodulin. Especially remarkable is in this respect the behavior of peptide 74−142 the phosphorylation of which is reduced by 80% upon formation of the holoenzyme and is stimulated 10-fold upon addition of polylysine to the holoenzyme. As noted above, this peptide also displays a phosphorylation pattern closer than those of peptides 68−106 and 54−106 to that of

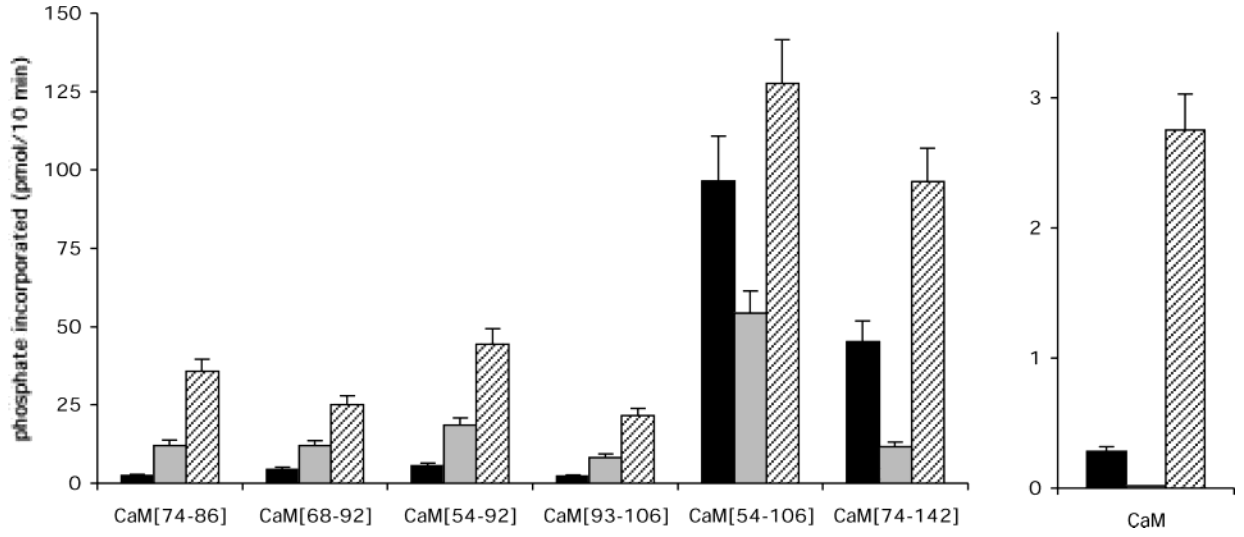


FIGURE 4: Effects of the $\beta$-subunit and of polylysine on the phosphorylation of calmodulin and of its synthetic fragments. The peptide (200 $\mu$M) and protein (10 $\mu$M) substrates were phosphorylated by CK2$\alpha$ (5 pmoles) either alone (black bars) or previously combined with equimolar amounts of $\beta$-subunit (gray bars) under conditions described in the Materials and Methods section except for the absence of NaCl. Polylysine, when present, was 330 nM (hatched bars). By increasing polylysine up to 1 $\mu$M, stimulation of phosphorylation was not significantly changed.

full-size calmodulin, as its Thr79/Ser81 phosphorylation predominates over that of Ser101, whereas the opposite applies to peptides 68−106 and 54−106 (see Table 4).

