activity, while HF-treated hCG is devoid of such activity (Moyle et al., 1975; Manjunath & Sairam, 1982; Chen et al., 1982). The procedure of removal of the terminal charged sugar should also apply to the crystallization of the other homologous glycoprotein hormones. However, since some of the homologous pituitary glycoprotein hormones are partially sulfated as well as sialylated, utilizing recombinant hormones that are expressed in systems that do not add sulfate or sialic acid may provide the best material for crystal growth. Such materials would require no chemical modifications and have the potential to produce crystals that might diffract X-rays to higher resolution than those described here. Such studies are now in progress.

ACKNOWLEDGMENTS

We acknowledge with gratitude the communication of results from Prof. Neil Issacs prior to publication. We also acknowledge helpful discussions with Dr. Francis J. Morgan.

REFERENCES

Birken, S., & Canfield, R. E. (1980) in *Chorionic Gonadotropin* (Segal, S. J., Ed.) pp 65–88, Plenum Press, New York.

Canfield, R. E., & Morgan, F. J. (1973) in *Methods in investigative and diagnostic endocrinology* (Berson, S. A., & Yalow, R. S., Eds.) Part III, p 727, North-Holland Publishing Co., Amsterdam.

Chen, H.-C., Shimohigashi, Y., Dufau, M. L., & Catt, K. J. (1982) *J. Biol. Chem. 257*, 1444.

Harris, D. C., Machin, K. J., Evin, G. M., Morgan, F. J., & Issacs, N. W. (1989) *J. Biol. Chem. 264*, 6705.

Kessler, M. J., Reddy, M. S., Shah, R. H., & Bahl, O. P. (1979) *J. Biol. Chem. 254*, 7901.

Keutmann, H. T., Johnson, L., & Ryan, R. J. (1985) *FEBS Lett. 185*, 333.

Laemmli, U. K. (1970) *Nature 227*, 680.

Loosfelt, H., Misrahi, M., Atger, M., Salesse, R., Vu Hai-Luu Thi, M. T., Jolivet, A., Guiochon-Mantel, A., Sar, S., Jallal, B., Garnier, J., & Milgrom, E. (1989) *Science 245*, 525.

Manjunath, P., & Sairam, M. R. (1982) *J. Biol. Chem. 257*, 7109.

Matzuk, M., Keene, J. L., & Boime, I. (1989) *J. Biol. Chem. 264*, 2409.

McFarland, K. C., Sprengel, R., Phillips, H. S., Köhler, M., Rosemblit, N., Nikolics, K., Segaloff, D. L., & Seeburg, P. H. (1989) *Science 245*, 494.

McPherson, A. (1982) *Preparation and analysis of protein crystals*, p 97, John Wiley & Sons, New York.

Merz, W. E. (1988) *Biochem. Biophys. Res. Commun. 156*, 1271.

Moyle, W. R., Bahl, O. P., & Marz, L. (1975) *J. Biol. Chem. 250*, 9163.

Perini, F., & Peters, B. P. (1982) *Anal. Biochem. 123*, 357.

Pierce, J. G., & Parsons, T. F. (1981) *Annu. Rev. Biochem. 50*, 465.

Puett, D., Ryan, R. J., & Stevens, V. C. (1982) *Int. J. Pept. Protein Res. 19*, 506.

Rosa, C., Amr, S., Birken, S., Wehmann, R., & Nisula, B. (1984) *J. Clin. Endocrinol. Metab. 59*, 1215.

Sairam, M. R. (1989) *FASEB J. 3*, 1915.

Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 197.

