BRAIN TYPE II CALCIUM AND CALMODULIN-DEPENDENT PROTEIN KINASE: PURIFICATION, CHARACTERIZATION AND MOLECULAR CLONING

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ABSTRACT

A combination of biochemical, immunochemical, and molecular biological techniques have been employed to purify and characterize a rat brain Ca^{2+}/calmodulin-dependent protein kinase. The enzyme, named type II Ca^{2+}/calmodulin-dependent protein kinase (type II CaM kinase), was identified in rat brain homogenates by its ability to phosphorylate site II on the synaptic vesicle associated protein synapsin I.

Type II CaM kinase has been purified 290 fold over crude homogenates and is found to be composed of multiple copies of two different subunits. Both subunits copurify with kinase activity and are coprecipitated with kinase activity by an anti-kinase monoclonal antibody. The two subunits have molecular weights of 50,000 (α) and 58,000/60,000 (β), and are present in a 3:1 α:β ratio. The type II CaM kinase holoenzyme has a sedimentation coefficient of 16.4 S, a Stokes radius of 95 Å, and a calculated molecular weight of 650,000. A dodecameric holoenzyme consisting of 9 α subunits and 3 β subunits has been proposed. The purified type II CaM kinase phosphorylates several substrates, in addition to synapsin I, at a significant rate, and may therefore be responsible for a number of neuronal responses to Ca^{2+}.

The α subunit of type II CaM kinase has a number of biochemical characteristics which are similar to the major protein component of a subcellular fraction which is derived from brain postsynaptic densities (PSDs). A direct comparison between the α subunit of type II CaM kinase and the major PSD protein using immunochemical and biochemical techniques has revealed that they are in fact very similar or identical proteins.

Two approaches have been taken to further characterize the subunits of type II CaM kinase at a molecular level. The first approach has been to isolate cDNA clones which code for the β subunit. A number of clones have been

isolated and sequenced. The amino acid sequence for the β subunit (predicted from the cDNA sequence) is homologous to several other protein kinases. Southern blot analysis with a β subunit cDNA indicates the existence of a type II CaM kinase multigene family. The second approach to the molecular characterization of the type II CaM kinase subunits has been to determine the amino acid sequence of peptides derived from the α subunit. Two regions of α subunit sequence have been determined, and both are found to be homologous to regions of β subunit amino acid sequence deduced from β subunit cDNA clones.

The molecular characterization of neuronal type II CaM kinase in vitro has both provided insight into the possible function of the enzyme in vivo and suggested experimental approaches which may eventually allow its in vivo function to be directly addressed.

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INTRODUCTION

The function of specialized cells in a wide variety of tissues is often regulated by extracellular signals such as neurotransmitters and hormones. These signals produce their effects by altering the intracellular concentration of second messengers in their target cells. Among the second messengers which have been identified are cAMP (Robinson et al., 1968), cGMP (Kuo and Greengard, 1970), calcium ion (Kretsinger, 1979), and diacylglycerol (Michell, 1983). The cyclic nucleotides and diacylglycerol appear to act exclusively by activating specific protein kinases which phosphorylate, and thereby regulate the function of specific substrate proteins. Calcium ion, on the other hand, acts as a second messenger by a variety of different mechanisms, often by way of the ubiquitous Ca^{2+} binding protein calmodulin (CaM) (Cheung, 1980). Among the enzymes regulated by Ca^{2+} are a cyclic nucleotide phosphodiesterase (Sharma et al., 1980), a protein phosphatase (calcineurin) (Stewart et al., 1982), an adenylate cyclase (Brostrom et al., 1978), and at least 5 types of protein kinase (Kennedy et al., 1986). All of these enzymes are involved, either directly or indirectly, in the regulation of protein phosphorylation, which is indicative of the general role of protein phosphorylation in second messenger regulatory cascades. In addition, several of these Ca^{2+} regulated enzymes are particularly concentrated in the nervous system where the number of different cell types and large amount of intercellular communication dictates abundant second messenger response systems.

The role of Ca^{2+} as an intracellular regulator was first demonstrated in skeletal muscle where Ca^{2+} is released from the sarcoplasmic reticulum during a muscle action potential (Katz, 1966) and binds to the regulatory protein tropinin C. Troponin C undergoes a conformational change allowing actin and myosin to interact and resulting in muscle contraction (Potter and Gergely, 1975). The

mechanisms of Ca^{2+} regulation in other systems is less clearly understood. In the nervous system, a number of important processes are regulated by Ca^{2+} including neurotransmitter release, neurotransmitter synthesis, and membrane exctiability, among others.

Ca^{2+} is absolutely required for neurotransmitter release. This requirement was first demonstrated by Katz and Miledi (1965) using a frog neuromuscular junction preparation. More detailed experiments on the role of Ca^{2+} in neurotransmitter release have relied on the squid stellate ganglion preparation. This preparation is well suited for these studies because of the large size of the neurons which allow both the presynaptic terminal and the postsynaptic cell to be penetrated with intracellular electrodes and voltage clamped. Studies by Llinas and colleagues (Llinas et al., 1982) have demonstrated that there is a nonlinear relationship between the presynaptic Ca^{2+} current and the amount of transmitter released, suggesting a cooperative mechanism for Ca^{2+} action. In addition, Llinas et al. (1981) have determined that there is only a 200 μsec delay between the influx of Ca^{2+} and the release of transmitter. These results place constraints on the molecules which may be involved in neurotransmitter release. For example, the speed with which transmitter is released makes it unlikely that enzymatic reactions are directly responsible for the fusion between synaptic vesicles and the plasma membrane, although such enzymatic reactions may modulate neurotransmitter release on a longer timescale. It is possible, therefore, that Ca^{2+} may initiate synaptic vesicle/plasma membrane fusion directly by binding to a specific synaptic vesicle receptor (Reichardt and Kelly, 1983), although such a mechanism has not been proven.

Ca^{2+} can regulate the membrane excitability of neurons by a number of different mechanisms. Specific potassium channels have been found in a variety of different neurons which are activated by intracellular Ca^{2+} (Meech, 1978).

When activated, these channels tend to reduce membrane excitability by maintaining the cell below the action potential initiation threshold. Intracellular Ca^{2+} also appears to inactivate voltage-sensitive Ca^{2+} channels (Hagiwara and Byerly, 1981). Recent experiments suggest that the Ca^{2+}/phospholipid-dependent protein kinase (C kinase) may activate certain voltage-sensitive Ca^{2+} channels in Aplysia bag cells (DeReimer et al., 1985). Ca^{2+} may also regulate neuronal excitability indirectly by regulating the activity of two different Ca^{2+} pumps, a plasma membrane pump activated by Ca^{2+}/CaM and a synaptic vesicle pump activated by a Ca^{2+}/CaM-dependent protein kinase (Goldin et al., 1983).

The synthesis of serotonin and catecholamine neurotransmitters can be regulated by Ca^{2+}. A Ca^{2+}/CaM-dependent protein kinase phosphorylates the rate limiting enzymes, tyrosine hydroxylase and tryptophan hydorxylase, involved in the biosynthesis of these transmitters, resulting in an increase in their catalytic activity (Yamauchi et al., 1981). In addition to the processes just described, Ca^{2+} has also been implicated in phototransduction (O'Brien, 1982), the determination of the neurotransmitter choice made by sympathetic neurons in culture (Walicke and Patterson), the initiation of fast axonal transport (Hammerschlag and Stone, 1982), and the regulation of the structure and function of several cytoskskeletal structures (Kirschner, 1978; Hathaway et al., 1981; Craig and Pollard, 1982). One way of sorting out the mechanisms underlying this increasingly complex array of cellular regulation in the nervous system is to identify and characterize the molecular targets for Ca^{2+} regulation. This includes the identification and characterization of Ca^{2+} regulated protein kinases and their substrates. This thesis reports on one aspect of this task, the purification and molecular characterization of a Ca^{2+} and calmodulin dependent protein kinase which is concentrated in rat brain.

The study of Ca^{2+}/CaM dependent protein phosphorylation in the nervous system was preceeded by the identification and characterization of two Ca^{2+}/CaM dependent protein kinases from non-neuronal tissue, phosphorylase kinase and myosin light chain kinase. The molecular characteristics of these two kinases will be briefly described in order to facilitate later comparisons.

Phosphorylase kinase is a central enzyme in the regulation of glycogen metabolism. It catalyzes the phosphorylation of glycogen phosphorylase converting it from an inactive form (phosphorylase b) to an active form (phosphorylase a) thus initiating the breakdown of glycogen (Cohen, 1978). Phosphorylase kinase has been purified from a number of different sources and its molecular properties characterized (Malencik and Fischer, 1982; Chan and Graves, 1984). It is made up of four copies of each of four subunits $(\alpha\beta\gamma\delta)_4$ (Shenolikar et al., 1979) and has a molecular weight of 1,300,000 (Cohen, 1973; Hayakawa et al., 1973). The α and β subunits both bind exogenous calmodulin (Picton et al, 1980) and are phosphorylated by cAMP-dependent protein kinase (Cohen, 1973; Walsh et al., 1971), indicating that they may play a regulatory role. The γ subunit appears to be catalytic (Chan and Graves, 1982) and the δ subunit is identical to calmodulin (Grand et al., 1981). Unlike other CaM dependent enzymes, the δ subunit is an integral comoponent of the holoenzyme complex and does not easily dissociate even in the absence of Ca^{2+} (Picton et al., 1980). Phosphorylase kinase has a relatively narrow substrate specificity and therefore may perform only a small number of specialized functions (Malencik and Fischer, 1982; Chan and Graves, 1984).

Myosin light chain kinase phosphorylates the regulatory P-light chains of myosin (Perrie et al., 1972). In smooth muscle and many non-muscle cells this phosphorylation is required for the expression of actin activated myosin ATPase activity (Adelstein and Eisenberg, 1980). The myosin ATPase activity is thought

to be responsible for the hydrolysis of ATP that accompanies smooth muscle contraction (Kamm and Stull, 1985). The role of myosin light chain phosphorylation in other cells, including platelets, skeletal muscle, and cardiac muscle, is yet to be determined. Myosin light chain kinase has been purified from a wide variety of tissues and species (Kennedy et al., 1986). In all cases the enzyme appears to be monomeric with molecular weights ranging from 72,000-150,000. The differences in molecular weights are probably due to tissue and species specific isozymes (Nunnally et al., 1985). Myosin light chain kinase is activated by the binding of Ca^{2+}/CaM and, like phosphorylase kinase, phosphorylates only a limited number of substrates and therefore may perform very specialized functions.

Both phosphorylase kinase and myosin light chain kinase are present in the brain (Osawa, 1973; Dabrowska and Hartshorn, 1978). However, their abundance cannot account for the rate or diversity of CaM dependent phosphorylation observed in brain homogenates. Two distinct neuronal CaM dependent protein kinases have been identified by their ability to phosphorylate the synaptic vesicle associated protein synapsin I (Kennedy and Greengard, 1981). One of these has been called CaM dependent protein kinase I because it phosphorylates site I on synapsin I, the same site phosphorylated by cAMP dependent protein kinase. The other enzyme, called type II CaM dependent protein kinase, phosphorylates site II on synapsin I and is the topic of this thesis. Based on the work described in this thesis, and the work from a number of different laboratories over the past three years, it appears that brain type II CaM kinase is a member of a family of structurally similar, but not identical, broad substrate specificity kinases which may regulate a variety of cellular functions (Kennedy et al., 1986). A discussion of this family of kinases will occupy the remainder of this introduction.

The members of this family of CaM dependent protein kinases have been purified from a number of different tissues and have the following properties in common. 1) They are multimeric proteins of M_r=300,000-700,000 composed of structurally related subunits of M_r=50,000-60,000. 2) All of the subunits appear to be catalytic, bind CaM, and are autophosphorylated. 3) They all have the same relatively broad substrate specificity. The members of this kinase family include the neuronal type II CaM kinase, CaM dependent glycogen synthase kinases from liver and skeletal muscle, and a number of incompletely characterized CaM kinases from a variety of tissues and species. The characteristics of each of these kinases will be described separately.

Brain type II CaM dependent kinase phosphorylates a variety of substrates and has been purified from rat brain independently by several groups. Substrates used during its purification include synapsin I (Bennett et al., 1983; McGuinness et al., 1983), tryptophan hydroxylase (Yamauchi and Fujisawa, 1983a), casein (Kuret and Schulman, 1984), tubulin (Goldenring et al., 1983), and smooth muscle myosin light chain (Fukunaga et al., 1982). In all cases, the reported molecular weights are large, ranging from 460,000-650,000. Reported sedimentation coefficients range from 13.7 to 16.5S, and Stokes radii from 81.3 to 96Å. With one exception, all the enzymes are reported to contain a prominent M_r=50,000 (α) subunit and less prominent M_r=58,000 (β') and M_r=60,000 (β) subunits in approximately a 3:1 ratio. The one exception is a report by Fukunaga et al. (1982) in which the enzyme appears to contain a single M_r=49,000 subunit. The α and β subunits of these enzymes both bind CaM (Bennett et al., 1983; Goldenring et al., 1983; Kuret and Schulman, 1984) and are labeled by 8-azido ATP (Lai et al., 1983), indicating that they both may be catalytic. Both subunits can be autophosphorylated (Bennett et al., 1983; Goldenring et al., 1983; Kuret and Schulman, 1984) with α and β incorporating a maximum of 2 and 3 moles of

phosphate per mole of subunit, respectively. Peptide mapping and immunological studies indicate that the α and β subunits are distinct peptides, with some homologous regions (Kennedy et al., 1983; Goldenring et al., 1983; McGuinness et al., 1983; Kelly et al., 1984). The two β subunits on the other hand are very similar, indicating that one may be derived from the other by proteolysis or posttranslational modification.

It is now apparent that there are at least two and perhaps more isozymes of the neuronal type II CaM kinase. The enzyme described above represents the most abundant form, the forebrain isozyme, which comprises about 1% of total brain protein (Erondu and Kennedy, 1985). Recently, the type II CaM kinase was isolated from cerebellum (McGuinness et al., 1985; Miller and Kennedy, 1985). Its physical characteristics are similar to those of the forebrain isozyme; however, their subunit compositions differ. The cerebellar enzyme contains α and β subunits similar or identical to those of the forbrain enzyme, but they are present in a different ratio (α:β is 1:4). The functional significance, if any, of these brain region specific isozymes is not known.

Recently, two CaM dependent glycogen synthase kinases have been purified. One of these enzymes, isolated from liver (Ahmad et al., 1982; Payne et al., 1983), is composed of approximately equal amounts of two subunits with M_r's=50,000 and 53,000. The holoenzyme has a Stokes radius of 70Å, a sedimentation coefficient of 10.6S (Payne et al., 1983), and a calculated molecular weight of 300,000. Thus it contains an average of three copies of each of the two subunits. Both types of subunit can be autophosphorylated with incorporation of up to four moles of phosphate per mole of subunit (Ahmad et al., 1982). A different CaM dependent glycogen synthase kinase has been purified from skeletal muscle (Woodgett et al., 1983). This enzyme is composed of two subunits with M_r's=58,000 and 54,000 in approximately a 4:1 ratio, both of which

are autophosphorylated. The molecular weight of the holoenzyme determined by sedimentation equilibrium is 696,000. Electron microscopic studies of negatively stained enzyme molecules revealed a hexagonal shape. These results taken together suggest that the holoenzyme is a dodecamer made up of two stacked hexagonal rings.

The brain type II CaM kinase has a number of similarities to the glycogen synthase kinases just described. Aside from the similarities in holoenzyme composition, the subunits have internal structural similarities. One monoclonal antibody generated against the brain type II CaM kinase reacts with both the α and β subunits of the brain enzyme, and also with both subunits of the skeletal muscle glycogen synthase kinase (McGuinness et al., 1983). In addition, one-dimensional phosphopeptide maps of the subunits of these two kinases revealed similarities between the β and β' subunits of the brain enzyme and the M_r=58,000 subunit of the skeletal muscle enzyme (McGuinness et al., 1983). Finally, direct comparison of the substrate specificities of the brain type II CaM kinase and the skeletal muscle glycogen synthase kinase indicated nearly identical substrate specificity (see below) (McGuinness et al., 1983; Woodgett et al., 1984).

A number of additional protein kinases that have characteristics similar to those just described have been identified in and/or purified from other tissues and species. A CaM stimulated protein kinase was purified from rat pancreas (Gorelick et al, 1983; Cohn et al., 1984) based on its ability to phosphorylate ribosomal protein S6. The purified kinase has a molecular weight of 600,000 measured by gel filtration and 300,000 measured by sucrose density sedimentation. It is composed of a single M_r=51,000 CaM-binding, autophosphorylatable subunit.

Another such CaM dependent kinase was recently partially purified from avian erythrocytes (Palfrey et al., 1984). It phosphorylates the same site on

synapsin I as the brain type II CaM kinase and has a molecular weight of greater than 500,000 determined by gel filtration. The partially purified preparation contains several polypeptides including a major CaM-binding and endogenously phosphorylated peptide (M_r=58,000) and two minor CaM-binding peptides (M_r's=50,000 and 54,000). Palfrey (1984) has also purified a protein kinase from mammalian heart made up of M_r=55,000 and M_r=75,000 autophosphorylatable subunits, and partially purified a CaM dependent synapsin I kinase activity from Torpedo electric organ (Palfrey et al., 1983). The latter activity appears to copurify with two major endogenous substrates (M_r's=52,000 and 54,000).

Finally, kinases homologous to the brain type II CaM kinase have been observed in two invertebrate species, Aplysia and Drosophila. The Aplysia nervous system contains an M_r=51,000 CaM binding, endogenously phosphorylated protein that reacts with a monoclonal antibody against rat brain type II kinase (DeRiemer et al., 1984). The phosphopeptide map of this protein is also similar to that of the rat enzyme. Thus, this protein is probably a subunit of an Aplysia type II CaM kinase. A CaM dependent kinase which phosphorylates synapsin I in homogenates of Drosophila heads is precipitated by a monoclonal antibody generated against the rat brain type II kinase (J. Wall and M. Kennedy, unpublished observations). The antibody precipitates three phosphoproteins (M_r's=60,000, 58,000 and 54,000) which may be the subunits of a Drosophila type II CaM kinase. The potential for physiological and genetic studies in these two systems should facilitate the study of the functional roles of this class of kinases.

The results discussed so far indicate that the family of broad specificity CaM kinases is a large one with wide tissue and species distribution. The exact relationship among the various isozymes is not well understood. Monoclonal antibodies have been useful in identifying and comparing kinase subunits from

various tissues and species (McGuinness et al., 1983; Palfrey et al., 1983; DeRiemer et al., 1984; Woodgett et al., 1984). The isolation of cDNA clones for the various subunits and the determination of their amino acid sequences should ultimately define the relationships among them. The isolation of a cDNA clone for the β subunit of brain type II kinase and the determination of its sequence is described in Chapter 3 of this thesis. The information from this clone has revealed regions of homology with other protein kinases (cAMP dependent protein kinase, phosphorylase kinase, myosin light chain kinase, and transforming tyrosine kinases) and should be very useful for comparisons when sequence information from the other broad specificity CaM kinases becomes available.

One of the most striking similarities among all of these kinases is their broad substrate specificity. Among the substrates phosphorylated at a significant rate are glycogen synthase, synapsin I, microtubule associated protein-2 (MAP_2), smooth muscle myosin light chain, tyrosine hydroxylase, tryptophan hydroxylase, myelin basic protein, casein, phosvitin, histone H1, histone H3, ribosomal protein S6, tau proteins, vimentin, and tubulin, and the kinase itself (autophosphorylation) (see Kennedy et al., 1986 for review). Differences in the reported substrate specificities of the various broad specificity kinases in early reports appear to have reflected differences in the preparation of the substrates. In two reports in which identical substrates were used for comparison of brain type II CaM kinase and skeletal muscle glycogen synthase kinase, nearly identical substrate specificities and relative rates of phosphorylation of the substrates were obtained for the two enzymes (McGuinness et al., 1983; Woodgett et al., 1984). However, the relative rates of phosphorylation of these in vitro substrates does vary greatly, and it remains to be determined which of them are actually substrates in vivo.

A number of the substrates undergo functional changes in vitro following their phosphorylation by the broad specificity CaM kinases. The phosphorylation of synapsin I results in a decrease in its binding to a high affinity binding site on synaptic vesicles (Huttner et al., 1983; Schiebler et al., 1983). The possibility that this synapsin I phosphorylation might regulate neurotransmitter release was suggested by recent experiments in which brain type II CaM kinase was injected into the presynaptic terminal of the squid giant synapse (Llinas et al., 1985). Phosphorylation of glycogen synthase by the broad specificity CaM kinases results in inactivation of the enzyme (Cohen, 1982). Brain type II CaM kinase phosphorylates smooth muscle myosin light chain at the same site as does myosin light chain kinase, and thus enables actin to activate the myosin ATPase (Edelman et al., 1986). Phosphorylation of MAP_2 by brain type II CaM kinase inhibits microtubule assembly (Yamamoto et al., 1983) and promotes microtubule disassembly (Yamauchi and Fujisawa, 1983b). Phosphorylation of two enzymes involved in catecholamine metabolism, tyrosine hydroxylase and tryptophan hydroxylase, allows them to interact with an "activator" protein and results in a two-fold increase in catalytic activity (Yamauchi et al., 1981; Vulliet et al., 1984). The autophosphorylation of the brain type II kinase results in a decrease in its overall catalytic activity, but a dramatic increase in its CaM independent activity (Miller and Kennedy, 1986). The generation of this CaM independent activity may allow the kinase to regulate neuronal processes on a time scale much longer than the generally short increases in intracellular Ca^{2+} concentration (Lisman, 1985). These in vitro studies suggest that the broad specificity CaM kinases may be involved in the regulation of glycogen synthesis, neurotransmitter synthesis and release, microtubule assembly, actomyosin interactions, and perhaps even long term regulation of neuronal processes. It will be exciting to determine which of these regulatory roles are played in vivo.

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CHAPTER 1

PURIFICATION AND CHARACTERIZATION OF A CALMODULIN-DEPENDENT PROTEIN KINASE THAT IS HIGHLY CONCENTRATED IN BRAIN

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Purification and Characterization of a Calmodulin-dependent Protein Kinase That Is Highly Concentrated in Brain*

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A calcium and calmodulin-dependent protein kinase has been purified from rat brain. It was monitored during the purification by its ability to phosphorylate the synaptic vesicle-associated protein, synapsin I. A 300-fold purification was sufficient to produce kinase that is 90–95% pure as determined by scans of stained sodium dodecyl sulfate-polyacrylamide gels and has a specific activity of 2.9 μmol of ^{32}P transferred per min/mg of protein. Thus, the kinase is a relatively abundant brain enzyme, perhaps comprising as much as 0.3% of the total brain protein.

The Stokes radius (95 Å) and sedimentation coefficient (16.4 S) of the kinase indicate a holoenzyme molecular weight of approximately 650,000. The holoenzyme is composed of three subunits as judged by their co-migration with kinase activity during the purification steps and co-precipitation with kinase activity by a specific anti-kinase monoclonal antibody. The three subunits have molecular weights of 50,000, 58,000, and 60,000, and have been termed α, β′, and β, respectively. The α- and β-subunits are distinct peptides, however, β′ may have been generated from β by proteolysis. All three of these subunits bind calmodulin in the presence of calcium and are autophosphorylated under conditions in which the kinase is active. The subunits are present in a ratio of about 3 α-subunits to 1 β/β′-subunit. We therefore postulate that the 650,000-Da holoenzyme consists of approximately 9 α-subunits and 3 β/β′-subunits.

The abundance of this calmodulin-dependent protein kinase indicates that its activation is likely to be an important biochemical response to increases in calcium ion concentration in neuronal tissue.

The functions of specialized cell types within different tissues are coordinated by a variety of extracellular agents. Each of these triggers a series of intracellular regulatory events that ultimately alter the functional state of the cell. The first step in the action of many regulatory agents is to increase the intracellular concentration of a second messenger, such as a cyclic nucleotide or calcium ion (1–3). A complete understanding of the general principles of cellular regulation will require a description of the molecular events triggered by each of these second messengers.

We are interested in the mechanisms of action of calcium ion in nervous tissue. A number of important neuronal processes are regulated by changes in the intracellular concentration of calcium (4–7). These changes are brought about both by the actions of specific extracellular agents such as neurotransmitters and neurohormones, and by electrical activity which activates voltage-sensitive calcium channels (8). Changes in calcium concentration are important in synaptic processes such as post-tetanic potentiation and long-term potentiation that involve modulation of the quantity of transmitter released per impulse (9–11). In certain neurons, the conductances of specific potassium (12) or calcium (13) membrane channels are regulated by intracellular calcium ion. Such transient changes in the strength of specific synapses, or in the electrical properties of individual neurons can modulate the flow of information through complex neuronal circuits (14). Information about the properties and distributions in different types of neurons of proteins that are regulated by physiological changes in calcium concentration will be necessary to understand, at the molecular level, the responses of various neurons, and of individual synaptic terminals, to changes in calcium flux.

It is generally accepted that the cyclic nucleotides regulate cell physiology primarily, if not exclusively, by activating protein kinases (15, 16). Distinct kinases that are activated by either cyclic AMP or cyclic GMP have been purified and characterized (17, 18). In contrast, the mechanisms by which calcium alters cellular functions appear to be quite diverse (19). Nevertheless, the importance of calcium-regulated protein kinases has recently been recognized (5, 20). Initial characterization of this distinct class of kinases has revealed that, unlike the cyclic AMP-dependent protein kinases, there are a number of calcium-regulated protein kinases that differ in several characteristics, including mechanism of regulation by calcium, substrate specificity, and tissue distribution (21–29).

Calcium and calmodulin-dependent protein kinase activities have been observed in crude brain homogenates and in various neuronal subcellular fractions (26, 27, 29–34). Although two well characterized calmodulin-dependent protein kinases from non-neuronal tissues, myosin light chain kinase, and phosphorylase kinase, are present in brain, they do not account for a large portion of brain calmodulin-dependent protein kinase activity (26, 27). This paper reports the purification and subunit structure of a distinct calmodulin-dependent protein kinase that is highly concentrated in brain. The enzyme was first observed in brain homogenates as a calmodulin-dependent kinase that phosphorylated synapsin I, a protein associated with synaptic vesicles (26, 35). For clarity, we continue to refer to the enzyme as "calmodulin-dependent synapsin I kinase," although we believe that it is likely to be involved in the phosphorylation and regulation of a number of other brain proteins.