It can be concluded therefore that the carboxyl terminal domain, with special reference to the region downstream from Ca-binding loop III, plays a critical role in rendering calmodulin phosphorylation both susceptible to down-regulation by the $\beta$-subunit and more critically dependent on polylysine. Pertinent to this may be the observation that this region of calmodulin is very acidic with nine aspartic and glutamic acids concentrated between residues 118 and 133. A similar acidic cluster is found in the N-terminal region of the $\beta$-subunit of CK2, where it has been shown to play a pseudosubstrate-like down-regulatory role, unless it interacts with polylysine and other polycationic peptides, thus accounting, at least in part, for the stimulatory effect of these compounds (*46*, *48*). It is also possible therefore that, in the absence of polylysine, repulsive electrostatic interactions between the N-terminal domain of the $\beta$-subunit and the C-terminal region of calmodulin may prevent the binding of calmodulin to CK2 holoenzyme and consequently its phosphorylation. Polylysine therefore would assist calmodulin phosphorylation in two ways, by removing intrasteric inhibition (as in the case of most substrates) and by overcoming specific repulsive interactions between calmodulin and the $\beta$-subunit. Pertinent to this may be the observation that peptide 74−142 (including the acidic cluster) is 20-fold more effective than peptide 74−86 to compete against CaM phosphorylation by CK2 holoenzyme in the presence of polylysine although the $K_m$ values of these peptides (as well as those of the other peptides analyzed in Figure 4) are almost identical (not shown).

To assess the plausibility of this model, we have run far-Western blot (FWB) experiments by overlaying transblotted $\beta$-subunit with calmodulin, either in the absence or presence of polylysine. As shown in Figure 5, only in the presence of polylysine positive interactions between calmodulin and the $\beta$-subunit become evident, supporting the view that in the absence of polylysine they are overcome by repulsive forces

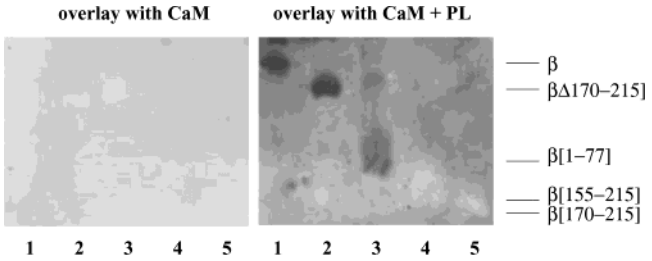


FIGURE 5: Polylysine is essential for the binding of calmodulin to the N-terminal region of $\beta$-subunit. Far-Western blots revealing interactions of calmodulin with CK2 $\beta$-subunit were performed as described in the Materials and Methods section. Either 2 $\mu$g of full-length and truncated $\beta$-subunit ($\beta\Delta$170−215) (lanes 1 and 2, respectively) or 2 nmol of the $\beta$ fragments 1−77, 155−215, and 170−215 (lanes 3, 4, and 5, respectively) were separated on SDS−PAGE and transferred to nitrocellulose membranes prior to being overlaid with calmodulin in the absence (left panel) or in the presence of 330 nM polylysine (right panel). Bound calmodulin was immunodetected by rabbit polyclonal anti-CaM antibodies.

between the two molecules. The polylysine responsive interacting region has been mapped to the N-terminal region of $\beta$ since positive FWB reaction has been also observed with a truncated form of $\beta$ lacking the C-terminal region ($\beta\Delta$170−215) and with a synthetic fragment reproducing the N-terminal domain of $\beta$ (1−77) but not with synthetic peptides encompassing its C-terminal region (170−215 and 155−215) as also shown in Figure 5.

These data strongly suggest that the $\beta$-subunit plays a dual function with respect to calmodulin phosphorylation by CK2 holoenzyme: in the absence of polycationic effectors repulsive electrostatic forces between its N-terminal region and the C-terminal domain of calmodulin, both negatively charged, hamper the association of calmodulin with and its phosphorylation by CK2 holoenzyme; in contrast, in the presence of effectors capable of masking the negative charges concentrated in the N-terminal region of the $\beta$-subunit, positive interactions with calmodulin prevail, accounting for the by far superior phosphorylation of calmodulin by CK2 holoenzyme as compared to CK2$\alpha$ subunit whenever polylysine is present (*25*). While the nature of the positive

