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Molecular Characterization of Muscle-specific Calmodulin-Dependent Protein Kinases

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Thesis submitted in partial fulfilment
of the requirements for the degree of
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à la mémoire de mon grand-père, Mastaï Palma "Pit" Plouffe

in memory of my grandparents, Teresa Frances and John Joseph Leddy

ABSTRACT

Calmodulin-dependent protein kinases play an important role in the regulation of muscle function. Activation of calmodulin-dependent protein kinase activity associated with rabbit skeletal muscle sarcoplasmic reticulum (SR) resulted in the phosphorylation of polypeptides of 450, 360, 165, 105, 89, 73, 60, 34 and 20 kDa. Radioligand binding studies indicated that a membrane-bound 60 kDa polypeptide contained both calmodulin (CaM) and ATP-binding domains. Under renaturing conditions on nitrocellulose blots, the 60 kDa polypeptide of the membrane exhibited CaM-dependent autophosphorylation activity, suggesting that it was a CaM-dependent protein kinase. In addition, a 73 kDa polypeptide was also found to bind CaM and undergo CaM-dependent autophosphorylation, suggesting that it too was a CaM-dependent protein kinase. CaM kinase II antibodies cross-reacted with the 60 kDa in Western blots and immunoprecipitated the 60 kDa polypeptide, along with the 360, 105, 89, 73, 34 and 20 kDa phosphoproteins from detergent-solubilized SR membranes. These same antibodies also detected a 73 kDa immunoreactive polypeptide in skeletal and cardiac muscle that could undergo autophosphorylation in a CaM-dependent manner. Subcellular distribution of the 60 kDa kinase indicated the specific association of the polypeptide with the junctional face membrane of SR. The 73 kDa polypeptide was found to be present in much less abundance compared with the 60 kDa polypeptide and was tightly associated with membrane and not cytosolic fractions. Studies have shown that a CaM kinase activity intrinsic to the membrane can inactivate the Ca^{2+} release channel of skeletal muscle

SR. Since our results demonstrated that the 60 and 73 kDa polypeptides of SR are CaM-dependent protein kinases, we postulated that these kinases, through their associations and phosphorylation activities, may be responsible for gating the Ca^{2+} release channel. However, the purified or membrane-associated preparation of the Ca^{2+} release channel was not phosphorylated by the purified CaM kinase preparation although it was a substrate for protein kinase C.

To gain further information on the CaM-dependent protein kinases in muscle, the cDNAs encoding the CaM-dependent protein kinases from skeletal muscle were cloned. A β isoform of the CaM kinase II (CaMKII) family was isolated and encoded a polypeptide of 60 kDa containing numerous functional features typical of the classical CaMKII β isoforms: kinase domain, ATP-binding site, autoinhibitory peptide and calmodulin-binding motif. A second clone, named SOCK, was sequenced and characterized as a novel isoform of CaM-dependent protein kinases. The cDNA sequence of SOCK contained an open reading frame of 2.0 kb coding for a polypeptide of 665 amino acids with a predicted molecular weight of 72,836. The predicted amino acid sequence of SOCK showed that it was similar to the classical β CaMKII isoform but contained a novel peptide sequence of 122 amino acids in the C terminus. This novel domain was enriched in proline residues and was homologous to SH3-binding domains containing the minimal consensus sequence P-X-X-P. Genomic cloning of SOCK indicated that the 122 amino acid sequence was generated via alternative splicing of three exons encoding the three proline-rich domains, two of which were highly homologous. GST-fusion proteins constructs revealed that SOCK, as well as the novel proline-rich

region, bound to SH3 domains of the src family of tyrosine kinases. Furthermore, the SH3 domains could specifically bind the 73 and 60 kDa CaM kinases from soluble membrane extracts implying that these polypeptides represented SOCK or SOCK-related polypeptides. The tissue distribution of SOCK indicated the presence of a 4.8 kb transcript expressed exclusively in skeletal, cardiac and smooth muscle but not in brain, liver, kidney or spleen. Cell fractionation studies, as well as confocal microcopy of epitope-tagged SOCK, showed that SOCK and src can be localized to similar cellular compartments in muscle cells. Binding of SOCK kinase to src resulted in the inhibition of CaM-dependent phosphorylating activity. The potential effect of SOCK expression was studied in embryonal carcinoma P19 cells that differentiate into different types of muscle (cardiac, skeletal and smooth) and neuroectoderm depending on the extracellular medium. P19 cells transfected with an expression plasmid carrying SOCK cDNA failed to differentiate along the mesodermal pathway but not the neuroectodermal pathway. These results indicate that SOCK represents a novel isoform of the β CaM kinase II family that is uniquely structured and may participate to integrate calcium and tyrosine kinase signalling pathways in muscle systems.

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" nos bras et nos jambes

sont d'érables, de pins et d'épinettes

nos visages

sont de soleil et de vent

nos rêves

envolées d'outardes et odeur du bois brûlé

nos joies

fleurs sauvages et ruisseaux d'eau claire"

Gens d'ici,
Jean-Marc Dalpé

"We shall not cease from exploration

And the end of all our exploring

Will be to arrive where we started

And know the place for the first time."

Little Gidding V, Four Quartets

Thomas Stearns Eliot

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LIST OF ABBREVIATIONS

°C	degree Celsius
AMP	adenosine monophosphate
bp	base pair
CaM	calmodulin
CaM K	calcium/calmodulin-dependent protein kinase
cDNA	complementary DNA
CHAPS	3-([cholamidopropyl]-dimethylammonio)-2-hydroxyl-1-propanesulfonate
Da	Daltons
DHP	dihydropyridine
DIFP	diisopropyl fluorophosphate
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GST	glutathione-s-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonate
IgG	immunoglobulin G
MEM	minimal essential medium

MLCK	myosin light chain kinase
NP-40	Nonidet P-40, nonyl phenoxy polyethoxy ethanol
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming units
PIPES	piperazine-N,N'-bis(ethanesulfonic acid)
PK	protein kinase
PMSF	phenylmethylsulfonyl fluoride
RPM	rotations per minute
RyR	ryanodine receptor
SDS	sodium dodecyl sulfate
SOCK	son of CaM kinase
SR	sarcoplasmic reticulum
SSC	standard citrate saline
TBS	tris buffered saline
TCA	tricholoroacetic acid
TRIS	tris(hydroxymethyl)aminomethane
Triton X-100	octylphenol ethylene oxide condensate
T-tubule	transverse-tubule
Tween-20	polyoxyethylene sorbitan monolaurate

A. INTRODUCTION

The development, growth and maintenance of multicellular organisms represents a series of complex transient interactions between a cell and its external environment. External signals activate membrane receptors that transduce the signal to selected downstream effectors via a cascade of covalent modifications to cellular proteins. Phosphorylation appears to represent one of the most important molecular mechanisms by which extracellular stimuli produce their biological response within the cell. Early studies of carbohydrate metabolism demonstrated that enzyme phosphorylation could have a regulatory role (Walsh *et al.*, 1968). Subsequent findings extended this concept, leading to the realization that this biochemical mechanism had widespread importance. Thus, it is clear today that protein phosphorylation is involved not only in the regulation of intermediary metabolism but also in the regulation of a wide variety of other important physiological functions such as neurotransmission, secretion, cellular homeostasis and muscle contraction and development (Walaas and Greengard, 1991).

A.1 Protein Kinases

Specialized phosphotransferases, named protein kinases, catalyze the protein phosphorylation reaction by transferring a phosphoryl group, usually the gamma phosphoryl group of ATP, to a specific amino acid in a targeted protein. Phosphorylation is a completely and readily reversible process. The removal of phosphate groups is catalyzed by phosphatases that reset proteins to their dephosphorylated state. At the biochemical level, the addition or

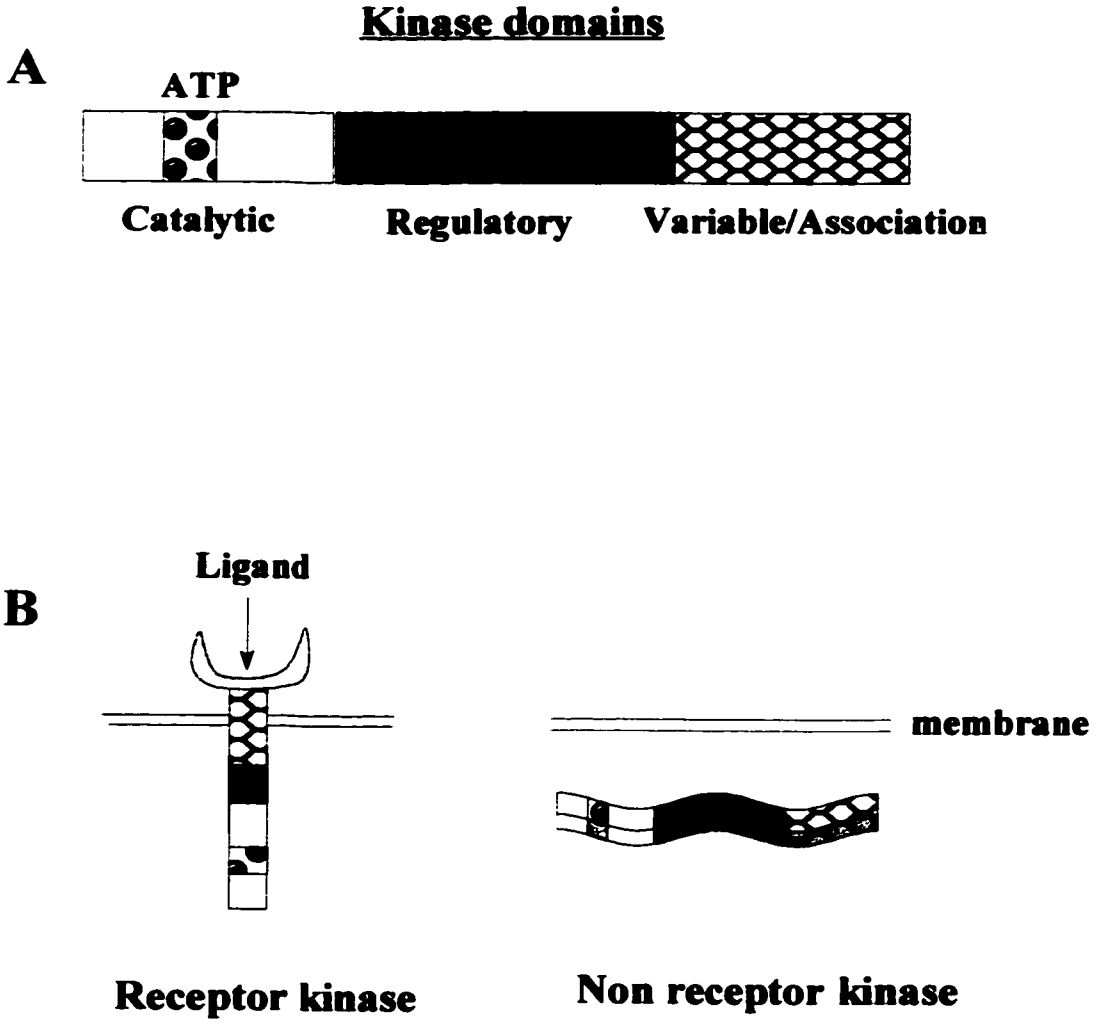
removal of a phosphate group results in the stimulation or inhibition of many proteins including enzymes, receptors, ion channels and structural proteins (Girault, 1993).