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The structure of the synapsin I kinase is different from either myosin light chain kinase or phosphorylase kinase (22–25). However, it is similar but not identical to a glycogen synthase kinase recently purified from liver (36, 37), and another calmodulin-dependent protein kinase recently purified from rat brain (38). Thus, it may be one of a class of related calmodulin-dependent protein kinases.

EXPERIMENTAL PROCEDURES

Materials—ATP, 3′,5′-cAMP, dithiothreitol, imidazole, EDTA, PMSF,[1] fast green FCF, Coomassie brilliant blue R, fibrinogen, bovine serum albumin, ovalbumin, phosphorylase *b*, carbonic anhydrase, soybean trypsin inhibitor, actin, alcohol dehydrogenase, glycogen synthase, trypsin, casein, phosvitin, arginine-rich histone, Type II hemoglobin, insulin, and transferrin were purchased from Sigma. Thyroglobulin, ferritin, catalase, Protein A and Sepharose 4B were purchased from Pharmacia Fine Chemicals. Leupeptin was purchased from Peninsula Laboratories, Inc. Myosin was purchased from Bethesda Research Laboratories. Lactoperoxidase was purchased from Calbiochem. Chymotrypsinogen was purchased from Worthington. [γ-^{32}P]ATP was purchased from ICN Nutritional Biochemicals. Na^{125}I and [^{3}H]3′,5′-cAMP were purchased from New England Nuclear. Trifluoperazine was obtained from Smith, Kline, and French. Ultrapure sucrose was purchased from Schwarz/Mann. DEAE-cellulose (DE-52) was purchased from Whatman. Nitrocellulose membranes (BA85, 0.45 μm) were purchased from Schleicher and Schuell. RPMI 1640 tissue culture medium, glutamine, penicillin, and streptomycin were purchased from Gibco Laboratories. Newborn calf serum was purchased from Irvine Scientific. Selenous acid (Specpure) was purchased from Johnson Matthey, Inc., Synapsin I was purified from bovine brain by a modification of the procedure of Ueda and Greengard (39), as described in Huttner *et al.* (40). The modifications were as follows. A crude brain particulate fraction was used at step 2, rather than the M-1 fraction. The pH 6 supernatant was adjusted to pH 8 and subjected to chromatography on CM-cellulose, hydroxylapatite and Sephadex G-100. The final purified fractions were concentrated by ammonium sulfate precipitation and dialyzed against 25 mM Tris-HCl, pH 7.0. Calmodulin was purified from bovine brain by the method of Watterson *et al.* (41). Microtubule protein prepared from rat brain by the method of Shelanski *et al.* (42), was a gift of James Soha of our laboratory. Gizzard myosin light chain was a gift of Dr. Robert Adelstein of the National Institutes of Health. Affinity-purified rabbit anti-calcineurin was a gift of Dr. Claude Klee of the National Institutes of Health. Calmodulin-Sepharose was prepared by the method of March *et al.* (43). Simonsen albino rats (140–160 g males) were purchased from Simonsen Laboratories.

Assay for Calmodulin-dependent Synapsin I Kinase Activity—Calmodulin-dependent synapsin I kinase was assayed, with minor modifications, as previously described (26, 35) at 30 °C in a reaction mixture (final volume, 100 μl) containing 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 μg of calmodulin, 50 μM [γ-^{32}P]ATP (0.5–2 × 10^3 cpm/pmol), varying amounts of enzyme, either 0.4 mM EGTA (minus calcium) or 0.4 mM EGTA/0.7 mM $CaCl_2$ (plus calcium), and either no synapsin I, or 10 μg of synapsin I. After preincubation for 1 min, the reaction was initiated by the addition of [γ-^{32}P]ATP, and terminated after 30 s by the addition of 50 μl of a "stop solution" containing 0.3 M EDTA and 2 mg/ml of bovine serum albumin (as a carrier) followed immediately by 1 ml of ice-cold 10% trichloroacetic acid. After 10 min at 4 °C, the reaction mixture was centrifuged in a Beckman microfuge B for 2 min, and the supernatant was removed by aspiration. The pellet was redissolved in 100 μl of 0.1 N NaOH and immediately reprecipitated with 1 ml of 10% trichloroacetic acid. After dissolving the final pellet in 100 μl of 0.1 N NaOH, 1 ml of Aquasol (New England Nuclear) was added and the radioactivity was quantitated by liquid scintillation spectrometry. Phosphorylation of synapsin I by the calmodulin-dependent kinase was measured as the difference between calcium-stimulated incorporation of ^{32}P into trichloroacetic acid-insoluble material in the absence and presence of synapsin I. In the later steps of the purification (after DEAE-cellulose chromatography), phosphorylation of synapsin I in the absence of calcium was minimal, and incorporation of ^{32}P into trichloroacetic acid-insoluble material was measured only in the presence of calcium. A previous report presented evidence that brain homogenates contained two calmodulin-dependent synapsin I kinase activities that recognized different sites in the synapsin I molecule. The kinase activities were named "30-kDa region" kinase and "10-kDa region" kinase, after the *Staphylococcus aureus* V8 protease peptides that contained the respective sites (26). In the present report, phosphorylation of synapsin I was measured at pH 8, rather than at pH 7. At this pH, 95% or more of the radioactive phosphate incorporated into synapsin I in the presence of calcium was present in the site recovered in the 30-kDa fragment, even in crude brain homogenates. Thus, this assay condition eliminated the need to subtract phosphorylation by the 10-kDa region kinase.

Phosphorylation of the Kinase Subunits—Incorporation of ^{32}P into the kinase subunits was measured under the conditions used to measure synapsin I kinase activity except that synapsin I was omitted from the assay, [γ-^{32}P]ATP was used at a lower concentration (10 μM) and higher specific activity (2–4 × 10^4 cpm/pmol), and the reaction was terminated after varying lengths of time by the addition of 50 μl of a stop solution containing 9% SDS, 6% (v/v) 2-mercaptoethanol, 15% (w/v) glycerol, 0.186 M Tris-HCl (pH 6.7) and a trace of bromphenol blue. The solution was then boiled for 2 min and 120 μl was subjected to SDS/PAGE. The gel was dried and the ^{32}P-labeled proteins were localized by autoradiography (26). When quantitation was necessary, the labeled bands were cut out of the dried gel, placed in liquid scintillation fluid, and subjected to liquid scintillation spectrometry.

Substrate Specificity—Incorporation of phosphate into various substrate proteins was measured under standard assay conditions using 60 ng of purified kinase and varying amounts of possible substrate proteins. The reaction was terminated after 30 s by addition of 50 μl of SDS stop solution. The samples were boiled for 2 min and subjected to SDS/PAGE. After the gels were stained and dried, ^{32}P-labeled proteins were located by autoradiography. For quantitation, labeled bands were cut out of the dried gel, placed in liquid scintillation fluid and subjected to liquid scintillation spectrometry.

Immunoprecipitation of Kinase Activity—Hybridomas that secrete monoclonal antibodies specific for the synapsin I kinase were selected from cells formed by fusion of NS1/SP2 myeloma cells with spleen cells from mice that had been immunized with kinase purified through the calmodulin-Sepharose step. Details of the preparation and selection of these hybridomas, including the one used in this study (VIE9, anti-kinase) will be published separately.[2] For immunoprecipitation experiments, VIE9 and a control hybridoma (VF3, anti-sodium channel, kindly supplied by Dr. Larry Fritz of Caltech) were grown to 2 × 10^5 cells/ml in RPMI 1640 medium supplemented with 20% calf serum, 2 mM glutamine, 1 mM pyruvate, 0.5 mM oxaloacetic acid, and 1 mg/ml of penicillin-streptomycin. The cells were harvested by centrifugation and washed once in an equal volume of the same medium without calf serum and with 5 μg/ml of insulin, 0.1 mg/ml of transferrin, 20 μM ethanolamine, and 0.4 μg/ml of H_2SeO_3 (selenium) (44). They were then resuspended in the supplemented serum-free medium and kept for 24 h at 37 °C in a CO_2-controlled incubator. Cells were removed by centrifugation from the medium which contained secreted antibodies. The antibodies were concentrated 100-fold by precipitation with 50% ammonium sulfate, and dialyzed against 40 mM Tris-HCl (pH 7.5).

For precipitation experiments, purified synapsin I kinase (15 μg) was added to 40 μg of either VIE9 anti-kinase antibody, control monoclonal antibody, or 40 mM Tris-HCl (pH 7.5) in a volume of 0.1 ml and incubated overnight on ice. The resulting immune complexes were pelleted by centrifugation in a Beckman microfuge B for 2.5 min and 10-μl aliquots of the supernatants were assayed for synapsin I kinase activity. The protein in the remaining supernatants was concentrated by precipitation with 10% trichloroacetic acid, dissolved in 1/3 diluted SDS stop solution, and adjusted to neutral pH with NaOH. Pellets containing the immune complexes were washed twice with phosphate-buffered saline, dissolved in 1/3 diluted SDS stop solution, and, together with the supernatants, subjected to SDS/PAGE.

Densitometric Scans of SDS-Polyacrylamide Gels—The purified kinase was subjected to SDS/PAGE and protein bands were stained with either Coomassie blue (0.075% in 25% isopropanol, 10% acetic acid) or fast green (0.5% in 50% methanol, 10% acetic acid) (45).

[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

[2] N. E. Erondu, M. B. Kennedy, V. Krieger, and C. B. Shapiro, manuscript in preparation.

Lanes were cut from the gels and scanned with a Beckman ACTA III recording spectrophotometer at 560 nm for Coomassie blue and 635 nm for fast green. Peak areas were quantitated using a Tektronix 4052 minicomputer interfaced to a Tektronix 4956 digitizing tablet. The areas of the major peaks were found to be linearly proportional to the amount of protein loaded on the gel over a range of 7.5–30 μg with the fast green stain. The linear range was more variable with the Coomassie blue stain, but was usually from 2–10 μg.

Immunoblots—Proteins from SDS gels were transferred onto nitrocellulose paper as described by Towbin *et al.* (46) in a Bio-Rad Trans-Blot Cell. After transfer, the nitrocellulose sheets were incubated with: 1) Buffer D (50 mM Tris (pH 7.4), 0.9% NaCl, 0.1% NaN_3) containing 5% hemoglobin (2 h); 2) the appropriate antibody diluted into Buffer D containing 1% hemoglobin (8–12 h); 3) wash buffer (Buffer D containing 0.5% hemoglobin) (1 h with 3 changes); 4) affinity-purified rabbit anti-mouse IgG diluted 1/500 into wash buffer (2 h); 5) wash buffer (1 h with 3 changes); 6) ^{125}I protein A ($2–3 \times 10^5$ cpm/ml) in wash buffer (2 h); 7) wash buffer (1 h with 3 changes). The nitrocellulose sheet was then dried, and bands containing ^{125}I were detected by autoradiography.

Other Procedures—Polyacrylamide gel electrophoresis in the presence of SDS was performed by the method of Laemmli (47). The stacking gel (2.5 × 16 cm × 1.5 mm) contained 3.5% acrylamide/0.09% bisacrylamide and the running gel (14 × 16 cm × 1.5 mm) contained 10% acrylamide/0.27% bisacrylamide. Molecular mass standards used in SDS/PAGE were: microtubule-associated protein$_2$, 300 kDa; phosphorylase *b*, 94 kDa; bovine serum albumin, 68 kDa; α-tubulin, 56 kDa; β-tubulin, 54 kDa; ovalbumin, 43 kDa; alcohol dehydrogenase, 41 kDa; carbonic anhydrase, 29 kDa; chymotrypsinogen, 25 kDa; soybean trypsin inhibitor, 21.5 kDa. The sedimentation coefficient ($s_{20,w}$) of the kinase was determined by sucrose density gradient centrifugation as described by Martin and Ames (48) with ovalbumin ($s_{20,w}$ = 3.5 S), fibrinogen ($s_{20,w}$ = 7.9 S), catalase ($s_{20,w}$ = 11.3 S) and thyroglobulin ($s_{20,w}$ = 19.2 S) as standards. The Stokes radius (Å) of the kinase was determined by gel filtration on Sepharose 4B using the analysis described by Porath (49). Protein standards used for calibration of the gel filtration column were: catalase, Stokes radius = 52 Å; ferritin, Stokes radius = 61 Å; thyroglobulin, Stokes radius = 85 Å; fibrinogen, Stokes radius = 107 Å; myosin, Stokes radius = 200 Å. Protein was determined by a modification of the method of Lowry *et al.* (50) with bovine serum albumin as a standard. Iodinated tryptic peptide maps were prepared by the method of Elder *et al.* (51). Calmodulin-stimulated phosphodiesterase was assayed as described by Rangel-Aldao *et al.* (52).

RESULTS

Purification of Calmodulin-dependent Synapsin I Kinase

The initial steps of the purification (through calmodulin affinity chromatography) were as previously described by Kennedy *et al.* (35) with modifications. In that study it was shown that synapsin I kinase is present in both soluble and particulate fractions of brain homogenates and that kinases partially-purified from each of the two fractions are indistinguishable by several criteria. For practical reasons, we have used as our enzyme source, the soluble fraction alone. Under the conditions described, it contains about 60% of the total synapsin I kinase activity. All purification steps were carried out at 0–4 °C.

Preparation of Crude Extract—Brains (1.4 g each) were removed from 90 rats and homogenized immediately by 12 up-and-down strokes with a Teflon/glass homogenizer at 900 rpm in 10 volumes of Buffer A (20 mM Tris (pH 7.5), 1 mM imidazole, 0.1 mM $CaCl_2$, 1 mM dithiothreitol, 0.1 mM PMSF) containing 1 mM $MgCl_2$, 25 mg/liter of soybean trypsin inhibitor and 1 mg/liter of leupeptin. The homogenate was centrifuged at 10,000 × *g* for 20 min and the resulting supernatant was centrifuged at 170,000 × *g* for 1 h.

DEAE-cellulose Chromatography—The 170,000 × *g* supernatant was brought to 1 mM iodoacetate, adjusted to pH 7.5, and loaded onto a DEAE-cellulose column (5 × 17 cm) previously equilibrated with Buffer A. The column was washed with one column volume of 0.05 M NaCl in Buffer A and was then developed with a 2-liter linear gradient of 0.05–0.30 M NaCl in Buffer A.

Ammonium Sulfate Fractionation—The DEAE column fractions containing synapsin I kinase activity were pooled, adjusted to 0.1 M Tris (pH 7.5), 1 mM dithiothreitol, 0.1 mM PMSF, and brought to 40% saturation by the addition of solid ammonium sulfate. After 3–12 h, precipitated protein was collected by centrifugation and redissolved in a small volume of Buffer B (40 mM Tris (pH 7.5), 0.2 mM $CaCl_2$, 1 mM dithiothreitol, 0.1 mM PMSF) containing 0.2 M NaCl.

Calmodulin Affinity Chromatography—The redissolved ammonium sulfate precipitate was cleared by centrifugation for 10 min at 10,000 × *g*, then applied to a calmodulin-Sepharose affinity column (1.5 × 7 cm) equilibrated with Buffer B containing 0.2 M NaCl. The column was then washed overnight with Buffer B containing 2 M NaCl (~120 ml), followed by one column volume of Buffer B. Synapsin I kinase was eluted with 40 mM Tris (pH 7.5), 2 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF. Recovery of enzyme activity from the column was 50–60% with a 13-fold purification.

Sepharose 4B Gel Filtration—The calmodulin-Sepharose column fractions containing synapsin I kinase activity were pooled and adjusted to 40% ammonium sulfate as described above. The precipitate was redissolved in a small volume of Buffer C (40 mM Tris (pH 7.5), 2 mM EGTA, 0.2 M NaCl, 1 mM dithiothreitol) containing 20% (v/v) glycerol and loaded on a Sepharose 4B column (0.75 × 120 cm) equilibrated with the same buffer. The column was developed at a flow rate of 1.8 ml/h. The elution profile obtained is shown in Fig. 1. It contains a single peak of kinase activity that coincides with the major protein peak. Analysis of the peak fractions by SDS/PAGE indicated that a major 50 kDa protein and two bands in the 60-kDa region (58 and 60 kDa) co-migrated with the peak of kinase activity (data not shown). This step resulted in a 2-fold purification with 40–50% recovery of activity.

Sucrose Density Gradient Centrifugation—The peak Sepharose 4B fractions were pooled and protein was concentrated by precipitation with 40% ammonium sulfate. The precipitate was redissolved in a small volume of Buffer C and loaded onto two 12-ml linear gradients of 5–20% sucrose in Buffer C. The gradients were subjected to centrifugation at 40,000 rpm for 12 h in a Beckman SW 40 Ti rotor. They were then fractionated with an Isco Model 184 density gradient fractionator. As shown in Fig. 2, the gradients contained a single major peak of both synapsin I kinase activity and protein. The minor peak closer to the bottom of the tube may represent an enzyme dimer. The peak fractions were pooled and concentrated by calmodulin affinity chromatography (to remove sucrose), followed by ammonium sulfate precipitation. The ammonium sulfate precipitate was dissolved in a small volume of Buffer C so that the protein concentration was 1–2 mg/ml. After this step, the kinase showed variable stability when stored at 0 °C, but could be stored at −80 °C for long periods with virtually no loss of activity.

The purification procedure is summarized in Table I. The concentrated sucrose pool represented a 290-fold purification over the homogenate with 1.2% recovery of activity from the homogenate and 2.9% recovery from the 170,000 × *g* supernatant. After the final step, 90–95% of the protein present was associated with peptides that co-migrated with kinase activity throughout the purification and were co-precipitated with kinase activity by an anti-kinase monoclonal antibody (see below).

One reason for the rather low recovery was a variable and unusually poor recovery after DEAE-cellulose chromatogra-

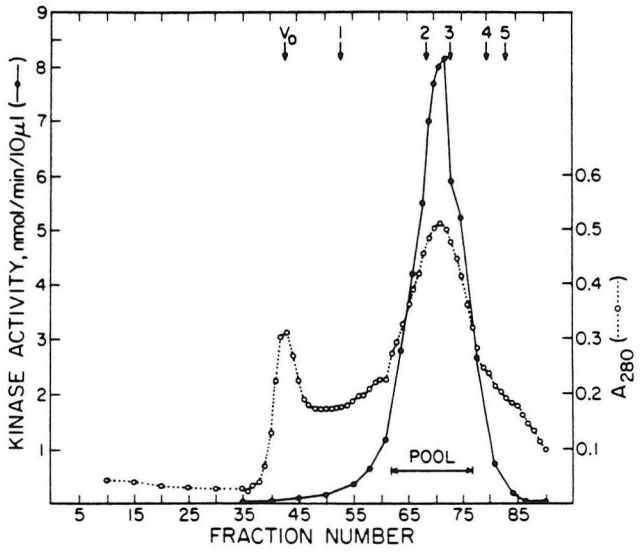

FIG. 1. **Sepharose 4B elution profile (purification step 7).** Gel filtration on Sepharose 4B was carried out as described in the text. Fractions of 0.6 ml were collected and assayed for synapsin I kinase activity. Aliquots of each fraction were diluted as required to obtain a linear rate of synapsin I phosphorylation in a 30-s assay. The elution positions of standard proteins were determined in separate runs by absorbance at 280 nm and are indicated by *arrows*. Standard proteins were: 1) myosin (Stokes radius = 200 Å); 2) fibrinogen (107 Å); 3) thyroglobulin (85 Å); 4) ferritin (61 Å); and 5) catalase (52 Å).

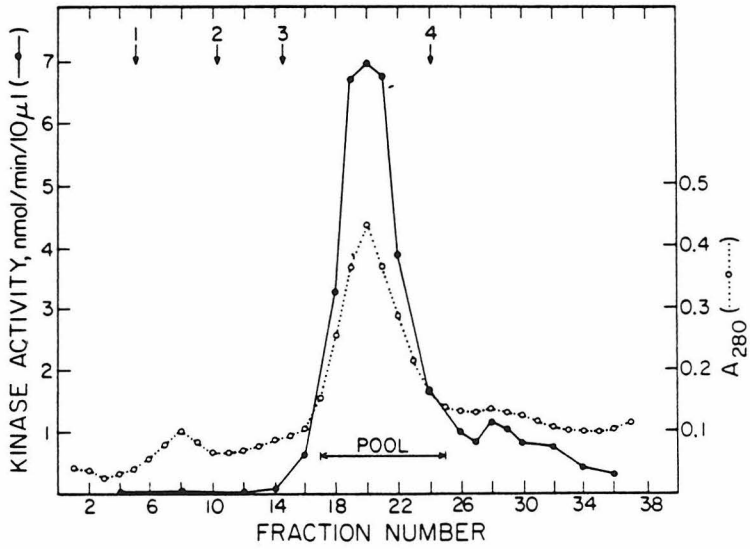

FIG. 2. **Sucrose density gradient sedimentation profile (purification step 8).** Sucrose density gradient centrifugation was carried out as described in the text. Fractions of 0.33 ml were collected (starting from the *top* of the gradient) and assayed for synapsin I kinase activity. Aliquots of fractions were diluted for assay as described in the legend of Fig. 1. The positions of standard proteins were determined on parallel gradients by absorbance at 280 nm and are indicated by *arrows*. Standard proteins were: 1) ovalbumin ($s_{20,w}$ = 3.5 S); 2) fibrinogen (7.9 S); 3) catalase (11.3 S); and 4) thyroglobulin (19.2 S).

TABLE I

Summary of purification of rat brain calmodulin-dependent synapsin I kinase

Step	Total activity[a]	Total protein[a]	*n*	Specific activity	Purification	Recovery
	μmol/min	*mg*		*μmol/min/mg*	*-fold*	%
1. Homogenate	132 ± 27	13,100 ± 1,900	9	0.010	1	100
2. 10,000 × *g* Supernatant	82.7 ± 23	3,660 ± 755	9	0.023	2.3	63
3. 170,000 × *g* Supernatant	55.8 ± 16	3,010 ± 747	9	0.019	1.9	42
4. DEAE-cellulose eluate	17.4 ± 6.7	725 ± 84	7	0.024	2.4	13
5. 40% Ammonium sulfate	17.4 ± 5.4	225 ± 45	7	0.077	7.7	13
6. Calmodulin-Sepharose eluate	9.5 ± 3.1	9.6 ± 3.1	7	0.99	99	7.2
7. Sepharose 4B eluate	3.8 ± 0.8	2.1 ± 0.5	5	1.81	181	2.9
8. Sucrose gradient pool	1.6 ± 0.7	0.55 ± 0.2	2	2.90	290	1.2

[a] Values shown are mean ± S.D. of *n* experiments.

phy. In large scale preparations, recovery at this step has varied from 18 to 40%. These recoveries are lower than those first reported for smaller scale preparations (35). This may be due to denaturation of the enzyme with time on the column. We have tried recombining the enzyme fraction with other pooled column fractions and with proteins stripped from the column by 2 M salt and have seen no stimulation of activity. Thus, we have no evidence that we have lost a factor required for maximal activity. Substitution of other DEAE-resins has not improved the recovery. Recoveries for subsequent purifi-

cation steps, as reported in Table I, include small losses during concentration of pooled fractions. Recoveries during the chromatography and centrifugation steps themselves were generally 50% or greater. In recent preparations, we have eliminated the calmodulin-Sepharose concentration step following sucrose density gradient centrifugation, and this has improved recovery.

Structure and Properties of the Calmodulin-dependent Synapsin I Kinase

Identification of the Kinase Subunits—The peptide composition of the enzyme fractions at each stage of the purification is shown in Fig. 3. The purified kinase fraction contained a major peptide band at 50 kDa, a less prominent band at 60 kDa, and minor bands at 58 and 45 kDa. Two lines of evidence indicate that the 50-, 58-, and 60-kDa peptides (termed α, β', and β respectively) are associated with enzyme activity and are present in a holoenzyme complex.

The amount of these three proteins peaked in the same fractions as kinase activity during gel filtration and sucrose density gradient centrifugation. An example is shown in Fig. 4. In this experiment, the purified kinase was subjected to analytical sucrose density gradient centrifugation. Fractions were assayed for synapsin I kinase activity and subjected to SDS/PAGE. The gels were stained with Coomassie blue and the amount of protein in each band was determined from the area of the optical density peaks on densitometric scans. Although the β and β'-bands were separated on the gel, the optical density peaks on the scans were not well resolved (see Fig. 6). The ratio of the β' "shoulder" to the β peak was constant, so that β and β'-bands were grouped together and treated as one "band" for this analysis. They will be referred to as the β/β'-band. As shown in Fig. 4, both the α and β/β'-bands peak in the same fraction as enzyme activity, and both of the peaks are asymmetric, tailing toward the bottom of the gradient. The exact ratio of the α to β/β'-bands across the gradient was slightly variable, but this probably reflects variability in the staining with Coomassie blue (see below), rather than true differences in the ratio of the bands.

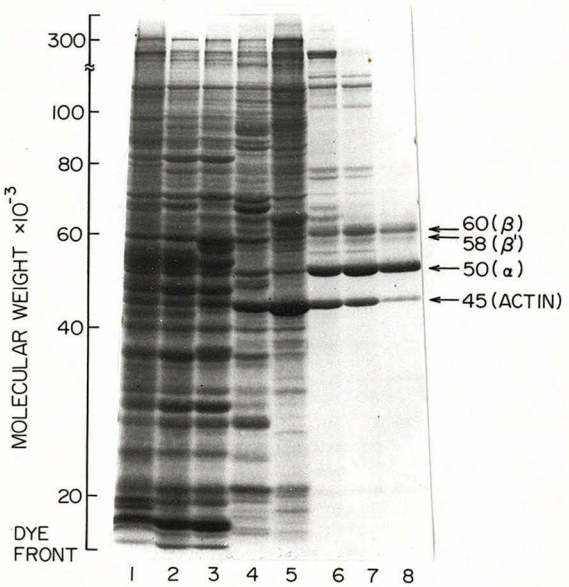

FIG. 3. **Purification of synapsin I kinase as monitored by SDS/PAGE.** Samples from each step of the purification were subjected to SDS/PAGE and stained with Coomassie blue. The purification step and amount of protein corresponding to each *lane* are as follows: *lane 1*, homogenate, 100 μg; *lane 2*, 10,000 × *g* supernatant, 100 μg; *lane 3*, 170,000 × *g* supernatant, 100 μg; *lane 4*, DEAE-cellulose eluate, 75 μg; *lane 5*, 40% ammonium sulfate precipitate, 75 μg; *lane 6*, calmodulin-Sepharose eluate, 15 μg; *lane 7*, Sepharose 4B eluate, 10 μg; *lane 8*, sucrose gradient pool, 7.5 μg.

