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Ca^{2+}/calmodulin-dependent protein kinase II: Identification of threonine-286 as the autophosphorylation site in the α subunit associated with the generation of Ca^{2+}-independent activity

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ABSTRACT **Autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II converts the enzyme to a Ca^{2+}-independent form. The time course for this conversion correlates with the autophosphorylation of a threonine residue located within a thermolytic phosphopeptide common to the α and β/β' subunits. In the present study, this site was identified in the α subunit. After autophosphorylation under conditions that produced near-maximal Ca^{2+}-independent activity, the α and β/β' subunits were separated by $NaDodSO_4$/PAGE, and the α subunit was cleaved with cyanogen bromide. The major phosphopeptide (CB-1), containing phosphothreonine as the only radiolabeled amino acid, was purified by reverse-phase high performance liquid chromatography and subjected to automated gas-phase Edman degradation. The sequence obtained, Xaa-Arg-Gln-Glu-Thr-Val-Asp-Xaa-Leu-Lys-Lys-Phe-Asn-Ala-Arg-Arg-Lys-Leu, represented the NH_2-terminal 18 residues (residues 282–299) of a 26-amino acid cyanogen bromide peptide predicted from the deduced primary structure of the α subunit and contained a consensus sequence for Ca^{2+}/calmodulin-dependent kinase II phosphorylation that included Thr-286. The sequences obtained for two phosphopeptides derived from secondary chymotryptic digestion of CB-1 confirmed that Thr-286 was the phosphorylated residue.**

Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (Ca^{2+}/CaM kinase II) is a multifunctional protein kinase that phosphorylates several substrate proteins including synapsin I, microtubule-associated protein 2, glycogen synthase, and tyrosine hydroxylase (refs. 1–3; for review, see ref. 4). Isozymes of Ca^{2+}/CaM kinase II, which have been purified from rat brain (1–3) and a variety of other tissues (4), all consist of high molecular weight complexes comprised of subunits of M_r 50,000 (α) and M_r 60,000/58,000 (β/β'). The subunits are autophosphorylated *in vitro* in the presence of Ca^{2+}/CaM and ATP, which results in the conversion of the enzyme to a Ca^{2+}-independent form (5–9). The generation of the Ca^{2+}-independent form coincides with the autophosphorylation of a threonine residue contained within a phosphopeptide common to the α and β/β' subunits (10).

The deduced amino acid sequences of the α (11, 12) and β/β' (13, 14) subunits of rat brain Ca^{2+}/CaM kinase II have been described. Based on the consensus phosphorylation site sequence recognized by Ca^{2+}/CaM kinase II in several substrates (15), a number of potential autophosphorylation sites are found in each subunit. Among these are Thr-286 (α subunit) and Thr-287 (β/β' subunit), which are adjacent to the putative CaM-binding domain present in each subunit. It has been speculated that these sites may be involved in the regulation of kinase activity (10, 11).

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In the present study, purified rat forebrain Ca^{2+}/CaM kinase II was autophosphorylated with [γ-^{32}P]ATP by using conditions that generate the Ca^{2+}-independent form of the enzyme. The subunits were separated and the α subunit was digested with cyanogen bromide (CNBr). A single, major ^{32}P-labeled phosphopeptide, purified by reverse-phase HPLC, was sequenced directly or was subjected to secondary digestion with chymotrypsin, after which the chymotryptic phosphopeptides were repurified and sequenced. These results provide direct evidence that Thr-286 is the autophosphorylation site in the α subunit of Ca^{2+}/CaM kinase II associated with the generation of Ca^{2+}-independence.