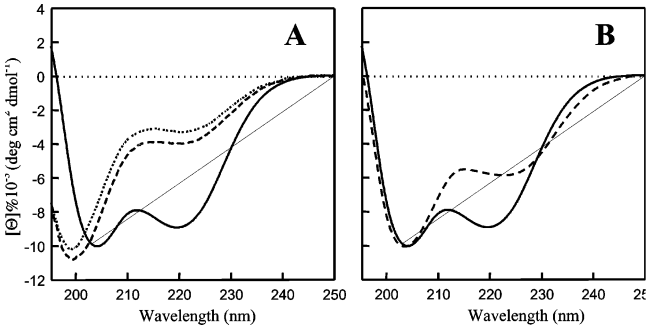


FIGURE 6: CD spectra of CaM[54−106] fragment are affected by $Ca^{2+}$ and Ser81 phosphorylation. Panel A shows the far-UV CD spectra of CaM[54−106] in the absence (•••) and in the presence of $Ca^{2+}$ (—). The spectrum recorded in the presence of $Mg^{2+}$ is also reported (− − −). Panel B shows the far-UV CD spectra of CaM[54−106] (—) and CaM[54−106]pS81 (− − −) both in the presence of $Ca^{2+}$. All the spectra were recorded in 10 mM Tris/HCl, 50 mM NaCl, and, when present, 5 mM $CaCl_2$ or 5 mM $MgCl_2$, pH 7.5. The spectra were then normalized as mean residue ellipticity as described in the Materials and Methods section.

interactions between $\beta$ and calmodulin in the presence of polybasic effectors remains a matter of conjecture, a plausible model would be that hydrophobic contacts between amphipathic helices may be involved. Note in this respect that both the N-terminal acidic stretch of $\beta$ (*49*) and the C-terminal acidic stretch of calmodulin (residues 118−133) (*43*) belong to amphipathic helices. This latter in particular corresponds to helix E of the Ca-binding EF domain IV the hydrophobic residues of which are exposed upon $Ca^{2+}$ binding (*50*).

*6. Phosphorylation of Ser81 Disrupts the Central Helix.* The structural effects of the phosphorylation of calmodulin are still unknown. The two phosphorylation sites at positions 79 and 81 are particularly interesting in this respect because they are located in a very flexible region of the central helix that connects the N-terminal and the C-terminal lobes of calmodulin. To date, more than 20 structures of CaM have been solved, using X-ray crystallography or NMR, in complex with different interactors. In these complexes, the central helix of CaM invariably plays a crucial role since its partial unfolding is instrumental to the conformational changes undergone by the whole molecule. Pertinent to this could be the observation that phosphorylation by CK2 may affect a class of recurrent motifs found at α-helices of "naturally unfolded" proteins (*51*). This prompted us to check whether phosphorylation of Ser81 might have structural consequences on the central helix of calmodulin.

First, a far-UV CD analysis was carried out with the unphosphorylated peptide CaM[54−106] that reproduces the whole central helix and the two flanking $Ca^{2+}$-binding loops II and III. The peptide adopts a substantial proportion of α-helical structure only in a buffer solution containing calcium ions, and this effect is calcium-specific, not being observed in the presence of $Mg^{2+}$ ions (Figure 6, panel A).

From a structural point of view, the behavior of the peptide CaM[54−106] resembles that of full-length calmodulin, of which the content in α-helix increases upon calcium binding (*52*, *53*). The phosphopeptide CaM[54−106]pS81, in which Ser81 was replaced by phosphoserine, in the presence of $Ca^{2+}$ ions adopts a conformation characterized by a lower content of α-helix as compared to its nonphosphorylated counterpart, as judged from the magnitude of the ellipticity at 222 nm (Figure 6, panel B). It can be concluded therefore that the