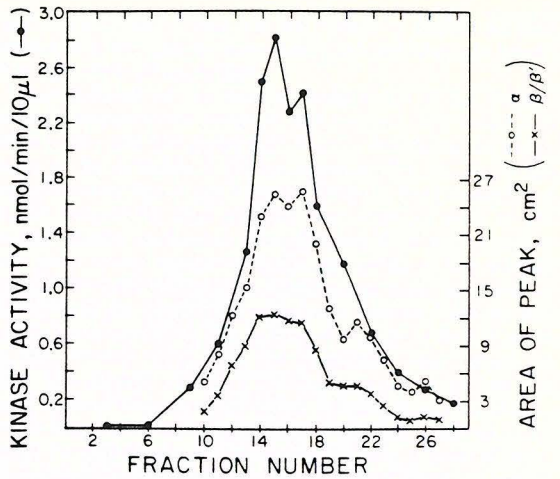

FIG. 4. **Co-migration of α and β/β' peptides with kinase activity during sucrose density gradient centrifugation.** A portion of the concentrated sucrose pool from purification step 8 (600 μg) was loaded on a 4.8-ml linear gradient of 5–20% sucrose in Buffer C. The gradient was subjected to centrifugation at 65,000 rpm for 4 h in a Beckman SW 65 Ti rotor. Fractions of 0.18 ml were collected and an aliquot of each was diluted and assayed for kinase activity as described in the legend of Fig. 1. A 75-μl aliquot of each fraction was subjected to SDS/PAGE. The gel was stained with Coomassie blue and the amount of α and β/β' peptide in each fraction was quantitated by densitometric scans.

Further evidence that these bands are indeed subunits of the kinase comes from the use of a monoclonal antibody raised against the partially purified kinase. Incubation of the kinase with an appropriate amount of this antibody, as described under "Experimental Procedures," resulted in the precipitation of 92–94% of the enzyme activity along with the α, β, and β' protein bands (Fig. 5). These same three proteins were also specifically precipitated from less pure enzyme fractions that contained several other proteins (data not shown). Incubation without antibody or with a control monoclonal antibody did not precipitate the enzyme activity or the protein bands. The results of the sucrose gradient experiment (Fig. 4) and the immunoprecipitation experiment (Fig. 5) indicate that the α, β, and β' proteins are contained in a complex that has calmodulin-dependent protein kinase activity.

The α and β enzyme subunits appear to be distinct peptides by two criteria. First, phosphopeptide maps of the phosphorylated forms of the two subunits are different and do not support the notion that, for example, the α-subunit was generated from the β-subunit by proteolysis (35). Second, two monoclonal antibodies that react with the α-subunit on immunoblots, do not react with the β or β'-subunits.[2] On the other hand, β' may have been generated from β by proteolysis, since phosphopeptide maps of these two subunits are consistent with this possibility (35). Further structural studies will be necessary to clarify the relationship of the two β-subunits.

The 45-kDa protein band contained in the purified kinase has been identified as actin by the following criteria (data not shown): 1) It co-migrated with skeletal muscle actin during

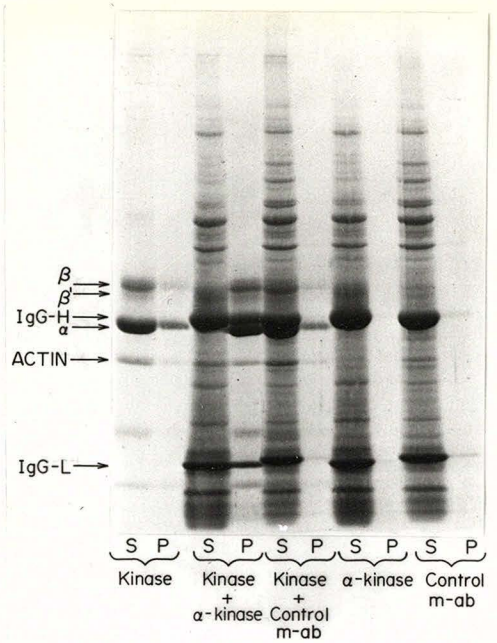

FIG. 5. **Immunoprecipitation of kinase activity.** Immunoprecipitation of kinase activity was carried out as described under "Experimental Procedures." The protein staining patterns of the supernatant (*S*) and pellet (*P*) from incubations containing kinase alone, kinase plus either anti-kinase antibody (*α-kinase*) or control monoclonal antibody (*m-ab*), and the two antibodies alone are shown. Kinase activity remaining in the supernatant after incubation with α-kinase antibody was 7.8% of the activity in the kinase control and 5.6% of the activity remaining in the supernatant after incubation with the control antibody. The lightly staining, low molecular weight bands seen in the kinase supernatant and in the immunoprecipitate are breakdown products which gradually appear after prolonged storage. These breakdown products are recognized by the α-kinase antibody on immunoblots and are precipitated with the enzyme activity (*kinase + α-kinase* pellet). *IgG-H* and *IgG-L* refer to the heavy and light chains of mouse IgG, respectively.

SDS/PAGE. 2) Radioiodination of the 45-kDa and skeletal muscle actin bands followed by digestion with trypsin produced essentially identical iodinated tryptic peptides. 3) A monoclonal antibody that recognizes only the 45-kDa protein on immunoblots, also recognized skeletal muscle actin. The amount of actin in the purified enzyme fractions has varied from 2 to 5% as measured by densitometry of stained gels (see Fig. 6). It appeared to be in the form of heterogeneous F-actin since it was distributed uniformly throughout both the gel filtration and sucrose gradient fractions. Because the amount of actin present was variable, and decreased in proportion to protein kinase with each purification step (see Fig. 1), we have concluded that it is a persistent contaminant and is not a stoichiometric part of the holoenzyme complex. However, we cannot rule out that the kinase has a specific but low affinity actin-binding site that results in the co-purification of actin. It is of interest that a portion of the actin was precipitated with enzyme activity by the anti-kinase monoclonal antibody (Fig. 5). This may have been the result of a specific interaction between actin and the enzyme, or could simply have been caused by trapping of F-actin in the antibody-enzyme matrix.

Two other low molecular weight protein bands appear slowly in the purified kinase when it is stored at 0 °C. These can be seen in Fig. 5, one slightly above and one below the light chain of IgG. These are not present in freshly purified kinase (Fig. 6), thus we have concluded that they are breakdown products of the kinase.

Determination of the Subunit Composition of the Holoenzyme—Densitometric scans of gels stained with both Coomassie blue and fast green were used to determine the molar ratio of the synapsin I kinase subunits (Fig. 6). As in the experiment of Fig. 4, the β and β'-subunits were treated as a single subunit for this analysis. The areas of each of the optical density peaks were normalized by dividing the area of the peak by the apparent molecular weight of the protein band. The molar ratio of the subunits was then calculated as the ratio of the normalized peak areas. Results from the fast green-stained gel (Fig. 6*A*) indicated a molar α to β/β' ratio of 3:1, whereas those from the Coomassie blue-stained gel (Fig. 6*B*) showed a ratio of 5:1. Coomassie blue-stained gels have shown variable subunit ratios (ranging from 2.5:1 to 5:1), while fast green-stained gels have consistently shown a 3:1 ratio. For this reason, we believe the 3:1 ratio is more reliable. Determination of the exact subunit ratio will await the results of protein sequencing experiments.

The approximate molecular weight of the kinase holoenzyme was determined from its hydrodynamic properties. The Stokes radius of the enzyme, as determined by gel filtration on Sepharose 4B (Fig. 1), was 95 Å, and the sedimentation coefficient ($s_{20,w}$), as determined by sucrose density gradient centrifugation (Fig. 2), was 16.4 S. From these values, a molecular weight of 650,000 and a frictional ratio of 1.67 was calculated for the kinase holoenzyme as described in Table II.

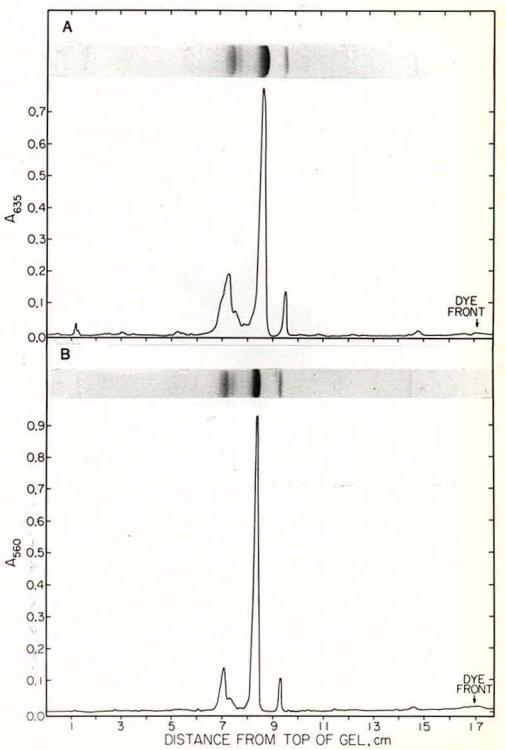

FIG. 6. **Densitometric scans of fast green and Coomassie blue-stained gels of the purified kinase.** *A*, fast green-stained gel (22.5 μg of purified kinase) and densitometric scan at 635 nm. *B*, Coomassie blue-stained gel (7.5 μg, of purified kinase) and densitometric scan at 560 nm.

TABLE II

Physical properties of rat brain calmodulin-dependent protein kinase

Property	Method of determination	Value
Stokes radius (a)[a]	Gel filtration	94.7 ± 1.2 Å
Sedimentation coefficient ($s_{20,w}$)[a]	Sucrose density gradient	16.4 ± 0.7 S
Molecular weight (M_r)[b]	Stokes radius and sedimentation coefficient	650,000
Frictional ratio (f/f_0)[c]	Stokes radius and sedimentation coefficient	1.67
Subunit structure (α:β'/β)	Molecular weight and subunit ratio	9:3 (M_r = 654,000) 10:2 (M_r = 644,000)

[a] Values shown are mean ± S.D. of five separate experiments.

[b] Calculated according to the equation

$$M_r = 6\pi\eta_{20,w} \cdot s_{20,w} \cdot a \cdot N/(1 - \bar{v}\rho_{20,w})$$

where N = Avogadro's number, $\eta_{20,w}$ = viscosity of water at 20 °C, $\rho_{20,w}$ = density of water at 20 °C, and $\bar{v}$ = partial specific volume, for which a value of 0.725 ml/g was assumed (53).

[c] Calculated according to the equation (53)

$$f/f_0 = a(4\pi N/3\bar{v}M_r)^{1/3}$$

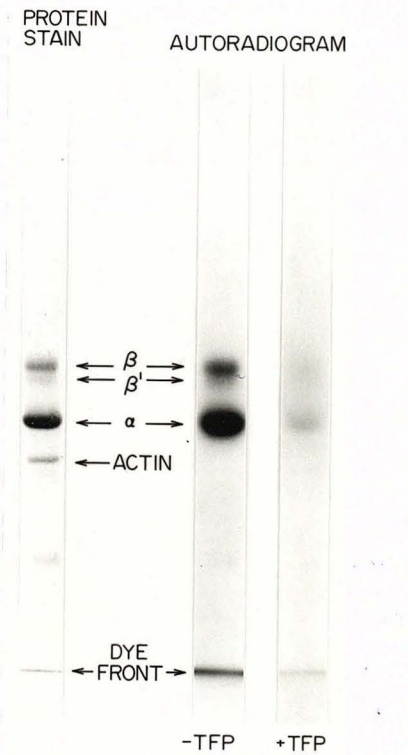

FIG. 7. **^{125}I Calmodulin binding to kinase subunits.** Purified calmodulin was iodinated by the lactoperoxidase method to a specific activity of 3.6×10^5 cpm/μg as described by Carlin *et al.* (54). ^{125}I calmodulin co-migrated with unlabeled calmodulin during SDS/PAGE and was able to activate the purified synapsin I kinase. Purified kinase (6 μg/lane) was subjected to SDS/PAGE. The binding of ^{125}I calmodulin to peptides in the gel was carried out as described by Carlin *et al.* (54) in the absence (−TFP) or presence (+*TFP*) of 1.0 mM trifluoperazine. Following ^{125}I calmodulin binding, the gels were stained with Coomassie blue, and labeled bands were detected by autoradiography.

These numbers indicate that the kinase is a large, somewhat elongated molecule made up of multiple subunits.

From the molecular weight of the enzyme and the ratio of its subunits, we can deduce the subunit structure of the holoenzyme. A subunit ratio (α:β/β') of 3:1 would indicate a holoenzyme consisting of nine α-subunits and three β/β'-subunits, whereas a molar ratio of 5:1 would indicate a holoenzyme consisting of 10 α-subunits and two β/β'-subunits. The physical properties of the kinase are summarized in Table II.

Identification of the Calmodulin-binding Subunits—Calmodulin-binding proteins in the purified kinase were identified by a calmodulin SDS/PAGE overlay technique (54). ^{125}I calmodulin bound specifically to the α, β, and β'-subunits (Fig. 7). The binding was nearly completely inhibited by trifluoperazine. Calmodulin did not bind to actin or to bovine serum albumin included in gels as a control (data not shown). The binding of ^{125}I calmodulin at the dye front may have been due to interaction with small breakdown products of the kinase. The amount of ^{125}I calmodulin bound to the α and β/β'-subunits was determined by γ-emission spectrometry of excised gel pieces. The ratio of ^{125}I bound to the α-subunit to that bound to the β/β'-subunit was 2.9:1. This result is consistent with a 3:1 subunit ratio, if each subunit has one calmodulin binding site/molecule.

Autophosphorylation of the Subunits—Incubation of the purified kinase under standard assay conditions resulted in phosphorylation of both the α- and β-subunits. The time course of this autophosphorylation is shown in Fig. 8. The incorporation of phosphate was both rapid and stoichiometrically significant. After 1 min, each subunit incorporated at least 1 mol of [^{32}P]phosphate/mol of protein. After 10 min, the α-subunit contained 2 mol of phosphate and the β-subunits 3 mol/mol of protein. As phosphorylation proceeded, labeled protein bands with apparent molecular weights of 64,000 and 54,000 gradually appeared. The appearance of

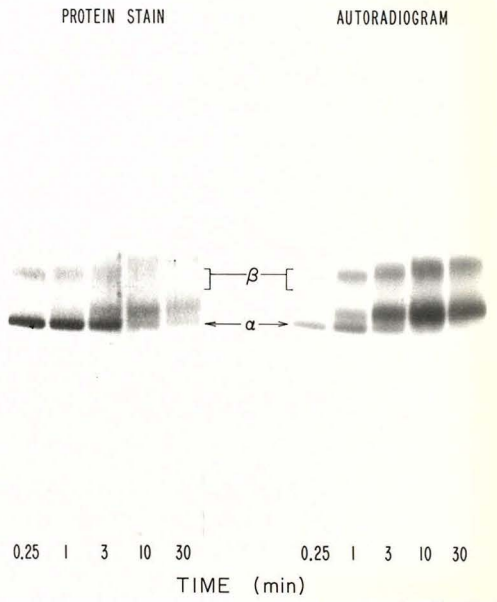

FIG. 8. **Autophosphorylation of kinase subunits.** Purified kinase (3.0 μg) was endogenously phosphorylated as described under "Experimental Procedures" for varying lengths of time. The enzyme subunits were separated by SDS/PAGE and stained with Coomassie blue. ^{32}P incorporation was detected by autoradiography.

TABLE III
Substrate specificity of rat brain calmodulin-dependent kinase

Substrate	Concentration in assay	Rate of calcium-stimulated phosphorylation of substrate
	mg/ml	%
Synapsin I	0.1	100[a]
Microtubule protein[b]	0.4	35
Smooth muscle myosin light chain	0.4	34
Arginine-rich histone[c]	0.1	18
Phosvitin	0.4	15
Casein	0.4	4
Phosphorylase *b*	0.4	0
Glycogen synthase	0.1	0

[a] The rate of calcium-stimulated phosphorylation of synapsin I was 57 pmol/min.
[b] The microtubule protein sample consisted of approximately 95% tubulin and 5% microtubule-associated protein (MAP). Only MAP_2 was phosphorylated.
[c] The arginine-rich histone sample consisted of approximately 30% histone H3, which was the only protein phosphorylated.

these bands was correlated with a decrease in both protein staining and ^{32}P content in the original α- and β-subunit bands. Our interpretation of these findings is that both subunits can be multiply phosphorylated. Phosphorylation of the first sites has no effect on mobility of the subunits on SDS gels, whereas phosphorylation of subsequent sites results in a considerable shift in mobility.

Substrate Specificity—In order to facilitate comparison of the synapsin I kinase to other protein kinases, the ability of the purified kinase to phosphorylate a number of substrates frequently used for assaying protein kinases was examined. As shown in Table III, microtubule protein, smooth muscle myosin light chain, histone H3, and phosvitin were all phosphorylated at a significant rate, whereas casein, phosphorylase *b*, and glycogen synthase were phosphorylated poorly or not at all. The microtubule protein consisted of both tubulin (95%) and microtubule-associated proteins (MAPs, 5%). Only MAP_2 was phosphorylated. The small amount of MAP_2 present in the assay and its relatively high incorporation of phosphate suggests that it is a particularly good substrate for the purified kinase. As a control, each of the substrates was included in an assay with synapsin I to determine if any component of the substrate was inhibiting kinase activity. The glycogen synthase (as purchased) had to be dialyzed against 40 mM Tris (pH 7.5) to remove an inhibitory component before being tested as a substrate. Ten times the usual concentration of calmodulin had to be used in the histone assays.

DISCUSSION

We have reported the purification to near homogeneity of a calcium and calmodulin-dependent protein kinase from rat brain. The kinase is a multisubunit complex with a molecular weight of about 650,000. It contains three subunits; α (50 kDa), β (60 kDa), and β' (58 kDa). The α- and β-subunits are distinct peptides, however β' may have been generated from β by proteolysis. All three of these peptides co-migrate with kinase activity during the purification steps and are co-precipitated with kinase activity by a specific anti-kinase monoclonal antibody. They all can bind calmodulin in the presence of calcium and are autophosphorylated under conditions in which the kinase is active. From the ratio of the subunits, and the holoenzyme molecular weight, we postulate a subunit composition for the kinase of approximately nine α-subunits and three β/β'-subunits.

The recovery of purified synapsin I kinase suggests that it is a relatively abundant brain enzyme constituting approximately 0.3% of the total brain protein. This estimate assumes that the losses of catalytic activity during each purification step were due to losses of enzyme protein rather than to a decrease in the activity of individual enzyme molecules. However, two additional lines of argument suggest that the estimate is essentially correct. First, the specific activity of the purified kinase is as high or higher than that of many other purified protein kinases (36–38, 55, 56). Thus, it seems unlikely that there has been a dramatic decrease in the activity of individual enzyme molecules during the purification. Second, a similarly high concentration of the α-subunit in brain homogenates has been measured by a radioimmunoassay utilizing the anti-kinase monoclonal antibodies.[2]

In an earlier paper (35), we suggested, on the basis of studies carried out with partially purified synapsin I kinase, that three, prominent "substrate" proteins for a calmodulin-dependent protein kinase in brain homogenates might actually be autophosphorylated subunits of the calmodulin-dependent synapsin I kinase itself. The characterization of the subunits of the purified kinase presented in this study confirms that all three of these substrate proteins are in fact part of the synapsin I kinase holoenzyme. Grab *et al.* (34) and Cohen *et al.* (57) have shown that a similar set of three substrate proteins are present in purified cerebral postsynaptic densities, fibrous structures that are located on the cytoplasmic surface of postsynaptic cells, in the region underlying the presynaptic terminal. One of these substrates is a prominent postsynaptic density protein termed the "major 52K PSD protein" by Kelly and Cotman (58). In a separate study, we have shown by biochemical and immunochemical criteria that the α-subunit of the synapsin I kinase is identical to this major 52K PSD protein (70). This suggests that the kinase may be concentrated in postsynaptic densities *in vivo* and thus may be involved in the regulation of postsynaptic as well as presynaptic processes. The presence of the kinase in postsynaptic densities could account for a portion of the synapsin I kinase that is associated with the particulate fraction of brain homogenates (35).

The α- and β-subunits of the kinase each incorporate at least two mol of phosphate/mol of protein in the presence of calcium, calmodulin, magnesium, and ATP. Incorporation of phosphate into the first site does not affect the mobility of the kinase subunits on SDS gels, whereas phosphorylation of additional sites causes a shift in their mobility (Fig. 8). Preliminary experiments have not yet revealed any large effect of this autophosphorylation on the kinetic or structural properties of the purified kinase. Another possible function of the autophosphorylation is the regulation of the association of the kinase with subcellular structures *in vivo*. We have previously noted that the synapsin I kinase is distributed between the soluble and particulate fractions in brain homogenates and that the kinases purified from each of the two fractions appear identical by several criteria (35). Recent experiments in which we examined the autophosphorylation of the kinase subunits in crude soluble and in crude particulate fractions suggested that the soluble form of the kinase, but not the particulate form, may already contain endogenous phosphate at one or more of the autophosphorylatable sites (data not shown). This supports the notion that autophosphorylation may regulate the intracellular location of the kinase.

The calmodulin-dependent synapsin I kinase is different in subunit composition and holoenzyme molecular weight from muscle myosin light chain kinase (23–25) and phosphorylase kinase (22), however, it is similar to two other recently char-

acterized calmodulin-dependent protein kinases (36–38). One of these is a calmodulin-dependent protein kinase from rat brain that phosphorylates smooth muscle myosin light chain and also other endogenous substrates. It was purified by Fukunaga *et al.* (38) and reported to be a 640-kDa holoenzyme composed of 11 to 14 identical 49 kDa subunits. It did not contain the 58- and 60-kDa proteins that we have termed β-subunits. The 49-kDa subunit was autophosphorylated in the presence of calcium, calmodulin, magnesium, and ATP. The recovery of this enzyme suggested that it could constitute as much as 0.1% of the total brain protein. The substrate specificity of this kinase resembles that of the synapsin I kinase. Both enzymes phosphorylate smooth muscle myosin light chain, microtubule-associated protein and arginine-rich histone. However, the kinase of Fukunaga *et al.* (38) phosphorylates casein well and phosvitin poorly, whereas the synapsin I kinase phosphorylates phosvitin at a moderate rate, and does not phosphorylate casein (Table III). Because of the close similarity of this protein kinase to the synapsin I kinase described in this report, we have made a comparison between the results of Fukunaga *et al.* (38) and our own, to determine whether the two enzymes might be related.

The first purification step used by Fukunaga *et al.* (38) was precipitation of protein at pH 6.1. This step left 99% of the calmodulin-dependent myosin light chain kinase activity in the supernatant. In contrast, we have found that precipitation at pH 6.1 leaves only about 10% of the calmodulin-dependent synapsin I kinase activity in the supernatant. The rest is irreversibly inactivated. This difference in recovery could mean either that the two protein kinase activities are properties of distinct molecules or that there is some difference in the methods of assay used by each group. Fukunaga *et al.* (38) used an incubation time of 10 min in their standard assay. We have found that the rate of phosphorylation of synapsin I falls off rapidly after about 30 s. Thus, the total incorporation of phosphate into synapsin I after 10 min is not a reflection of the initial enzymatic rate, and does not accurately measure the amount of enzyme present. In order to see whether the same situation might hold for phosphorylation of myosin light chain in brain homogenates, we determined the recovery of calmodulin-dependent myosin light chain kinase activity after pH 6.1 precipitation using both 30-s and 10-min assays. The assays were performed as described by Fukunaga *et al.* (38) with gizzard myosin light chains as substrate. We found an 80% recovery using a 10-min assay, and a 20–25% recovery using a 30-s assay (data not shown). Thus, it is possible that Fukunaga *et al.* (38) overestimated the recovery of kinase activity during their purification.

The major difference between the purification procedure used by Fukunaga *et al.* (38) and our own was this pH 6.1 precipitation step. Their other steps included chromatography methods similar to our own. We think that it is possible that their protein kinase contains α-subunits identical to those we have described, but has lost the β-subunits, perhaps at the acid precipitation step. Although a molecular weight of 640,000 was estimated for their enzyme from gel filtration data, its hydrodynamic properties indicate that it is actually smaller than the synapsin I kinase described in this report. Its Stokes radius, determined by gel filtration, is 81 Å and its sedimentation coefficient, determined by sucrose density gradient centrifugation, is 15.3 S (38), whereas the comparable values for the synapsin I kinase are 95 Å and 16.4 S. The molecular weight of the Fukunaga kinase, calculated from both its Stokes radius and its sedimentation coefficient as described in Table II, would be ~512,000, 140,000 less than the value of 650,000 calculated for the synapsin I kinase. This apparent difference in size is consistent with the notion that the difference between the two kinases could be the presence or absence of the β-subunits.