Identification of the Site on Calcineurin Phosphorylated by Ca^{2+}/CaM-Dependent Kinase II: Modification of the CaM-Binding Domain

Todd M. Martensen,*,‡ Brian M. Martin,§ and Randall L. Kincaid‡

Section on Immunology, Laboratory of Physiologic and Pharmacologic Studies, National Institute on Alcohol Abuse and Alcoholism, Rockville, Maryland 20852, and Molecular Neurogenetics Section, Clinical Neurosciences Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland 20892

Received September 1, 1989; Revised Manuscript Received October 4, 1989

ABSTRACT: The catalytic subunit of the Ca^{2+}/calmodulin- (CaM) dependent phosphoprotein phosphatase calcineurin (CN) was phosphorylated by an activated form of Ca^{2+}/CaM-dependent protein kinase II (CaM-kinase II) incorporating approximately 1 mol of phosphoryl group/mol of catalytic subunit, in agreement with a value previously reported (Hashimoto et al., 1988). Cyanogen bromide cleavage of radiolabeled CN followed by peptide fractionation using reverse-phase high-performance liquid chromatography yielded a single labeled peptide that contained a phosphoserine residue. Microsequencing of the peptide allowed both the determination of the cleavage cycle that released [^{32}P]phosphoserine and the identity of amino acids adjacent to it. Comparison of this sequence with the sequences of methionyl peptides deduced from the cDNA structure of CN (Kincaid et al., 1988) allowed the phosphorylated serine to be uniquely identified. Interestingly, the phosphoserine exists in the sequence Met-Ala-Arg-Val-Phe-Ser(P)-Val-Leu-Arg-Glu, part of which lies within the putative CaM-binding site. The phosphorylated serine residue was resistant to autocatalytic dephosphorylation, yet the slow rate of hydrolysis could be powerfully stimulated by effectors of CN phosphatase activity. The mechanism of dephosphorylation may be intramolecular since the initial rate was the same at phosphoCN concentrations of 2.5–250 nM.

Protein phosphorylation/dephosphorylation is involved in a variety of neuronal signal transduction mechanisms (Hemmings et al., 1989). When effector molecules interact with a specific cell surface receptor, they, through a direct interaction or second messenger release, alter the activity of multifunctional protein kinases and phosphoprotein phosphatases. These enzymes mediate signal transduction through

‡ National Institute on Alcohol Abuse and Alcoholism.
§ National Institute of Mental Health.

covalent modification of their substrates that in turn alter the physiological properties of effector-responsive cells. Ca^{2+}/calmodulin- (Ca^{2+}/CaM[1]) dependent protein kinase II [reviewed by Colbran et al. (1989), Schulman and Lou (1989), Kennedy et al. (1987), and Nairn et al. (1985)] is believed to play an important role in neurotransmission since the kinase is highly concentrated in synaptic densities, and phosphorylation of neuronotypic substrates is correlated with activation of synaptic activity.

One of the major phosphoprotein phosphatase activities in neural tissues is a Ca^{2+}/CaM-regulated phosphatase called calcineurin (CN) or protein phosphatase 2B [reviewed by Klee and Cohen (1988) and Tallant and Cheung (1986)]. The enzyme exists in brain in concentrations ~20 times that of muscle and catalyzes in vitro the dephosphorylation of several brain-specific phosphoproteins (King et al., 1984). CN is a heterodimer containing a catalytic (M_r ~60 000) and a regulatory (M_r ~18 000) subunit; the latter is a Ca^{2+}-binding protein structurally similar to calmodulin (Klee et al., 1988). The phosphatase is an Fe^{3+} and Zn^{2+} metalloprotein containing covalently bound phosphate (King & Huang, 1984). The enzymatic activity can be regulated by a variety of effectors. Ca^{2+}/CaM binding to the enzyme activates the phosphatase activity that in vitro is accompanied by a time-dependent deactivation process whose molecular mechanism is not understood. Also, the enzymatic activity of the phosphatase can be potently stimulated by certain divalent metals such as Mn^{2+}, Ni^{2+}, Co^{2+}, and Mg^{2+}; furthermore, these metals are important in maintaining the Ca^{2+}/CaM-activated state. Recently, the identification of a "consensus site" for Ca^{2+}/CaM-kinase II phosphorylation in the catalytic subunit was reported from inspection of the deduced primary structure of the CaM-binding domain of the catalytic subunit (Kincaid et al., 1988). The sequence of the putative CaM-binding site was very similar to that of myosin light chain kinase where the phosphorylation of a serine residue near the CaM-binding site (Lukas, et al., 1986) accounts for altered Ca^{2+}/CaM activation of the enzyme (Conti & Adelstein, 1981).