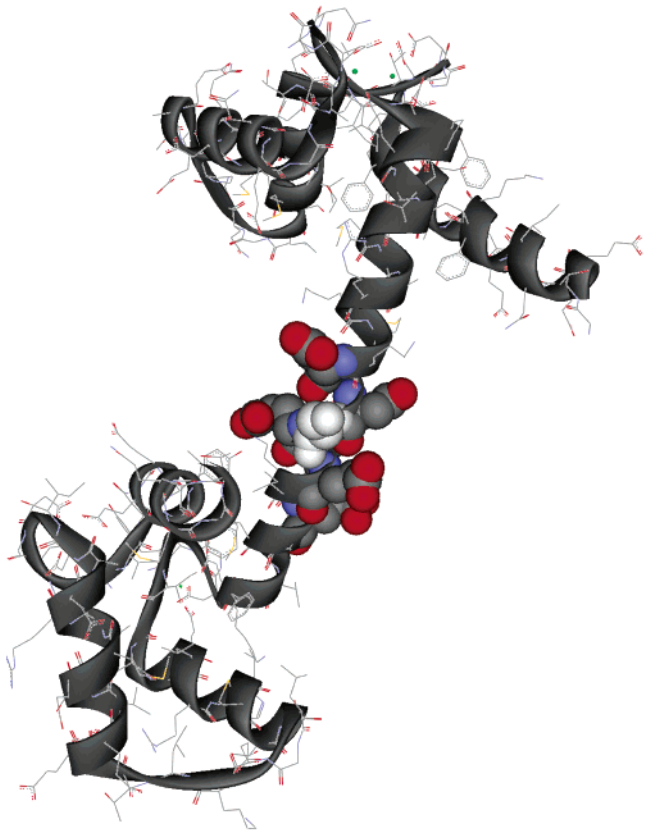

FIGURE 7: Ser81 surrounded by acidic side chains in the central helix of calmodulin drawn from the crystal structure of holo-calmodulin (pdb code 1cll) (*43*) by using DS ViewerPro 5.0 (Accelerys Inc.) software. Ser81 is shown in white. Oxygen atoms of the surroundings carboxylic side chains are in red.

phosphorylation of Ser81 destabilizes the central helix. The inspection of the crystal structure of holo-CaM (pdb code 1cll) (*43*), in which Ser81 is surrounded by negatively charged residues (Asp78, Asp80, Glu82, Glu83, Glu84, and Glu87) (see Figure 7), suggests that such a destabilization is in part due to electrostatic repulsion, although more specific electrostatic interactions of the phosphate of Ser81 with basic side chains nearby could also facilitate unfolding.

## CONCLUSIONS

The data presented show that the phosphorylation of CaM by CK2 at its physiological phosphoacceptor residues (Thr79, Ser81, and Ser101) is a complex event under the control of multiple factors, notably the integrity of the CaM molecule, the balance between CK2 catalytic and regulatory subunits, and the presence of endogenous effectors that by mimicking the in vitro effect of polylysine make possible CaM phosphorylation by CK2 holoenzyme. Only a large fragment of CaM including the central helix and the whole C-terminal domain (74−142) resembles full-size CaM in that its phosphorylation is drastically inhibited by the $\beta$-subunit and is dramatically stimulated by polylysine. Moreover, similar to calmodulin phosphorylated in vivo (*13*), the 74−142 fragment undergoes a minor yet detectable bisphosphorylation at both Thr79 and Ser81 residues upon incubation with CK2. Its overall phosphorylation pattern, however, is still significantly different from that of full size CaM (Table 4, compare 74−142 with CaM), suggesting that the absolute integrity of the CaM molecule is required for its correct

targeting by CK2 either α subunit or holoenzyme. This latter appears to be mediated by the N-terminal domain of the $\beta$-subunit, which binds the C-terminal region of CaM through a polylysine-dependent interaction. Ser81, once phosphorylated, promotes the unfolding of the central helix, a structural alteration that is likely to account at least in part for the functional consequences of CaM phosphorylation. This is especially evident with the peptide 54−106, composed of the central helix flanked by the $Ca^{2+}$ binding loops II and III, the structural alterations of which upon Ca binding are reminiscent of full-size calmodulin (see Figure 6A). Thus the unfolding of the central helix (implicated in the binding of CaM-dependent enzymes) upon Ser81 phosphorylation supports the view that CK2 regulates the interaction of CaM with its protein partners, in agreement with a number of previous observations (*8*).