The evidence that the β-subunits are a part of the complex that contains synapsin I kinase activity comes from two distinct experiments and is quite strong (see "Results" and previous "Discussion"). However, there have been reports that under some circumstances, protein kinases can form multienzyme complexes with other functionally related molecules (59, 60). We therefore sought to determine whether the β-subunits might be one of two previously described calmodulin-binding proteins with molecular weights of about 60,000, calcineurin A, or calmodulin-dependent cyclic nucleotide phosphodiesterase. Calcineurin is an abundant brain protein thought to have protein phosphatase activity (61). Calcineurin A, its larger, calmodulin-binding subunit, has a molecular weight of 61,000 (62). We obtained polyclonal affinity-purified rabbit anti-calcineurin from Dr. Claude Klee and tested by the immunoblot procedure (see "Methods") for the presence of calcineurin at various stages in the purification of the kinase. Calcineurin was easily detected in the 170,000 × *g* brain supernatant but was not detectable in the final purified kinase preparation (data not shown). Calmodulin-dependent cyclic nucleotide phosphodiesterase is a dimer of two identical calmodulin-binding 59-kDa subunits (63, 64). When purified from brain, it has a specific activity of 160–300 μmol/min/mg and is quite stable. We tested for the presence of this protein at various stages in the purification of the kinase by measuring phosphodiesterase activity according to the method of Rangel-Aldao *et al.* (52). We found a level of calcium-activated phosphodiesterase activity in the 170,000 × *g* supernatant that was consistent with that reported by other laboratories (63, 64). However, the specific activity in the purified kinase was less than 0.008 μmol/min/mg, the limit of detection in this assay (data not shown). Thus, the β-subunits do not appear to be either calcineurin A or calmodulin-dependent phosphodiesterase. It should be emphasized, however, that we do not know the specific functions within the kinase holoenzyme of the α- or the β-subunits. Thus, the possibility remains that one of the subunits may have a function that is not directly involved in catalysis of protein phosphorylation.

The second, recently characterized, calmodulin-dependent protein kinase that resembles synapsin I kinase is glycogen synthase kinase. It is a holoenzyme of about 300,000 Da, composed of roughly equal amounts of autophosphorylatable subunits of 50 and 53 kDa (36, 37). The association of autophosphorylatable, 50–53 kDa subunits into a multimeric holoenzyme is reminiscent of the structure of synapsin I kinase. However, there appear to be significant differences in the substrate specificities of the two enzymes. For example, although both of them phosphorylate smooth muscle myosin light chain (36, 37), synapsin I kinase does not phosphorylate glycogen synthase (see Table III). In addition, casein is a poor substrate for synapsin I kinase (Table III), but a good substrate for glycogen synthase kinase (Ref. 36, but see Ref. 37). Nevertheless, the structural similarities of these two kinases suggest that they may be members of a family of related calmodulin-dependent protein kinases that have evolved from a common precursor.

Although the physiological role of the calmodulin-dependent synapsin I kinase is not yet known, several of its properties suggest that it is involved in the regulation of both pre- and postsynaptic functions. For example, one of its brain substrate proteins, synapsin I, is located primarily in synaptic terminals (65, 66) where it is specifically associated with synaptic vesicles (67). Phosphorylation of synapsin I by the calmodulin-

dependent synapsin I kinase can be triggered both *in vivo* and *in vitro* by depolarization of terminals in the presence of calcium (68, 69). In addition, we have recently shown that the synapsin I kinase itself may be a prominent constituent of certain brain postsynaptic densities based on the identity of one of its subunits with the "major 52K postsynaptic density protein" first described by Kelly and Cotman (58, 70). The molecular characterization of synapsin I kinase presented in this report, and the generation of antibodies that specifically recognize it,[2] will aid in the elucidation of its full range of substrate proteins, and of its distribution in different parts of the nervous system and within individual nerve cells. Such information should be useful in the development of a molecular model for synaptic function and its regulation.

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CHAPTER 2

BIOCHEMICAL AND IMMUNOCHEMICAL EVIDENCE THAT THE "MAJOR POSTSYNAPTIC DENSITY PROTEIN" IS A SUBUNIT OF A CALMODULIN-DEPENDENT PROTEIN KINASE

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Biochemical and immunochemical evidence that the "major postsynaptic density protein" is a subunit of a calmodulin-dependent protein kinase

(synaptic structure/brain phosphoproteins/synaptic regulation)

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ABSTRACT **By three criteria, two biochemical and one immunochemical, the major postsynaptic density protein (mPSDp) is indistinguishable from the 50-kilodalton (kDa) α subunit of a brain calmodulin-dependent protein kinase. First, the two proteins comigrate on NaDodSO$_4$/polyacrylamide gels. Second, iodinated tryptic peptide maps of the two are identical. Finally, a monoclonal antibody (6G9) that was raised against the protein kinase binds on immunoblots to a single 50 kDa band in crude brain homogenates and to both the α subunit of the purified kinase and the mPSDp from postsynaptic density fractions. The purified kinase holoenzyme also contains a 60-kDa subunit termed β. A comparison of the peptide map of β with the maps of 60-kDa proteins from the postsynaptic density fraction suggests that β is present there but is not the only protein present in this molecular weight range. These results indicate that the calmodulin-dependent protein kinase is a major constituent of the postsynaptic density fraction and thus may be a component of type I postsynaptic densities.**

Many synaptic junctions in the central nervous system contain a prominent specialized structure called the "postsynaptic density" (PSD) (1–5). When viewed by electron microscopy, it is a fibrous, electron-opaque thickening lying opposite the presynaptic terminal, on the cytoplasmic side of the postsynaptic membrane. The morphology of postsynaptic densities is variable. Some are thick [20–60 nm (5)] and appear to cover the entire postsynaptic surface area, whereas others are thin, often discontinuous, patches (1, 6). The former have been termed type I PSDs, while the latter are called type II (1). Some investigators have suggested that different types of densities are associated with synapses of particular types. For example, type I PSDs most often occur in synapses that are thought to be excitatory (1, 7, 8), whereas type II PSDs are seen in synapses thought to be inhibitory (9, 10). This hypothesis suggests that morphologically distinct PSDs may also contain distinct proteins that serve specialized functions associated with their particular transmitter type.

In order to understand the structure and function of PSDs, various research groups have developed subcellular fractionation methods to isolate highly enriched preparations of PSD-like material (11–13). These procedures involve osmotic lysis of a subcellular fraction enriched in synaptosomes, followed by purification of junctional membrane complexes and extraction of membrane components by treatment with detergent. The detergent-insoluble residue, purified by density gradient fractionation, consists of electron-opaque, fibrous, disk-shaped structures, 20–60 nm thick and 200–500 nm in diameter (11–15). These structures are similar in appearance and staining characteristics to type I PSDs in intact fixed tissue, and they are not produced by detergent treatment of other subcellular organelles such as mitochondria or myelin (11). Thus, this subcellular fraction is considered to be highly enriched in type I PSDs.

The PSD fraction contains 20–30 proteins (13, 16). Among them are the cytoskeletal proteins tubulin (17, 18), actin (18, 19), and fodrin (20). It has been proposed that they are primarily structural, serving to anchor membrane or cytosolic proteins in the region of the postsynaptic membrane (21). The fraction is enriched in cyclic nucleotide phosphodiesterase activity (22, 23), calmodulin (24), and both cAMP- (25–27) and calmodulin-dependent protein kinase activities (27–29). Immunocytochemical evidence suggests that a calmodulin-dependent protein phosphatase, calcineurin, may also be located in PSDs *in situ* (30, 31). The presence of these regulatory enzymes suggests a role for the PSD in regulation of postsynaptic properties, such as receptor clustering, receptor sensitivity, or gating of ion fluxes. The major component of PSD fractions, making up 10–30% of the total protein, is a 50-kilodalton (kDa) protein of unknown function termed the "major PSD protein" (mPSDp) (18). This protein binds calmodulin (32, 33) and is also a substrate for a calmodulin-dependent protein kinase (28, 32).

We have recently purified and characterized a brain calmodulin-dependent protein kinase (34) that phosphorylates synapsin I (35, 36) as well as other brain proteins (34, 37). We will refer to it as synapsin I kinase to distinguish it from other calmodulin-dependent protein kinases. It is found in both soluble and particulate fractions of brain homogenates (37) and is a relatively abundant enzyme, making up as much as 0.3% of the total brain protein. When purified from the soluble fraction, it is a 650-kDa holoenzyme composed of three subunits with molecular masses of 50, 58, and 60 kDa (termed α, β', and β, respectively) (34). The α and β subunits are distinct peptides; however, β' may have been generated from β by proteolysis. The holoenzyme contains approximately nine α subunits and three β/β' subunits. All of the subunits bind calmodulin and are autophosphorylated under conditions in which the kinase is active. They are labeled with ^{32}P in endogenously phosphorylated brain homogenates (34, 37–39), where they account for a major portion of the endogenous "substrates" for calmodulin-dependent protein kinase in the 50- to 60-kDa region of NaDodSO$_4$/polyacrylamide gels (37).

Proteins of the same molecular weights are phosphorylated by a calmodulin-dependent protein kinase in purified PSD preparations (28, 29). The 52-kDa phosphoprotein is the major PSD protein referred to earlier. The identities of the 58- and 60-kDa phosphoproteins have not been established. These pro-

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Abbreviations: PSD, postsynaptic density; mPSDp, major postsynaptic density protein; kDa, kilodalton(s); INT, *p*-iodonitrotetrazolium violet; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; SM, synaptic membrane.

teins are similar to the phosphorylated proteins observed in crude brain particulate fractions (38). Consequently, Grab *et al.* (28) have suggested that the phosphorylated proteins in this molecular mass range in brain particulate fractions are probably PSD in origin.

The similarities between the 50-, 58-, and 60-kDa proteins phosphorylated in PSD fractions and the subunits of synapsin I kinase suggested that the two sets of proteins might be identical. To test this possibility, we have compared the proteins in PSD preparations with the subunits of synapsin I kinase by immunochemical and biochemical methods. We report here that the mPSDp is indistinguishable by three criteria from the α subunit of calmodulin-dependent synapsin I kinase. In addition, the β subunit of the kinase is present in the PSD fraction, although it is not the only protein present in the 60-kDa molecular mass range.

METHODS

Materials. $Na^{125}I$ (carrier free) was purchased from New England Nuclear. Dithiothreitol, *N,N*-bis(2-hydroxyethyl)glycine (Bicine), *p*-iodonitrotetrazolium violet (INT), sodium phosphate (mono- and dibasic), succinic acid, hemoglobin (bovine type II), mouse IgG, and napthol blue black (amido black) were purchased from Sigma. Trypsin treated with 1-tosylamido-2-phenylethyl chloromethyl ketone was purchased from Worthington, ultrapure sucrose from Schwarz-Mann, sodium *N*-lauroylsarcosinate from ICN, and staphylococcal protein A from Pharmacia. Cellulose-coated thin-layer chromatography plates were purchased from Eastman, and nitrocellulose membranes (BA85, 0.45 μm pore diameter) from Schleicher & Schuell. NS1/SP2 myeloma cells were a gift of Jeremy Brockes. BALB/c ByJ mice were purchased from The Jackson Laboratory, Simonsen albino rats (140- to 160-g males) from Simonsen Laboratories (Gilroy, CA), and New Zealand female rabbits from Lab Pets (Rosemead, CA).

Rabbit anti-mouse IgG antiserum was obtained from rabbits immunized by repeated subcutaneous injections with mouse IgG in Freund's adjuvant and was affinity purified by chromatography on Sepahrose 4B coupled with mouse IgG (40). ^{125}I-Labeled protein A (2–3 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) was prepared by the chloramine-T method (41). Calmodulin-dependent synapsin I kinase was prepared as described by Bennett *et al.* (34).

Preparation of Synaptic Membrane (SM) and PSD Fractions. SM and PSD fractions were prepared from 15 rats by the method of Cotman *et al.* (11). The PSD pellet adheres to glass and plastic; therefore, to maximize recovery of protein, it was resuspended in a small volume of 1% $NaDodSO_4$/2 mM Bicine. The reduction of INT by mitochondria that is used to increase their density produces an insoluble precipitate called formazan that adheres to the SM and PSD fractions. This precipitate interfered with determination of protein by the Lowry method (42). However, we estimate from Coomassie blue-stained gels that we obtained 40–60 mg of SM protein and 200–300 μg of PSD protein.

Selection of Hybridoma 6G9. Hybridomas that secrete monoclonal antibodies specific for synapsin I kinase were selected from a fusion of NS1/SP2 myeloma cells with spleen cells of BALB/c ByJ mice that had been immunized with kinase purified through the calmodulin-Sepharose step (34, 37). Details of the preparation and selection of these hybridomas will be published separately. Hybridoma 6G9 was initially selected by a solid-phase radioimmunoassay similar to that described by Moore *et al.* (43) in which culture supernatants were tested for the presence of antibodies that bound to the crude antigen. 6G9 culture supernatant was then tested for binding to individual peptides by the immunoblot procedure described below. The 6G9 hybridoma was subcloned, then ascites fluid was prepared by injection of $1–3 \times 10^6$ cells intraperitoneally into pristane-primed BALB/c ByJ mice. The antibody was partially purified by precipitation with 50% saturated ammonium sulfate, redissolved to a protein concentration of 20 mg/ml, and dialyzed against 20 mM Tris·HCl, pH 7.0.

Immunoblots. Proteins from $NaDodSO_4$/polyacrylamide gels were transferred onto nitrocellulose paper as described by Towbin *et al.* (44). After transfer, the nitrocellulose sheets were incubated with (*i*) buffer D (50 mM Tris·HCl, pH 7.4/0.9% NaCl/0.1% NaN_3) containing 5% hemoglobin (2 hr); (*ii*) 6G9 partially purified ascites fluid diluted into buffer D containing 1% hemoglobin (8–12 hr); (*iii*) wash buffer (buffer D containing 0.5% hemoglobin) (1 hr with three changes); (*iv*) rabbit anti-mouse IgG (2–7 μg/ml) in wash buffer (2 hr); (*v*) wash buffer (1 hr with three changes); (*vi*) ^{125}I-labeled protein A ($2–3 \times 10^5$ cpm/ml) in wash buffer (2 hr); (*vii*) wash buffer (1 hr with three changes). The nitrocellulose sheet was dried, and bands containing ^{125}I were detected by autoradiography.

Other Procedures. Peptide mapping was performed by the method of Elder *et al.* (45) as modified by Kelly and Cotman (18). Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was performed by the method of Laemmli (46). Stacking gels were 3.5% acrylamide/0.09% bisacrylamide. Running gels were 10% acrylamide/0.27% bisacrylamide. Molecular mass standards were phosphorylase *b*, 94 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa. Crude rat brain homogenate was prepared as described by Bennett *et al.* (34) and subjected to centrifugation at $10,000 \times g$ for 10 min to remove unbroken cells and nuclei.

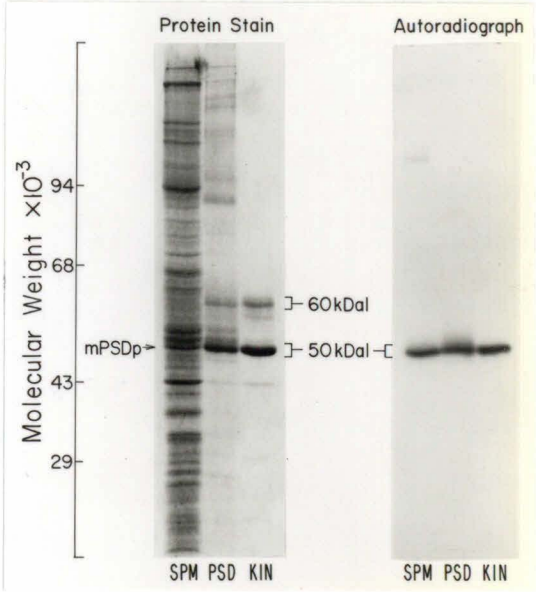

FIG. 1. Immunochemical relationship between the α subunit of synapsin I kinase and the mPSDp. (*Left*) The SM fraction (5 μl, ≈70 μg), the PSD fraction (30 μl, ≈25 μg), and synapsin I kinase (KIN, 6 μg) purified to 95% homogeneity (34) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and stained with Coomassie blue. The position of mPSDp is noted. (*Right*) The SM fraction (5 μl), the PSD fraction (7 μl, ≈6 μg), and purified synapsin I kinase (1 μg) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, transferred to nitrocellulose, and tested for reaction with antibody 6G9 (diluted 1:500).

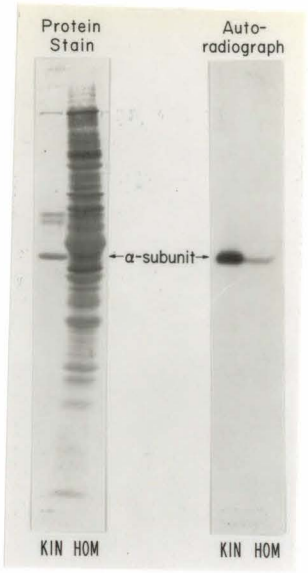

FIG. 2. Binding specificity of antibody 6G9 tested by the immunoblot method. Synapsin I kinase (KIN, 6 μg), partially purified through the calmodulin-Sepharose step (34, 37), and crude rat brain homogenate (HOM, 150 μg) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis in duplicate lanes and transferred to nitrocellulose paper. One pair of lanes (*Left*) was stained with amido black (44). The other pair (*Right*) was tested for reaction with antibody 6G9 (diluted 1:1000) by the immunoblot method. The calmodulin-Sepharose purified kinase is approximately 50% pure. The major band in the 50-kDa region is the α subunit (37). This is the preparation that was originally used as antigen for the generation of antibody 6G9.

RESULTS

The SM and PSD fractions prepared as described in *Methods* had protein compositions (Fig. 1 *Left*) similar to those published by Kelly and Cotman (16). The protein previously defined as mPSDp migrated with the α subunit of purified synapsin I kinase (marked 50 kDa).

For immunochemical comparison of the α subunit and the mPSDp, we used a monoclonal antibody (6G9) that binds with high affinity to the α subunit of the kinase on immunoblots (Figs. 1 and 2) and recognizes a single protein band of the same molecular weight in crude brain homogenates (Fig. 2). Proteins from the SM and PSD fractions were tested for binding to 6G9 as described for Fig. 1 *Right*. The results indicated that the epitope recognized by 6G9 is contained in the mPSDp as well as the α subunit of the kinase. Since 6G9 appears to react with only one protein band even in crude brain homogenates, its binding to both the α subunit and the mPSDp is strong evidence that the two are chemically related.

Additional and independent evidence that the two proteins are closely related was obtained by comparison of their iodinated tryptic peptide maps. Gel pieces containing the two proteins were cut from gels similar to those pictured in Fig. 1. The proteins were iodinated within the gel and digested with trypsin as described (18). Maps of the resulting iodinated peptides were indistinguishable (Fig. 3). In two separate experiments, both major and minor peptides were identical. The maps resemble those published by Kelly and Cotman of the mPSDp cut from $NaDodSO_4$ gels of the PSD fraction (18). This experiment provides further evidence that the mPSDp and the α subunit of the kinase are closely related and suggests that they may be identical.

The PSD fraction contained protein bands in the 60-kDa region that migrated near the position of the β subunit of the purified synapsin I kinase (Fig. 1, marked 60-kDa). We do not yet have a monoclonal antibody that reacts with the β subunits of the kinase on immunoblots, so we were unable to make an immunochemical comparison of the β subunits and the 60-kDa proteins of the PSD fraction. However, we have compared the two by peptide mapping. The regions marked by the 60-kDa bracket in Fig. 1 were cut from gels of the PSD fraction and the purified kinase. Iodinated tryptic peptide maps of the proteins are shown in Fig. 4. The eight major peptides in maps of the β subunit (marked with arrows) appeared to be prominent in maps of the 60-kDa proteins from the PSD fraction. Thus, a protein related to the β subunit is apparently present in the PSD fraction, but it is not the only protein present in this molecular mass range.

DISCUSSION

We have shown that the protein termed the major PSD protein (18) has several similarities to the α subunit of a recently purified brain calmodulin-dependent protein kinase, synapsin I

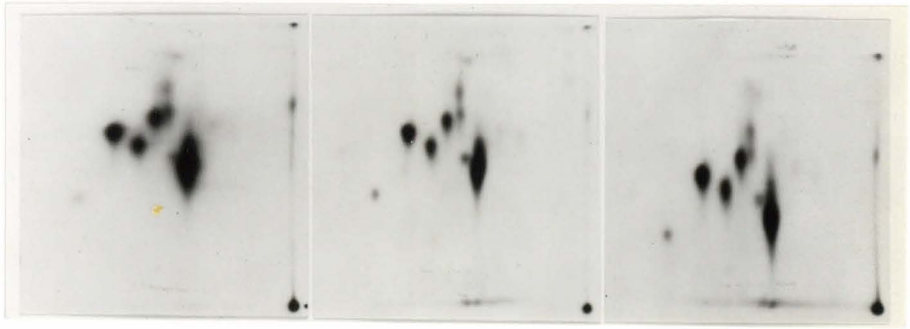

FIG. 3. Iodinated tryptic peptides of the α subunit of synapsin I kinase and the mPSDp. The PSD fraction (30 μl, ≈25 μg) and purified synapsin I kinase (6 μg) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. The bands marked 50 kDa in Fig. 1 were cut from the gel. Iodinated tryptic peptide maps of each were prepared as described (18). Two microliters of peptide solution was applied to the first two plates; 1.5 μl of each was applied to the third. The origin was at the lower right. The anode was to the right and the cathode to the left; chromatography was from bottom to top.

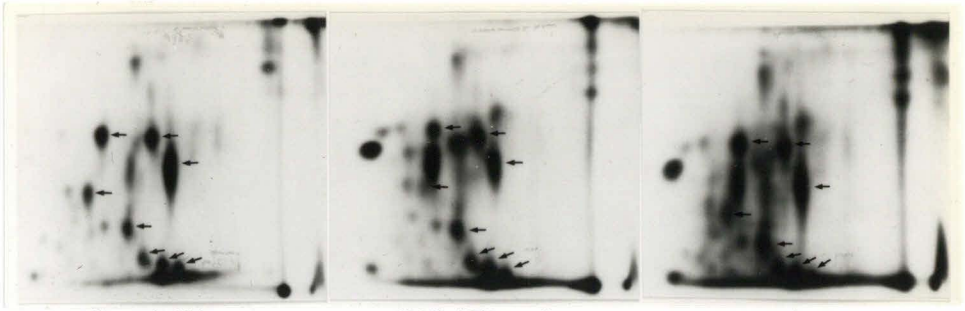

FIG. 4. Iodinated tryptic peptides of the β subunit of synapsin I kinase and 60-kDa proteins from the PSD fraction. The bands marked 60-kDa in Fig. 1 were cut from the same gel used in the experiment of Fig. 3. Iodinated tryptic peptide maps were prepared from each; 2 μl of peptide solution was applied to the plates. Electrophoresis and chromatography were as in Fig. 3.

kinase (34). They have the same mobility on $NaDodSO_4$/polyacrylamide gels. They both bind calmodulin (28, 34) and are phosphorylated in the presence of calcium, calmodulin, Mg^{2+}, and ATP (30, 34). They are both recognized by monoclonal antibody 6G9. Finally, iodinated tryptic peptide maps of the two are identical. These similarities do not rule out small differences between them in amino acid sequence or covalent modification. Nevertheless, they indicate that the two proteins are closely related and may be identical. One of the other two subunits of synapsin I kinase, β, also appears to be present in the PSD fraction. A third subunit, β', occurs in lower amounts than the other two in the kinase holoenzyme and may be a proteolytic product of β (34, 37). We have preliminary evidence, based on peptide maps, that β' is also present in the PSD fraction (data not shown).

The α subunit and the mPSDp are similar in two additional respects not described in this report. Immunochemical measurements indicate that, like the mPSDp (27, 47), the α subunit is much less concentrated in cerebellum than in the forebrain (48). Also, like the mPSDp (18), the α subunit streaks in the isoelectric focusing dimension of two-dimensional gels, and its recovery is low (data not shown). This probably occurs because the kinase holoenzyme is not completely dissociated by nonionic detergents.

Although several laboratories have shown that the PSD fraction contains structures that correspond in morphology and staining characteristics to postsynaptic densities, the possibility of artifactual association of proteins with this fraction during homogenization or treatment with detergent has not been completely ruled out (although see ref. 13). Thus, we cannot yet firmly conclude that the mPSDp/α subunit is associated with PSDs *in vivo*. Immunocytochemical experiments would be helpful in confirming this conclusion, but antibodies to the mPSDp are not produced when the PSD fraction is used as immunogen (49). We have, however, been able to produce antibodies to the α subunit by using soluble synapsin I kinase holoenzyme as immunogen. These should be useful in determining the location of this protein in intact tissue.

When it was originally described, it was postulated that the mPSDp might be a specific marker for the PSD (18, 50). However, Flanagan *et al.* (51) recently presented evidence that it may exist in soluble as well as particulate form. Our results support this idea, since we have previously shown that the α subunit is present in both soluble and particulate fractions of brain homogenates and appears to exist in a dynamic equilibrium between them (37). Kelly and Cotman (52) and Ratner and Mahler (21) have demonstrated that the mPSDp is crosslinked to PSD proteins by disulfide bonds. Thus, disulfide bonds are probably involved in the association of the kinase with the PSD fraction. They have proposed that control of formation of these bonds may be involved in regulation of the assembly and function of PSDs *in vivo*. It is possible that the extent of disulfide bonding may be influenced by autophosphorylation or dephosphorylation of the kinase subunits.

Evidence from other laboratories suggests that the synapsin I kinase is also associated with synaptic vesicles. For example, synapsin I, a principle substrate of the kinase, is primarily associated with vesicles (25, 53). Moreover, highly enriched vesicle fractions contain an endogenous protein kinase activity that phosphorylates synapsin I at the sites that are phosphorylated by purified synapsin I kinase (54, 55). In addition, partially purified synaptic vesicles from rat brain (56) and *Torpedo* electric organ (55) contain endogenous substrates for calmodulin-dependent protein kinase that have the same molecular weights as the autophosphorylated subunits of synapsin I kinase. The presence of the synapsin I kinase in several subcellular compartments would be consistent with the notion that it is involved in the regulation of many functions (34).

The Stokes radius of the synapsin I kinase holoenzyme is 95 Å, as determined by gel filtration (34), thus its diameter in solution is approximately 200 Å. Electron micrographs of isolated PSDs show that they contain particles with a median diameter of 180 Å that appear to be held together by a lattice of fibers (13–15). The similarity of the two diameters suggests that the kinase holoenzyme could be equivalent to the PSD "particles." This idea is supported by the observation that PSDs isolated from the cerebellum lack both the mPSDp and the particles (27).