MATERIAL AND METHODS

Materials. Ca^{2+}/CaM kinase II was purified from rat forebrain as described (3). Calmodulin was prepared (16) from frozen rabbit brains obtained from Pel-Freez. Synapsin I was prepared from bovine brain as described (17). [γ-^{32}P]ATP was purchased from New England Nuclear. CNBr, triethylamine, and trifluoroacetic acid were purchased from Pierce. α-Chymotrypsin was purchased from Cooper Biomedical (Malvern, PA). Thermolysin was purchased from Calbiochem–Behring. Reverse-phase HPLC columns were purchased from Rainin [Woburn, MA; Vydac C_{18} (45 × 25 cm) and Brownlee C_4 (0.21 × 3 cm)] and from J. T. Baker [Phillipsburg, NJ; Bakerbond wide-pore C_4 (0.45 × 25 cm)]. Plastic-backed cellulose thin-layer chromatographic plates were purchased from Kodak. P-81 phosphocellulose paper was purchased from Whatman (Hillsboro, OR).

Autophosphorylation of Ca^{2+}/CaM Kinase II. Autophosphorylation of Ca^{2+}/CaM kinase II was performed and measured essentially as described (10), except that final concentrations of 15 mM $CaCl_2$, calmodulin at 100 μg/ml, and 3 μM [γ-^{32}P]ATP (25 mCi/μmol; 1 Ci = 37 GBq) were used in the reaction mixture. For analytical experiments, Ca^{2+}/CaM kinase II was autophosphorylated for 10 sec, 1 min, and 5 min and the effect of autophosphorylation on enzyme activity was determined by using bovine synapsin I as substrate (10). For the preparative scale experiment, ≈1 mg of purified enzyme was autophosphorylated under the conditions described above for 10 sec at 0°C.

CNBr Cleavage of Ca^{2+}/CaM Kinase II. The ^{32}P-labeled α and β/β' subunits of Ca^{2+}/CaM kinase II were separated by $NaDodSO_4$/PAGE in 10% polyacrylamide gels (18), localized by autoradiography of the unfixed, dried gel, and excised. Reswollen gel pieces were pooled, frozen with liquid N_2, and then thawed and homogenized in 2 ml of 10 mM NH_4HCO_3/1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 15,000 × *g* for 15 min and the supernatants were lyophilized. The eluted protein was treated with chloroform/methanol (19) and traces of Na-

Abbreviations: CaM, calmodulin; Ca^{2+}/CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II.

$DodSO_4$ were removed by ion-pair extraction (20). The precipitated protein was redissolved in 0.2 ml of 70% (vol/vol) formic acid containing CNBr at 2 mg/ml and incubated for 72 hr under N_2 in the dark at room temperature. Additional 1-mg aliquots of CNBr were added at 24 and 48 hr. The reaction mixture was then diluted 1:5 with H_2O and lyophilized.

Purification of ^{32}P-Labeled Phosphopeptides of the α Subunit of Ca^{2+}/CaM Kinase II. The lyophilized CNBr digest of the α subunit of Ca^{2+}/CaM kinase II was treated with 0.1% NH_4OH for 15 min to convert COOH-terminal homoserine lactone to the carboxyl form (21). The digest was then lyophilized, dissolved in 200 μl of 50% (vol/vol) formic acid/0.05% trifluoroacetic acid and fractionated by reverse-phase HPLC with a Vydac C_{18} column. The peptides were eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Fractions were collected and monitored for radioactivity by Cerenkov counting. Peptide peaks were monitored by UV detection at 210 nm. A Bakerbond C_4 column was used for the further purification of the CB-1A pool.

Amino Acid Sequence Determination. The amino acid sequence of ^{32}P-labeled phosphopeptides was determined as described (22) by using an Applied Biosystems (Foster City, CA) AB-470 gas-phase sequencer. Identification of phenylthiohydantoin amino acid derivatives was accomplished with C_{18} reverse-phase HPLC and was semi-quantitative.

Phosphorylation of Synthetic Peptides. Synthetic peptides were prepared by Meng Ho (The Rockefeller University), the Rockefeller University Protein Sequencing Facility, and the Yale University Protein Chemistry Facility by using the Merrifield solid-phase procedure (23). Mass spectrometry was used to confirm the structure of each peptide and the concentration of peptides was determined by amino acid analysis. The phosphorylation of synthetic peptides was carried out as described (3) except that 200 μM [γ-^{32}P]ATP was used and various concentrations of peptide replaced synapsin I. Peptide phosphorylation was linear with respect to time and enzyme concentration. Reactions were carried out for 0.5–5.0 min and stopped by the addition of 50 μl of 30% (vol/vol) acetic acid. The P-81 phosphocellulose paper assay was used to quantitate the incorporation of phosphate into the peptide (3). Kinetic parameters were derived from double-reciprocal plots.