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## REFERENCES

1. Klee, C. B., and Vanaman, T. C. (1992) Calmodulin, *Adv. Prot. Chem. 35*, 213−321.
2. Chim, D., and Means, A. R. (2000) Calmodulin: a prototypical calcium sensor, *Trends Cell Biol. 10*, 322−328.
3. Srinivasan, R., Gillett, C. E., Barnes, D. M., and Gullick, W. J. (2000) Nuclear expression of the c-erbB-4/HER-4 growth factor receptor in invasive breast cancers, *Cancer Res. 60*, 1483−1487.
4. Cohen, P., and Klee, C. B., Eds. (1988) *Molecular aspects of cellular regulation*, Vol. 5, Elsevier, Amsterdam.
5. Babu, Y. S., Sack, J. S., Greenhough, T. J., Buge, C. E., Means, A. R., and Cook, W. J. (1985) Three-dimensional structure of calmodulin, *Nature 315*, 37−40.
6. James, P., Vorherr, T., and Carafoli, E. (1995) Calmodulin-binding domains: just two faced or multi-faceted? *Trends Biochem. Sci. 20*, 38−42.
7. Jurado, L. A., Chockalingam, P. S., and Jarrett, H. W. (1999) Apocalmodulin, *Physiol. Rev. 79*, 661−682.
8. Benaim, G., and Villalobo, A. (2002) Phosphorylation of calmodulin. Functional implications, *Eur. J. Biochem. 269*, 3619−3631.
9. Greif, D. M., Sacks, D. B., and Michel, T. (2004) Calmodulin phosphorylation and modulation of endothelial nitric oxide synthase catalysis, *Proc. Natl. Acad. Sci. U.S.A. 101*, 1165−1170.
10. Sacks, D. B., Davis, H. W., Williams, J. P., Sheehan, E. L., Garcia, J. G., and McDonald, J. M. (1992) Phosphorylation by casein kinase II alters the biological activity of calmodulin, *Biochem. J. 283*, 21−24.
11. Sacks, D. B., Mazus, B., and Joyal, J. L. (1995) The activity of calmodulin is altered by phosphorylation: modulation of calmodulin function by the site of phosphate incorporation, *Biochem. J. 312*, 197−204.
12. Quadroni, M., L'Hostis, E. L., Corti, C., Myagkikh, I., Durussel, I., Cox, J., James, P., and Carafoli, E. (1998) Phosphorylation of calmodulin alters its potency as an activator of target enzymes, *Biochemistry 37*, 6523−6532.
13. Leclerc, E., Corti, C., Schmid, H., Vetter, S., James, P., and Carafoli, E. (1999) Serine/threonine phosphorylation of calmodulin modulates its interaction with the binding domains of target enzymes, *Biochem. J. 344*, 403−411.
14. Corti, C., Leclerc L'Hostis, E., Quadroni, M., Schmid, H., Durussel, I., Cox, J., Dainese Hatt, P., James, P., and Carafoli, E. (1999) Tyrosine phosphorylation modulates the interaction of calmodulin with its target proteins, *Eur. J. Biochem. 262*, 790−802.
15. Colca, J. R., DeWald, D. B., Pearson, J. D., Palazuk, B. J., Laurino, J. P., and McDonald, J. M. (1987) Insulin stimulates the phosphorylation of calmodulin in intact adipocytes, *J. Biol. Chem. 262*, 11399−11402.
16. Blackshear, P. J., and McNeill-Haupt, D. (1989) Evidence against insulin-stimulated phosphorylation of calmodulin in 3T3-L1 adipocytes, *J. Biol. Chem. 264*, 3854−3858.
17. Joyal, J. L., and Sacks, D. B. (1994) Insulin-dependent phosphorylation of calmodulin in rat hepatocytes, *J. Biol. Chem. 269*, 30039−30048.
18. Quadroni, M., James, P., and Carafoli, E. (1994) Isolation of phosphorylated calmodulin from rat liver and identification of the in vivo phosphorylation sites, *J. Biol. Chem. 269*, 16116−16122.
19. Plancke, Y. D., and Lazarides, E. (1983) Evidence for a phosphorylation form of calmodulin in chicken brain and muscle, *Mol. Cell. Biol. 3*, 1412−1420.
20. Nakajo, S., Hayashi, K., Daimatsu, T. Tanaka, M., Nakaya, K., and Nakamura Y. (1986) Phosphorylation of rat brain calmodulin in vivo and in vitro, *Biochem. Int. 13*, 687−693.