The identification of the mPSDp as a subunit of calmodulin-dependent synapsin I kinase will allow us to use antibodies to the kinase to test for its presence in PSDs in intact tissue. If its presence there is confirmed, it will strengthen the idea that PSDs play an important role in postsynaptic regulation by calcium ion.

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CHAPTER 3

SELECTION AND CHARACTERIZATION OF cDNA CLONES FOR THE β SUBUNIT OF NEURONAL TYPE II Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE

Abstract

cDNA clones coding for the β subunit of neuronal type II Ca^{2+}/calmodulin-dependent protein kinase have been isolated and characterized. The initial clone was isolated from a λGT11 expression library by immunological screening and was able to hybrid select message for both kinase subunits. Northern blot analysis indicated that it reacted strongly with the β subunit message and weakly with the α subunit message. The λGT11 clone was used to screen a λGT10 library to obtain additional overlapping cDNA clones with longer inserts. DNA sequence analysis of these clones revealed a single long open reading frame (1626 nucleotides) with 62 nucleotides 5' of the first start codon, and 149 nucleotides 3' of the stop codon. Primer extension analysis indicated that the 5' end of the message extended 134 nucleotides beyond the end of the cDNA clones so far isolated. The open reading frame codes for a protein consisting of 542 amino acid residues and with a molecular weight of 60,333. The amino terminal half of the predicted β subunit sequence is homologous to several other protein kinases including phosphorylase kinase, myosin light chain kinase, and cAMP-dependent protein kinase. Potential ATP binding, calmodulin binding, and autophosphorylation sites within the β subunit sequence were identified. Southern blot analysis indicated the existence of a multigene family related to the β subunit of neuronal type II Ca^{2+}/calmodulin-dependent protein kinase.

Calcium ion acts as a second messenger in the nervous system by regulating a variety of processes (Greengard, 1981; Reichardt and Kelly, 1983). One way of determining the mechanisms of Ca^{2+} action is through the molecular characterization of the proteins which are regulated by Ca^{2+}. I am interested in the molecular characterization of one such Ca^{2+} target protein, a neuronal Ca^{2+}/calmodulin-dependent protein kinase. The enzyme I have been studying is very abundant, comprising up to 1% of total brain protein (Erondu and Kennedy, 1985), and has been named type II Ca^{2+}/calmodulin-dependent protein kinase (type II CaM kinase). It has been purified independently by several different laboratories employing a number of different substrates to monitor the purification. The substrates used included synapsin I (Bennett et al., 1983; McGuinness et al., 1983), tryptophan hydroxylase (Yamauchi and Fujisawa, 1983), tubulin (Goldenring et al., 1983), casein (Kuret and Schulman, 1984), and smooth muscle myosin light chain (Fukunaga et al., 1982). From the variety of in vitro substrates for the type II CaM kinase, it is apparent that it may be involved in mediating a number of neuronal responses to Ca^{2+}. In addition, two related calmodulin-dependent glycogen synthase kinases have been purified from liver (Ahmad et al., 1982; Payne et al., 1983) and skeletal muscle (Woodgett et al., 1983). These enzymes have subunit compositions, holoenzyme structures, and substrate specificities which are similar to those of the neuronal type II CaM kinase. Threrefore, these enzymes may make up a family of related calmodulin-dependent protein kinases expressed in a variety of tissues.

The following observations about the type II CaM kinase have raised questions about its structure which can best be approached at the nucleic acid level. It is these observations and questions which have been the motivation behind the quest for cDNA clones for the type II CaM kinase. 1) The neuronal type II CaM kinase is composed of two types of subunits with molecular weights

of 50,000 and 60,000, termed α and β, respectively (Bennett et al., 1983). The two subunits appear to be distinct peptides, although immunological and peptide mapping studies suggest they are related (Goldenring et al., 1983; Kennedy et al., 1983; McGuinness et al., 1983; Kelly et al., 1984). Detailed protein sequence information for the two subunits (obtained from cDNA clones) would be very useful in determining their relationship. 2) Different brain regions contain different isozymes of the type II CaM kinase. The different isozymes are composed of identical or nearly identical subunits (α and β) assembled in dramatically different ratios (McGuinness et al., 1985; Miller and Kennedy, 1985). The mechanism by which brain region specific isozymes are generated, be it expression of region specific subunit genes, differential expression of one gene for each subunit, or posttranslational assembly of the subunits, could be addressed with cDNA clones. 3) The β subunit displays varying degrees of microheterogeneity in purified preparations from different brain regions (Bennett et al., 1983; McGuinness et al., 1985; Miller and Kennedy, 1985). cDNA clones for the β subunit would be very useful in determining if this microheterogeneity is the result of different genes for each peptide, differential RNA processing, or differential posttranslational modification of the peptides. 4) A number of different tissues express kinases which appear to be related to the neuronal type II CaM kinase (McGuinness et al., 1983; Woodgett et al., 1983). How closely these other kinases are related at the nucleotide sequence level could be addressed with cDNA clones. 5) The activity of neuronal type II kinase is regulated by calmodulin binding and by autophosphorylation (Miller and Kennedy, 1986). The identification of calmodulin binding and autophosphorylation sites within the primary sequence of the kinase (determined from cDNA clones) could be very useful in future functional studies involving site directed mutagenesis or site specific antibodies.

In this report, the isolation and characterization of cDNA clones for the β subunit of neuronal type II CaM kinase is described. Clones containing 62 nucleotides of 5' untranslated sequence, 1626 nucleotides of coding sequence, and 149 nucleotides of 3' untranslated sequence have been isolated and sequenced. The amino acid sequence of the β subunit has been deduced from the cDNA sequence and is found to be homologous to other protein kinases. The β subunit cDNA clones described in this study will be very useful in addressing the questions about the kinase structure outlined above.

Materials and Methods

Materials. Restriction enzymes, DNA polymerase I, DNA polymerase I Klenow fragment, alkaline phosphatase, T4 DNA ligase, actinomycin D, dithiothreitol, and calf liver tRNA were from Boehringer Mannheim Biochemicals. DNAse I was from Worthington, and Moloney murine leukemia virus reverse transcriptase was from Bethesda Reasearch Laboratories. Oligo dT-cellulose, deoxynucleotide triphosphates, dideoxynucleotide triphosphates, M13 hybridization probe primer, and polynucleotide kinase were purchased from Pharmacia P-L Biochemicals. [α^{32}P] dATP and [^{35}S] methionine were from Amersham, and carrier-free [^{125}I] NaI and [γ^{32}P] ATP were from ICN Nutritional Biochemicals. Bovine serum albumin (Pentax fraction V) was from Miles Scientific. Protein A and protein A-sepharose were from Pharmacia Fine Chemicals, and isopropyl-β-D-thiogalactopyranoside (IPTG) was from Calbiochem. Nitrocellulose filters (BA85, 0.45 μm) were purchased from Schleicher and Schuell.

cDNA libraries. Both a λGT10 (Huynh et al., 1985) and a λGT11 (Young and Davis, 1983a) cDNA library were provided by David Anderson of Columbia University. Both libraries were constructed from cDNA synthesized by oligo dT

priming poly A^+ RNA isolated from the whole brains of three month old rats. Second strand synthesis was carried out using RNase H and DNA polymerase I as described by Gubler and Hoffman (1983).

Immunological screening of λGT11 library. Immunological screening of a rat brain λGT11 cDNA library was performed essentially as described by Moon et al. (1985). Overnight cultures (200 µl) of E. coli LE 392 or Y1088 (Young and Davis, 1983b) were infected with 50,000 plaque-forming units from the λGT11 rat brain cDNA library, diluted with 7 ml of L-top agar, and plated on 150 mm L-plates. The plates were incubated at room temperature for 30 min, and then at 37° until plaques became just detectable (3-4 h). At this time, nitrocellulose filters (impregnated with 10 mM IPTG for Y1088) were placed onto the top agar, and the plates were inverted and incubated at 37° an additional 10-12 h. The filters were then removed from the plates and washed with TBS (150 mM NaCl, 15 mM Tris pH 7.5, 1 mM EDTA, 5 mM NaN_3) for 15 min to remove bacterial debris. The filters were then incubated in G buffer (TBS plus 0.1% gelatin) at 37° for 2 h and then with GT buffer (G buffer plus 0.1% tween 20) at 37° for an additional 2 h.

Two different rabbit polyclonal antisera were used to screen the λGT11 library. The antisera were produced by multiple subcutaneous injections of purified forebrain type II CaM kinase (Annette) or of electrophoretically purified β subunit (Darcy) in phosphate buffered saline containing an equal weight of polyinosinic-polycytidylic acid. Prior to screening, the antisera were preadsorbed against overnight cultures of LE392 and Y1088 (100 µl antiserum plus 100 µl culture) for 30 min at room temperature. The bacteria were removed by centrifugation and the antisera were diluted in GT buffer to their final concentration (1 in 500 dilution for Annette and 1 in 100 dilution for Darcy). The diluted antisera were then preadsorbed with nitrocellulose filter replicas of

plates infected with λGT11 containing no cDNA insert (50,000 plaques/plate) for 3 h at room temperature. The preadsorbing filters were removed and the cDNA library filters were added and incubated for 2 h at room temperature. Unbound antibody was removed by 6 washes with GT buffer for 15 min each. The filters were then incubated with ^{125}I-labeled Protein A in GT buffer (10^6 cpm/ml) for 2 h at room temperature followed by 6 washes with GT buffer as before. Antibody reacting plaques were identified by autoradiography, recovered, and rescreened. The initial screening of 250,000 plaques with Annette antiserum identified 9 potential positives, of which 5 subsequently rescreened positively with both Annette and Darcy antisera. These 5 represent 2 different cDNA inserts (see Results).

Screening of λGT10 library by plaque hybridization. Overnight cultures of E. coli C600 Δhfl (200 μl) were infected with 50,000 plaque-forming units from the λGT10 rat brain cDNA library, diluted with 7 ml L-top agar, and plated on 150 mm L-plates. The plates were inverted and cultured at 37° for 10-12 h. The DNA from the bacteriophage plaques was transferred to nitrocellulose filters and denatured as described by Benton and Davis (1977). The filters were prehybridized with 5X SET (1X SET is 0.15 M NaCl, 30 mM Tris pH 8.0, 20 mM EDTA), 10X Denhardts (1X Denhardts is 0.02% bovine serum albumin, 0.02% SDS, 0.02% Ficoll, 0.02% polyvinylpolypyrolidone), 50 mM sodium phosphate buffer pH6.8 (prehybridization buffer I) for 2 h at 42°, followed by 50% formamide, 5X SET, 1X Denhardts, 20 mM sodium phosphate, 50 μg/ml denatured and sheared salmon sperm DNA (prehybridization buffer II) for 2 h at 42°. The filters were then hybridized with ^{32}P-labeled probe in prehybridization buffer II for 20-24 h at 42°. Filters were washed three times with 1X SSPE (1X SSPE is 180 mM NaCl, 1 mM EDTA, 1 mM NaH_2PO_4, pH 7.7), 0.1% SDS at room temperature and then four times with 0.1X SSPE, 0.1% SDS at 42°. Plaques

hybridizing with the probe were detected by autoradiography, isolated, and rescreened. The primary screen of 400,000 plaques with a 600 base pair restriction fragment of clone λ11β5 identified 52 potential positives of which 26 rescreened positively.

In vitro translation and immunoprecipitation. Rabbit reticulocyte lysate was prepared according to the method of Jackson and Hunt (1983) and was used to translate either total RNA, poly A^+ RNA, or hybrid selected RNA using [^{35}S] methionine to label in vitro synthesized proteins. In vitro translation products were diluted with 3 volumes of NET (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM EDTA) containing 1% NP-40 and then pretreated with one volume of protein A-sepharose beads (added as a 1:1 suspension in NET containing 1% NP-40) and 0.2 volumes of rabbit anti-mouse IgG (1.5 mg/ml) for 2 h at 4° with constant mixing. The beads were sedimented by brief centrifugation in a microfuge and the supernatant was transferred to a fresh tube. The supernatant was incubated with 80 ng of pure forebrain kinase and 2.5 µl of monoclonal antibody 4A11 (50% ammonium sulfate cut of ascites fluid, 20 mg protein/ml) for 4 h at 0°. An additional 7.5 µl of rabbit anti-mouse IgG and 40 µl of protein A-sepharose were added and the reaction was incubated at 4° for 2 h with constant mixing. The beads were again sedimented and the supernatant discarded. The beads were washed 6 times with NET containing 1% NP-40 and then twice with NET. The immunoprecipitated proteins were released from the beads by boiling in SDS gel loading buffer and analyzed by 10% polyacrylamide SDS gel electrophoresis. The ^{35}S-labeled proteins were detected by fluorography.

Positive hybrid-selection of RNA. Positive hybrid-selection of RNA was performed essentially as described by Moon et al. (1985). Single-stranded DNA (10 µg) from M13 subclones with inserts in either the coding or anti-coding orientation were bound to nitrocellulose filters. The filters were baked in a

vacuum oven at 80° for 2 h, boiled in water, prehybridized in 50 µl 50% formamide, 0.1 M Pipes pH 6.4, 0.6 M NaCl, 2 mM EDTA, 50 µg/ml poly(A), and 100 µg/ml tRNA at 54° for 1 h and then for 1 h in the above solution supplemented with 150 ng of globin mRNA. The prehybridized filters were incubated for 10 h at 54° in 50 µl of prehybridization solution supplemented with 200 µg forebrain total RNA and without poly(A), tRNA, and globin mRNA. After the hybridization, the filters were washed twice for 15 min with prehybridization solution containing poly(A) and tRNA. The filters were then washed 12 times with 1X SSPE, 0.5% SDS containing 25 µg/ml tRNA at 65° and twice with 20 mM Tris, 0.1 mM EDTA pH 7.6 containing 25 µg/ml tRNA at 20°. The RNA was eluted from the filters by boiling in 100 µl water containing 50 µg/ml tRNA, concentrated by ethanol precipitation, and translated in vitro. The in vitro translation products were analyzed by immunoprecipitation as described above.

Northern blots. RNA was purified from whole rat brain or rat brain regions (forebrain and cerebellum) by extraction with guanidine thiocyanate as described by Chirgwin et al. (1979). Poly A^+ RNA was isolated by two cycles of adsorption to and elution from oligo dT cellulose (Aviv and Leder, 1972). Poly A^+ RNA was size fractionated by electrophoresis through 1% agarose gels containing 2.2 M formaldehyde and transferred to nitrocellulose filters as described (Maniatis et al., 1982). Conditions of prehybridization and hybridization were as described for plaque hybridization.

Southern blots. High molecular weight DNA was isolated from rat brain as described by Blin and Stafford (1976). 10 µg of DNA was digested with restriction enzymes EcoRI, BamHI, and HindIII (10-20 U) for 6 h in the buffers recommended by the manufacturer. The digested DNA was then size fractionated by electrophoresis through 1% agarose, denatured with NaOH, and transferred to nitrocellulose filters as described (Maniatis et al., 1982).

Conditions for prehybridization and hybridization were as described for plaque hybridization.

Subcloning into bacteriophage M13. cDNA inserts were excised from the λGT10 clones with EcoRI, isolated by agarose gel electrophoresis (Dretzen et al., 1981), and ligated into the EcoRI site of the M13 vectors mp18 and mp19. The cDNA inserts from the λGT11 clones could not be excised with EcoRI because the EcoRI site at one end of each of the cDNAs had been lost. For this reason, the cDNA inserts (along with about 1 kb of λ flanking sequence on each end) were excised with the restriction enzymes KpnI and SacI, isolated by agarose gel electrophoresis, and ligated into the M13 vectors mp18 and mp19 which had been cut with the same restriction enzymes. The initial M13 subclones were used to determine a detailed restriction map of the cDNAs, and appropriate restriction fragments were isolated and ligated into compatible mp18 and mp19 vectors for sequencing (Messing, 1983).

DNA sequencing. Single stranded template DNA from the M13 subclones was isolated from bacteriophage purified by equilibrium sedimentation in a CsCl gradient. The DNA sequence was determined for both strands by the dideoxy chain termination method (Sanger et al., 1977) using either a 15 nucleotide universal primer or an 18 nucleotide β subunit specific primer and [α^{32}P] dATP as the label. The DNA samples were resolved on 4% polyacrylamide gels (0.2mm thick, 80cm long) run at 3000 V for 4 or 16 h. Approximately 400-500 nucleotides could be read from each pair of loadings.

Primer extension. An 18 nucleotide primer complimentary to a region of the RNA near the putative start codon (see Results) was synthesized by Dr. S. Horvath of Caltech. The primer was labeled at the 5' end using polynucleotide kinase and [γ^{32}P] ATP as described (Meikoth and Wahl, 1984). The ^{32}P-labeled primer (20 ng) was hybridized to 10 μg of forebrain poly-A^+ or poly-A^- RNA at

37° for 30 min in 0.17 M NaCl. The RNA and primer were ethanol precipitated and redissolved in double distilled H_2O. The samples were then brought to 50 mM Tris pH 7.5, 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 0.5 mM of each deoxynucleotide triphosphate, 50 μg/ml actinomycin D, and 33,000 U/ml Moloney murine leukemia virus reverse transcriptase, and incubated at 37° for 1 h. The reaction was stopped by addition of EDTA, extracted with chloroform, and the DNA ethanol precipitated. The primer extension products were resolved on a 10% polyacrylamide sequencing gel and detected by autoradiography.

Hybridization probes. Hybridization probes were prepared from the single stranded DNA of the M13 subclones by extension of a hybridization probe primer with DNA polymerase I Klenow fragment in the presence of [α^{32}P] dATP as described by Messing (1983). Probes with specific activities of 1-2x10^8 cpm/μg of insert were obtained.

Sequence analysis. DNA sequence data was analyzed using a DNA Sequence Analysis Program on an IBM personal computer. A homology search of the National Biomedical Research Foundation protein database was performed on the Bionet computer system using the SEARCH (Dayhoff et al., 1983) and IFIND programs (Brutlag et al., 1982).

Results

Isolation of type II CaM kinase cDNA clones. To isolate cDNA clones coding for the type II CaM kinase, a rat brain cDNA library constructed in the expression vector λGT11 was screened with two anti-kinase polyclonal antisera. The antisera used to screen the library were generated against either pure forebrain kinase or SDS polyacrylamide gel purified β subunit. Both antisera reacted strongly with the β subunit and weakly with the α subunit on immunoblots (Miller and Kennedy, 1985). Two cDNA clones were isolated.

Fusion proteins made both of them react with both antisera. The reaction of the fusion proteins with the antisera was blocked by the addition of purified forebrain kinase to the screening reaction. The two clones, called λ11β5 and λ11β9, had insert sizes of 1.0 and 0.6kb respectively. Restriction mapping indicated that the λ11β9 insert was contained entirely within the λ11β5 insert. For this reason, only the λ11β5 clone was characterized further.

Hybrid selection. To confirm that the λ11β5 clone coded for the type II CaM kinase, a positive hybrid selected translation was performed. In order to do the hybrid selection experiment, I needed a way to identify the in vitro translated kinase subunits. One of a series of anti-kinase monoclonal antibodies generated in our lab, antibody 4A11, was able to immunoprecipitate type II CaM kinase from brain homogenates and appeared to react with both of the kinase subunits in solution (N. Erondu, unpublished observations). This antibody was therefore a good candidate for use in the immunoprecipitation of the kinase subunits from an in vitro translation reaction. In vitro translation of total brain RNA followed by immunoprecipitation of the translation products with monoclonal antibody 4A11 resulted in the specific precipitation of both in vitro labeled kinase subunits (Fig. 1, lane 1). Control immunoprecipitations carried out in the absence of antibody 4A11 (rabbit anti-mouse IgG and protein A-sepharose only) did not precipitate these two peptides (Fig.1, lane 2). The two peptides specifically precipitated by antibody 4A11 consistently had a 5% higher mobility on SDS gels than the kinase subunits from purified preparations (data not shown). This difference may be due to a posttranslational modification of the subunits, such as phosphorylation, which does not occur during in vitro synthesis. For the hybrid selection experiment, the λ11β5 clone insert was subcloned in two different orientations into the single stranded bacteriophage vectors M13 mp18 and M13 mp19 so that the single stranded form of one of the

subclones had the insert in an anti-coding orientation (and therefore would be able to select mRNA) and the other subclone had the insert in a coding orientation (and would be unable to select mRNA). The single stranded DNA from both M13 subclones was isolated, bound to nitrocellulose filters, and hybridized with total rat brain RNA. The hybrid selected RNA was translated in vitro and the translation products immunoprecipitated with antibody 4A11. The λ11β5 cDNA selected message for both subunits in one orientation (Fig. 1, lane 4) and for neither subunit in the opposite orientation (Fig. 1, lane 3). The orientation of these subclones (coding or anti-coding) was confirmed by Northern blot and nucleotide sequence analysis.

Northern blots. The fact that the λ11β5 clone could select message for both subunits has two potential explanations: 1) both subunits are coded for by a single mRNA, or 2) there is a high degree of sequence homology between the two subunit messages. In order to distinguish between these possibilities, a Northern blot analysis was performed. This analysis takes advantage of the difference in distribution of the two kinase subunits in different brain regions (Erondu and Kennedy, 1985; McGuinness et al.,1985; Miller and Kennedy, 1985). The α subunit is much more abundant in the forebrain than in the cerebellum, whereas the β subunit is distributed equally between the forebrain and cerebellum. This difference in the subunit distribution is also reflected in the distribution of the subunit messages. Equal amounts of forebrain and cerebellar RNA were translated in vitro and the translation products immunoprecipitated with antibody 4A11. As seen in Fig. 2A, the α subunit is much more prominent in translation products of forebrain RNA whereas the β subunit is equally abundant in translation products of forebrain and cerebellar RNA. The distribution of the messages recognized by clone λ11β5 was determined by probing Northern blots containing equal amounts of forebrain and cerebellar poly-A^+ RNA with a ^{32}P-

labeled λ11β5 probe. The probe reacted strongly with a 4.8kb message which is equally abundant in the forebrain and cerebellum (Fig. 2B, thick arrow). In addition, the probe reacted weakly with a 5.4kb message which is more abundant in the forebrain than in the cerebellum (Fig. 2B, thin arrow). The size (5.4kb) and distribution (forebrain>>cerebellum) of the latter message is identical to that of a message recognized by oligonucleotide probes generated from α subunit protein sequence data (see Chap. 4). From these results, it is concluded that the λ11β5 clone codes for the β subunit, but is also capable of hybridizing with α subunit nucleotide sequences.

Isolation of additional clones. Since the λ11β5 clone insert was only 1.0kb in length, it could not code for the entire β subunit protein. For this reason, a λGT10 rat brain cDNA library was screened with a λ11β5 probe in order to obtain overlapping clones with longer inserts. Twenty-three additional clones were isolated, of which those with the longest inserts were subcloned into M13 for restriction mapping and sequence determination.

Sequence determination. The restriction map and sequencing strategy for the β subunit coding clones is shown in Fig 3. Restriction map analysis of the λ11β5 clone and the various λ10β clones produced a consistent map of about 1.8kb (Fig. 3). Although a number of clones extended outside of this 1.8kb region, no consistent restriction map was obtained for the flanking regions, and they may represent cloning artifacts generated by the ligation of two or more unrelated sequences during the construction of the library. Because of the potential for this artifact in the λGT10 library (D. Anderson, personal communication), only restriction sites and sequences which were obtained from at least two independent clones were included in the overall restriction map (Fig. 3) and the composite nucleotide sequence (Fig.4). The sequencing strategy illustrated in Fig. 3 indicates the direction and extent of sequencing carried out

on each β subunit clone. The thin arrows indicate sequencing reactions carried out on M13 subclones containing the entire cDNA insert or a specific restriction fragment of the insert and employing a universal sequencing primer. The thick arrows indicate the sequencing reactions carried out on M13 subclones containing the entire cDNA insert and employing a β subunit specific oligonucleotide primer. A single long open reading frame was detected and is indicated by the striped region of the restriction map in Fig. 3. The restriction map and nucleotide sequence were completely consistent between overlapping regions of the various β subunit clones (within the 1.8kb region) with two exceptions. 1) Two independent λ10β clones (λ10β8-3 and λ10β4-6a) had an internal HincII restriction fragment which was about 50 bases shorter than an internal HincII fragment in all of the other β subunit clones (see Fig. 3). The sequence of the HincII fragment from these two clones was determined, and both were found to have the same 45 base deletion with sequences identical to the other β subunit clones on either side of the deletion. This deletion may be the result of alternative splicing of the β subunit mRNA (see discussion). 2) The same two clones (λ10β8-3 and λ10β4-6a) had sequences at their ends (hatched areas in Fig. 3) that were completely unrelated to the sequences in the two corresponding regions of the other β subunit clones and may be the result of the cloning artifact mentioned above.

The composite nucleotide sequence from the various β subunit clones is shown in Fig. 4 along with the deduced amino acid sequence for the longest open reading frame. Each position in the sequence of 1840 nucleotides has been determined an average of 5.3 times and all of the sequence has been determined for both strands. The putative start codon is at position 63 in the sequence and has the appropriate neighbor sequences to be a functional start codon (a purine three positions before and one position after the AUG codon) (Kozak, 1983).

However, there are no stop codons in any of the reading frames 5' of the putative start codon, and primer extension analysis using an oligonucleotide primer which hybridizes near the putative start codon indicates that the 5' end of the message (or a strong reverse transcriptase stop signal) is 134 nucleotides beyond the 5' end of the sequence I have determined (Fig. 3). Therefore, the potential exists that the true start codon is closer to the 5' end of the mRNA, and that the amino acid I have designated number 1 is actually an internal methionine. An open reading frame extends from nucleotide 63 to 1689 and codes for 542 amino acids. The molecular weight of the protein encoded by this open reading frame is 60,333, in close agreement with the molecular weight of 60,000 estimated for the β subunit from its mobility on SDS polyacrylamide gels (Bennett, et al., 1983). The other two reading frames have a total of 11 and 20 stop codons over this region. An additional 149 nucleotides of 3' untranslated region extends beyond the termination codon. No polyadenylation signal was detected in the 3' untranslated region. This is not very surprising since the Northern blot results indicate that the message is much longer than is required to code for the β subunit and this additional sequence is likely to be in the form of a very long (>2kb) 3' untranslated region. The sequence underlined in Fig. 4 is the region deleted from clones λ10β8-3 and λ10β4-6a. The deletion codes for 15 amino acids and does not disrupt the open reading frame.