Recently, CN was shown to be phosphorylated by the autophosphorylated (Ca^{2+}/CaM-independent) form of Ca^{2+}/CaM-kinase II (Hashimoto et al., 1988). The reaction was inhibited when Ca^{2+}/CaM was added, apparently by CaM interaction with the substrate CN through a substrate-directed effect; this agreed with earlier observations (Singh et al., 1987).

We undertook the present study to identify the site on CN phosphorylated by Ca^{2+}/CaM-kinase II and to examine whether the modification could regulate phosphatase activity or specificity since phosphorylation with protein kinase C (Lim Tung, 1986) or casein kinase (Singh et al., 1987) did not appear to affect phosphatase activity.

Experimental Procedures

CN and CaM were purified from bovine brain by published procedures (Kincaid et al., 1986; Kincaid, 1987). Protein phosphatase inhibitor-1 was purified from rabbit skeletal muscle and phosphorylated by the procedure of Cohen et al. (1988). The CaM-independent form of CaM-kinase II prepared by limited proteolysis of the autophosphorylated kinase and repurification on Cibacron blue–Sepharose (Kwiatkowski & King, 1989) was kindly provided by Dr. Marita King, Department of Chemistry, The Ohio State University, Columbus, OH. This treatment results in an activated kinase preparation that has lost the autophosphorylation domain and is Ca^{2+}/CaM-independent. The enzyme was stored in small aliquots at −80 °C. The catalytic subunit of cAMP protein kinase was purchased from Sigma, and New England Nuclear supplied [γ-^{32}P]ATP. Other materials were as follows: Sephadex G-50 (Pharmacia), phosphocellulose papers (Whatman), and X-Omat film (Kodak). All chemicals were of reagent grade.

Phosphorylation of CN. CN was phosphorylated by a procedure similar to that of Hashimoto et al. (1988). For phosphorylation studies, CN stored at −20 °C in 40% glycerol at 2 mg/mL was diluted 10-fold into 50 mM Hepes, pH 7.3, containing bovine serum albumin (1 mg/mL), Mg^{2+} (10 mM), Ca^{2+} (0.5 mM), EGTA (0.1 mM), and ATP (0.1 mM) [specific activity = (1–5) × 10^6 cpm/nmol]. Sufficient Ca^{2+}/CaM-kinase II was added to achieve maximal phosphorylation during incubations of 5–10 min. Incorporation of ^{32}P into CN was determined by scintillation counting of samples from reactions spotted on phosphocellulose discs and washed in H_3PO_4 (Witt & Roskoski, 1977).

Cyanogen Bromide Digestion of [^{32}P]CN. Radiolabeled CN was precipitated and washed in 15% trichloroacetic acid from CaM-kinase II reactions lacking bovine serum albumin. The precipitate (~0.2 mg) was taken up in 97% formic acid (0.1 mL) and dialyzed against 70% formic acid at 4 °C in a collodion bag (Thomas Instruments) prior to the addition of CNBr. CNBr was added to 30 mM (100 times the concentration of methionine residues, ~14 residues/mol of CN catalytic subunit), and the digestions were incubated in the dark for 10 h at 30 °C. The digest was dried in a speed-vac and dissolved in 80% formic acid.

Purification of the ^{32}P-Labeled Phosphopeptide of CN and Amino Acid Sequence Analysis. The solubilized CNBr digest was diluted in 0.1% trifluoroacetic acid and fractionated by reverse-phase HPLC with a (Vydac) C_4 column. The phosphorylated peptide was eluted with a linear gradient of acetonitrile (5–90%) in 0.1% trifluoroacetic acid. Collected fractions were monitored for radioactivity by Cerenkov counting. The peak fractions were dried and subsequently applied to a C_{18} reverse-phase column that was developed similarly. Amino acid sequence analyses were carried out as previously described for tryptic peptides (Kincaid et al., 1988) on an Applied Biosystems 470A gas-phase sequenator and a Model 120A phenylthiohydantoin amino acid analyzer. A Beckman spinning-cup sequenator was also utilized to enable the radioactivity eluted after each Edman degradation cycle to be measured.