21. Meggio, F., Brunati, A. M., and Pinna, L. A. (1987) Polycation-dependent, $Ca^{2+}$-antagonized phosphorylation of calmodulin by casein kinase-2 and spleen tyrosine protein kinase, *FEBS Lett. 215*, 241−246.
22. Sacks, D. B., and McDonald, J. M. (1992) Effects of cationic polypeptides on the activity, substrate interaction, and autophosphorylation of casein kinase II: a study with calmodulin, *Arch. Biochem. Biophys. 299*, 275−280.
23. Song, Q., Combest, W. L., and Gilbert, L. I. (1994) Spermine and polylysine enhanced phosphorylation of calmodulin and tubulin in an insect endocrine gland, *Mol. Cell. Endocrinol. 99*, 1−10.
24. Meggio, F., and Pinna, L. A. (2003) One-thousand-and-one substrates of protein kinase CK2? *FASEB J. 17*, 349−368.
25. Meggio, F., Boldyreff, B., Marin, O., Marchiori, F., Perich, J. W., Issinger, O. G., and Pinna, L. A. (1992) The effect of polylysine on casein-kinase-2 activity is influenced by both the structure of the protein/peptide substrates and the subunit composition of the enzyme, *Eur. J. Biochem. 205*, 939−945.
26. Antonelli, M., Daniotti, J. L., Rojo, D., Allende, C. C., and Allende, J. E. (1996) Cloning, expression, and properties of the α′ subunit of casein kinase 2 from zebrafish (*Danio rerio*), *Eur. J. Biochem. 241*, 272−279.
27. Marin, O., Meggio, F., and Pinna, L. A. (1999) Structural features underlying the unusual mode of calmodulin phosphorylation by protein kinase CK2: a study with synthetic calmodulin fragments, *Biochem. Biophys. Res. Commun. 256*, 442−446.
28. Meggio, F., Donella Deana, A., and Pinna, L. A. (1981) Endogenous phosphate acceptor proteins for rat liver cytosolic casein kinases, *J. Biol. Chem. 256*, 11958−11961.
29. Rhyner, J. A., Koller, M., Durussel-Gerber, I., Cox, J. A., and Strehler, E. E. (1992) Characterization of the human calmodulin-like protein expressed in *Escherichia coli*, *Biochemistry 31*, 12826−12832.
30. Sarno, S., Boldyreff, B., Marin, O., Guerra, B., Meggio, F., Issinger, O.-G., and Pinna, L. A. (1995) Mapping the residues of protein kinase CK2 implicated in substrate recognition: mutagenesis of conserved basic residues in the alpha-subunit, *Biochem. Biophys. Res. Commun. 206*, 171−179.
31. Ruzzene, M., and Pinna, L. A. (1999) Assay of protein kinases and phosphatases using specific peptide substrates, in *Protein Phosphorylation − A practical approach*. (Hardie, D. G., Ed.) pp 221−253, Oxford University Press, London.
32. Perich, J. W., Meggio, F., Reynolds, E. C., Marin, O., and Pinna, L. A. (1992) Role of phosphorylated aminoacyl residues in generating atypical consensus sequences which are recognized by casein kinase-2 but not by casein kinase-1, *Biochemistry 31*, 5893−5897.
33. Boyle, W. J., Van der Geer, P., and Hunter, T. (1991) Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates, *Methods Enzymol. 201*, 110−149.
34. Goetz, C., Wagner, P., Issinger, O.-G., and Montenarh, M. (1996) p21WAF1/CIP1 interacts with protein kinase CK2, *Oncogene 13*, 391−398.
35. Holmes, C. F. (1987) A new method for the selective isolation of phosphoserine-containing peptides. *FEBS Lett. 215*, 21−24.
36. Meyer, H. E., Swiderek, K., Hoffmann-Posorske, E., Korte, H., and Heilmeyer, L. M., Jr. (1987) Quantitative determination of phosphoserine by high-performance liquid chromatography as the phenylthiocarbamyl-S-ethylcysteine. Application to picomolar amounts of peptides and proteins, *J. Chromatogr. 397*, 113−121.
37. Resing, K. A., Johnson, R. S., and Walsh, K. A. (1995) Mass spectrometric analysis of 21 phosphorylation sites in the internal repeat of rat profilaggrin, precursor of an intermediate filament associated protein, *Biochemistry 34*, 9477−9487.