Sequence homologies. The amino acid sequence deduced from the β subunit cDNA clones was compared to the sequences in the National Biomedical Research Foundation protein sequence data base. The top 9 alignment scores were all protein kinases or protein kinase-related proteins. The top three scores were for the γ subunit of phosphorylase kinase, myosin light chain kinase, and the catalytic subunit of cAMP-dependent protein kinase. The alignment of these three sequences with the β subunit sequence is shown in Fig. 5. The region of

homology begins near (but not at) the amino terminal of the β subunit and extends approximately 300 amino acids towards the carboxyl terminal. Gaps were inserted to optimize the alignment. The β subunit of type II CaM kinase (amino acids 18-312) is identical with the γ subunit of phosphorylase kinase (amino acids 23-316), myosin light chain kinase (amino acids 65-344), and the catalytic subunit of cAMP-dependent protein kinase (amino acids 46-335) at 38%, 31%, and 27% of their amino acid positions, respectively. If conservative amino acid substitutions are included in the comparison (Dayhoff, 1978), the homology percentages go up to 58% for the γ subunit of phosphorylase b kinase, 52% for mysoin light chain kinase, and 48% for the catalytic subunit of cAMP-dependent protein kinase. The putative ATP binding site for the β subunit was identified as Lys-43 (marked by an asterisk in Fig. 5) by analogy with the ATP binding residue chemically identified for other protein kinases (see next section). The amino acid sequence beyond amino acid 313 of the β subunit was not significantly homologous to the various protein kinases or to any other protein sequences in the database.

Functional domains. The β subunit amino acid sequence was analyzed for primary and secondary structural features which may identify potential functional sites and domains. A diagram of the β subunit of the type II CaM kinase which illustrates the potential functional domains is shown in Fig. 6. The region of homology between the β subunit and the other protein kinases is indicated by the striped bar. This region is likely to represent the catalytic domain. Indeed, the putative ATP binding residue (Lys-43) is within this region and its position is indicated in Fig. 6 by the circled lysine (K) residue with an asterisk above it. This lysine is in a position corresponding to the lysine which is labeled by the ATP analogue p-fluorosulfonylbenzoyl 5'-adenosine in cAMP-dependent protein kinase (Zoller et al., 1981), cGMP-dependent protein kinase

(Hashimoto et al., 1982) and the Rous sarcoma virus protein kinase (Kamps et al., 1984). The lysine labeled in each kinase may be in the vicinity of the normal binding site for the γ-phosphate of ATP. An alanine is present two amino acids on the amino terminal side of the labeled lysine (Ala-X-Lys) and a cluster of glycines with the sequence Gly-X-Gly-X-X-Gly has been identified 16-28 amino acids on the amino terminal side of the labeled lysine. This cluster of glycines is found in a number of different protein kinases and other nucleotide binding proteins and has been proposed to be involved in ATP binding (Kamps et al., 1984). The corresponding position within the β subunit has the sequence Gly-Lys-Gly-Ala-Phe-Ser. The third glycine in the cluster is replaced by a serine in the β subunit sequence. The third glycine is also replaced by a serine in the corresponding region of sequence from the γ subunit of phosphorylase b kinase (Reimann et al., 1984), so the third glycine may not be required for ATP binding. No homology was found between the β subunit amino acid sequence and the sequences believed to be involved in nucleotide binding in a number of GTP binding proteins (Halliday, 1983). A comparison of the predicted secondary structure of the various protein kinases with the X-ray crystal structure of the catalytic subunit of cAMP-dependent protein kinase (which will be available soon; S. Taylor, personal communication) should help in the identification of other conserved structural regions within the catalytic domain which may be functionally important.

Analysis of the kinetics of the type II CaM kinase autophosphorylation reaction indicates that there may be additional catalytic ATP binding sites (S. Miller, unpublished observation). Such potential ATP binding sites were identified by searching the β subunit amino acid sequence for the concensus sequence of Ala-X-Lys for protein kinase ATP binding sites, with lysine being the ATP binding residue. The positions of such potential ATP binding sites are

indicated by the circled lysines in Fig. 6 and correspond to lysines at positions 146, 153, 267, and 410. None of these sites have the sequence Gly-X-Gly-X-X-Gly present 16-18 residues on the amino terminal side of the lysine. Since this cluster of glycines may be important in nucleotide binding (Kamps et al., 1984), it is not clear which, if any, of these potential sites represent actual ATP binding sites.

Both subunits of the type II CaM kinase undergo autophosphorylation with α and β incorporating 2 and 3 moles of phosphate per mole of subunit, respectively (Bennett et al., 1983). The phosphorylation occurs on both serine and threonine residues (Goldenring et al., 1983; S. Miller, unpublished observations). A number of potential autophosphorylation sites have been identified within the β subunit sequence. The general properties of phosphorylation sites for other protein kinases have been determined (Kemp et al., 1975; Tessmer et al., 1977; Glass and Krebs, 1982; Kemp et al., 1983; Turner et al., 1985). A basic amino acid (arginine or lysine) is usually required 2 or 3 residues on the amino terminal side of the phosphorylated amino acid (serine or threonine). In studies of the substrate specificities of two calmodulin-dependent glycogen synthase kinases which are related to the neuronal type II CaM kinase, it was concluded that the sequence Arg-X-X-Ser or Arg-X-X-Thr is required for phosphorylation (Payne et al., 1983; Pearson et al., 1985). The arginine in the sequence could be replaced by lysine, although the resulting peptide is phosphorylated less efficiently (Pearson et al., 1985). The sites within the β subunit amino acid sequence which fit this requirement are indicated by the boxed serine (S) and threonine (T) residues in Fig. 6 and correspond to serines at positions 51, 315, 363, and 395, and threonines at positions 36, 262, 287, 321, 356, and 510. Since the activity of the type II CaM kinase is regulated by autophosphorylation (Miller and Kennedy, 1986), and this regulation is potentially very important to the physiological

function of the enzyme, it will be important to identify which of the potential β subunit phosphorylation sites is involved in this regulation.

Potential calmodulin binding sites from a number of CaM-dependent protein kinases, including skeletal and smooth muscle myosin light chain kinase (Blumenthal et al., 1985; Lukas et al., 1986) and phosphorylase b kinase (Lukas et al., 1986), have been identified. There is no concensus primary sequence emerging for the calmodulin binding site. However, there is a secondary structural feature, a basic amphiphilic α-helix, common to all calmodulin binding peptides (Cox et al., 1985). A strongly basic region within the β subunit sequence was identified which had an α-helix forming potential (amino acids 295-315). A two-dimensional projection (Schiffer and Edmunson, 1967) of this region indicates that it contains a potential amphiphilic structure that has three arginine residues and one lysine residue on its polar surface. The apolar surface contains three alanine residues, a leucine, and an isoleucine residue. The positions of this region within the β subunit structure is indicated by the hatched bar in Fig. 6. This region has only limited homology with other protein kinases. However, it is close in the primary sequence to the putative calmodulin binding domains in the skeletal myosin light chain kinase sequence (amino acids 342-360) (Blumenthal et al., 1985) and in the phosphorylase b kinase γ subunit sequence (amino acids 322-345) (Lukas et al., 1986). The sequences of these putative calmodulin binding domains are underlined in the sequence comparisons illustrated in Fig. 5. It remains to be determined if the potential β subunit calmodulin binding site is actually involved in the regulation of the type II CaM kinase by calmodulin.

Also illustrated in Fig. 6 is the region of sequence which is encoded by the deletion identified in two of the β subunit cDNA clones (λ10β8-3 and λ10β4-6a). This region (indicated by the dotted bar) may be missing from a β subunit-related

protein which may have been synthesized from an alternatively spliced mRNA (see Discussion). Also shown is the position of sequence homology between the β subunit and two portions of amino acid sequence obtained from α subunit-derived peptides (black bars) (see Chap. 4).

Southern blots. Genomic southern blots probed with clone λ10β5-2 (a clone including the entire coding region) indicated the existence of a potential β subunit multigene family. Blots of three different restriction digests of genomic DNA were probed in 50% formamide at 42° and washed in 0.1X SSPE and 0.1% SDS at various temperatures (Fig. 7). Multiple bands were detected in all three restriction digests under low stringency washing conditions (42°). At higher stringency (55°) fewer bands were detected, and at the highest stringency tested (65°) only 1 band was detected in the EcoRI digest, and 2 bands each in the BamHI and HindIII digests. These results suggest the existence of multiple genes or pseudogenes with varying degrees of homology with the β subunit cDNA sequence. These related sequences may include genes coding for the α subunit of neuronal type II CaM kinase and/or related kinases expressed in non-neuronal tissues (see Discussion).

Discussion

I have isolated a number of cDNA clones which appear to code for the β subunit of neuronal type II CaM kinase. The first two clones were isolated from a λGT11 expression library screened with two anti-kinase polyclonal antisera. The longer of these two clones (λ11β5) was able to hybrid select message for both subunits (Fig. 1). This result suggests that either both subunits are encoded by one message, or there is enough nucleotide sequence homology between the messages for the two subunits for the cDNA to hybridize to both. The latter explanation appears to be correct, since Northern blot analysis indicates that

clone λ11β5 is able to hybridize to two brain messages (Fig. 2B). The strongest reaction is with a 4.8 kb message whose distribution between the forebrain and cerebellum is the same as that of the β subunit and the β subunit message. The λ11β5 clone also reacts weakly with a 5.4 kb message whose distribution between forebrain and cerebellum is the same as that of the α subunit. From these results, it was concluded that clone λ11β5 codes for the β subunit, but is also capable of hybridizing with α subunit sequences.

Additional β subunit cDNA clones were isolated from a λGT10 library probed with a ^{32}P-labeled restriction fragment of clone λ11β5. These clones included 1840 nucleotides of β subunit cDNA sequence and contained a single long open reading frame (Fig. 3). However, primer extension analysis indicates that the β subunit cDNA sequence compiled in Fig. 4 does not include the 5' 134 nucleotides of the β subunit message. For this reason, the start codon for β subunit translation cannot be definitively assigned. The first start codon within the sequence I have determined is at position 63 and is followed by an open reading frame 1626 nucleotides long. This open reading frame codes for a protein 542 amino acids long with a molecular weight of 60,333. This molecular weight is in close agreement with that expected for the β subunit. Two methods could be used to determine if the 542 amino acid protein predicted from the cDNA sequence corresponds to the entire β subunit. 1) Additional cDNA or genomic clones which extend to the 5' end of the message could be isolated and their sequence determined. If no other start codons are found and/or there are stop codons in all three reading frames 5' of the previously identified putative start codon, then the protein predicted from the current sequence information is likely to be correct. 2) A cDNA clone coding for the entire coding region (i.e., λ10β5-2) could be cloned into a plasmid vector containing the SP 6 promoter (i.e., pSP 64). An mRNA corresponding to the cDNA could then be synthesized in

vitro using SP6 RNA polymerase (Green et al., 1983) and then translated in vitro in a rabbit reticulocyte lysate (Jackson and Hunt, 1983). The products of the translation could then be compared to authentic β subunit synthesized from brain poly-A^+ RNA. If the two proteins are the same size, then the predicted coding region is likely to be correct.

An additional 149 nucleotides of sequence has been determined 3' of the stop codon. Surprisingly, all of the β subunit cDNA clones analyzed so far have their 3' end within about 10 bases of each other. This site is not likely to be very close to the actual 3' end of the message, since the 3' untranslated region is probably greater than 2 kb in length. In addition, no poly-T region representing the primer used to make the cDNAs was found at the 3' end of any of the clones. Two possible explanations for these findings are: 1) that a strong DNA polymerase I stop signal is located at this position in the 3' untranslated sequence so that the second strand of the cDNAs all terminated near this site, and 2) that nearby sequences are susceptible to self priming (for example, a T-rich sequence which might bind the mRNA's 3' poly-A tail) which resulted in first strand cDNA synthesis beginning near this site.

Two different λ10β clones (λ10β8-3 and λ10β4-6a) each had an identical 45 base deletion in the middle of the long open reading frame (see Fig. 3, Fig. 4, and Fig. 6). The region on either side of the deletion was identical to that of the other β subunit clones. The deletion does not disrupt the long open reading frame, and may be the result of alternative splicing of the β subunit message. The sequence at each end of the deletion is consistent with it being an exon which has been spliced out (Mount, 1982). If the putative start and stop codons from the other β subunit cDNAs are used, then the alternatively spliced message would code for a protein of M_r=58,000. This is the same size as a protein present in purified kinase preparations, termed β', which appears to be closely related to

the β subunit (Kennedy et al., 1983; Miller and Kennedy, 1985). Additional experiments, including detailed proteolytic mapping of the β and β' subunits, S1 nuclease protection of brain RNA with β subunit cDNAs, and analysis of the structure of the β subunit gene, will be required to determine if differential splicing is responsible for the generation of the β' subunit.

The amino acid sequence deduced from the β subunit cDNA clones is homologous to several other protein kinases (Fig. 5 and Fig. 6). The homologies are confined to the amino terminal portion of the β subunit indicating that this region may be responsible for the basic functions of all kinases, including the binding and transfer of the γ-phosphate from ATP to a particular substrate. Indeed, the protein kinase ATP binding site concensus sequence of Ala-X-Lys is present near the beginning of the homologous region and Lys-43 is in a position homologous to the ATP binding lysine residue chemically identified in cAMP and cGMP-dependent protein kinases and the Rous sarcoma virus protein kinase (Hashimoto et al., 1982; Zoller et al., 1981; Kamps et al., 1984). cDNA clones which code for the amino terminal half of the β subunit react more strongly on Northern blots with the α subunit message than do cDNA clones coding for the carboxyl terminal domain of the β subunit (data not shown). This indicates that the homology between the α and β subunits may be higher in the region coding for their catalytic domains. Alignment scores were higher for the calmodulin-dependent protein kinases (phosphorylase kinase and myosin light chain kinase) than for the cyclic nucleotide dependent kinases or the oncogene tyrosine kinases. This indicates that the calmodulin-dependent protein kinases are more closely related to each other than to other protein kinases. The sequences outside the homologous region are likely to be involved in functions unique to each particular kinase such as binding to regulatory components, or interaction with other kinase subunits. A number of potential functional domains within the

β subunit amino acid sequence have been identified (Fig. 6). These include possible additional ATP binding sites, potential autophosphorylation sites, and a potential calmodulin binding domain (see Results). It remains to be experimentally determined which of these sites is actually involved in the regulation of the type II CaM kinase.

Southern blot analysis of rat genomic DNA suggests the existence of a kinase multigene family (Fig. 7). Potential members of this family include both subunits of neuronal type II kinase, and the subunits of skeletal muscle and liver calmodulin-dependent glycogen synthase kinase. All of the subunits of these enzymes are in the same molecular weight range (50-60,000), bind calmodulin, are autophosphorylated, and are structurally related (Kennedy et al., 1986). It remains to be determined how many of these other enzymes are homologous enough to be detected as related nucleotide sequences. Detailed Southern blot analysis using probes from different regions of the cDNA and different conditions of stringency should help to define the number and relatedness of the multiple kinase genes or pseudogenes.

Experiments with the β subunit cDNA clones described in this chapter are beginning to address the questions about the type II CaM kinase structure outlined in the Introduction. The hybrid selection and Northern blot results indicate that the α and β subunits are definitely related at the nucleotide sequence level. Determination of the precise amount of homology between the two subunits will require the isolation of cDNA clones for the α subunit. The in vitro translation and immunoprecipitation experiment with forebrain and cerebellar RNA suggests that the different ratios of the two subunits in different brain regions is controlled at the transcriptional level. It has not been determined, however, if the transcripts in the different brain regions are derived from a single gene or multiple genes for each subunit. The mechanism for the

generation of multiple β-related proteins remains unclear. The sequence analysis of two β subunit cDNA clones suggests a possible differential splicing mechanism for the generation of some of the β subunit microheterogeneity. Again, the possibility of multiple genes coding for the different proteins or differential post-translational modification of the proteins cannot be ruled out. Southern blot analysis indicates the existence of multiple sequences related to the β subunit cDNA. Some of these may represent sequences for related kinases expressed in other rat tissues. Finally, a number of potential ATP binding, calmodulin binding, and autophosphorylation sites within the deduced β subunit amino acid sequence have been identified. The identification of these functional domains may allow the function of the type II CaM kinase to be addressed directly.

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Figure 1. Immunoprecipitation of type II CaM kinase subunits and hybrid selection of type II CaM kinase subunit mRNAs. Lanes 1 and 2: 30 µg of forebrain total RNA was translated in vitro in 100 µl of a rabbit reticulocyte lysate translation mixture containing 70 µCi [^{35}S]methionine for 90 min at 30°. The translation products were immunoprecipitated as described in Materials and Methods with monoclonal antibody 4A11 (lane 1) or with no primary antibody (lane 2). Lanes 3 and 4: The λ11β5 cDNA insert was subcloned into the single stranded bacteriophage M13 in both orientations and used to hybrid select mRNA as described in Materials and Methods. Hybrid selected RNA was translated in vitro in 30 µl of a rabbit reticulocyte lysate translation mixture containing 21 µCi of [^{35}S]methionine for 90 min at 30°. The translation products were immunoprecipitated with monoclonal antibody 4A11 as described in Materials and Methods. The immunoprecipitated peptides from the in vitro translation products of RNA hybrid selected by the λ11β5 cDNA in either a coding (lane 3) or anti-coding orientation (lane 4) are shown.

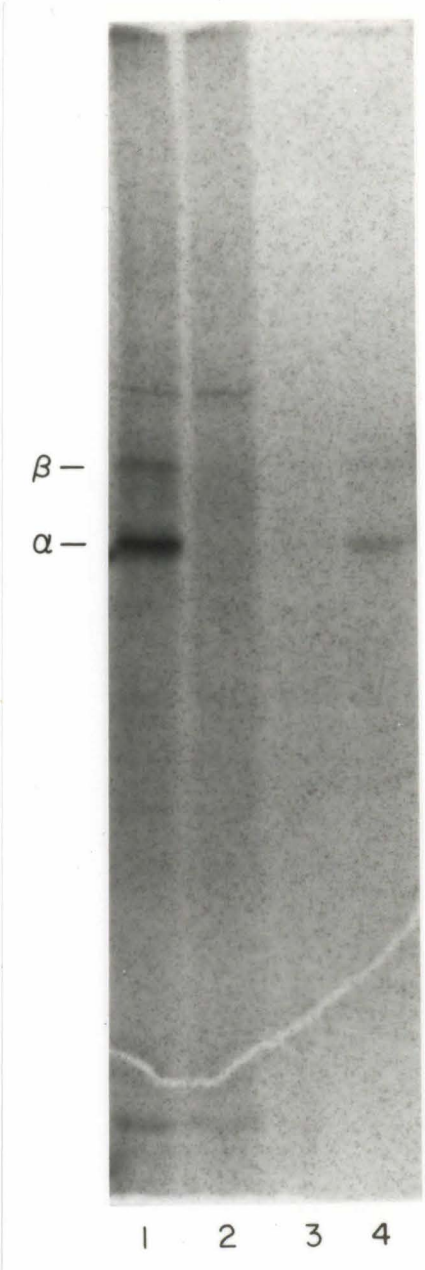
β —
α —
1
2
3
4

Figure 2. Immunoprecipitation and Northern blot analysis of cerebellar and forebrain RNA. A. 15 μg of cerebellar (CER) and forebrain (FB) total RNA were translated in vitro in 90 μl of a rabbit reticulocyte lysate translation mixture containing 63 μCi of [^{35}S]methionine for 90 min at 30°. The translation products were immunoprecipitated with monoclonal antibody 4A11 as described in Materials and Methods. B. 5 μg of cerebellar (CER) and forebrain (FB) poly-A$^+$ RNA was resolved on a 1% agarose gel containing 2.2 M formaldehyde and transferred to a nitrocellulose filter. The filter was probed with 0.5 μg of a ^{32}P-labeled single stranded M13 subclone of λ11β5 (subclone BH-19, 600 bases long) as described in Materials and Methods. The probe was labeled to a specific activity of 2x10^8 cpm/μg of insert. The position of the major and minor reacting bands are indicated by the thick and thin arrows, respectively. The molecular weight markers used were E. coli and sea urchin ribosomal RNAs.

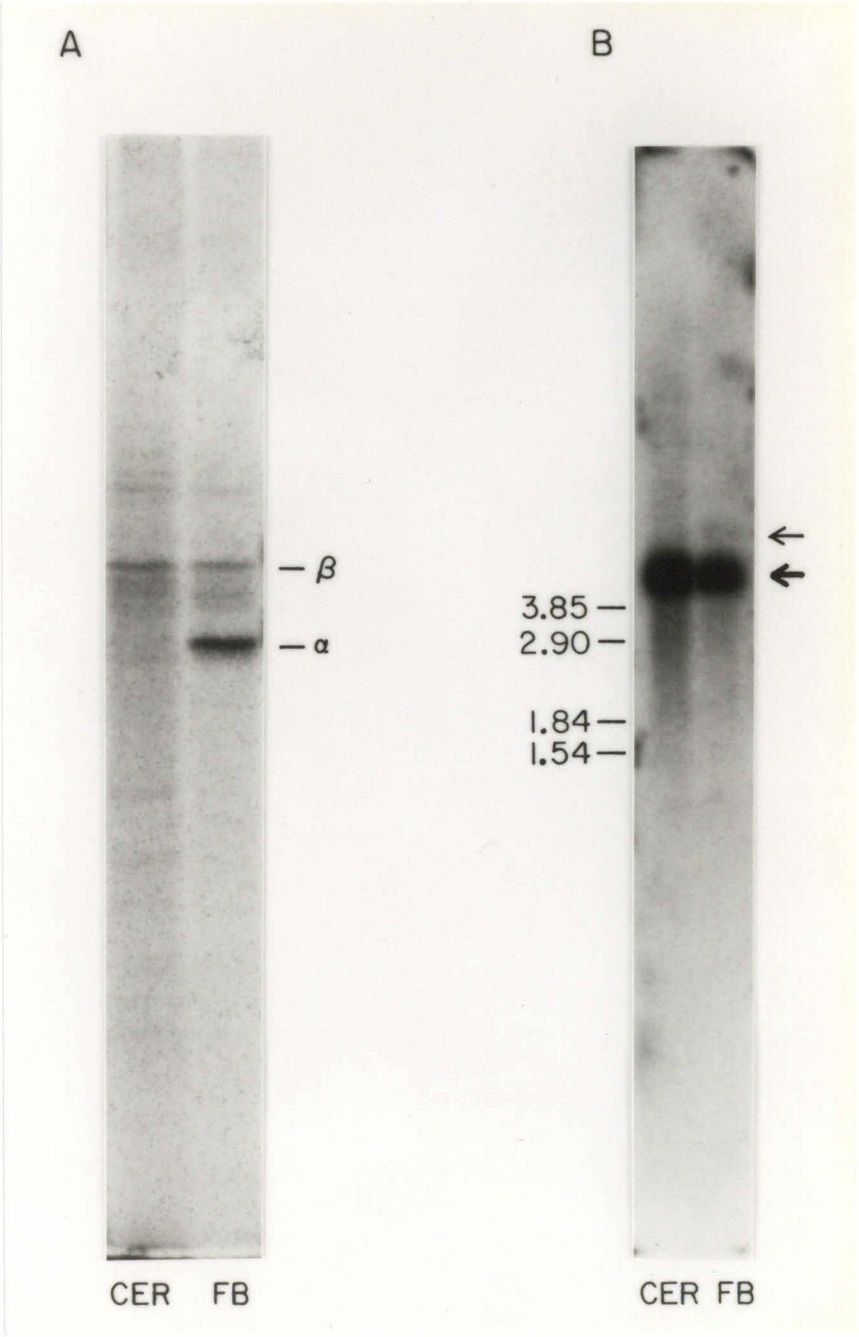
A
B
β
α
3.85
2.90
1.84
1.54
CER FB
CER FB

Figure 3. Restriction map and sequencing strategy of the β subunit cDNA clones. The composite restriction map for the various β subunit cDNA clones is illustrated at the top with the region of vertical stripes indicating the longest open reading frame. The sequencing strategy for each clone is shown separately. The arrows below each clone indicate the direction and extent of each sequencing reaction. Arrows pointing to the left represent sequence determined for the coding strand, and arrows pointing to the right represent sequence determined for the anti-coding strand. The thin arrows represent sequencing reactions primed with a universal sequencing primer, and the thick arrows represent sequencing reactions primed with a β subunit specific oligonucleotide. For each clone, the solid bar represents the region for which the sequence was identical between at least two independent clones (1.84 kb total). The open bar represents regions of the clones which extended beyond the 1.84 kb region and which were not consistent between independent clones. These regions probably represent a cloning artifact (see Results). The hatched bar represents sequences within the 1.84 kb region which were inconsistent with at least two other independent clones. The distance from the end of the cDNA sequence to the 5' end of the β subunit message was determined by primer extension, and is indicated at the bottom by the arrow labeled primer extension.