Miscellaneous Techniques. Two-dimensional thermolytic phosphopeptide mapping and phospho amino acid analysis were performed as described (10). Protein content was determined by the dye-binding method of Bradford (24).

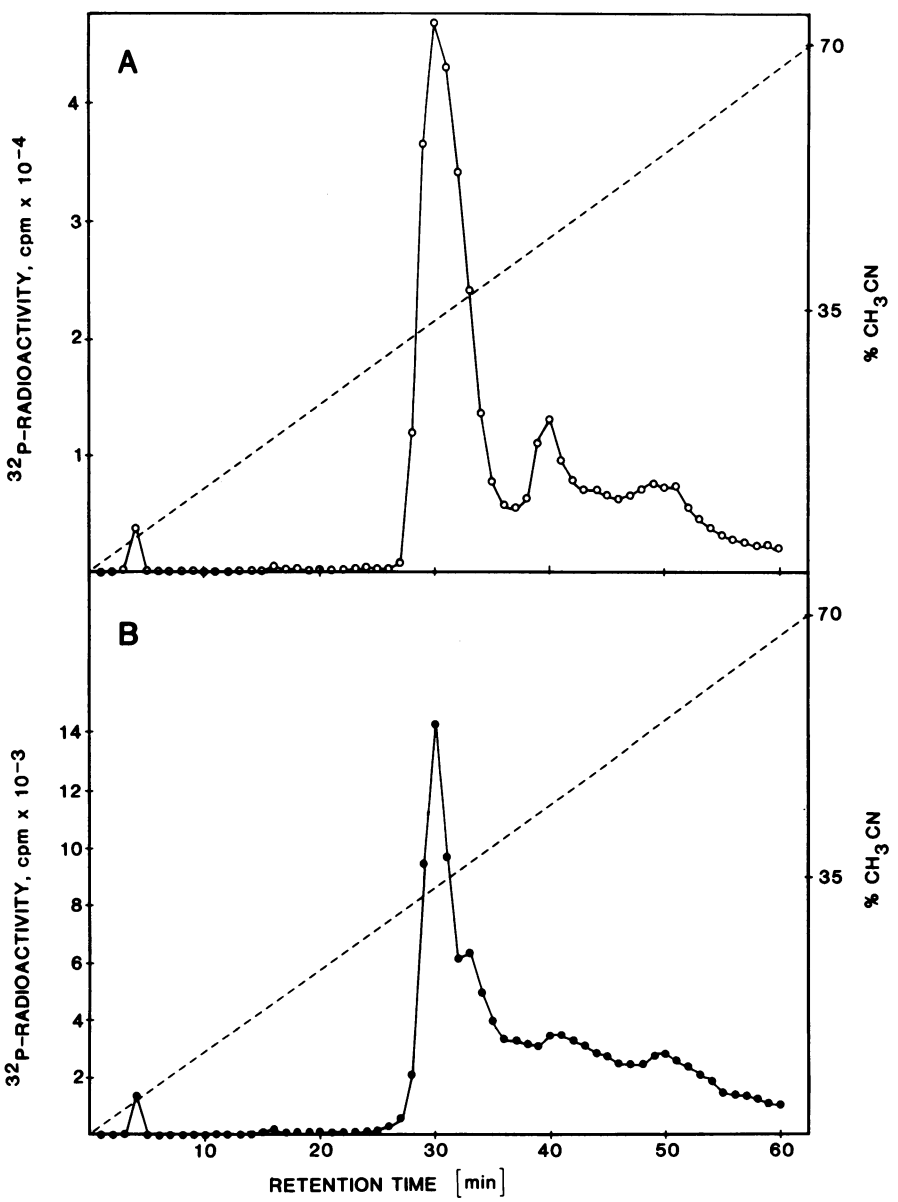

FIG. 1. HPLC elution profiles of phosphopeptides derived from analytical scale CNBr cleavage of the α and β/β′ subunits of Ca^{2+}/CaM kinase II. Purified Ca^{2+}/CaM kinase II was autophosphorylated for 10 sec at 0°C. The α (*A*) and β/β′ (*B*) subunits were separated by $NaDodSO_4$/PAGE, eluted from the gel, and cleaved with CNBr. The fragments were redissolved in 50% (vol/vol) formic acid/0.05% trifluoroacetic acid and separated by using C_{18} HPLC with a linear gradient of 0–70% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid over 60 min at a flow rate of 1 ml/min. ● and ○, ^{32}P radioactivity; - - - - -, concentration of acetonitrile (CH_3CN).

RESULTS

Reverse-Phase HPLC Mapping of CNBr Peptides Derived from Autophosphorylated Ca^{2+}/CaM Kinase II. Results from our laboratory (10) have demonstrated that the autophosphorylation of a threonine residue located within the thermolytic phosphopeptide 1/1', common to both the α and β/β' subunits of Ca^{2+}/CaM kinase II, correlated with the generation of the Ca^{2+}-independent form of the enzyme. In an initial attempt to determine the amino acid sequence surrounding this autophosphorylated threonine residue, pilot studies were performed at an analytical scale in which Ca^{2+}/CaM kinase II was autophosphorylated for various