Dephosphorylation of CN. Radiolabeled CN was chromatographed over Sephadex G-50 in 50 mM Hepes, 30 mM NaCl, 1 mM EDTA, and 2 mg/mL bovine serum albumin. The peak fractions were pooled and diluted with an equal volume of glycerol and stored at −20 °C. For dephosphorylation studies, the sample was diluted 10-fold to ~40 nM in the same buffer lacking EDTA with the effectors indicated. Reactions (100 μL) were terminated by the addition of 50 μL of bovine serum albumin (10 mg/mL in 0.1 M HCl), followed by 0.85 mL of 15% trichloroacetic acid. After 5 min on ice samples were centrifuged and the supernatants assayed for radioactivity by scintillation counting.

Miscellaneous Techniques. Partial acid digestion of [^{32}P]CN, purification of [^{32}P]phosphoamino acids, thin-layer electrophoresis at pH 3.5, and assay of molybdate-extracted [^{32}P]P_i in butanol–benzene were carried out as described by

[1] Abbreviations: CaM, calmodulin; CN, calcineurin; CaM-kinase II, Ca^{2+}/CaM-dependent protein kinase II; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

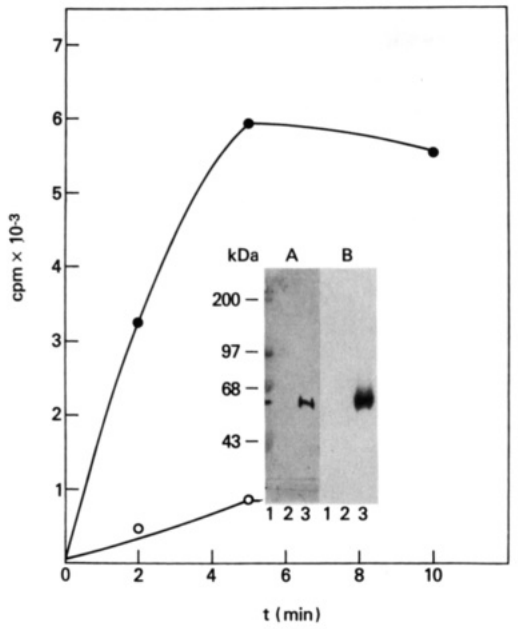

FIGURE 1: Phosphorylation of CN by CaM-kinase II: autoradiography and immunostaining. CaM-kinase was incubated alone (○, lane 2) or with CN (●, lane 3) in reaction mixtures containing Ca^{2+}, Mg^{2+}, and [γ-^{32}P]ATP (see Experimental Procedures). Samples were removed at the indicated times to determine incorporated radioactivity. At 10 min a portion of each incubation was denatured in sodium dodecyl sulfate and then applied to a polyacrylamide gel. After development of the gel the proteins were transferred electrophoretically to nitrocellulose; then the blot was probed with anti-CN antibodies. Inset: (A) immunoblot; (B) autoradiogram of (A). Prestained molecular weight markers containing 40 ng of authentic bovine brain CN are shown in lane 1.