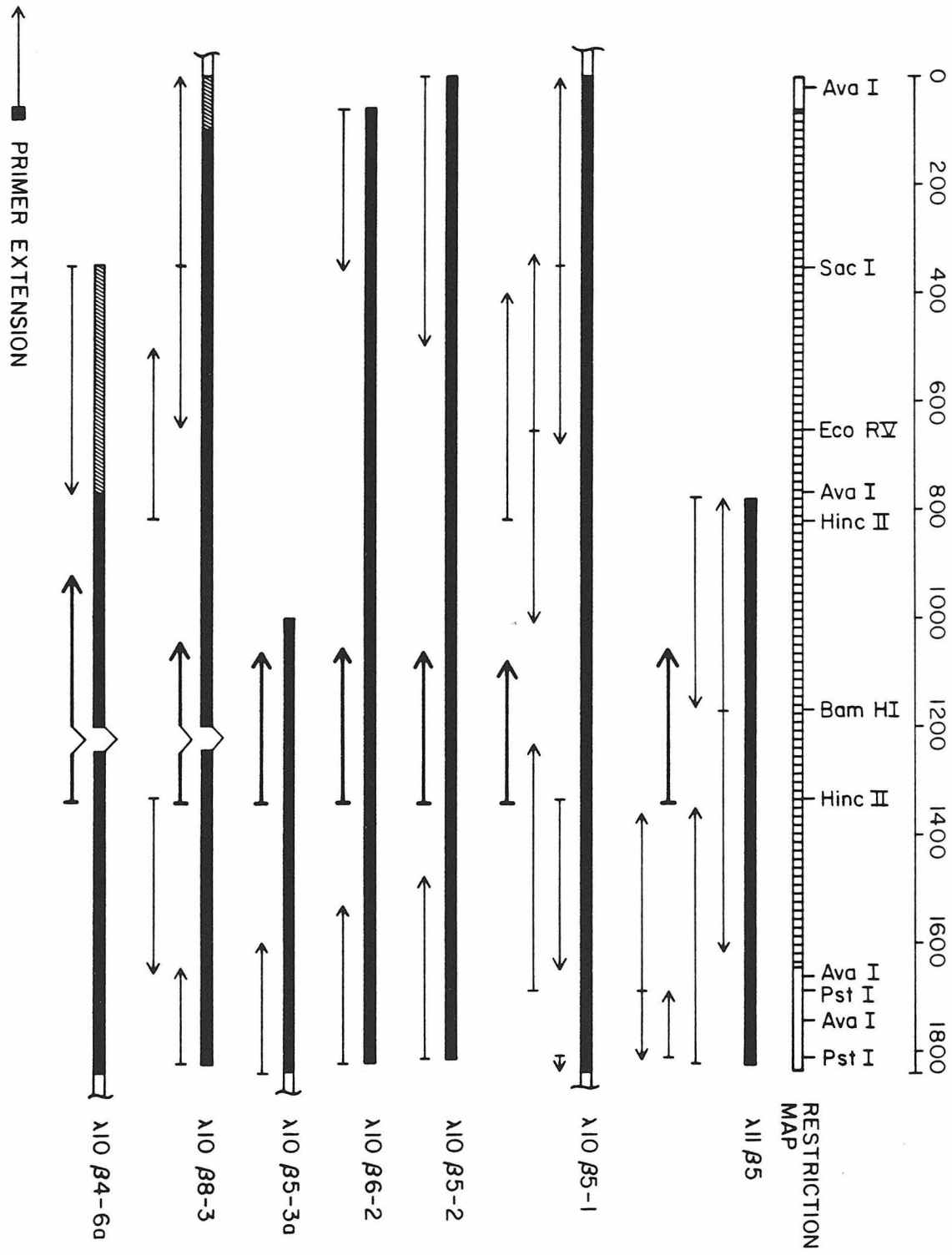
0
200
400
600
800
1000
1200
1400
1600
1800
Ava I
Sac I
Eco RV
Ava I
Hinc II
Bam HI
Hinc II
Ava I
Pst I
Ava I
Pst I
RESTRICTION MAP
λ11 β5
λ10 β5-1
λ10 β5-2
λ10 β6-2
λ10 β5-3a
λ10 β8-3
λ10 β4-6a
PRIMER EXTENSION