Several hundred different genes coding for protein kinases and phosphatases have now been identified. All kinases are thought to derive from a single ancestral gene and share a basic modular structure (Hunter, 1991). Each polypeptide module or domain is a distinct structural unit that imparts certain functional properties to the enzyme. Protein kinases generally contain at least four broadly defined modules: an ATP-binding domain, a catalytic domain, a regulatory domain and a variable or association domain (Fig. 1). These domains are often, though not necessarily, located on a single polypeptide chain. They can be assembled to form either cytoplasmic, membrane-bound or receptor kinases. By their very nature, protein kinases must contain a domain that binds to the ATP molecule and a domain that transfers the phosphate to the substrate. The ATP-binding domain of protein kinases is characterized by a consensus sequence of eight invariant amino acids of which a lysine residue is critical for kinase activity. By contrast, the catalytic domain is much larger, although it does not usually exceed three hundred amino acids in length (Hanks *et al.*, 1988). Catalytic domains contain the polypeptide sequence responsible for facilitating the phosphate transfer between ATP and substrate. Because protein kinases often have multiple substrates, it has become more convenient to classify them according to the amino acids they target. Such phosphoryl acceptors include the alcohol group of the amino acids serine and threonine, as well as the phenol group of tyrosine. The resulting protein kinases classes are serine/threonine kinases, tyrosine kinases and dual specificity kinases that target both serine/threonine and tyrosine residues. Of these three classes, serine/threonine kinases have

Figure 1 Modular Domains of Protein Kinases

(A) Protein kinases possess a modular structure of juxtaposed amino acid domains that form characteristic ATP-binding sites, catalytic domains, regulatory segments, variable and association domains.

(B) The protein kinase polypeptides can be localized to the cytosol or consist of transmembrane proteins. The transmembrane protein kinases often possess a specialized extracellular regulatory domain capable of binding ligands such as growth factors. This has led to their appellation as receptor kinases as opposed to their cytosolic, non-receptor counterparts.


Kinase domains
A
ATP
Catalytic
Regulatory
Variable/Association
B
Ligand
membrane
Receptor kinase
Non receptor kinase

been the most extensively studied (Edelman *et al.*, 1987). Two prominent members of this class, protein kinase A and protein kinase C, play pivotal roles in cellular signal transduction mediated by cyclic AMP and calcium/phospholipid, respectively. Other members of the serine/threonine kinase class include the calmodulin-dependent protein kinases, such as the multifunctional CaM kinase II or the highly specific myosin light chain kinase. The identification of a second class of protein kinases followed with the discovery that the transforming protein of Rous sarcoma virus, $pp60^{v\text{-}src}$, was a tyrosine kinase (Collett *et al.*, 1980). Some members of the tyrosine kinase gene family act as receptors for growth factors (EGF, PDGF, insulin) and serve functional roles in controlling growth and differentiation (Bolen, 1993; Fantl *et al.*, 1993). The dual specificity kinases were identified much later in mammals and yeast. These kinases demonstrate the ability to autophosphorylate on serine, threonine and tyrosine residues. Some examples include STY kinase, which is thought to be important in cellular growth and differentiation, and MEK (MAPK/ERK) which plays a predominant role in mitogen-activated signal transduction pathways (Douville *et al.*, 1994). Each of these classes of serine/threonine and/or tyrosine protein kinases contain catalytic domains that share a high degree of homology. Specific amino acid clusters often flank the targeted amino acid residues to form the basis of consensus phosphorylation sequences for each class of kinase (Pearson and Kemp, 1991).

The greatest area of diversity between classes of kinases is exhibited in the regulatory and association domains. The regulatory and association domains of kinases consist of a variety of non-catalytic domains often present in other non-kinase molecules. For example, in the serine/threonine protein kinase family, some regulatory domains include calcium and

calmodulin-binding sites in calmodulin-dependent protein kinases (CaMK), phospholipid-binding sites in protein kinase C (PKC) and cyclic nucleotide-binding domains in cyclic-AMP (PKA) and cyclic-GMP (PKG) dependent protein kinases (Edelman *et al.*, 1987). Furthermore, in many protein kinases, the regulatory domain contains a pseudo-substrate sequence that binds to the catalytic site and prevents access of external substrates to the catalytic site. Phosphorylation of the catalytic site or binding of a second messenger can relieve this internal inhibitory effect by inducing a conformational change in the kinase's polypeptide structure (Girault, 1993). Other regulatory domains are characteristic of the tyrosine kinase receptors. These include conserved extracellular regulatory domains that interact with a variety of growth factors such as EGF, PDGF, FGF and insulin (Fantl *et al.*, 1993).

Both receptor and non-receptor tyrosine kinases alike are also characterized by the presence of SH2 and SH3 association domains, so named for the src homology 2 and src homology 3 domains. The SH2 and SH3 domains direct protein-protein interactions through high affinity interactions with specific phosphotyrosine containing peptides and proline-rich regions, respectively (Pawson, 1994). Serine/threonine protein kinases also possess association domains. These domains are involved in the oligomerization of kinase monomers or the attachment to subcellular locations via anchoring proteins such as AKAP for protein kinase A and KAP for the calmodulin-dependent protein kinase II (Bayer *et al.*, 1996; Scott, 1997).

The great diversity of protein kinase families has resulted from the variations in juxtaposition of these four basic functional modules. This modular structure lends some

common properties to all kinases while providing the greater degree of flexibility required for targeting across different subcellular compartments and regulating a wide array of physiological functions such a neurotransmitter release, hormonal secretion, muscle contraction and cellular growth and differentiation. A full molecular understanding of the role of protein phosphorylation in these various tissues will require the identification and characterization of the protein phosphorylation systems present within them.

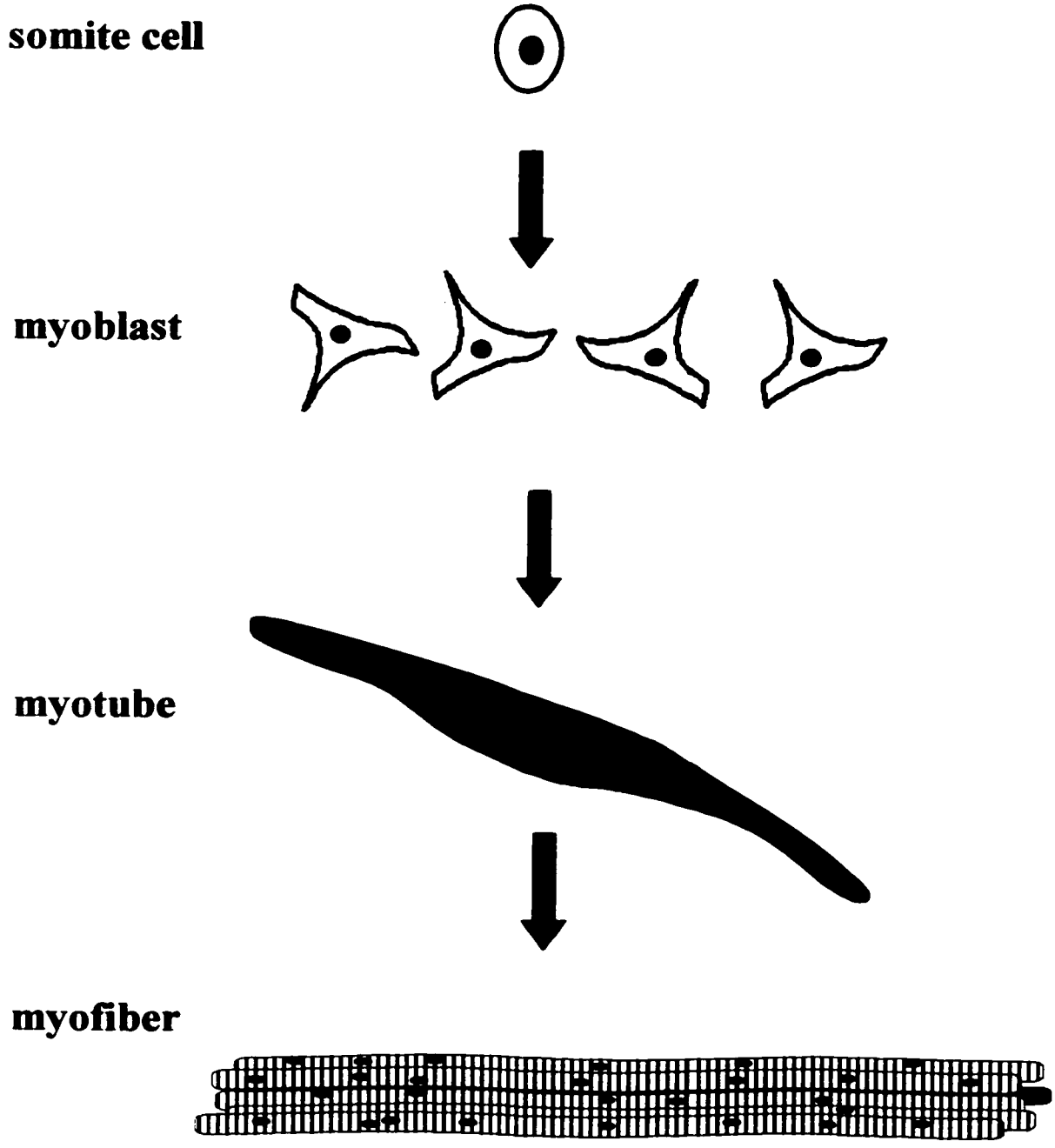
A.2 Muscle Structure and Function

Muscle tissue makes up almost half the human body's mass. As the body's contractile engine, muscle plays a vital role in circulation, breathing, digestion and locomotion. Muscle has evolved complex intracellular cascades to control the contractions triggered by the nervous system and adapt its output in response to the environment. Among these cascades, protein phosphorylation is a major mechanism that would be well utilized for regulation. Because of its relative abundance, organized intracellular structure, adaptability and elaborate signalling systems, muscle tissue provides an ideal source for the study of such phosphorylation systems.