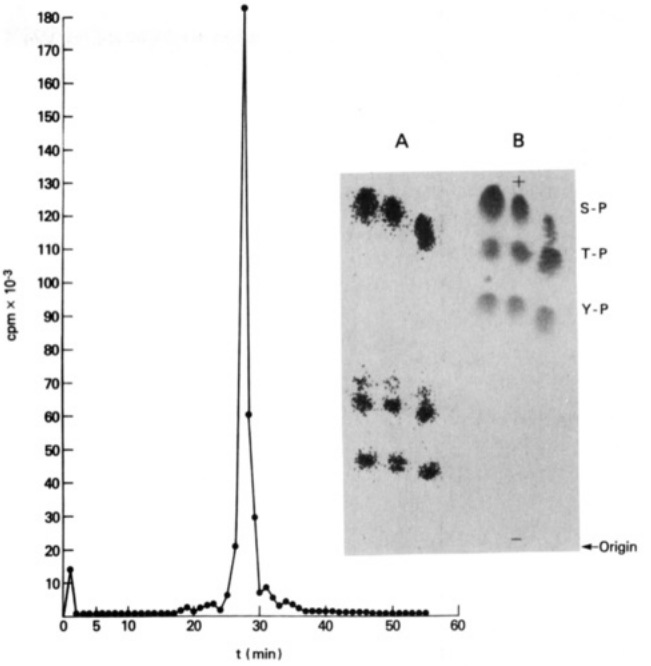

FIGURE 2: Reverse-phase (C_4) HPLC elution profile of radioactivity from CNBr digest of [^{32}P]CN and [^{32}P]phosphoamino acid identification. The CNBr digest was chromatographed on a C_4 Vydac column with a 5–90% acetonitrile gradient over 55 min. Samples of the peak tube and of each adjacent tube were subjected to partial acid hydrolysis followed by electrophoresis at pH 3.5 to identify the radiolabeled amino acid. Inset: (A) radioactive image (Betascope 603, Betagen Corp., Bedford, MA) of the electropherogram of hydrolysates containing the phosphoamino standards phosphoserine (S-P), phosphothreonine (T-P), and phosphotyrosine (Y-P); (B) ninhydrin stain of samples with the identification of the phosphoamino acid as indicated. CNBr digestion, reverse-phase HPLC, partial acid digestion, and thin-layer electrophoresis were performed as described under Experimental Procedures.

Martensen (1984). Western blotting and immunodetection of CN by antibodies were carried out as described by Kincaid (1988).

Results and Discussion

Activated CaM-kinase II readily phosphorylated CN in reaction mixtures containing [γ-^{32}P]ATP, Mg^{2+}, and Ca^{2+} at neutral pH (Figure 1), in agreement with the observations of Hashimoto et al. (1988). Utilization of the autophosphorylated/proteolyzed kinase preparation (see Experimental Procedures) avoided contamination of the CN preparation with ^{32}P-labeled kinase. No radioactive band was observed in sodium dodecyl sulfate–polyacrylamide gels in control incubations of the kinase (Figure 1). Incubation of CN with the kinase resulted in the labeling of a protein that migrated identically with the 60-kDa subunit of CN and, when blotted to nitrocellulose, cross-reacted with anti-CN antibodies. The incorporation of radioactivity enabled the calculation of an average phosphoryl group content of 0.9 mol/mol of CN from two separate preparations.

Reverse-Phase HPLC Purification of a CNBr Peptide Derived from Phosphorylated CN and Identification of the Phosphoamino Acid. The identification of the phosphorylated amino acid(s) labeled by Ca^{2+}/CaM-kinase has not previously been reported. We use both trypsin and CNBr digestions of labeled CN followed by HPLC to determine the number of radiolabeled peptides and the identity of the phosphoamino acids. Trypsin digestion of denatured phosphoCN yielded large radiolabeled peptides that were retained by 16% polyacrylamide gels, while CNBr digestion produced smaller peptides such that 67% of the radioactivity migrated ahead of the dye front on 16% polyacrylamide gels. When the CNBr-digested [^{32}P]CN was fractionated on a C_4 reverse-phase column (Figure 2), 58% of the radioactivity applied to the column eluted in a single peak termed CN-1. Phosphoamino acid analysis of fractions within CN-1 showed only phosphoserine as the labeled amino acid. On one occasion the peak seen in Figure 2 was split, which was attributed to homoserine lactone hydrolysis. Since phosphoserine residues are labile to base, alkaline treatment of the CNBr digest to hydrolyze homoserine lactone was avoided to protect the degradation of phosphoserine residues.