amounts of time at 0°C. The phosphorylated subunits were then purified and either digested with different proteases or cleaved with CNBr, and the resulting peptides were analyzed by two-dimensional peptide mapping or HPLC. Under the assay conditions employed, autophosphorylation of Ca^{2+}/CaM kinase II for 10 sec resulted in the generation of near-maximal Ca^{2+}-independent kinase activity, with a time course similar to that described with lower concentrations of Ca^{2+} and CaM in the reaction mixture (data not shown). The stoichiometry of ^{32}P incorporation after the 10-sec reaction was somewhat higher (0.26 mol of phosphate per mol of α subunit; 0.4 mol of phosphate per mol of β/β' subunit) than was reported (10). Two-dimensional thermolytic phosphopeptide maps of both the α and β/β' subunits showed the presence of peptide 1/1' (data not shown). However, the large excess of thermolysin required for complete digestion of the protein precluded the use of this protease for the purpose of producing and isolating a pure phosphopeptide that could be used for direct sequence analysis. Digestion of either subunit with trypsin, chymotrypsin, or endoprotease Arg-C also required very high protease/substrate ratios (>10:1) to produce distinct, limit phosphopeptides.

Chemical cleavage with CNBr of the isolated subunits of Ca^{2+}/CaM kinase II autophosphorylated for various times was performed. Attempts to characterize the CNBr phosphopeptides by two-dimensional peptide mapping were unsatisfactory. The low solubility of the phosphopeptides