During development, skeletal muscle derives from the somites of the embryo (Hauschka, 1994). The somites are masses of primordial muscle tissue that occur at intervals along the length of the embryo. As development proceeds within the somites, primitive muscle precursor cells, called myoblasts, appear and slowly begin to aggregate. The fusion of neighbouring myoblasts form small multinucleated cells known as myotubes (Ontell and Kozeka, 1984). The myotubes not only lengthen as an increasing number of myoblasts fuse

Figure 2 Schematic Representation of Skeletal Muscle Development

Embryonic cells in the somite undergo determination and differentiation into myoblasts. Myoblasts multiply and terminally differentiate by fusing into multinucleated myotubes that mature into myofibers. Each developmental stage is associated with specific myogenic factors.


somite cell
myoblast
myotube
myofiber

end to end but also grow in diameter as their core fills with newly synthesized contractile proteins which force the centrally located nuclei to the periphery (Fig. 2).

There are two general classes of muscle in the body: striated and smooth. Both are named for the characteristic appearance of the individual cells making up the muscle tissue when viewed through a light microscope. Striated muscle cells appear to have closely spaced, crosswise striations that are absent in smooth muscle cells. Smooth muscle is found in the gut, circulatory system, uterus and other areas requiring slow and maintained contractions. Striated muscle cells are found in skeletal muscle and the heart. Skeletal muscles are attached to and cover the skeleton and are primarily involved in locomotion and posture. Although cardiac muscle shares some properties with skeletal muscle, it is often considered as a class unto itself because the biochemical and electrical properties of its cell are quite different from skeletal muscle. For example, one important difference in cardiac muscle cells is the presence of gap junctions that interconnect the cardiac cells both chemically and electrically.

At greater magnifications, the individual striated muscle cells consist of many bundles of smaller fibers called myofibrils. It is the myofibrils that are responsible for the striated appearance of the muscle because they exhibit a repeating pattern of crosswise light and dark stripes. A single myofibril is composed of many short structural units, known as sarcomeres, which are arranged in an end to end fashion. The proteins at the junctions between sarcomeres form the Z line. Thus, a sarcomere extends along a myofibril from one Z line to the next Z line and represents the minimal contractile unit of a muscle. The organization of individual contractile proteins making up a sarcomere is a key feature of muscle contraction (Huxley, 1972). The contractile proteins consist of both thick and thin filaments. The thick

filaments are aggregates of myosin, a protein consisting of a long fibrous tail connected to a globular head. The thin filaments are largely made up of polymerized actin protein plus two other polypeptides, troponin and tropomyosin.. Each actin molecule in the chain contains a binding site that can interact specifically with a site on the globular head of myosin. Tension is developed by the thin filaments being pulled along the thick filaments by ATP-dependent processes involving the cyclic interactions between thick and thin filaments of muscle myosin and actin triggered by the elevation of intracellular calcium (Huxley, 1990). In smooth muscle, the development of tension is also triggered by calcium but differs from striated muscle in that a phosphorylation of myosin by myosin light chain kinase is required to initiate contraction.

There has been a renewed appreciation of the importance of calcium in various aspects of cell regulation, growth and differentiation (Clapham, 1995). In a typical mammalian cell, the intracellular calcium concentration is about 0.1 μM or about ten thousand times lower than the plasma calcium concentration . The maintenance of low intracellular calcium levels is essential to the survival of organisms that are driven by phosphate-based energy metabolism. ATP metabolism yields free inorganic phosphate that can combine with high levels of intracellular calcium to form toxic hydroxyapatite precipitates within the cells. Calcium acts as a common trigger in a wide diversity of biological processes (Tsien and Tsien, 1990). It acts as a second messenger by relaying the electrical and chemical messages that arrive at a cell's surface membrane to the biochemical machinery within the cell. Early work on both nerve and muscle physiology implicated calcium as an important intracellular trigger molecule (Heilbrunn, 1940). In fact, tension development in mammalian muscle was one of

the earliest biological processes shown to be regulated by calcium (Ebashi and Endo, 1968).

During excitation of the muscle cell, depolarization of the plasma membrane is thought to lead to the release of calcium from intracellular stores. The elevated cytosolic calcium activates the contractile apparatus. The contraction terminates as the calcium is pumped back into the stores via the various calcium transport mechanisms. Because calcium is closely linked to signal transduction, and in the case of muscle, determines the state of contraction, its cytosolic level in cells must be strictly controlled. Unlike other signal transduction molecules such as cAMP, calcium cannot be metabolized and its levels must be therefore be tightly regulated by other means. In muscle, the cytosolic levels of calcium are regulated by various membrane systems, such as the sarcolemma, transverse tubules, sarcoplasmic reticulum and mitochondria (Fig. 3). The process of excitation-contraction coupling in skeletal muscle begins with the depolarization signal originating from neural stimulation of the muscle (Ebashi, 1991). This electrical depolarization spreads across the plasma membrane of muscle fibers, or sarcolemma, and penetrates into the muscle fibers along the transverse-tubules (t-tubules), which are specialized invaginations of the plasma membrane. The t-tubules are particularly important in excitation-contraction coupling since they transmit the electrical signal to the sarcoplasmic reticulum (SR) and trigger calcium release. These t-tubules contain a high density of voltage sensors. These voltage sensors are L-type Ca^{2+} channels that detect the passing depolarization and relay the signal to the intracellular Ca^{2+} stores. It is thought that, in skeletal muscle, this occurs through direct physical interactions between the voltage sensors of the transverse tubules and the large protruding foot structures of the intracellular sarcoplasmic reticulum (Catterall, 1991). The sarcoplasmic reticulum

forms an extensive network of intracellular membranes that stores Ca^{2+}. The region of sarcoplasmic reticulum membrane that is in direct apposition with the transverse tubules is known as the junctional face. The junctional face membrane is particularly enriched in the large foot structures which contain the Ca^{2+} release channels of SR. The Ca^{2+} release channels, having been stimulated by the voltage sensors, open and allow Ca^{2+} to flow freely down its concentration gradient from the sarcoplasmic reticulum into the cytosol where it interacts with the proteins of the contractile apparatus to promote contraction (Lytton and MacLennan, 1991).

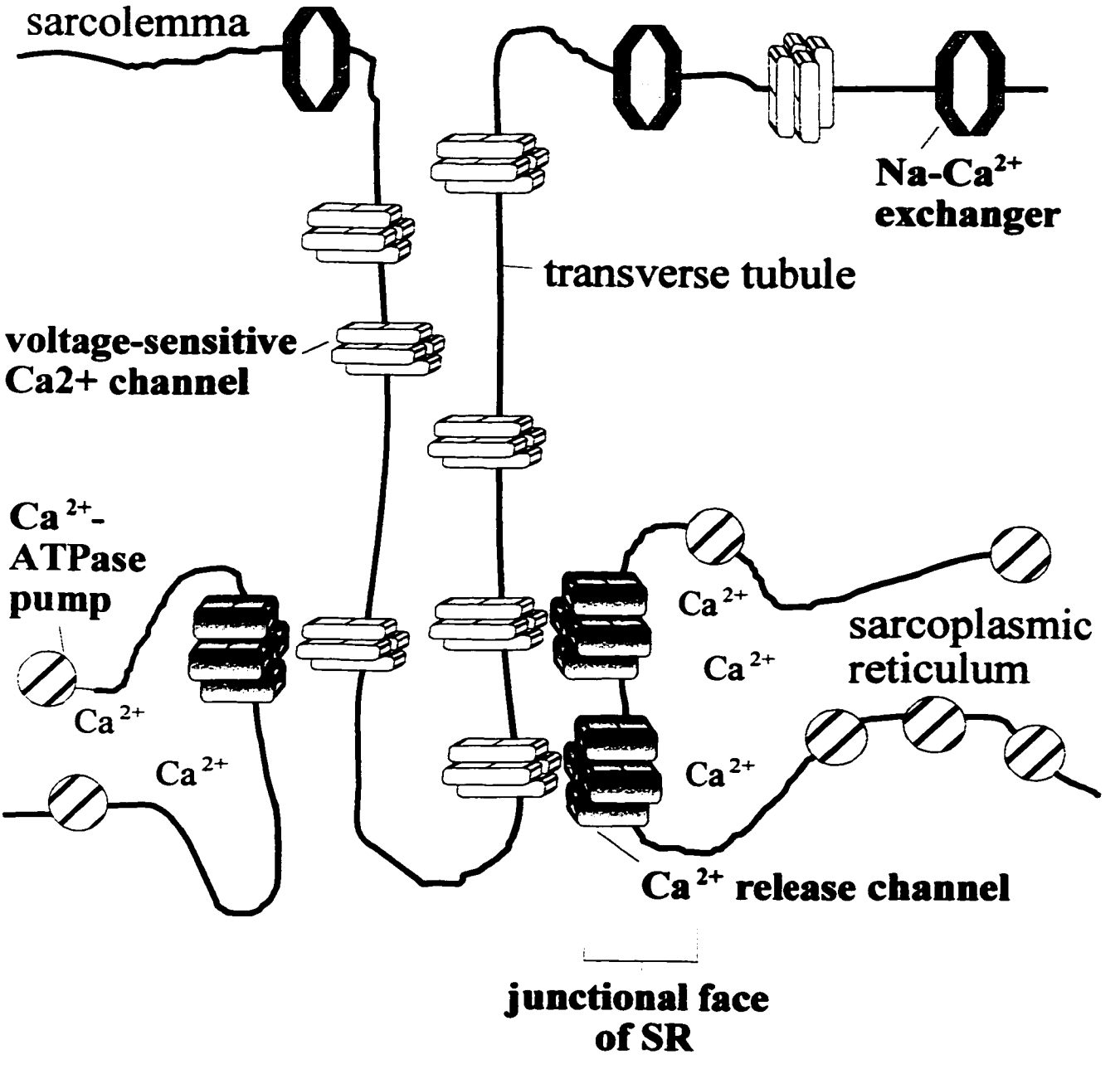
In the cardiac muscle, the process of excitation-contraction coupling differs slightly. The voltage sensors of the transverse tubules act as voltage-sensitive Ca^{2+} channels, allowing intracellular calcium levels to rise in response to the depolarization of the plasma membrane. The rise in intracellular calcium stimulates the opening of the Ca^{2+} release channels of the sarcoplasmic reticulum in a process described as calcium-induced calcium release (Fleischer and Inui, 1989). In both skeletal and cardiac muscle, the contraction is extinguished as the released calcium is removed by Ca^{2+} pumps located in the SR and the Na^{+}-Ca^{2+} exchanger in the plasma membranes. The calcium ions are stored within the lumen of the sarcoplasmic reticulum complexed with the high-capacity calcium-binding protein, calsequestrin.