38. Hunt, D. F., Yates, J. R., 3rd, Shabanowitz, J., Winston, S., and Hauer, C. R. (1986) Protein sequencing by tandem mass spectrometry, *Proc. Natl. Acad. Sci. U.S.A. 83*, 6233−6237.
39. Marin, O., Meggio, F., Marchiori, F., Borin, G., and Pinna, L. A. (1986) Site specificity of casein kinase-2 (TS) from rat liver cytosol. A study with model peptide substrates, *Eur. J. Biochem. 160*, 239−244.
40. Joyal, J. L., Crimmins, D. L., Thoma, R. S., and Sacks, D. B. (1996) Identification of insulin-stimulated phosphorylation sites on calmodulin, *Biochemistry 35*, 6267−6275.
41. Sacks, D. B., Davis, H. W., Crimmins, D. L., Persechini, A., and McDonald, J. M. (1992) Casein kinase II-catalysed phosphorylation of calmodulin is altered by amino acid deletion in the central helix of calmodulin, *Biochem. Biophys. Res. Commun. 188*, 754−759.
42. Marchiori, F., Meggio, F., Marin, O., Borin, G., Calderan, A., Ruzza, P., and Pinna, L. A. (1988) Synthetic peptide substrates for casein kinase 2. Assessment of minimum structural requirements for phosphorylation, *Biochim. Biophys. Acta 971*, 332−338.
43. Chattopadhyaya, R., Meador, W. E., Means, A. R., and Quiocho, F. A. (1992) Calmodulin structure refined at 1.7 Å resolution, *J. Mol. Biol. 228*, 1177−1192.
44. Keil, B. (1992) *Specificity of proteolysis*, p 335, Springer-Verlag, Berlin−Heidelberg−New York.
45. Rekha, N., and Srinivasan, N. (2003) Structural basis of regulation and substrate specificity of protein kinase CK2 deduced from the modelling of protein−protein interactions, *BMC Struct. Biol. 3*, 4−17.
46. Meggio, F., Boldyreff, B., Issinger, O.-G., and Pinna, L. A. (1994) Casein kinase 2 down-regulation and activation by polybasic peptides are mediated by acidic residues in the 55−64 region of the beta-subunit. A study with calmodulin as phosphorylatable substrate, *Biochemistry 33*, 4336−4342.
47. Pinna, L. A. (2002) Protein kinase CK2: a challenge to canons, *J. Cell Sci. 115*, 3873−3878.
48. Boldyreff, B., Meggio, F., Pinna, L. A., and Issinger, O.-G. (1994) Efficient autophosphorylation and phosphorylation of the $\beta$-subunit by casein kinase-2 require the integrity of an acidic cluster 50 residues downstream from the phosphoacceptor site, *J. Biol. Chem. 269*, 4827−4831.
49. Chantalat, L., Leroy, D., Filhol, O., Nueda, A., Benitez, M. J., Chambaz, E. M., Cochet, C., and Dideberg, O. (1999) Crystal structure of the human protein kinase CK2 regulatory subunit reveals its zinc finger-mediated dimerization, *EMBO J. 18*, 2930−2940.
50. Finn, B. E., Evenas, J., Drakenberg, T., Waltho, J. P., Thulin, E., and Forsen, S. (1995) Calcium-induced structural changes and domain autonomy in calmodulin, *Nat. Struct. Biol. 2*, 777−783.
51. Zetina, C. R. (2001) A conserved helix-unfolding motif in the naturally unfolded proteins, *Proteins 44*, 479−483.
52. Klee, C. B. (1977) Conformational transition accompanying the binding of $Ca^{2+}$ to the protein activator of 3′-5′-cyclic adenosine monophosphate phosphodiesterase, *Biochemistry 16*, 1017−1024.
53. Szilak, L., Moitra, J., Krylov, D., and Vinson, C. (1997) Phosphorylation destabilizes alpha-helices, *Nat. Struct. Biol. 4*, 112−114.

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