Purification and Amino Acid Sequencing of Phosphopeptide CN-1. The CN-1 peak from the C_4 column, when fractionated on a C_{18} reverse-phase column, yielded a single radioactive peak (CN-1′) that coincided with the elution of the major A_{225}-absorbing material (data provided to reviewers). This peak was utilized for both gas-phase and spinning-cup protein sequencing methodologies. Gas-phase sequencing of CN-1′ yielded the following structures: (1) Ala-Arg-Val-Phe-Xxx-Val-Leu-Arg, which coincided with residues 193–200 of the deduced primary structure of a clone containing the CaM-binding domain of mouse brain and with a hexapeptide sequence of a tryptic fragment of bovine brain CN (Kincaid et al., 1988), and (2) Tyr-Arg-Lys-Ser-Thr-Thr-Gly-Phe, corresponding to residues 77–85 of the same clone. We then determined the position of the radioactive residue in the peptide (i.e., the cycle at which radioactivity was released from the peptide) by monitoring the eluate/wash from the spinning-cup sequencer (Table I). In three separate experiments with two preparations of [^{32}P]CN, radioactivity appeared during the fifth cycle of the Edman degradation, consistent with the presence of phosphoserine at residue 5 of the first sequence.

Table I: Release of Radioactivity during Edman Degradation of Phosphopeptide Fraction CN-1′: Comparison with Deduced Amino Acid Sequences of Methionyl Peptides of the C-Terminal Region of CN[a]

		Position of residue following methionine						
		1	2	3	4	5	6	7
	cpm	67	47	48	32	550	230	120
Peptide	1	M-C-D-I-L-W-S-D						
	2	M-Y-R-K-S-Q-T-T						
	3	M-N-I-R-Q-F-N-C						
	4	M-N-V-F-T-W-S-L						
	5	M-L-V-N-V-L-N-I						
	6	M-A-R-V-F-S-V-L						
	7	M-L-P-S-G-V-L-S						
	8	M-P-P-R-R-D-A-M						
	9	M-P-S-D-A-N-L-N						

[a] Peptide CN-1′ was sequenced, as described under Experimental Procedures, by gas-phase and spinning-cup methodologies to determine the amino acids and radioactivity released at each cycle. One peptide sequence found, A-R-V-F-X-V-L-R, showed no residue in position 5 (X), consistent with the degradation of phosphoserine and the elution of radioactivity (cpm eluted in each cycle is shown in the top line of the table). Octapeptide sequences of methionine peptides from the C-terminal region of CN were obtained from the cDNA structure (Kincaid et al., 1988). Letters correspond to single-letter amino acid abbreviations. The underlined sequence corresponds to that phosphorylated by CaM-kinase II. All other methionyl peptides in the remainder of the deduced sequence for the catalytic subunit of CN lacked a serine residue in the fifth position (R. L. Kincaid, submitted for publication).

The sequence obtained from the phosphorylated peptide was present in the deduced sequence of the C-terminal domain of CN where the position of each serine relative to methionine is known. It was thus possible to identify the position of the phosphoamino acid. Inspection of Table I shows that only one peptide contains a serine residue five amino acid residues from a methionine breakpoint. Furthermore, this serine residue is found in a sequence considered to be a "consensus site" for Ca^{2+}/CaM-kinase (Pearson et al., 1985) which is juxtaposed with the CaM-binding site.[2] These results indicate that this sequence represents the major phosphorylation site on CN recognized by Ca^{2+}/CaM-kinase and provides a molecular mechanism (substrate directed) for the inhibitory effects of Ca^{2+}/CaM on the phosphorylation reaction described by others. This phosphorylation site is located approximately 110 residues from the C-terminus, consistent with the observation of Hashimoto et al. that trypsinization of radiolabeled CN yielded an unlabeled peptide of M_r ~43 000 that lacked CaM-binding activity. Thus, the phosphorylated site and the CaM-binding activity reside within a terminal domain of molecular mass ~17 000.

Autodephosphorylation of [^{32}P]CN: Effect of Dilution and Divalent Metals. The dephosphorylation rate of radiolabeled phosphoCN was measured after removal of Ca^{2+}, Mg^{2+}, and [^{32}P]ATP from the preparation by gel filtration (Figure 3). The dephosphorylation activity at 250 nM was similar to that at 25 or 2.5 nM, and the observed change in rate with time appeared to be independent of substrate loss. Since no decrease

[2] A synthetic peptide of 24 amino acids that includes this site inhibited the interaction of biotinylated CaM with CN on Western blots (Kincaid & Martin, 1989) and also blocked CaM-dependent phosphoprotein phosphatase activity (T. M. Martensen and R. L. Kincaid, unpublished data).