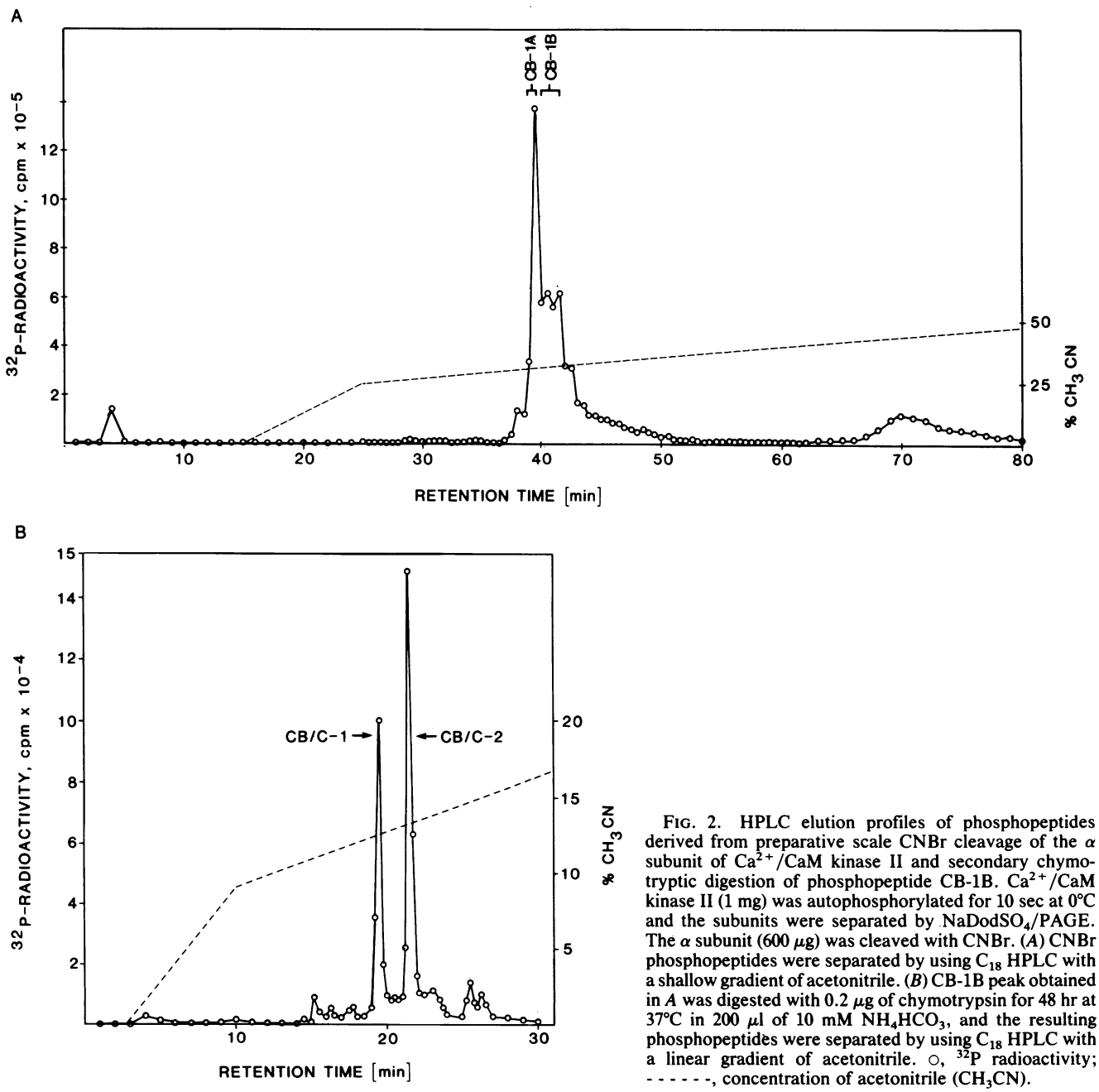

FIG. 2. HPLC elution profiles of phosphopeptides derived from preparative scale CNBr cleavage of the α subunit of Ca^{2+}/CaM kinase II and secondary chymotryptic digestion of phosphopeptide CB-1B. Ca^{2+}/CaM kinase II (1 mg) was autophosphorylated for 10 sec at 0°C and the subunits were separated by $NaDodSO_4$/PAGE. The α subunit (600 μg) was cleaved with CNBr. (*A*) CNBr phosphopeptides were separated by using C_{18} HPLC with a shallow gradient of acetonitrile. (*B*) CB-1B peak obtained in *A* was digested with 0.2 μg of chymotrypsin for 48 hr at 37°C in 200 μl of 10 mM NH_4HCO_3, and the resulting phosphopeptides were separated by using C_{18} HPLC with a linear gradient of acetonitrile. ○, ^{32}P radioactivity; - - - - -, concentration of acetonitrile (CH_3CN).