A.3 Phosphorylation and Regulation of Muscle Function

Intracellular calcium levels and excitation-contraction coupling hinge on the activity of four important proteins: the L-type Ca^{2+} channel of sarcolemma, the Ca^{2+} ATPase, the Na^{+}-Ca^{2+} exchanger and the Ca^{2+} release channels of the sarcoplasmic reticulum. All these

Figure 3 Subcellular Components Associated with Excitation-Contraction Coupling in Muscle

The plasma membrane, or sarcolemma, of skeletal muscle possesses specialized invaginations (transverse tubules) that are responsible for delivering the depolarization signal deep within the myofibers. Voltage-sensitive Ca^{2+} channels detect the passing depolarization and have been proposed to interact with the Ca^{2+} release channels to trigger release of Ca^{2+} from the sarcoplasmic reticulum of skeletal muscle. The released Ca^{2+} is removed from the cytoplasm by powerful Ca^{2+}ATPase pumps of the sarcoplasmic reticulum and Na^{+}/Ca^{2+} exchangers of the sarcolemma. The junctional face refers to the segment of sarcoplasmic reticulum membrane that is highly enriched in Ca^{2+} release channels and is in direct apposition to the transverse tubule.


sarcolemma
Na-Ca2+ exchanger
transverse tubule
voltage-sensitive Ca2+ channel
Ca 2+- ATPase pump
Ca 2+
Ca 2+
Ca 2+
Ca 2+
Ca 2+
sarcoplasmic reticulum
Ca 2+ release channel
junctional face of SR

polypeptides detect changes in calcium levels through sensitive Ca^{2+}-binding sites present within their polypeptide structure. These Ca^{2+}-binding sites consist of peptide domains that bind calcium with six oxygen atoms provided by glutamate and aspartate residues (McPhalen *et al.*, 1991). These Ca^{2+}-binding residues are often flanked by two helical domains, homologous to the E and F domains of parvalbumin, that together form EF hands (Carafoli and Penniston, 1985). The complex level of regulation required by these four polypeptides is provided in part by a variety of phosphorylation systems. Protein phosphorylation is particularly useful as a regulator of muscle activity because its reversible nature is well suited to the cyclical changes in second messenger concentrations during muscle contraction.

The L-type Ca^{2+} channel is a member of the voltage-sensitive Ca^{2+} channel family. In muscle, these channels are localized to the sarcolemma and are particularly enriched in the transverse tubules. The L-type Ca^{2+} channel is composed of the $\alpha 1$, $\alpha 2$-δ, β and γ subunits (Catterall, 1991). The $\alpha 1$ subunit is a 170 kDa transmembrane polypeptide which contains a binding site for dihydropyridines and forms the functional pore of the Ca^{2+} channel. The postulated transmembrane structure suggests that each $\alpha 1$ subunit consists of four hydrophobic domains. Each hydrophobic domain contains a positively charged S4 region that acts as a voltage-sensor (Catterall, 1993). The $\alpha 2$-δ, β and γ subunits act as accessory subunits that modulate the activity of the $\alpha 1$ pore-forming subunit (Gregg *et al.*, 1996; Mikami *et al.*, 1989). The regulation of the L-type Ca^{2+} channel by protein phosphorylation is widely known as an important physiological control mechanism. The $\alpha 1$ polypeptide sequence contains consensus sites for phosphorylation by protein kinases such as the cAMP-dependent protein kinase (PKA). Phosphopeptide analysis of the $\alpha 1$ subunit of skeletal

muscle revealed three sites of selective phosphorylation for PKA: Ser687, Ser1757 and Ser1854 (Rotman *et al.*, 1995). In cardiac muscle, only Ser1928 on the α1 cardiac subunit has been identified as a potential site for PKA phosphorylation (De Jongh *et al.*, 1996). More recently, studies have shown that PKA-mediated phosphorylation of the α1 and the resulting increase in calcium currents requires that PKA be tethered to the membrane by the anchoring protein AKAP79 (Gao *et al.*, 1997). AKAP is a kinase anchoring protein that acts as a platform for the assembly of the protein kinase A, calcineurin and protein kinase C signalling polypeptides (Bayer *et al.*, 1996). Our understanding of the regulation of the L-type Ca^{2+} channel by cAMP-dependent phosphorylation is notably important as it relates to the physiological regulation of cardiac function by the sympathetic nervous system. Beta-adrenergic agents, such as norepinephrine, act through the cAMP-dependent phosphorylation system to increase calcium entry into the cardiac cell via the L-type Ca^{2+} channels and thus increase the rate and force of contraction of the heart. Phosphorylation sites for PKA and PKC have also been identified on the β subunits of the cardiac Ca^{2+} channel but the precise role of this phosphorylation in regulating channel activity remains to be investigated (Puri *et al.*, 1997).

The Na^{+}-Ca^{2+} exchanger represents another major Ca^{2+}-binding protein located in the sarcolemma of cardiac muscle. It plays an important role in controlling intracellular calcium levels by promoting the extrusion of intracellular calcium in exchange for extracellular sodium. The Na^{+}-Ca^{2+} exchanger exhibits phosphorylation exclusively on serine residues in response to protein kinase C activators. Protein kinase C-dependent phosphorylation results in the upregulation of the Na^{+}-Ca^{2+} exchanger activity, decreased intracellular calcium levels

and, as a result, negative inotropic effects in cardiac muscle (Iwamoto *et al.*, 1996). In contrast, beta agonist stimulation, which activates cAMP production and the PKA pathway, suppressed Na^{+}-Ca^{2+} exchanger activity in cardiomyocytes (Fan *et al.*, 1996). Much as for the L-type Ca^{2+} channel, the stimulation of PKA activity results in increases in intracellular calcium and therefore force of contraction. In a clinical setting, this increased contractile force is potentially very beneficial for the patient with congestive heart failure (inotropic effect).

The Ca^{2+} ATPase is a powerful, ATP-dependent calcium pump that makes up almost two thirds of the proteins found in sarcoplasmic reticulum (MacLennan *et al.*, 1985). With each muscle contraction, these pumps are responsible for returning the released Ca^{2+} to their intracellular sarcoplasmic reticulum stores. Although the Ca^{2+} ATPase was demonstrated to be phosphorylated by a CaM kinase on Ser38, the pump is not thought to be a substrate for direct regulation by phosphorylation (Toyofuku *et al.*, 1994). The activity of the Ca^{2+} ATPase rather appears to be regulated through its interactions with phospholamban. Phospholamban consists of five monomers bound by noncovalent interactions. The phospholamban polypeptide can be phosphorylated in vivo by both PKA and CaM kinases (Jones *et al.*, 1985; Fujii *et al.*, 1989). When phospholamban protein is phosphorylated, both the Vmax and the calcium affinity of the pump are increased. In the heart, the increased Ca^{2+} ATPase activity resulting from β-adrenergic stimulation and increased PKA activity leads to the more rapid muscle relaxation times required to accommodate the increased heart rate (chronotropic effect) and force of contraction (inotropic effect). This increased relaxation capacity of the heart has been termed the lusitropic effect.

The Ca^{2+} release channel of the sarcoplasmic reticulum is a tetramer of four large 560 kDa polypeptide subunits with large N-terminal domains. These domains form the basis of the foot structures that associate with the junctional face membrane of SR which is in close apposition to the transverse tubules (Lytton and MacLennan, 1991). The Ca^{2+} release channel binds to the neutral plant alkaloid, ryanodine, and possesses numerous binding sites for regulatory factors. Micromolar levels of calcium and millimolar levels of ATP and caffeine are all known to stimulate the opening of the Ca^{2+} release channel whereas Mg^{2+}, ruthenium red, calmodulin, dantrolene and spermine inhibit the channel (Fleischer and Inui, 1989). The regulation of calcium release through phosphorylation is much less well understood. A number of studies have shown that the Ca^{2+} release channel from both cardiac and skeletal muscle is a substrate for cAMP- and cGMP-dependent protein kinases as well as protein kinase C (Takasago *et al.*, 1991). In the cardiac Ca^{2+} release channel, Ser 2809 has been identified as the major site of phosphorylation by CaM-dependent protein kinases that activates opening of the channel (Witcher *et al.*, 1991). Whether a similar CaM kinase activity is directed towards the skeletal muscle Ca^{2+} release channel remains a controversial issue as the combination of reported studies reflect significant discrepancies (Hain *et al.*, 1994). Perhaps the differences in the amino acids surrounding the corresponding phosphorylation site (Ser2843) accounts for the observed tissue differences. Out of twenty-five neighbouring amino acid residues, fourteen have been altered in the skeletal muscle form of the release channel. Modulation of the Ca^{2+} release channel can also occur through associations with accessory proteins such as sorcin. Sorcin is a Ca^{2+}-binding protein which co-immunoprecipitates with the Ca^{2+} release channel and decreases its open probability.

Phosphorylation of sorcin by PKA significantly decreases the ability of sorcin to modulate the Ca^{2+} release channel and provides an additional level of modulation of Ca^{2+} release channel activity (Lokuta *et al.*, 1997).

These describe but a few of the multiple phosphorylation events required to maintain tight controls on the levels of cytosolic calcium. Many of the stated phosphorylation activities have been extensively studied in cardiac muscle but much less well characterized in skeletal and smooth muscle systems.

A.4 Calmodulin-dependent Protein Kinases and Muscle Function

The action of intracellular calcium ions is not limited to the regulation of muscle contraction. Once inside the cell, calcium can elicit several biochemical responses by stimulating calcium-sensitive signalling pathways such as the protein kinase C phosphorylation system (Hug and Sarre, 1993). Calcium also binds to receptors that integrate and transmit the calcium signals further downstream. Calmodulin is one such Ca^{2+}-binding polypeptide that is activated upon binding calcium. Calmodulin (CaM) can bind up to four calcium ions and appears to be the most common translator of intracellular calcium signals (Wylie and Vanaman, 1988). The calcium/CaM complex in turn activates a number of polypeptides, including protein kinases called CaM-dependent protein kinases.