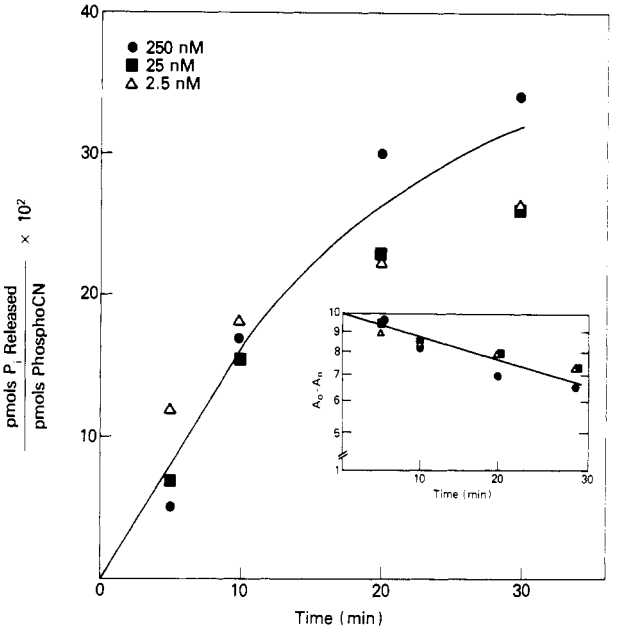

FIGURE 3: Time course of [^{32}P]CN autodephosphorylation. [^{32}P]CN in 50 mM Hepes, 2 mg/mL bovine serum albumin, 30 mM NaCl, and 0.1 mM EDTA, pH 7.2, was diluted to 250 (●), 25 (■), and 2.5 (△) nM, respectively, and incubated at 37 °C. Samples were removed at the indicated times for analysis of trichloroacetic acid soluble radioactivity. Data are expressed as the percentage of total phosphoCN remaining at the concentrations indicated. (Inset) Semilog plot of the dephosphorylation of [^{32}P]CN at each concentration: A_0, original radioactivity normalized to 100%; A_n, radioactivity released as inorganic phosphate. The experiment was repeated twice with an independent preparation of phosphoCN at 4 and 40 nM with the same result.

Table II: Effectors of [^{32}P]CN Autodephosphorylation[a]

sample	(cpm + effector)/(cpm − effector)	sample	(cpm + effector)/(cpm − effector)
no addition	1.0	Mg^{2+}, 1 mM	2.1
EGTA, 1 mM	1.3	Ca^{2+}, 1 mM	2.0
EDTA, 1 mM	1.3	Ni^{2+}, 1 mM	4.2
Mn^{2+}, 1 mM	3.0		

[a] [^{32}P]CN was diluted to 40 nM (see Experimental Procedures) with or without the compounds indicated and incubated at 32 °C for 10 min. Data are the ratio of [^{32}P]P_i released with effector present to that obtained with no effector present. Ratios shown are representative of two or three experiments. Maximal loss of substrate radioactivity (5000–10 000 cpm) was 35%.

in autodephosphorylation activity at 2.5 nM phosphoCN was observed compared to the rate at 250 nM, this would suggest that the K_m is less than 2 nM if dephosphorylation were intermolecular as was suggested (Hashimoto et al., 1988). This value would be 800 times lower than the K_m of the best phosphoprotein substrate, protein phosphatase inhibitor-1. Alternatively, the dephosphorylation mechanism may be intramolecular and therefore concentration independent since a semilog plot of the dephosphorylation of [^{32}P]CN with time at three concentrations appears to fit a straight line (Figure 3, inset). The value for the dephosphorylation rate was 0.12 nmol of P_i min^{-1} mg^{-1} at 37 °C, which agrees well with the value of Hashimoto et al. (1988) (0.09 nmol of P_i min^{-1} mg^{-1} at 30 °C). Autodephosphorylation was stimulated by Mn^{2+}, Ca^{2+}, and Ni^{2+} (see Table II). Since these metals stimulate catalysis, this may be their mechanism of action; however, they could also affect the degree of "exposure" of the phosphoserine residue, making it a better substrate. This might also explain the weak stimulatory action of EDTA and EGTA. This unusual dephosphorylation mechanism and its regulation by