resulted in a broad smear of radioactivity surrounding the origin (data not shown) and was consistent with the observation that a minimum of 25% (vol/vol) formic acid was required to solubilize the phosphopeptides after CNBr cleavage of either subunit. Therefore, analytical scale CNBr digests of the α and β/β' subunits were individually fractionated by C_{18} reverse-phase HPLC and the elution profile of [^{32}P]phosphopeptides was determined (Fig. 1). For the enzyme sample that was autophosphorylated for 10 sec at 0°C, a major peak (CB-1) was eluted at ≈30 min for either subunit. For each subunit, this peak accounted for ≈60% of the recovered radioactivity. Phospho amino acid analysis of CB-1 from either subunit identified phosphothreonine as the only radiolabeled amino acid present. The peak shape of CB-1 was somewhat variable for both subunits, with the frequent appearance of a descending "shoulder." Extending the autophosphorylation reaction to 1 min or 5 min resulted in the increased prominence in both subunits of a radioactive peak that was eluted at 39–40 min (data not shown). The time course for appearance of this peak did not correlate with the time course of generation of Ca^{2+}-independent enzyme activity.

Based on these analytical results, we decided to use HPLC purification of peptides derived from CNBr cleavage of the purified α and β/β' subunits after preparative-scale autophosphorylation of Ca^{2+}/CaM kinase II for 10 sec at 0°C. However, the limited amount of β/β' subunit present in the rat forebrain kinase precluded the complete identification of the autophosphorylation site in this subunit.

Purification and Amino Acid Sequencing of Phosphopeptide CB-1 from the α Subunit of Ca^{2+}/CaM Kinase II. Approximately 600 μg of α subunit protein was cleaved with CNBr. After NH_4OH treatment, the digest was solubilized in 50% (vol/vol) formic acid/0.05% trifluoroacetic acid and separated on a Vydac C_{18} column with a gradient of acetonitrile (Fig. 2*A*). The major peak of radioactivity was divided into two pools. The CB-1A pool included the fractions that were eluted as the sharp peak from 38.5 to 39.5 min (27% of recovered radioactivity) and the CB-1B pool included the shoulder of the major peak that was eluted at 39.5–42.5 min (30% of recovered radioactivity). Further purification of CB-1A was attempted in two other solvent systems, 0–70% (vol/vol) acetonitrile in 10 mM potassium phosphate (pH 2.5) and 0–70% (vol/vol) acetonitrile in 20 mM triethylamine acetate (pH 6). However, CB-1A was not eluted from a Brownlee C_4 column under these conditions. Therefore, CB-1A was rechromatographed in the trifluoroacetic acid/acetonitrile solvent system on a Bakerbond C_4 column and then on a Vydac C_{18} column. Stringent pooling of the peak radioactive fractions resulted in a final yield of ≈20 pmol of CB-1A. Gas-phase sequencing of this phosphopeptide yielded the primary structure Xaa-Arg-Gln-Glu-Thr-Val-Asp-Xaa-Leu-Lys-Lys-Phe-Asn-Ala-Arg-Arg-Lys-Leu (Fig. 3*C*). This sequence, corresponding to residues 282–299 of the deduced primary sequence of the α subunit (11, 12), represented the 18 NH_2-terminal residues contained in a 26-amino acid peptide that would be derived from CNBr cleavage of the α subunit at Met-281 and Met-307 (Fig. 3*B*). CB-1A migrated at the dye-front in 15% polyacrylamide gels, as expected for a peptide of this size (data not shown). If CB-1A had not terminated at Met-307, then cleavage at the next potential site, Met-372, would have produced a 10-kDa fragment that would migrate with a significantly lower R_f in 15% polyacrylamide gels. Therefore, it is virtually certain that CB-1A was the 26-amino acid peptide predicted from the deduced sequence.