Schulman and Greengard (1978) were the first to identify CaM as a calcium-dependent, dose-dependent activator of protein kinase activity. Since then, CaM has been shown to be an important activator of a great number of protein kinases (Nairn and Picciotto, 1994). These enzymes share many common structural features, such as conserved catalytic

and regulatory domains, but differ in their activation by calmodulin, their regulation by autophosphorylation, their localization and their interactions with substrate polypeptides. Much like other protein kinases, the activity of CaM-dependent protein kinases can be modulated by a regulatory domain within the polypeptide. The regulatory domain acts as a pseudo substrate that blocks access of the substrate or ATP to the catalytic site (Colbran *et al.*, 1989). An additional level of regulation is provided by the phosphorylation state of the catalytic domain. For example, autophosphorylation of a specific threonine residue (Thr 286) in CaM-dependent protein kinase II transforms the kinase to a calcium and CaM-independent enzyme (Schulman, 1993).

The CaM-dependent protein kinases are often classified according to their substrate specificity. Some CaM-dependent protein kinases, such as myosin light chain kinase, phosphorylase kinase and elongation factor 2 kinase (EF-2), phosphorylate only one or a few substrates. Of these, phosphorylase kinase was the first polypeptide to be identified as a kinase and contributes to the regulation of glycogen metabolism (Skuster *et al.*, 1980). Myosin light chain kinase (MLCK) was the first enzyme shown to respond to CaM and is involved in the regulation of muscle contraction and cytoskeleton structure (Edelman *et al.*, 1987). MLCK phosphorylates myosin light chain in smooth muscle, where it actively regulates excitation-contraction coupling, and skeletal muscle, where it influences the rate and extent of isometric twitch tension. A third specific kinase, EF-2 kinase or CaM kinase III, specifically phosphorylates elongation factor 2 that is necessary for the extension of polypeptide chains (Nairn and Picciotto, 1994). Other CaM-dependent protein kinases are said to be multifunctional as they target a number of different substrates. Such

multifunctional CaM kinases include CaM kinase I, II, IV that are involved in the metabolism of carbohydrates, lipids and amino acids, the synthesis and release of neurotransmitters, the regulation of ion channels, cytoskeleton, gene expression and cell growth and proliferation (Walaas and Greengard, 1991). CaM kinase I phosphorylates site 1 of the neuronal protein synapsin I (Picciotto *et al.*, 1996). CaM kinase V, Ia and Ib are all closely related isoforms of CaM kinase I whose activities appear to be regulated by a CaM-dependent protein kinase kinase (Ito *et al.*, 1994; Tokumitsu *et al.*, 1995). CaM kinase IV, also named CaM kinase Gr in reference to cerebellum granules, is detected predominantly in the brain but also in the thymus, testis, spleen and T cells (Means *et al.*, 1997).

The most extensively studied multifunctional CaM kinase is CaM kinase II (Braun and Schulman, 1995). CaM kinase II, although found in many tissues, was originally identified in brain where it is believed to act post synaptically to potentiate the response of glutamate receptors (Shenolikar *et al.*, 1986; McGlade-McCulloh *et al.*, 1993). It has also been associated with effects on cell growth and differentiation. CaM kinase II has been shown to affect multiple points in the mammalian cell cycle, including the initiation of the S phase and both initiation and completion of the M phase (Takuwa *et al.*, 1995), as well as influence the migration of vascular smooth muscle (Pauly *et al.*, 1995). Overexpression of CaM kinase II in PC12 cells also alters cell growth and morphology (Masse and Kelly, 1997). Interestingly, the expression of certain CaM kinase II isoforms is dependent on the differentiation state of cells. In muscle, Hoch *et al.* (1998) have demonstrated the differentiation-dependent isoform expression of the CaM kinase II delta isoform in embryonic rat heart-derived cell lines.

Although four different CaM kinase II isoform families have been defined to date

(α,β,γ,δ), all share a number of common features. Each isoform contains a catalytic domain, a regulatory domain (with autoinhibitory and calmodulin-binding sites) and an association domain (Hanson and Schulman, 1992). The kinase autophosphorylates on a threonine residue within the regulatory domain that frees the kinase of its CaM requirements. This intermolecular autophosphorylation increases the affinity of calmodulin for the kinase's binding site and is thought to decode information obtained from calcium transients (Thiel *et al.*, 1988; Schulman *et al.*, 1992). The CaM kinase II holoenzyme adopts a complex quaternary structure composed of five to twelve subunits of different isoforms. Electron microscopy studies suggest that these subunits associate via their C-terminal association domains with the catalytic domains facing outward in a wheel-like structure (Kanaseki *et al.*, 1991; Bennett *et al.*, 1983). The major differences between the four isoforms are mostly localized to a variable domain situated between the regulatory and association domains.

The β isoforms of the CaM kinase II gene family have been shown to be multifunctional in that they phosphorylated numerous cellular substrates (Hanson and Schulman, 1992). At least five members of the β isoform family have been investigated: the classical β and β' isoforms predominantly studied in brain tissue (Bennett and Kennedy, 1987; Bulleit *et al.*, 1988), their embryonal counterparts, βe and β'e (Tombes and Krystal, 1997), and the β3 isoform expressed in pancreas (Urquidi and Ashcroft, 1995).

CaM kinase II isoforms, although mostly cytosolic, have been shown to associate with membrane and cytoskeletal structures and implicated in cellular functions such as regulation of ion transport and cytoskeletal dynamics (McNeill and Colbran, 1995; Lu *et al.*, 1994; McGlade-McCulloh *et al.*, 1993; Nishimoto *et al.*, 1991). However, the precise nature of

these kinase activities and how they may associate with these diverse structures remain elusive. The role of CaM-dependent protein kinases in muscle is of particular interest since the levels of intracellular calcium fluctuate with every contraction-relaxation cycle. A CaM-dependent protein kinase activity has been shown to associate with skeletal and cardiac muscle membranes and been proposed to regulate the process of excitation-contraction coupling (Kim and Ikemoto, 1986; MacLennan *et al.*, 1984; Tuana *et al.*, 1987; Jett *et al.*, 1987). The exact mechanisms by which the CaM-dependent protein kinase can target the membrane calcium transport system remains unclear. It has been postulated that changes in the level of phosphorylation of SR proteins might regulate changes in ionic permeability of the SR membranes (Campbell and MacLennan, 1982; Chiesi and Carafoli, 1982; MacLennan *et al.*, 1984; Tuana and MacLennan, 1984; Kim and Ikemoto, 1986; Morii *et al.*, 1987). More specifically, a CaM-dependent protein kinase activity that has been shown to associate with the SR was proposed to gate the Ca^{2+} release channel in response to increased Ca^{2+} activity during muscle activation (MacLennan *et al.*, 1984). The CaM kinase phosphorylates several proteins and is a minor component of the SR with a stoichiometry resembling the ryanodine receptor rather than the Ca^{2+} ATPase (Campbell and MacLennan, 1982; Chiesi and Carafoli, 1983; Tuana and MacLennan, 1984). The isolated CaM kinase complex consists of the 89, 73, 60, 34 & 20 kDa phosphoproteins and partial proteolysis revealed that these phosphoproteins were distinct entities (Tuana and MacLennan, 1988). The 60 kDa polypeptide appears to be the major CaM-binding polypeptide in SR (Leddy *et al.*, 1993; Vale, 1988) and is phosphorylated on serine and threonine residues in a calmodulin-dependent manner (Campbell and MacLennan, 1982). CaM-dependent phosphorylation has been

correlated with the extent of inhibition of Ca^{2+} release from SR (Kim and Ikemoto, 1986). Patch clamp studies have implied that a protein kinase activity intrinsic to the SR membrane could inactivate the Ca^{2+} release channel (Wang and Best, 1992). The closure of the Ca^{2+} release channel by phosphorylation could be prevented by a CaM kinase II inhibitory peptide suggesting the CaM kinase activity of SR was involved in channel gating. In addition, a Ca^{2+}/CaM independent kinase activity associated with SR has also been described to phosphorylated polypeptides of 62, 42 and 20 kDa (Campbell and Shamoo, 1980).

A.5 Statement of the Problem

A growing number of studies point to a role for CaM-dependent protein kinase phosphorylation in the regulation of excitation-contraction coupling in muscle cells (Campbell and MacLennan, 1982; Chiesi and Carafoli, 1982; MacLennan *et al.*, 1984; Tuana and MacLennan, 1984; Kim and Ikemoto, 1986; Morii *et al.*, 1987). While a CaM kinase activity has been shown to reside in the sarcoplasmic reticulum, its precise characterization and function remains to be defined. We have undertaken to further characterize, at the biochemical and molecular level, the protein kinases implicated in the regulation of muscle membrane function. The biochemical characterization of the CaM kinases led to the definition of some notable differences in the properties of CaM kinases in muscle as compared to the well-characterized brain isoforms. The molecular characterization led to the identification of SOCK, a novel CaM kinase isoform that appears to be uniquely structured to integrate intracellular signalling pathways involving calcium and protein tyrosine kinases.

B. MATERIALS and METHODS

B.1 Preparation of Rabbit Skeletal Muscle Fractions

Microsomal membranes were prepared from New Zealand White rabbits (Charles River) as described by Chu *et al.*(1988). Rabbits were sacrificed by cervical dislocation and the fast twitch skeletal muscle from the back and hind legs were excised and placed on ice. All subsequent procedures were executed in a temperature-controlled room (4°C) to reduce proteolytic degradation. The pH of solutions was measured at room temperature and all solutions were chilled to 4°C prior to use. Unless otherwise indicated, protease inhibitors were added to all buffers, just prior to use, in final concentrations of 0.3 mM phenylmethane sulfonyl fluoride (PMSF), 1 mM benzamidine, 1 mM iodoacetamide and 0.5 μM pepstatin. All chemicals were of reagent grade and obtained from Sigma Chemical Co., BDH or other local suppliers.