Figure 4. Nucleotide sequence and predicted amino acid sequence of the β subunit of type II CaM kinase. The sequence was compiled by the analysis of the overlapping β subunit cDNA clones illustrated in Fig. 3. The predicted amino acid sequence for the longest open reading frame is shown below the DNA sequence. The sequence underlined (nucleotides 1194-1238) is the region deleted in clones λ10β8-3 and λ10β4-6a.

```
   GG AGCCGGAGTC GCCGCCGCCC GAGCGCAGCC GAGCGCACGC CGAGCCCAAT CGCCACCGCC      62

ATG GCC ACC ACG GTG ACC TGC ACC CGT TTC ACG GAC GAG TAC CAG CTA TAC GAG     116
Met Ala Thr Thr Val Thr Cys Thr Arg Phe Thr Asp Glu Tyr Gln Leu Tyr Glu      18

GAT ATT GGC AAG GGG GCT TTC TCT GTG GTC CGA CGC TGT GTC AAG CTC TGC ACC     170
Asp Ile Gly Lys Gly Ala Phe Ser Val Val Arg Arg Cys Val Lys Leu Cys Thr      36

GGC CAT GAG TAT GCA GCT AAG ATC ATT AAC ACC AAG AAG CTG TCA GCT AGA GAT     224
Gly His Glu Tyr Ala Ala Lys Ile Ile Asn Thr Lys Lys Leu Ser Ala Arg Asp      54

CAC CAG AAG CTG GAG AGG GAG GCT CGG ATC TGC CGC CTG CTG AAG CAT TCC AAC     278
His Gln Lys Leu Glu Arg Glu Ala Arg Ile Cys Arg Leu Leu Lys His Ser Asn      72

ATT GTA CGC CTC CAT GAC AGC ATC TCT GAA GAG GGC TTC CAC TAC CTG GTC TTC     332
Ile Val Arg Leu His Asp Ser Ile Ser Glu Glu Gly Phe His Tyr Leu Val Phe      90

GAC CTG GTC ACT GGT GGG GAG CTC TTT GAA GAC ATT GTG GCG AGA GAG TAC TAC     386
Asp Leu Val Thr Gly Gly Glu Leu Phe Glu Asp Ile Val Ala Arg Glu Tyr Tyr     108

AGT GAG GCT GAC GCC AGT CAC TGT ATC CAG CAG ATC CTG GAG GCT GTT CTC CAT     440
Ser Glu Ala Asp Ala Ser His Cys Ile Gln Gln Ile Leu Glu Ala Val Leu His     126

TGT CAC CAA ATG GGG GTC GTC CAC AGA GAC CTC AAG CCT GAA AAC CTC CTG CTC     494
Cys His Gln Met Gly Val Val His Arg Asp Leu Lys Pro Glu Asn Leu Leu Leu     144

GCC AGC AAA TGC AAA GGG GCC GCA GTG AAA CTG GCA GAC TTC GGC CTG GCC ATC     548
Ala Ser Lys Cys Lys Gly Ala Ala Val Lys Leu Ala Asp Phe Gly Leu Ala Ile     162

GAG GTT CAG GGA GAC CAG CAG GCA TGG TTT GGA TTT GCG GGA ACA CCA GGC TAC     602
Glu Val Gln Gly Asp Gln Gln Ala Trp Phe Gly Phe Ala Gly Thr Pro Gly Tyr     180

CTG TCT CCC GAA GTT CTT CGG AAG GAG GCC TAT GGC AAA CCA GTG GAT ATC TGG     656
Leu Ser Pro Glu Val Leu Arg Lys Glu Ala Tyr Gly Lys Pro Val Asp Ile Trp     198

GCA TGT GGG GTG ATC CTG TAT ATC CTG CTG GTG GGA TAC CCA CCT TTC TGG GAT     710
Ala Cys Gly Val Ile Leu Tyr Ile Leu Leu Val Gly Tyr Pro Pro Phe Trp Asp     216

GAG GAC CAG CAC AAG CTG TAC CAG CAG ATC AAG GCT GGG GCC TAT GAC TTC CCA     764
Glu Asp Gln His Lys Leu Tyr Gln Gln Ile Lys Ala Gly Ala Tyr Asp Phe Pro     234

TCC CCC GAG TGG GAC ACC GTT ACC CCT GAA GCC AAA AAC CTC ATC AAC CAG ATG     818
Ser Pro Glu Trp Asp Thr Val Thr Pro Glu Ala Lys Asn Leu Ile Asn Gln Met     252

TTG ACC ATC AAC CCC GCC AAG CGC ATC ACG GCC CAC GAG GCC CTG AAG CAC CCA     872
Leu Thr Ile Asn Pro Ala Lys Arg Ile Thr Ala His Glu Ala Leu Lys His Pro     270

TGG GTC TGC CAA CGA TCC ACG GTG GCC TCC ATG ATG CAC AGA CAG GAG ACT GTG     926
Trp Val Cys Gln Arg Ser Thr Val Ala Ser Met Met His Arg Gln Glu Thr Val     288

GAA TGT CTG AAG AAG TTC AAT GCA AGG AGG AAG CTC AAG GGA GCC ACT CTC ACC     980
Glu Cys Leu Lys Lys Phe Asn Ala Arg Arg Lys Leu Lys Gly Ala Ile Leu Thr     306

ACT ATG CTG GCC ACA CGG AAT TTC TCA GTG GGC AGA CAG ACC ACC GCT CCG GCC    1034
Thr Met Leu Ala Thr Arg Asn Phe Ser Val Gly Arg Gln Thr Thr Ala Pro Ala     324

ACA ATG TCC ACC GCG GCC TCC GGC ACC ACC ATG GGG CTG GTG GAA CAA GCC AAG    1088
Thr Met Ser Thr Ala Ala Ser Gly Thr Thr Met Gly Leu Val Glu Gln Ala Lys     342

AGT TTA CTC AAC AAG AAA GCA GAC GGA GTC AAG CCC CAG ACA AAC AGC ACC AAA    1142
Ser Leu Leu Asn Lys Lys Ala Asp Gly Val Lys Pro Gln Thr Asn Ser Thr Lys     360

AAC AGC TCG GCC ATC ACC AGC CCC AAA GGA TCC CTC CCT CCT GCC GCC CTG GAG    1196
Asn Ser Ser Ala Ile Thr Ser Pro Lys Gly Ser Leu Pro Pro Ala Ala Leu Glu     378

CCT CAA ACC ACC GTT ATC CAT AAC CCA GTG GAC GGC ATT AAG GAA TCT TCC GAC    1250
Pro Gln Thr Thr Val Ile His Asn Pro Val Asp Gly Ile Lys Glu Ser Ser Asp     396

AGC ACC AAC ACA ACC ATA GAG GAC GAA GAT GCC AAA GCC CGG AAG CAG GAA ATC    1304
Ser Thr Asn Thr Thr Ile Glu Asp Glu Asp Ala Lys Ala Arg Lys Gln Glu Ile     414

ATC AAG ACC ACA GAG CAG CTC ATC GAG GCC GTC AAC AAC GGC GAC TTT GAG GCC    1358
Ile Lys Thr Thr Glu Gln Leu Ile Glu Ala Val Asn Asn Gly Asp Phe Glu Ala     432

TAT GCG AAA ATC TGT GAC CCA GGC CTG ACC TCA TTT GAG CCT GAA GCT CTG GGC    1412
Tyr Ala Lys Ile Cys Asp Pro Gly Leu Thr Ser Phe Glu Pro Glu Ala Leu Gly     450

AAC CTG GTC GAA GGG ATG GAT TTC CAC AGA TTC TAC TTT GAG AAC CTG CTG GCC    1466
Asn Leu Val Glu Gly Met Asp Phe His Arg Phe Tyr Phe Glu Asn Leu Leu Ala     468

AAG AAC AGC AAG CCG ATC CAC ACC ACT ATC CTG AAC CCG CAC GTG CAC GTC ATC    1520
Lys Asn Ser Lys Pro Ile His Thr Thr Ile Leu Asn Pro His Val His Val Ile     486

GGC GAG GAT GCA GCC TGC ATC GCT TAC ATC CGC CTC ACA CAG TAC ATC GAC GGC    1574
Gly Glu Asp Ala Ala Cys Ile Ala Tyr Ile Arg Leu Thr Gln Tyr Ile Asp Gly     504

CAG GGC AGA CCC CGC ACC AGC CAG TCC GAA GAG ACC CGT GTG TGG CAC CGC CCC    1628
Gln Gly Arg Pro Arg Thr Ser Gln Ser Glu Glu Thr Arg Val Trp His Arg Pro     522

GAC GGC AAG TGG CAG AAT GTC CAT TTC CAC TGC TCG GGC GCT CCA GTG GCC CCA    1682
Asp Gly Lys Trp Gln Asn Val His Phe His Cys Ser Gly Ala Pro Val Ala Pro     540

CTG CAG TGA GAGCTGCGCC TGGTTTCACC GGACAGAGTT GGTGTTTGGA GCCCAGCCGC        1741
Leu Gln  *                                                                542

CCTCGGGCGC ACGGCCTGCC TGTCGCATGT TTGTGTCTGC CTCGTCCCCT CCCCTGGTGC        1801

CTGTGTCTGC AGAAAAACAA GACCAGATGT GATTTGTTT                                1840
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Figure 5. Amino acid sequence alignment of the β subunit of type II CaM kinase (type II-β), the γ subunit of phosphorylase b kinase (PbK-γ), skeletal muscle myosin light chain kinase (MLCK), and the catalytic subunit of cAMP-dependent protein kinase (cAK-C). The sequence of PbK-γ, MLCK, and cAK-C are from Reimann et al. (1984), Takio et al. (1985), and Shoji et al. (1981), respectively. The residue numbers (indicated in parentheses) begin with the amino terminal amino acid of each kinase, except for MLCK for which only the sequence of the carboxyl terminal portion of the protein has been published. For MLCK the numbering of Takio et al. (1985) is used. Sites of amino acid identity between the β subunit sequence and the other kinases are enclosed in a box, and gaps inserted to optimize the alignment are indicated by a dash. The putative ATP binding site is at Lys-43 of the β subunit sequence and is marked with an asterisk. The putative calmodulin binding domains for the β subunit of the type II CaM kinase (amino acids 295-315), the γ subunit of phosphorylase b kinase (amino acids 322-345), and skeletal muscle myosin light chain kinase (amino acids 342-260), are underlined.

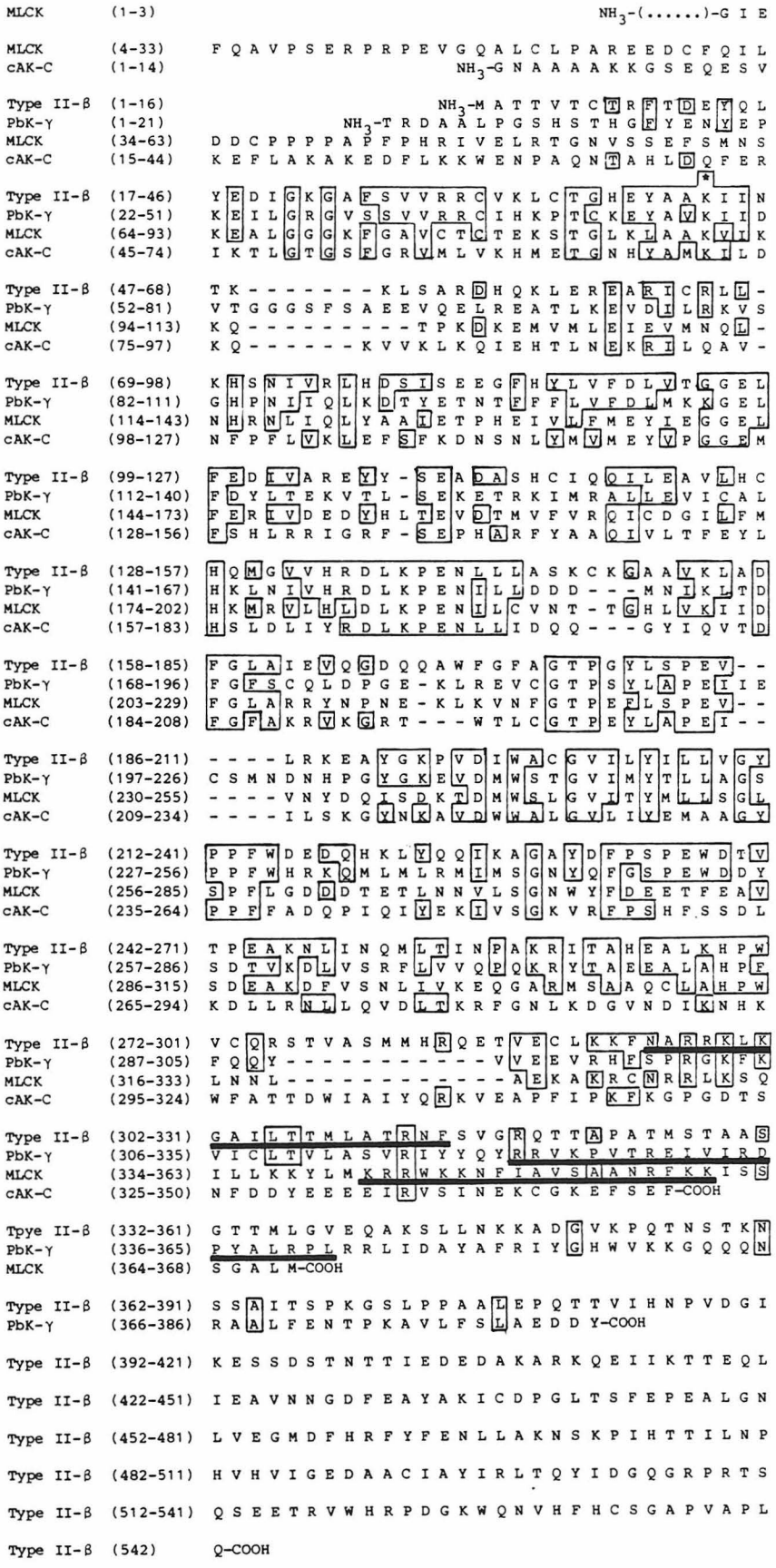
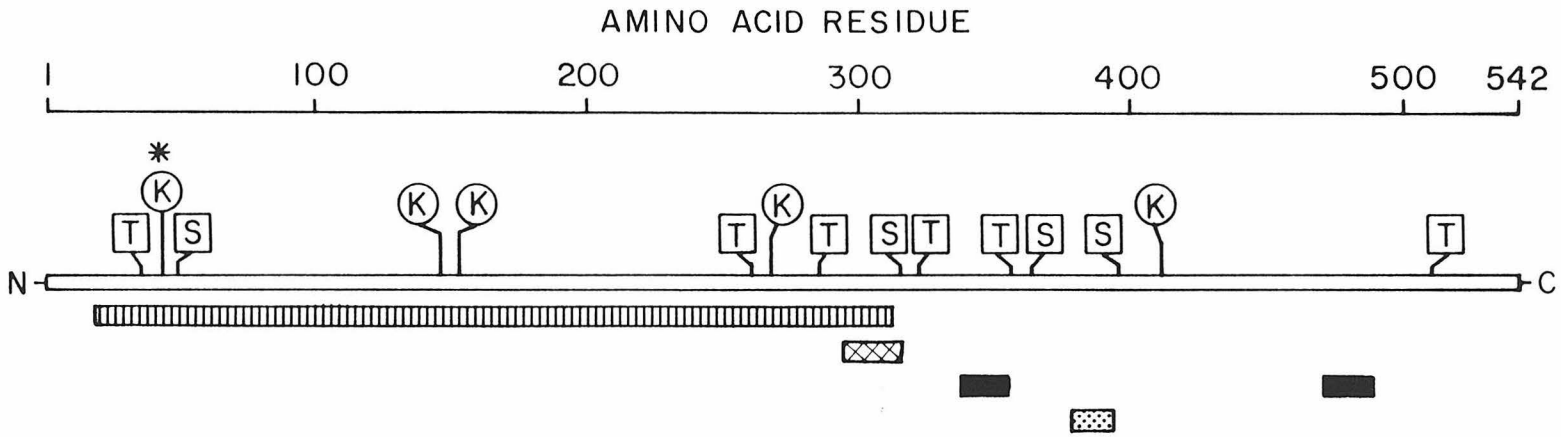

MLCK (1-3) NH3-(.....)-G I E
MLCK (4-33) F Q A V P S E R P R P E V G Q A L C L P A R E E D C F Q I L
cAK-C (1-14) NH3-G N A A A A K K G S E Q E S V
Type II-β (1-16) NH3-M A T T V T C T R F T D E Y Q L
PbK-γ (1-21) NH3-T R D A A L P G S H S T H G F Y E N Y E P
MLCK (34-63) D D C P P P P A P F P H R I V E L R T G N V S S E F S M N S
cAK-C (15-44) K E F L A K A K E D F L K K W E N P A Q N T A H L D Q F E R
Type II-β (17-46) Y E D I G K G A F S V V R R C V K L C T G H E Y A A K I I N
PbK-γ (22-51) K E I L G R G V S S V V R R C I H K P T C K E Y A V K I I D
MLCK (64-93) K E A L G G G K F G A V C T C T E K S T G L K L A A K V I K
cAK-C (45-74) I K T L G T G S F G R V M L V K H M E T G N H Y A M K I L D
Type II-β (47-68) T K - - - - - - - K L S A R D H Q K L E R E A R I C R L L -
PbK-γ (52-81) V T G G G S F S A E E V Q E L R E A T L K E V D I L R K V S
MLCK (94-113) K Q - - - - - - - - - - T P K D K E M V M L E I E V M N Q L -
cAK-C (75-97) K Q - - - - - - - K V V K L K Q I E H T L N E K R I L Q A V -
Type II-β (69-98) K H S N I V R L H D S I S E E G F H Y L V F D L V T G G E L
PbK-γ (82-111) G H P N I I Q L K D T Y E T N T F F F L V F D L M K K G E L
MLCK (114-143) N H R N L I Q L Y A A I E T P H E I V L F M E Y I E G G E L
cAK-C (98-127) N F P F L V K L E F S F K D N S N L Y M V M E Y V P G G E M
Type II-β (99-127) F E D I V A R E Y Y - S E A D A S H C I Q Q I L E A V L H C
PbK-γ (112-140) F D Y L T E K V T L - S E K E T R K I M R A L L E V I C A L
MLCK (144-173) F E R I V D E D Y H L T E V D T M V F V R Q I C D G I L F M
cAK-C (128-156) F S H L R R I G R F - S E P H A R F Y A A Q I V L T F E Y L
Type II-β (128-157) H Q M G V V H R D L K P E N L L L A S K C K G A A V K L A D
PbK-γ (141-167) H K L N I V H R D L K P E N I L L D D D - - - M N I K L T D
MLCK (174-202) H K M R V L H L D L K P E N I L C V N T - T G H L V K I I D
cAK-C (157-183) H S L D L I Y R D L K P E N L L I D Q Q - - - G Y I Q V T D
Type II-β (158-185) F G L A I E V Q G D Q Q A W F G F A G T P G Y L S P E V - -
PbK-γ (168-196) F G F S C Q L D P G E - K L R E V C G T P S Y L A P E I I E
MLCK (203-229) F G L A R R Y N P N E - K L K V N F G T P E F L S P E V - -
cAK-C (184-208) F G F A K R V K G R T - - - W T L C G T P E Y L A P E I - -
Type II-β (186-211) - - - - L R K E A Y G K P V D I W A C G V I L Y I L L V G Y
PbK-γ (197-226) C S M N D N H P G Y G K E V D M W S T G V I M Y T L L A G S
MLCK (230-255) - - - - V N Y D Q I S D K T D M W S L G V I T Y M L L S G L
cAK-C (209-234) - - - - I L S K G Y N K A V D W W A L G V L I Y E M A A G Y
Type II-β (212-241) P P F W D E D Q H K L Y Q Q I K A G A Y D F P S P E W D T V
PbK-γ (227-256) P P F W H R K Q M L M L R M I M S G N Y Q F G S P E W D D Y
MLCK (256-285) S P F L G D D D T E T L N N V L S G N W Y F D E E T F E A V
cAK-C (235-264) P P F F A D Q P I Q I Y E K I V S G K V R F P S H F S S D L
Type II-β (242-271) T P E A K N L I N Q M L T I N P A K R I T A H E A L K H P W
PbK-γ (257-286) S D T V K D L V S R F L V V Q P Q K R Y T A E E A L A H P F
MLCK (286-315) S D E A K D F V S N L I V K E Q G A R M S A A Q C L A H P W
cAK-C (265-294) K D L L R N L L Q V D L T K R F G N L K D G V N D I K N H K
Type II-β (272-301) V C Q R S T V A S M M H R Q E T V E C L K K F N A R R K L K
PbK-γ (287-305) F Q Q Y - - - - - - - - - - - V V E E V R H F S P R G K F K
MLCK (316-333) L N N L - - - - - - - - - - - - A E K A K R C N R R L K S Q
cAK-C (295-324) W F A T T D W I A I Y Q R K V E A P F I P K F K G P G D T S
Type II-β (302-331) G A I L T T M L A T R N F S V G R Q T T A P A T M S T A A S
PbK-γ (306-335) V I C L T V L A S V R I Y Y Q Y R R V K P V T R E I V I R D
MLCK (334-363) I L L K K Y L M K R R W K K N F I A V S A A N R F K K I S S
cAK-C (325-350) N F D D Y E E E E I R V S I N E K C G K E F S E F-COOH
Tpye II-β (332-361) G T T M L G V E Q A K S L L N K K A D G V K P Q T N S T K N
PbK-γ (336-365) P Y A L R P L R R L I D A Y A F R I Y G H W V K K G Q Q Q N
MLCK (364-368) S G A L M-COOH
Type II-β (362-391) S S A I T S P K G S L P P A A L E P Q T T V I H N P V D G I
PbK-γ (366-386) R A A L F E N T P K A V L F S L A E D D Y-COOH
Type II-β (392-421) K E S S D S T N T T I E D E D A K A R K Q E I I K T T E Q L
Type II-β (422-451) I E A V N N G D F E A Y A K I C D P G L T S F E P E A L G N
Type II-β (452-481) L V E G M D F H R F Y F E N L L A K N S K P I H T T I L N P
Type II-β (482-511) H V H V I G E D A A C I A Y I R L T Q Y I D G Q G R P R T S
Type II-β (512-541) Q S E E T R V W H R P D G K W Q N V H F H C S G A P V A P L
Type II-β (542) Q-COOH

Figure 6. Potential functional domains of the β subunit of the type II CaM kinase. A diagram of the β subunit protein is shown. The positions of potential ATP binding and autophosphorylation sites are indicated on the protein map. The domains corresponding to other potential structural or functional characteristics of the β subunit are indicated by the bars beneath the protein map.



POTENTIAL ATP BINDING SITE: A X (K)

POTENTIAL AUTOPHOSPHORYLATION SITES: $\begin{matrix}R\\K\end{matrix}$ XX $\begin{matrix}[S]\\[T]\end{matrix}$

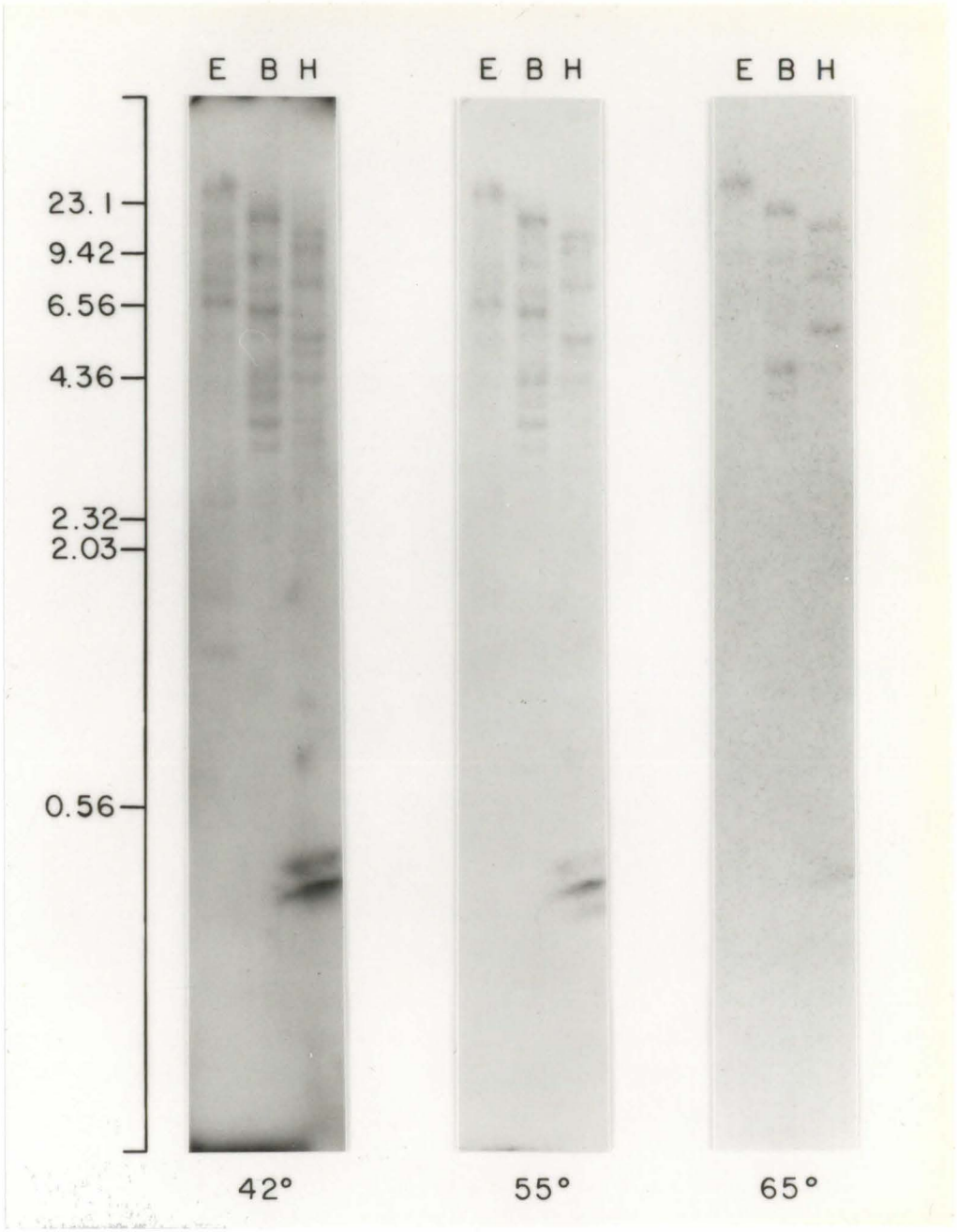
HOMOLOGOUS TO OTHER KINASES:

POTENTIAL CALMODULIN BINDING DOMAIN:

HOMOLOGOUS TO α SUBUNIT AMINO ACID SEQUENCE:

POTENTIALLY DELETED IN β′ SUBUNIT:

Figure 7. Southern blots. 10 μg of rat brain DNA was digested with EcoRI (E), BamHI (B), and HindIII (H), fractionated on a 1% agarose gel, denatured and transferred to a nitrocellulose filter. The filter was probed with 0.5 μg of ^{32}P-labeled single stranded M13 clone λ10β5-2 ($2x10^8$ cpm/μg of insert) and washed in 0.1X SSPE, 0.1% SDS at the temperatures indicated. The migration of λ-HindIII DNA markers is indicated at the left.


E B H
E B H
E B H
23.1
9.42
6.56
4.36
2.32
2.03
0.56
42°
55°
65°

CHAPTER 4

AMINO ACID SEQUENCE AND OLIGONUCLEOTIDE PROBES FOR THE α SUBUNIT OF NEURONAL TYPE II Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE

Abstract

In order to facilitate the comparison between the α and β subunits of neuronal type II Ca^{2+}/calmodulin dependent protein kinase, proteolytic peptides derived from the α subunit have been isolated and partially sequenced. The first 16 amino acids of a 20,000 Dalton chymotryptic peptide were determined and used to generate two α subunit specific oligonucleotide probes. The probes react with a 5.4 kb message which is more abundant in the forebrain than in the cerebellum, consistent with the distribution of the α subunit protein. The chymotryptic peptide sequence is identical to a short region of β subunit sequence at 9 out of 16 positions. Nineteen amino acids of "mixed sequence" was determined for a 7,000 Dalton tryptic peptide. This sequence is identical to a region of β subunit sequence at 10 out of 19 positions.

As described in the previous chapter, cDNA clones coding for the β subunit of type II CaM kinase have been isolated and characterized. In order to understand the relationship between the α and β subunits, and to continue the molecular characterization of the type II CaM kinase, I have been trying to isolate cDNA clones coding for the α subunit. Because the immunological screening of a λGT11 expression library failed to isolate any α subunit clones (Chap. 3), a second general approach has been taken. This second general approach involves the determination of some amino acid sequence for the protein of interest, the generation of oligonucleotide probes predicted from the amino acid sequence, and the use of the oligonucleotides to screen a cDNA library for hybridizing sequences. This approach has been used successfully to isolate cDNA clones for many proteins, including the subunits of the nicotinic acetylcholine receptor (Noda et al., 1982; Noda et al., 1983a; Noda et al., 1983b), and the sodium channel (Noda et al., 1984). In this chapter, the determination of a portion of the amino acid sequence for the α subunit of the type II CaM kinase and the generation of α subunit specific oligonucleotide probes is described.

Materials and Methods

Materials. Trypsin (TPCK treated) was from Worthington and chymotrypsin was from Sigma. [γ^{32}P] ATP was purchased from ICN Nutritional Biochemicals. Oligo dT-cellulose and polynucleotide kinase were from Pharmacia P-L Biochemicals. PEI cellulose thin layer chromatography plates containing a fluorescent indicator were from E-M Laboratories. Dithiothreitol and tRNA were from Boeringer Mannheim Biochemicals. Nitrocellulose filters (BA85, 0.45 μm) were purchased from Schleicher and Schuell. Synthetic oligonucleotides were prepared by Dr. Suzanna Horvath of Caltech.

Peptide isolation and sequencing. Purified forebrain kinase (770 µg) was digested with trypsin (3.1 µg) at 30° for 2 h or purified forebrain kinase (400 µg) was digested with chymotrypsin (800 ng) at 30° for 1.5 h. The proteolyzed sample was brought to 2% SDS and 3% β-mercaptoethanol, placed in a boiling water bath for 2 min, and loaded onto an HPLC gel filtration column. The absorbance of the column effluent was monitored at 214 nm and the peak fractions were pooled and analyzed on 15% polyacrylamide SDS gels. The purified peptides were then dialyzed against 10 mM NH_4HCO_3, 0.02% SDS for 40 h, lyophilized, and redissolved in 50 µl of double distilled H_2O. Approximately 1 nmole of each peptide was subjected to automated Edman degradation on a gas phase sequenator (Hunkapiller et al., 1983). PTH-derivatized amino acids were identified by reversed-phase HPLC (Hunkapiller and Hood, 1983). The peptide isolation and protein sequence determination was done in collaboration with Jim Hurley of Caltech.

Oligonucleotide purification and labeling. Oligonucleotides were resolved on a preparative 20% polyacrylamide gel containing 7 M urea. The most abundant oligonucleotide was localized by placing a thin layer chromatography plate containing a fluorescent indicator under the gel and illuminating with a short wave uv lamp from above. The appropriate region of the gel was excised and cut into small pieces with a razor blade. The oligonucleotide was eluted into 3 ml of 0.5 M NH_4 acetate, 0.1 M Mg acetate, 1 mM EDTA, and 0.1% SDS at 37° overnight. The gel pieces were removed by centrifugation and the elution buffer concentrated to 200 µl by butanol extraction. The oligonucleotide was then desalted on a 4 ml Sepharose G-25 column, lyophilized, and redissolved in double distilled H_2O to a concentration of 1 mg/ml.

Oligonucleotides (1 µg) were end labeled in a reaction containing 70 mM Tris pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 10 µM [γ^{32}P] ATP (7000

Ci/mmole), and 4 U polynucleotide kinase at 37° for 30 min. Unincorporated label was removed by chromatography on a 300 µl DEAE-cellulose column as described (Meinkoth and Wahl, 1984). Probes were labeled to a specific activity of 2-10x10^8 cpm/µg.

Northern blots. RNA was purified from rat brain regions (forebrain and cerebellum) by extraction with guanidine thiocyanate as described by Chirgwin et al. (1979). Poly-A^+ RNA was isolated by two cycles of adsorption to and elution from oligo dT-cellulose. (Aviv and Leder, 1972). RNA was size fractionated by electrophoresis through 1% agarose gels containing 2.2 M formaldehyde and transferred to nitrocellulose filters as described (Maniatis, et al., 1982). The filters were prehybridized in 0.9 M NaCl, 6 mM EDTA, 90 mM Tris pH 7.5, 0.1% SDS, and 100 µg/ml homochromatography mix I (randomly cleaved tRNA described by Jay, et al., 1974) for 2 h at 68°. The filters were hybridized at 42° for 20 h in the above buffer containing the ^{32}P-labeled oligonucleotide probe (3x10^6 cpm/ml). The filters were then washed 5 times for 5 min each with 6X SSC (1X SSC is 0.15 M NaCl, 15 mM Na citrate) at 42° and exposed to X-ray film.

Results

Peptide generation and sequencing. Peptides were generated by proteolytic digestion of the forebrain kinase holoenzyme and isolated by HPLC gel filtration chromatography. Three peptides were isolated with sufficient yield and purity to be suitable for sequencing. These were: 1) a 30,000 Dalton tryptic peptide, 2) a 7,000 Dalton tryptic peptide, and 3) a 20,000 Dalton chymotryptic peptide. The abundance of all three peptides in the original digest and their yield after purification indicated that they were all derived from the α subunit. Sequence analysis indicated that the 30,000 Dalton tryptic peptide has a blocked

amino terminal. This peptide may represent the amino terminal peptide of the α subunit, since the amino terminal of the α subunit is blocked, both in a native state and as isolated from SDS gels (M. Bennett and B. Conti-Tronconi, unpublished observation). The 7,000 Dalton tryptic peptide generated a "mixed sequence" (multiple residues in most of the sequencing cycles, see Fig. 3) indicating the existence of multiple peptide species in the sample. Sequence analysis of the 20,000 Dalton chymotryptic peptide yielded the first 16 amino acids of this peptide. This sequence was used to generate two α subunit specific oligonucleotide probes.

Oligonucleotide probes. The amino acid sequence determined for the 20,000 Dalton chymotryptic peptide is shown in Fig. 1 (middle). This amino acid sequence was reverse translated into all the possible mRNA sequences which could code for it. Two partially overlapping regions of the mRNA sequence were chosen for the generation of complementary oligonucleotide probes. These two regions were chosen because of their relatively low codon redundancy. One of the probes synthesized (MK-I) was complementary to all possible codons for amino acids 8-13 of the peptide (excluding the third nucleotide of the Gly-13 codon) and consisted of a mixture of 32 septadecamers (Fig. 1, top). The other probe (MK-II) was complementary to most of the possible codons for amino acids 11-16 of the peptide (excluding the third nucleotide of the Glu-16 codon) and consisted of a mixture of 48 septadecamers (Fig. 1, bottom). In order to reduce the redundancy of probe MK-II, three nucleotides at two positions in the codon sequence (the third nucleotides for the Gly-13 and the Val-14 codons) were eliminated from the probe because of their low frequency of usage (see Fig. 1).

Northern blots. Both oligonucleotide probes reacted with a 5.4kb message present in brain poly-A^+ RNA, but not in brain poly-A^- or liver poly-A^+ RNA (data not shown). This result indicates that the guesses made when making probe

MK-II were probably correct. The distribution of the message recognized by probe MK-I between the forebrain and cerebellum is shown in Fig. 2. The message is much more abundant in the forebrain than in the cerebellum, as is the α subunit protein and message (see Chapter 3, Fig. 2). The size (5.4kb) and distribution (forebrain >> cerebellum) of this message is identical to that of a message recognized by a β subunit cDNA probe at low stringency (see Chap. 3). These results lend further support to the idea that the oligonucleotide probes are α subunit specific.

Sequence homology. The amino acid sequence from the 20,000 Dalton chymotryptic peptide was compared to the β subunit amino acid sequence deduced from the β subunit cDNA clones described in Chap. 3. A region of significant homology was identified from amino acids 339-354 of the β subunit, in which 9 out of 16 amino acids were identical to the α subunit peptide (Fig. 3A). The 7,000 Dalton tryptic peptide "mixed sequence" was also compared to the β subunit sequence. Amino acids 471-489 of the β subunit were identical to the 7,000 Dalton tryptic peptide in 10 out of 19 positions (Fig 3B). These positions of homology within the β subunit sequence are in a region which does not show significant homology to any other protein kinase.

Discussion

Amino acid sequence for peptides derived from the α subunit of neuronal type II CaM kinase has been determined and been used to generate α subunit specific oligonucleotide probes. The α subunit specificity of the oligonucleotide probes was predicted from the abundance of the peptide sequenced, and was confirmed by the reaction of the probes on Northern blots with a message of the expected size and distribution of the α subunit message (Fig. 2). The sequence determined for two different α subunit-derived peptides was homologous with

regions of the β subunit sequence deduced from β subunit cDNA clones (Fig. 3). The regions of homology were in the carboxyl terminal half of the β subunit, the region which was not homologous with the other protein kinases (see Chap. 3, Fig. 6). This indicates that the α and β subunits of the type II CaM kinase are homologous in more regions than just the putative catalytic domain, and are perhaps homologous throughout their entire lengths.

The α subunit specific oligonucleotide probes were used to screen two different λGT10 cDNA libraries (a total of 500,000 clones) in an attempt to isolate α subunit cDNA clones. No clones which consistently reacted positively with both oligonucleotide probes were isolated. Since the α subunit is about three times as abundant as the β subunit, and 24 different β subunit cDNAs were isolated from one of the λGT10 libraries (Chap. 3), one would have expected to isolate many α subunit cDNA clones. There are a number of possible reasons for why I was unable to do so. The most likely reason is the length of the α subunit mRNA. Only about 1.5 kb of sequence is required to code for the α subunit protein (50,000 Daltons) yet the message is about 5.4 kb in length. This additional sequence is likely to be in the form of a long 3' untranslated region. During cDNA synthesis, this entire untranslated region would have to be reverse transcribed before reaching the coding sequence, and at least a portion of the coding sequence would have to be transcribed in order for the cDNA to be detected by the oligonucleotide probes. If the first strand cDNA synthesis was not efficient, or if there was a strong reverse transcriptase stop signal in the 3' untranslated region, then the α subunit coding sequence may not have been transcribed. The reason for the large number of β subunit clones isolated may have been because of an adventitious priming of first strand synthesis near the coding region (see Discussion in Chap. 3).

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Figure 1. Strategy for the production of α subunit specific oligonucleotide probes. The amino acid sequence determined for the 20,000 Dalton chymotryptic peptide is shown in the center. All possible codons coding for residues 8-13 (excluding the third nucleotide of the Gly-13 codon) are shown above the amino acid sequence, and all possible codons coding for residues 11-16 (excluding the third nucleotide of the Glu-16 codon) are shown below the amino acid sequence. Probe MK-I (top) is complementary to all possible codons for residues 8-13 and is a mixture of 32 septadecamers. Probe MK-II (bottom) is complementary to the codons for residues 11-16 and is a mixtrue of 48 septadecamers. In order to reduce the redundancy of probe MK-II, three of the possible codons were left out because of their low frequency of use. These codons are indicatied by the parentheses around one of the nucleotides in the third position of the Gly-13 codon, and two of the nucleotides in the third position of the Val-14 codon.

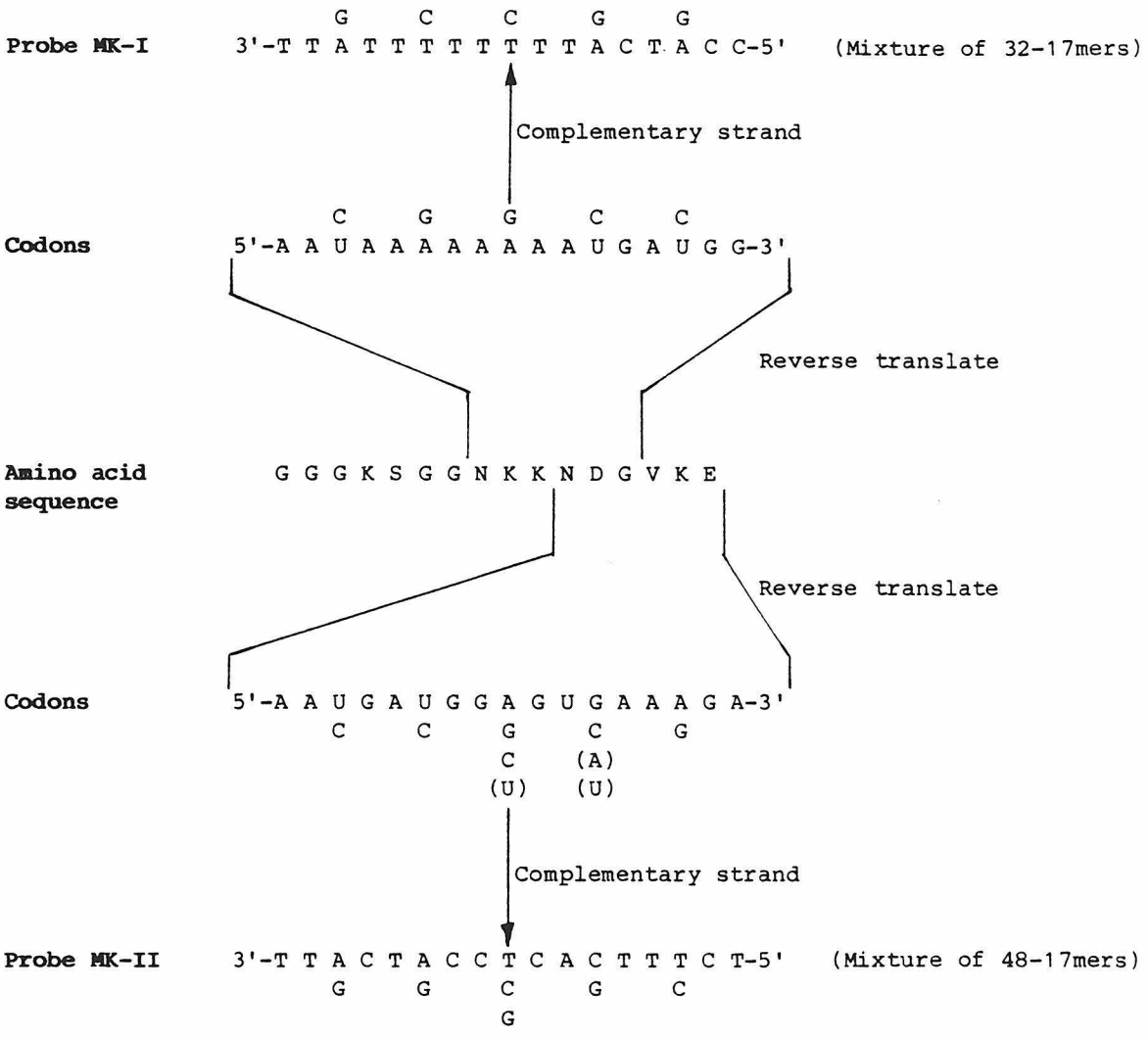
Probe MK-I
3'-T T A T T T T T T T A C T A C C-5'
(Mixture of 32-17mers)
G C C G G
Complementary strand
Codons
5'-A A U A A A A A A A A U G A U G G-3'
C G G C C
Reverse translate
Amino acid sequence
G G G K S G G N K K N D G V K E
Reverse translate
Codons
5'-A A U G A U G G A G U G A A A G A-3'
C C G C G
C (A)
(U) (U)
Complementary strand
Probe MK-II
3'-T T A C T A C C T C A C T T T C T-5'
(Mixture of 48-17mers)
G G C G C
G

Figure 2. Northern blot. 5 μg of forebrain (FB) and cerebellar (CER) poly-A^+ RNA was resolved on a 1% agarose gel containing 2.2 M formaldehyde and transferred to a nitrocellulose filter. The filter was probed with 0.5 μg ^{32}P-labeled MK-I ($2x10^8$ cpm/μg) as described in Materials and Methods. The molecular size markers were E. coli and sea urchin ribosomal RNAs.

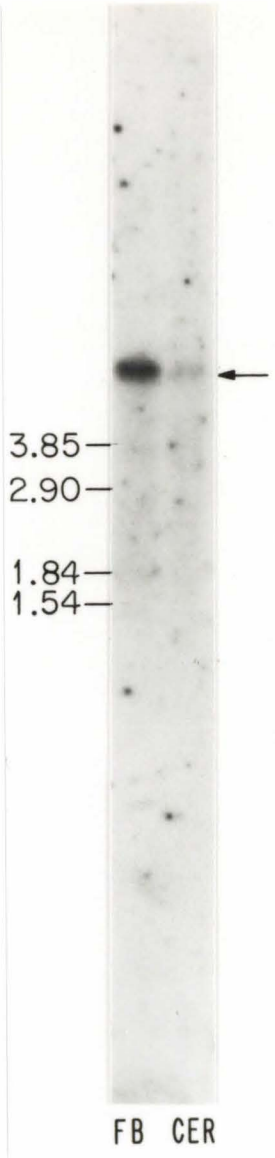
3.85
2.90
1.84
1.54
FB
CER

Figure 3. α and β subunit sequence homology. A. The alignment of the 20,000 Dalton chymotryptic peptide sequence with the β subunit sequence (amino acids 339-354) is shown. Residues which are identical in the two sequences are enclosed in a box. B. The alignment of the 7,000 Dalton tryptic peptide mixed sequence with the β subunit sequence (amino acids 471-489) is shown. Residues which are identical in the two sequences are enclosed in a box.

A

20K chymotryptic peptide G G G K S G G N K K N D G V K E

β-subunit (339-354) E Q A K S L L N K K A D G V K P

B

7K tryptic peptide (mixed sequence)

```
          K           I   L Q           L
  K L S K E V   V Q P E I               I
A I P V A M T E L N Y H V H L M G D E
```

β-subunit (471-489) S K P I H T T I L N P H V H V I G E D

CONCLUSION

The molecular characterization of neuronal type II Ca^{2+}/calmodulin-dependent protein kinase (type II CaM kinase) in vitro has provided insight into the possible function of the enzyme in vivo. Type II CaM kinase is far more highly concentrated in the forebrain than in phylogenetically older brain regions (Erondu and Kennedy, 1985). The hippocampus has the highest concentration of the enzyme, and is an area of the brain where long-term potentiation, a form of synaptic plasticity, is easily elicited (Turner et al., 1982; Lynch and Baudry, 1984). Recent experiments by Miller and Kennedy (1986) have demonstrated that autophosphorylation of the type II CaM kinase causes a portion of its activity to become independent of Ca^{2+}. This property of the enzyme may allow it to act as a molecular switch, and fills some of the criteria for a hypothetical model of memory storage proposed by Lisman (1985). These observations suggest that the type II CaM kinase may be involved in activity dependent changes in synaptic efficacy.

The broad substrate specificity of the type II CaM kinase suggests that it may be involved in the regulation of a number of neuronal processes including neurotransmitter release (Huttner et al., 1983; Llinas et al., 1985), neurotransmitter synthesis (Yamauchi et al., 1981), cytoskeletal structure and interactions (Yamamoto et al., 1983; Yamauchi and Fujisawa, 1983; Edelman et al., 1986) and glycogen metabolism (Cohen, 1982). The general distribution of the kinase throughout neurons (Ouimet er al., 1984) is also suggestive of multiple functional roles in vivo.

Experiments which directly address function have recently become feasible. Potential functional domains (including ATP binding, calmodulin binding, and autophosphorylation sites) have been identified within the amino acid sequence of the β subunit (this thesis) and corresponding domains should be

identified for the α subunit in the near future. Antibodies generated against synthetic peptides corresponding to these functional domains can be generated and tested for their effects on kinase activity in vitro (Herrera et al., 1985). If the antibodies cross-react with the type II CaM kinase from Aplysia or squid, then the function of the enzyme in these species could be addressed by injecting antibodies which block a particular in vitro function into well characterized neurons and observing any effects on their physiology. Additional functional studies could be performed by inhibiting kinase activity in a cell line by the introduction of antisense message (Kim and Wold, 1985) or by generating specific deletion mutants of the cDNAs and expressing them in a cell line such as cos-1 (Meijlink et al., 1985; Pelletier and Sonenberg, 1985). These studies, and others, will be very useful in eventually establishing the role of type II CaM kinase in the regulation of various neuronal processes by Ca^{2+}.

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