It is likely that the autophosphorylation site was Thr-286, since the sequence flanking this residue, Arg-Gln-Glu-Thr^{286}, conforms to the Ca^{2+}/CaM kinase II substrate consensus sequence, Arg/Lys-Xaa-Xaa-Ser/Thr (15). However, two additional threonine residues, Thr-305 and Thr-306, were present within the predicted sequence of CB-1A. To eliminate the possibility that either of these residues was the autophosphorylation site, the CB-1B pool was digested with chymotrypsin. The chymotryptic digest was chromatographed on a Vydac C_{18} column with the trifluoroacetic acid/acetonitrile solvent system (Fig. 2*B*). Two radioactive peaks were eluted at retention times of 19.5 min (CB/C-1) and 21.5 min (CB/C-2), respectively, and together accounted for ≈75% of the recovered radioactivity. Each peak, representing ≈2 pmol of phosphopeptide, was subjected to gas-phase sequencing. The two phosphopeptides yielded similar sequences, confirming the identification of Thr-286 as the autophosphorylation site (Fig. 3 *D* and *E*). The sequence obtained for CB/C-1 was Xaa-Arg-Gln-(Glu/Ala)-Xaa-Val-Asp-Xaa-Leu and the sequence obtained for CB/C-2 was Xaa-Xaa-Gln-Glu-Thr-Val-Asp. Although the identity of the residue at each position was not established, it was apparent that the phosphopeptides CB/C-1 and CB/C-2 contained the NH_2-terminal residues of the longer CB-1A phosphopeptide and were most likely derived from chymotryptic cleavage of CB-1B at Leu-290 or Phe-293. Since CB/C-1 and CB/C-2 were eluted at earlier retention times than was CB-1B from the C_{18} column and since CB/C-1 and CB/C-2 migrated as distinct, basic phosphopeptides in two-dimensional mapping experiments in contrast to CB-1B, it is clear that cleavage of the parent phosphopeptide had occurred. Thus the results determined by direct peptide sequencing indicated that Thr-286 is the autophosphorylation site in the α subunit of Ca^{2+}-CaM kinase II associated with the generation of Ca^{2+}-independence.

Phosphorylation of Synthetic Peptides Containing the Thr-286 Autophosphorylation Site. To further explore the characteristics of autophosphorylation of Thr-286, several synthetic peptides were examined for their ability to serve as substrates for the Ca^{2+}/CaM-dependent form of the enzyme. The peptide Met-His-Arg-Gln-Glu-Thr(*P*)-Val-Asp-Cys-Leu-Lys-NH_2, representing residues 281–291 of the α subunit, was

A. Met-His-Arg-Gln-Glu-Thr-Val-Glu-Cys-Leu-Lys-Lys-Phe-Asn-Ala-Arg-Arg-Lys-Leu-Lys-Gly-Ala-Ile-Leu-Thr-Thr-Met-Leu-

B. Met-His-Arg-Gln-Glu-Thr-Val-Asp-Cys-Leu-Lys-Lys-Phe-Asn-Ala-Arg-Arg-Lys-Leu-Lys-Gly-Ala-Ile-Leu-Thr-Thr-Met-Leu-

C. X*-Arg-Gln-Glu-Thr-Val-Asp- X*-Leu-Lys-Lys-Phe-Asn-Ala-Arg-Arg-Lys-Leu-

D. X*-Arg-Gln-Glu-Ala+- X*-Val-Asp- X*-Leu

E. X*- X*-Gln-Glu-Thr-Val-Asp

FIG. 3. Summary of amino acid sequences in the region surrounding Thr-286/287 of Ca^{2+}/CaM kinase II. (*A*) Deduced amino acid sequence (residues 282–309) of the β/β' subunit. (*B*) Deduced amino acid sequence (residues 281–308) of the α subunit. (*C*) Amino acid sequence of phosphopeptide CB-1A. (*D*) Amino acid sequence of phosphopeptide CB/C-1. (*E*) Amino acid sequence of phosphopeptide CB/C-2. *, The amino acid residue at this cycle was not identified; †, phenylthiohydantoin derivatives of glutamic acid and alanine were detected in equal amounts at this cycle; X, Xaa.