Muscle was rinsed to remove any blood and trimmed of residual tendons, fat and connective tissue. The tissue was then chopped into small (3 cm) cubes and fifty gram portions of muscle were minced using 3 x 5 s pulses of an Osterizer blender. The minced tissue was homogenized in 250 mL of buffer (5 mM Tris-HCl, pH 7.4 and 0.3 M sucrose) for 3 x 15 s with a Polytron PT 10/35 homogenizer (Brinkmann Instruments) at half maximal setting, taking care to cool the blades between pulses. All centrifuges and rotors were purchased from Beckman Instruments Inc. (Palo Alto, CA). Both rotors and centrifuge chambers were precooled to 4°C before centrifugation. The muscle homogenate was

centrifuged for 20 min at 11 000 g and the supernatant was further centrifuged for 60 min at 135 000 g to obtain the cytosolic fraction that represents this supernatant fraction. The pellet from the 11 000 g spin was resuspended in the same buffer using 15 passes of a Teflon pestle and Potter-Elvehjem tissue grinder. The resuspended pellet was homogenized and centrifuged a second time as described above. The pellet was discarded and this second supernatant was filtered through cheesecloth to remove insoluble lipids and denatured proteins. The filtrate was centrifuged for 60 min at 110 000 g. The pellets were resuspended and combined to form the crude microsomal fraction. 100 µL aliquots were saved for assaying the protein concentration and the remaining solution was snap frozen in liquid nitrogen and stored at -80°C.

The crude microsomal fraction was further purified into light and junctional SR fractions. Eight millilitres of crude microsomal fraction was loaded on top of a sucrose step gradient constructed of 2.5 mL of 45% (bottom), 4 mL of 38%, 4 mL of 34%, 4 mL of 32% and 2.5 mL of 27% sucrose (weight/weight) in 5 mM Tris-HCl, pH 7.4. Centrifugation was then carried out overnight at 70 000 g in a SW28 swinging bucket rotor. The fractions appearing as protein bands appearing at the step interfaces were collected using a Pasteur pipette, diluted two fold with buffer and centrifuged for 45 min at 178 000 g in a 60 Ti rotor. The pellets were resuspended, quick frozen in liquid nitrogen and stored at -80°C. The membranes enriched at each interface have been previously characterized by marker enzyme activity and receptor binding studies (Chu *et al.*, 1988). The light SR has been found to collect at the 32/34% interface while the heavy SR (or junctional terminal cisternae) appears at the 38/45% interface as demonstrated by the enrichment in Ca^{2+}-ATPase activity and

ryanodine receptor binding respectively.

The junctional face membranes of SR were isolated as described by Costello *et al.* (1988). The junctional terminal cisternae fraction was adjusted to a final concentration of 3.3 mg/mL and 1 mM $CaCl_2$ using 0.3 M sucrose, 5 mM HEPES, pH 7.4. Triton X-100 was added to a final concentration of 0.5% (volume/volume), mixed thoroughly and incubated for 20 min on ice. The mixture was centrifuged for 40 min at 110 000 g. The supernatant was discarded and the pellet was resuspended in buffered solution containing 2 mM EDTA. After a 10 min incubation, the mixture was centrifuged as above. The supernatant was again discarded and the pellet containing junctional face membrane was resuspended and stored at -80°C. For further comparison, a transverse-tubule enriched fraction was prepared according to Doucet and Tuana (1991) and was a generous gift of Dr. J.-P. Doucet. Protein concentrations were determined with the BioRad detergent compatible (DC) Protein Assay Kit.

B.2 Identification of CaM-Binding Polypeptides

CaM-binding proteins were identified by an overlay technique essentially as described (Carlin *et al.*, 1981). Protein samples were subjected to electrophoresis using a 10% highly porous SDS-PAGE system (Doucet *et al.*, 1990) which permits the rapid and efficient transfer of proteins to nitrocellulose while maintaining a broad degree of resolution. The proteins resolved on gels were transferred to nitrocellulose electrophoretically in 50 mM Tris, 150 mM glycine, 0.1% SDS (Towbin *et al.*, 1979). The nitrocellulose was blocked for 60 min with 3% powdered milk (Carnation) in 200 mM NaCl and 20 mM Tris-HCl, pH 7.2 and

incubated overnight at room temperature with 2.5 μCi [^{125}I]Bolton-Hunter-labelled CaM (DuPont-New England Nuclear) in the presence of 1 mM $CaCl_2$ or 2 mM EGTA. The membranes were washed three times 10 min in the same buffer without skim milk and exposed to X-ray film for autoradiography. Autoradiography was performed using Kodak X-Omat AR film (Eastman-Kodak Ltd) and a DuPont Cronex Lightning Plus enhancing screen. All electrophoresis chemicals and molecular weight standards used in SDS-PAGE were purchased from Bio-Rad. The nitrocellulose membranes had a pore size 0.2 μm and were obtained from Technical Marketing (Ottawa, ON).

B.3 CaM-dependent Phosphorylation Assay

Phosphorylation of samples was carried out at 23°C in the kinase buffer: 50 mM Pipes, pH 7.0, 10 mM $MgCl_2$, 10 mM NaF, 0.2 mM EGTA, 0.5 mM $CaCl_2$, 10-50 μM [γ-^{32}P]ATP (3000 Ci/mmol, DuPont NEN) in the presence or absence of 0.6 μM CaM. After a 90 s incubation, the reactions were terminated with SDS loading buffer containing 8 M urea, 3% SDS, 100 mM DTT, 0.005% bromophenol blue and 70 mM Tris-HCl, pH 6.7. Samples were boiled at 100°C for 2 min and analysed quantitatively in SDS-PAGE. These assay conditions are similar to those previously described for CaM-dependent protein kinase assays by Campbell and MacLennan (1982).

B.4 Renaturation of CaM-dependent Autophosphorylation Activity

Proteins were separated by SDS-PAGE in 10% polyacrylamide and electroblotted to nitrocellulose (Doucet and Tuana, 1991) except that methanol was omitted in the transfer

buffer. The autophosphorylation assay was carried out essentially as described (Celenza and Carlson, 1986) with the following modifications. The nitrocellulose was initially blocked with 5% powdered skim milk in 30 mM HEPES, pH 7.5 for 30 min. Proteins bound to the nitrocellulose were denatured for 1 hour at 25°C with 20 mL of 7 M guanidine-HCl, 50 mM DTT, 2 mM EDTA, 0.25% powdered skim milk in 50 mM Tris-HCl (pH 8.3) and were then allowed to renature for 16 hours at 4°C in 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 2 mM DTT, 2 mM EDTA, 0.1% NP40 and 0.25% powdered milk. Following the renaturation, the nitrocellulose was again blocked with 5% powdered milk in 30 mM HEPES, pH 7.5 for 30 min at 25°C to reduce background and then incubated with the kinase buffer: 0.03 μM [γ-^{32}P]ATP (3000 Ci/mmol)and 0.25% powdered milk in the presence and absence of 0.6 μM CaM. After several washes with 30 mM HEPES, pH 7.5 and 0.25% powdered milk at 25°C, the nitrocellulose was washed for 10 min at 4°C with a 1 N KOH solution to remove non-specific [γ-^{32}P]ATP binding and dried for autoradiography.

The protocol was subsequently modified to improve the renaturation of CaM-dependent protein kinases present in low abundance. In this modified protocol, samples were mixed with loading buffer prepared without reducing agents (ie. dithiothreitol) and were no longer boiled prior to fractionation by SDS-PAGE.

B.5 Isolation of the CaM Kinase Complex

Rabbit skeletal muscle sarcoplasmic reticulum membranes were isolated as described above and were extracted with EGTA as described by Tuana and MacLennan (1984). The kinase complex was isolated according to Tuana and MacLennan (1988) with the following

modifications. The EGTA-extracted membranes were suspended in 10 mM Tris-HCl, pH 7.4 and solubilized in 0.2 % Nonidet P-40 (NP40) for 15 min on ice. Following centrifugation for 45 min at 120 000 g, the supernatant was adjusted to 1 mM free calcium and loaded on a calmodulin (CaM) agarose affinity column (Sigma Chemical Co.) equilibrated with 0.15 M NaCl, 0.1 % NP40 and 50 mM Tris-HCl, pH 7.4 (buffer A) containing 0.1 mM $CaCl_2$. After 60 min of incubation at 4°C, the column was washed extensively with the same buffer to remove non-specific binding. The CaM-binding proteins were eluted with buffer A containing 2 mM EGTA. The polypeptide composition of the eluted fractions was visualized by silver staining of SDS-PAGE gels (Morrissey, 1981). The CaM kinase from cardiac SR was isolated in a similar manner.

B.6 ATP Photoaffinity Labelling

Photoaffinity labelling of the CaM affinity-purified kinase complex was carried out according to the method of Nairn and Greengard (1987). Assay conditions were similar to those used for the phosphorylation assay except that 10 nM azido-ATP replaced [^{32}P]ATP. To minimize signal from non-specific binding, skeletal muscle membranes were first solubilized with detergent and partially purified by CaM-affinity chromatography to obtain isolated CaM kinase complexes. CaM affinity purified kinase complexes were incubated at room temperature for 5 min with 10 nM azido[^{32}P]ATP then placed on ice and irradiated for 2 min with ultraviolet light which cross-links the azido[γ-^{32}P]ATP to the ATP-binding polypeptide. Specific binding was displaced in the presence of 1 μM excess unlabelled ATP. The reactions were terminated with SDS-PAGE loading buffer and the samples were

subjected to SDS-PAGE and autoradiography to detect the labelled polypeptides.

B.7 Immunostaining of Rabbit Skeletal Muscle Fractions

Rabbit skeletal muscle fractions were isolated as described above. The 'pyrophosphate wash' fraction was obtained as follows. Heavy SR membranes were resuspended in a solution of 20 mM sodium pyrophosphate, 20 mM NaH_2PO_4 and 1 mM $MgCl_2$, pH 7.1 and incubated for 15 min. The mixture was centrifuged at 110 000 g for 40 min and the supernatant was kept as the 'pyrophosphate wash' fraction. Muscle fractions were size-fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% powdered skim milk in Tris Buffered Saline (TBS; 10 mM Tris-HCl, pH 7.2, 150 mM NaCl) for 60 min. The blots were rinsed with TBS and incubated for 1-2 h with the RU16 anti-(CaM kinase II) antibody diluted in TBST (TBS+0.05% Tween) with 1% powdered milk. The blots were rinsed 3 X 10 min with TBST and incubated with alkaline phosphatase-conjugated anti-rabbit IgG (1:3000) for 60 min. Following 3 X 10 min washes in TBST and a final 2 min wash in TBS, the immunoreactive polypeptides were visualized with 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 160 μg/mL) and nitro blue tetrazolium (NBT; 330 μg/mL) in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 10 mM NaCl, 5 mM $MgCl_2$). The colour development was halted with a solution of 1 mM EDTA in TBS. RU16 is an affinity-purified rabbit polyclonal antibody directed toward the following sequence, a.a. 444-468: TSFEPEALGNLVEGMDFHRFYFENL, found in brain CaM kinase II. RU16 was a generous gift from Dr. Andrew Czernik (Rockefeller University).