effectors require further study to establish the physiological significance. Additionally, whether phosphorylated CN is a preferred substrate for other phosphoprotein phosphatases needs to be determined.

References

Cohen, P., Foulkes, G. H., Holmes, C. F. B., Nimmo, G. A., & Tonks, N. K. (1988) *Methods Enzymol. 159*, 427–436.

Colbran, R. J., Schworer, C. M., Hashimoto, Y., Fong, Y.-L., Rich, D. P., Smith, M. K., & Soderling, T. R. (1989) *Biochem. J. 258*, 313–325.

Conti, M. A., & Adelstein, R. S. (1981) *J. Biol. Chem. 256*, 3178–3181.

Hashimoto, Y., King, M. M., & Soderling, T. R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7001–7005.

Hemmings, H. C., Jr., Nairn, A. C., McGuinness, T. L., Huganir, R. L., & Greengard, P. (1989) *FASEB J. 3*, 1583–1592.

Kennedy, M. B., Bennett, M. K., Erondu, N. E., & Miller, S. G. (1987) *Calcium Cell Funct. 7*, 61–107.

Kincaid, R. L. (1987) *Methods Enzymol. 139A*, 3–19.

Kincaid, R. L. (1988) *Methods Enzymol. 159*, 627–651.

Kincaid, R. L., & Martin, B. M. (1989) in *Calcium Protein Signaling. Proceedings of the Sixth International Symposium on Calcium in Health and Disease* (Hidaka, H., Ed.) pp 347–358, Plenum Press, New York.

Kincaid, R. L., Manganiello, V. C., Odya, C. E., Osborne, J. C., Jr., Stith-Coleman, I. E., Danello, M. A., & Vaughan, M. (1984) *J. Biol. Chem. 259*, 5158–5166.

Kincaid, R. L., Nightingale, M. S., & Martin, B. M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8983–8987.

King, M. M., & Huang, C. Y. (1984) *J. Biol. Chem. 259*, 8847–8856.

King, M. M., Huang, C. Y., Chock, P. B., Nairn, A. C., Hemmings, H. C., Jr., Chan, K.-F., & Greengard, P. (1984) *J. Biol. Chem. 259*, 8080–8083.

Klee, C. B., & Cohen, P. (1988) *Mol. Aspects Cell. Regul. 5*, 225–248.

Klee, C. B., Draetta, G. F., & Hubbard, M. J. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 149–200.

Kwiatkowski, A. P., & King, M. M. (1989) *Biochemistry 28*, 5380–5385.

Lim Tung, H. Y. (1986) *Biochem. Biophys. Res. Commun. 138*, 783–788.

Lukas, T., Burgess, W., Prendergras, F., Lau, W., & Waterson, D. M. (1986) *Biochemistry 25*, 1458–1463.

Martensen, T. M. (1984) *Methods Enzymol. 107*, 3–22.

Nairn, A. C., Hemmings, H. C., Jr., & Greengard, P. (1985) *Annu. Rev. Biochem. 54*, 931–976.

Pearson, R. B., Woodget, J. R., Cohen, P., & Kemp, B. E. (1985) *J. Biol. Chem. 260*, 14471–14476.

Schulman, H., & Lou, L. L. (1989) *Trends Biochem. Sci. 14*, 62–66.

Singh, T. J., & Wang, J. H. (1987) *Biochem. Cell Biol. 65*, 917–921.

Tallant, E. A., & Cheung, W. Y. (1986) *Calcium Cell Funct. 6*, 71–112.

Witt, J. J., & Roskoski, R., Jr. (1975) *Anal. Biochem. 66*, 253.