found to be a good substrate, with an apparent K_m value of 130 μM and a V_{max} of 13 μmol·min^{-1}·mg^{-1}. This value compares favorably with values obtained for other peptide substrates containing phosphorylation site sequences present in other substrates for Ca^{2+}/CaM kinase II, such as synapsin I [bovine phospho site 3 peptide (17), Tyr-Arg-Gln-Gly-Pro-Pro-Gln-Lys-Pro-Pro-Gly-Pro-Ala-Gly-Pro-Thr-Arg-Gln-Ala-Ser(*P*)-Gln-Ala-Gly-Pro-NH_2; K_m = 50 μM and V_{max} = 6.5 μmol·min^{-1}·mg^{-1}; this study] and glycogen synthase [glycogen synthase-(1–10) peptide, Pro-Leu-Ser-Arg-Thr-Leu-Ser(*P*)-Val-Ser-Ser-NH_2; K_m = 7.5 μM and V_{max} = 12.5 μmol·min^{-1}·mg^{-1}; ref. 15]. These results are also in agreement with those obtained by Payne *et al.* (25) in which the phosphorylation of a peptide representing residues 281–290 of the α subunit was examined. A nonphosphorylatable analog of Ca^{2+}/CaM kinase II α subunit-(281–291), Met-His-Arg-Gln-Glu-Ala-Val-Asp-Cys-Leu-Lys-NH_2, was an inhibitor of Ca^{2+}/CaM kinase II with a K_i value of 250 μM when a peptide, residues 281–291 of the α subunit, was used as substrate.

DISCUSSION

This study confirms that the initial autophosphorylation of Ca^{2+}/CaM kinase II on threonine residues is associated with the generation of the Ca^{2+}-independent form of the enzyme. This autophosphorylation site is contained within the thermolytic peptide 1/1′, common to both α and β/β' subunits (10). We have directly identified this site in the α subunit of rat forebrain Ca^{2+}/CaM kinase II as Thr-286. The sequence Arg-Gln-Glu-Thr^{286} conforms to the consensus sequence Arg/Lys-Xaa-Xaa-Ser/Thr recognized by Ca^{2+}/CaM kinase II. As predicted (10, 11), this site is adjacent to the putative CaM-binding domain of Ca^{2+}/CaM kinase II.

The limited quantities of starting material prevented the direct identification of the initial threonine autophosphorylation site in the β/β' subunit. Nevertheless, it is very likely that autophosphorylation occurs at Thr-287, based on results showing that the phosphopeptide patterns generated from CNBr cleavage of the α and β/β' subunits are identical and that the thermolytic peptide 1/1′ is common to both subunits. This would be expected since the proposed site in the β/β' subunit, Thr-287, is located within a sequence that is homologous with that of the α subunit, differing by only one amino acid (Glu-289 replacing Asp-288; Fig. 3 *A* and *B*).

This study suggests that phosphorylation of Thr-286, adjacent to the CaM-binding domain of Ca^{2+}/CaM kinase II, is involved in the generation of the Ca^{2+}-independent form of the kinase. Although the molecular mechanism of this conversion is unknown, it is attractive to postulate that the autophosphorylation at Thr-286 (Thr-287 in the β/β' subunit) might alter internal inhibitory constraints on the catalytic domain of the kinase. Thus autophosphorylation of this site may maintain a structure that is functionally equivalent to that which occurs upon the binding of Ca^{2+}/CaM. Once autophosphorylation of Thr-286/287 occurs, the requirement for Ca^{2+}/CaM to maintain the active conformation of the enzyme would then be relieved. It has been suggested that other protein kinases, including myosin light chain kinase (26) and protein kinase C (27), contain a pseudosubstrate domain that prevents binding of substrate to the catalytic site of the enzyme. It is possible that the regulatory mechanism involving autophosphorylation of Thr-286/287 relieves a similar form of pseudosubstrate inhibition in Ca^{2+}/CaM kinase II, although there is no evidence that Thr-286/287 occupies the catalytic site in the inactive enzyme. In fact it has been suggested (25) that amino acid residues 290–296 of the α subunit of Ca^{2+}/CaM kinase II may function as such a pseudosubstrate domain.

The conversion to the Ca^{2+}-independent form of the enzyme occurs at low overall stoichiometry of phosphorylation (3–4 mol/mol of holoenzyme) (6, 7). Cooperative interactions between kinase subunits might, therefore, be affected by Thr-286/287 autophosphorylation. In addition, it has been suggested that autophosphorylation is an obligatory step in the activation of the enzyme by CaM (28). The direct identification of Thr-286 as the initial autophosphorylation site in the α subunit of Ca^{2+}/CaM kinase II should contribute to the elucidation of the various mechanisms involved in regulation of this enzyme.

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