B.8 Inhibition of CaM Kinase Activity

CaM-dependent protein kinase assays were performed on heavy SR membranes as described above with the addition of varying concentrations of the CaM-dependent protein kinase antagonist, CaM kinase II fragment (290-309). Phosphorylated heavy SR membranes were subjected to SDS-PAGE. The SDS-PAGE gels were stained with Coomassie Blue, dried on filter paper and exposed for autoradiography. The 60 kDa phosphorylated polypeptide was localized by autoradiography, cut out of the dried gel and the radioactivity was quantified by liquid scintillation counting. Results are expressed as percent inhibition of the radioactivity incorporated in the 60 kDa polypeptide.

B.9 Immunoprecipitation of the CaM-dependent Protein Kinase of Skeletal Muscle

Rabbit skeletal muscle microsomes were phosphorylated as described above in the presence and absence of 0.6 μM CaM. The solubilized fractions were incubated for 60 min at 25°C or overnight at 4°C with the RU16 antibody raised against the multifunctional CaM kinase II. The antigen-antibody complexes were isolated by adsorption to protein A-agarose. The antigen-antibody-protein A agarose pellets were washed three times in 200 mM NaCl, 0.5% NP40 and 10 mM Tris-HCl, pH 7.4 then once in the same buffer without detergent. NP40-solubilized fractions were also subjected to a preadsorption step using control rabbit serum and protein-A-agarose. The proteins were eluted from the protein A beads by boiling for 5 min with SDS loading buffer and subjected to SDS-PAGE and autoradiography.

B.10 Preparation of Antibodies Against the 60 kDa Polypeptide

The 60 kDa CaM-binding protein was isolated from a CaM affinity column and gel purified. Ten micrograms of purified 60 kDa polypeptide was injected into guinea pigs to raise antibodies and a 5 μg boost was administered at two-four week intervals. Serum from immunized guinea pigs and preimmune serum was obtained and stored at -70 °C until use.

B.11 Isolation and Phosphorylation of Ryanodine Receptor/Ca^{2+}-release Channel

The ryanodine receptor from skeletal muscle was purified as described by Lai *et al.* (1988) with the following modifications. Skeletal muscle heavy SR membranes were isolated in the presence of 2.5 mM EGTA and 1 mM diisopropyl fluorophosphate (DIFP). The membranes were solubilized (1.3mg/mL) and labelled for 2 h at 25°C in binding buffer (1 M NaCl, 150 μM $CaCl_2$, 100 μM EGTA, 25 mM NaPIPES pH 7.1, 1 mM DIFP) containing 1.6% CHAPS, 3 mg/mL phosphatidylcholine, 2 mM ATP and 300 nM [9,21-^{3}H] ryanodine (2Ci/mmol) (DuPont-New England Nuclear). The detergent-solubilized membranes were centrifuged at 100 000 g for 20 min to remove insoluble material. The solubilized polypeptides (1.25 mg/mL) were loaded on a 5-20% linear sucrose density gradient in binding buffer containing 0.9% CHAPS, 3 mM AMP and 4 mg/mL phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) (PC:PE:PS=16:3:1) and were centrifuged in a Beckman SW28 rotor at 26 000 RPM for 16h at 4°C. Gradient fractions were analysed for radioactivity by liquid scintillation counting and for protein composition by silver-staining of SDS-PAGE gels (Morrissey, 1981). The peak ryanodine binding fractions were pooled and concentrated.

For the phosphorylation assay, samples were phosphorylated as described above. Heat-inactivated muscle membranes were generated by incubating muscle fractions at 60°C for 15 min to disrupt any endogenous kinase activity. The purified protein kinase C was kindly donated by Dr. Michael Walsh (University of Calgary) and initial preparations of purified ryanodine receptor from skeletal muscle were provided by Dr. Éric Rousseau (Université de Sherbrooke).

B.12 Cloning of CaM-dependent Protein Kinases in Human Skeletal Muscle

Human skeletal muscle oligo dT primed Lambda Zap II cDNA library was obtained from Stratagene. The cDNA library was plated according to the manufacturer's recommendations on twenty plates (size 150 mm x 15 mm). Each plate contained fifty thousand plaque forming units (PFU). Colony/plaque screen hybridization was performed using nylon transfer membranes (DuPont NEF-978Y) for the screening. The transfer membranes were probed with an α-[^{32}P]-dCTP labelled cDNA probe.

The cDNA representing the CaM kinase II β isoform was prepared as follows. Rat brain RNA was isolated using the Tripure isolation reagent as recommended by the manufacturer (Boehringer Mannheim). cDNA was synthesized in 20 µL reaction buffer utilizing 1 µg of total RNA. The RNA was denatured for 10 min at 70°C and reverse transcription was performed at 42°C for 1 h following the manufacturer's recommendations (Gibco BRL). The first strand cDNA was subsequently used as a template in 50 µL PCR reaction (10 µL cDNA, 5 µL 10X Taq polymerase buffer, 2 µL of 10 µM stock of each primer #1 and primer #2, 1 µL of dNTP mix -10mM of each of the dNTPs - and 0.5 µL Taq

DNA polymerase enzyme). The nucleotide sequences for the primers were as follows: Primer #1: (5' CTG AAG AAG TTC AAT GCA AGG AGG 3'); Primer #2: (5' GCA GCA GGT TCT CAA AGT AGA ATC 3') and were selected to amplify the variable region of greatest diversity within the CaM kinase II family. The probe was prepared using the Rediprime random primer labelling kit (Amersham) to contain approximately $1x10^6$ CPM/mL hybridizing solution. Hybridization solutions contained 10% polyethylene glycol, 1.5X SSPE and 7% SDS. Nylon membrane containing plaques were hybridized overnight at 65°C. The membranes were washed once with 0.5X SSC, 0.1% SDS for 15 min and twice with 0.2X SSC, 0.1 % SDS at 50°C for 1 h each wash. The membranes were then exposed to Kodak Biomax MR films overnight at -70°C with two intensifying screens. Positive cDNAs identified on tertiary screening were isolated and subcloned into pBluescript SK- by in vivo excision as outlined by the manufacturer (Stratagene).

Plasmid isolation were routinely purified from overnight cultures by the alkaline lysis method (Birnboim and Doly, 1979). For sequencing reactions, plasmid DNA was also isolated using the Qiagen kit. Qiagen purified plasmid DNA was denatured with 0.1 volume of 0.2 M NaOH for 10 min at room temperature. The denatured plasmid DNA was neutralized with 0.1 volume of 3 M sodium acetate, pH 5.2 and was precipitated with ethanol. The DNA pellet was then used for dideoxy chain terminator sequencing (Sanger *et al.*, 1977) using Sequenase DNA polymerase (Amersham) with the M13F and M13R primers. The section of the clone containing the novel insert was also sequenced using an automated ABI sequencer. Nucleotide sequences were analyzed with the following computer software: Seqaid II (University of Kansas) and BLAST (Altschul *et al.*, 1997).

The Rapid Amplification of cDNA Ends (RACE) protocol was utilized as described by the manufacturer (Gibco-BRL) to obtain the 5' end of the sequence. PCR was performed on poly dCTP-tailed cDNA prepared from human skeletal muscle RNA using a gene-specific primer (GSP1: nt 1101-1083 of SOCK), nested primer (GSP2: nt 1009-1028 of SOCK) as well as the GibcoBRL anchor primer provided for this purpose. The PCR products were resolved by agarose gel electrophoresis, excised and purified using the Geneclean II resin (Biocan). The purified PCR products were subcloned using the TA cloning kit (Invitrogen) into the pCRII vector. Four clones were sequenced and two on both strands. The sequence of each clone was completed using sequence-specific internal primers (Table 1).

The full length nucleotide sequence was generated as follows. RACE clone #3 was used as a template for PCR to amplify a 630 bp fragment using primers R5 and R3, cut with EcoR1 and Pst1 and ligated to the 9 cHM restriction fragment cut with Pst1. The ligation mix was amplified by PCR using the R5 and R6 primers and the product was then digested, cut with EcoR1 and cloned into pTZ19 (Pharmacia).

The human skeletal muscle CaM-dependent protein kinase nucleotide sequence was submitted to Genbank and its sequence accession number is U23460. This clone was subsequently named SOCK, an acronym for Son of CaM Kinase.

B.13 Cloning of Mouse SOCK and β CaM Kinase II Isoform

To identify members of the CaM kinase family in mouse, a mouse skeletal muscle λgt11 cDNA library (Clontech ML3006b, lot #39055, prepared from the oligo dT and

Table 1. Primers for Sequencing of SOCK and β CaM Kinase II cDNA.

The primers are identified according to their corresponding nucleotide sequences in SOCK. The size (in base pairs) and orientation of each primer is also noted.

Primer name	Size (bp)	SOCK nucleotide sequence	Orientation
P1	24	946-968	5'
P2	24	1820-1843	3'
pro1-1	23	1306-1328	5'
pro1-2	26	1671-1690	3'
pro1-3	20	1820-1839	3'
YPRO-F	25	1306-1330	5'
YPRO-R	18	1652-1669	3'
18C-T2	18	932-949	5'
18C-T3	18	1250-1267	5'
8C-T3	20	1935-1954	3'
8C-T4	20	2008-2027	3'
8C-T5	18	1673-1690	3'
8C-T6	18	1426-1443	3'
18-J3	17	1249-1265	5'
7C-F	18	2102-2119	3'
R1	20	890-909	3'
R2	20	75-94	5'
R3	20	678-697	3'
R4	19	304-322	5'
R5	25	75-92	5'
R6	27	2049-2071	3'
GSP1	19	1083-1101	3'
GSP2	20	1009-1028	3'

random-primed mRNA pooled from 200 BALB/c males, ages 8-12 weeks) was screened with the same cDNA probe as described above. The screening of one million PFUs in the library yielded many positives including one full length clone, referred to as 11D and a second clone, 10 MM, that was slightly smaller in size. The positive cDNA clones were amplified by PCR using gt11-F (5' TGG AGC CCG TCA GTA TCG GCG -3') and gt11-R (5' GTA GCG ACC GGC GCT CAG CTG 3') primers, digested with EcoR1 and ligated to EcoR1 digested pTZ19 (Pharmacia). The open reading frames of both clones were sequenced completely using primer-directed sequencing.

B.14 Genomic Organization of SOCK

The mouse genomic library in the Lambda FIX II vector was prepared from the liver of 4 to 8 week old female mice (Stratagene 129SUJ). One million PFUs were screened. Colony/plaque screen hybridization transfer membranes (DuPont) were probed with the [^{32}P] dCTP labelled novel proline-rich region of SOCK (nt 1310-1690 of SOCK). Phage DNA from positive clones was prepared as previously described (Sambrook *et al.* 1989) using XL1-Blue MRA (P2) as a host. DNA was digested with different restriction enzymes and analyzed by Southern blot hybridized with the same probe used to screen the library. Positive fragments were subcloned into appropriately digested pTZ19 and sequenced from both ends. Potential exon/intron boundaries within the genomic structure were identified by divergence from the cDNA sequence and detection of GT..AG consensus site elaborated for the ends of intronic sequences.

B.15 RNA Isolation and Northern Blot Analysis

Rabbits were sacrificed by cervical dislocation and the indicated tissues were excised and snap frozen in liquid nitrogen. Total cellular RNA was extracted using the Tripure kit according to the manufacturer's instructions (Boehringer Mannheim). RNA samples were size-fractionated on formaldehyde-agarose gels and Northern blot transfer on nylon membranes (MSI MAGNA) was carried out overnight using 10X SSC (1X SSC = 0.15 M NaCl, 0.015 sodium citrate, pH 7.0) (Sambrook *et al.*, 1989). The blots were rinsed with DEPC-treated water and the RNA cross-linked by UV radiation. The blots were prehybridized for 1 hour at 42°C in hybridization solution containing 50% deionized formamide, 0.1% SDS, 200 μg/mL sheared salmon sperm DNA, 6X SSC, 5X Denhardt's Solution (1% Ficoll, 1% polyvinylpyrrolidine, 1% bovine serum albumin). After prehybridization, the various blots were hybridized overnight with randomly primed α-[^{32}P]-dCTP labelled cDNA probes and were then washed under conditions of low stringency (0.1% SDS, 1X SSC) or high stringency (0.1% SDS, 0.1XSSC) as required to minimize background signal. Films were exposed for autoradiography at -70°C for 1-3 days using two intensifying screens. After hybridizing with one probe, each membrane was stripped and sequentially reprobed with a cDNA probe for glyceraldehyde-3-phosphate dehydrogenase to insure equal loading of the samples. The cDNA fragments used as hybridization probes for these analyses were as follows: 1) rabbit brain CaM kinase II β isoform 500 bp fragment flanked by primers #1 and #2 (Bennett and Kennedy, 1987); 2) human skeletal muscle SOCK novel proline-rich domain, 366 bp segment; 3) glyceraldehyde-3-phosphate dehydrogenase; 4) myogenin; 5) non-specific actin probe consisting of a 0.6 Kb Pst1 fragment containing the last exon of the

human cardiac actin gene (Rudnicki *et al.*, 1988).

B.16 GST-Fusion Proteins

GST fusion proteins containing SOCK and the novel proline-rich region were constructed. A SOCK fusion protein construct was created by ligating the full length SOCK restriction fragment to the pGEX3xΔB vector. A fusion protein directed to the novel proline-rich region was constructed by PCR amplification using the primers Pro-1 (nt 1306-1328 of SOCK) and Pro-2 (nt 1690-1671 of SOCK) which flank the novel region. The PCR fragment was cloned into TA cloning vector (Invitrogen), digested with EcoR1 and the released fragment was cloned into the pGEX 2TK vector. This vector codes for the glutathione-S-transferase (GST) polypeptide from *Shistosoma manosi* that is predicted to have a molecular weight of 26 kDa. The ligated clones were sequenced to ensure that the inserted fragment was fused in frame with the GST polypeptide. DH5α bacteria containing the recombinant protein were induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) to activate protein expression. The GST fusion proteins were isolated on glutathione-agarose beads (Pharmacia) following sonication in TBST with 1%Nonidet P-40. Solubility of the fusion protein during sonication was increased with the addition of 1.5% sarkosyl at the expense of kinase activity. GST-srcSH3 and GST-fyn(B)SH3 fusion proteins were isolated in a similar manner using constructs generously provided by Dr. John Bell (University of Ottawa).

B.17 Immunoreactivity of GST-SOCK

One (1) mL aliquots of bacterial culture expressing GST-SOCK were collected at 1h intervals and centrifuged to remove the supernatant nutrient broth. The pellet was resuspended in SDS loading buffer and 20 μL was fractionated by SDS-PAGE. The immunoreactivity of the expressed GST-constructs to the anti-(CaM KII) antibody was investigated by Western blot analysis using the RU16 antibody at dilution of 1:1000 in TBS. The expressed GST-SOCK fusion protein was cleaved with Factor Xa to release the expressed SOCK polypeptide from the GST moiety. The immunoreactivity of the released polypeptide to the anti-(CaM Kinase II) antibody was investigated by Western blot analysis as described above.

B.18 Associations of SOCK with Polypeptides of Rabbit Skeletal Muscle

GST-SOCK, GST-SOCK SH3-binding domain, GST-src SH3 and GST-fyn SH3 fusion proteins were affinity-purified on glutathione-agarose beads. Rabbit skeletal muscle cytosol was prepared as described above. Solubilized membrane fractions were prepared by incubating microsomes with 1% NP40 for 15 min and removing the insoluble material by centrifugation at 100 000g.

For the association assays, 500 μg aliquots were pre-cleared with 5 μg of GST then incubated with 1μg GST-fusion protein or GST alone (on agarose beads) from 3 h to overnight at 42°C. The fusion protein beads were then washed four times in 5 mL of TBST, centrifuged, resuspended and analysed by Western blots. Western blotting was performed using the RU16 antibody or the c-src antibody (Santa Cruz) at a dilution of 1:1000. The c-src

antibody (N-16) is a rabbit affinity-purified polyclonal antibody raised against a peptide corresponding to amino acid residues 3-18, SSKSKPKDPSQRRRSLEP, within the amino terminal region of the human src gene product. The reaction was visualized with either alkaline phosphatase-conjugated anti-rabbit IgG or peroxidase-conjugated anti-mouse IgG.

B.19 Tissue and Subcellular Distribution of the Immunoreactive 73 kDa Polypeptide

The tissue distribution of CaM Kinase II isoforms was investigated. Microsomal fractions from rabbit brain, skeletal muscle and heart were prepared. Tissue was excised from rabbits sacrificed by cervical dislocation and was homogenized in a buffer containing 0.3 M sucrose, 10 mM Tris-HCl, pH 7.4. The homogenates were centrifuged at 110 000 g for 45 min. The resulting supernatants were referred to as cytosol whereas the resuspended pellets formed the microsomal fractions. The cytosol and microsomal fractions were subjected to SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was blocked with 5% skim milk in TBS buffer, pH 7.2. Western blotting was performed using the RU16 anti-(CaMKII) antibody at a dilution of 1 to 5000 in TBST. Each lane contained two hundred and fifty micrograms of protein.

To characterize the nature of the association of the CaM kinase II immunoreactive polypeptides with membrane fractions, skeletal muscle microsomes were further purified into heavy SR membranes as described. Heavy SR membranes were extracted with the following buffers: 2 mM EGTA; 0.6 M KCl; 20 mM pyrophosphate buffer; 1% octyl glucoside. Each extracted mixture was centrifuged at 110 000 g to obtain supernatant and extracted pellet fractions. These samples were analysed by Western blot using the anti-(CaMKII) antibody

at a dilution of 1:1000 in TBS.

The co-distribution of c-src tyrosine kinase and CaM kinase II was also investigated by Western blot analysis with c-src and RU16 antibody (1:1000 dilution) on rabbit skeletal muscle cytosol and microsomes prepared as described above.

B.20 Immunofluorescence Staining of SOCK in C2C12 Cells

A myc-epitope tagged SOCK protein was constructed by subcloning the full length SOCK cDNA into the human myc epitope of Bluescript KS +MTG encoding the peptide sequence MEGKLISEEDL (Roth *et al.*, 1991). The cDNA encoding the fusion protein was excised and subcloned into the pCDNA3 (Invitrogen) vector.

C2C12 myoblast cells (Yaffe and Saxel, 1977) were cultured in a α-minimal essential medium (MEM) supplemented with 10% fetal bovine serum (Cansera) in an incubator with 5% CO_2, 95% air in a humidified atmosphere at 37°C. The cells were transfected with the myc-SOCK pCDNA3 vector by the calcium phosphate protocol (Sambrook *et al.*, 1989) and stable clones were selected with G418. Clones were assayed for expression of the exogenous transcript by Western blot analysis. When cells were differentiated, the cells were cultured until 75% confluent then the culture medium was changed to α-MEM supplemented with 10% horse serum (Gibco BRL). The culture medium was changed every 24 hours for the first two days of differentiation and every 48 hours thereafter. The differentiation of the cultured cells was observed for 5 days.

For immunofluorescence studies, undifferentiated C2C12 cells were grown on matrigel-coated coverslips in α-MEM supplemented with 10% FBS. The coverslips were

washed with PBS and then fixed for 20 min in Lana's fixative consisting of 4% (w/v) paraformaldehyde and 12% (v/v) saturated picric acid in 160 mM sodium phosphate buffer pH 6.9. Myc-tagged SOCK was visualized with the 9E10 anti-myc monoclonal antibody (Evan *et al.*, 1985). Anti- myc used at a 1:50 dilution in PBS containing 0.3% Triton X-100 (PBST). Slides were incubated for 3 h at room temperature in a humidified chamber and then rinsed three times 10 min in PBS. Cy-3 conjugated sheep anti-mouse IgG (Sigma) was diluted 1:100 in PBST and incubated for 30 min at 37°C.

For double immunofluorescence studies, myc-tagged SOCK was immunostained with 9E10 monoclonal mouse antibody (1:50) and src tyrosine kinase was immunostained with anti-c-src rabbit polyclonal antibody (1:100) as described above. The secondary antibodies, mixed and prepared the night before, contained fluorescein isothiocyanate (FITC) labelled donkey anti-rabbit IgG (1:25; Amersham) and biotin-conjugated donkey anti-mouse IgG (1:50) and were incubated for 45min at 37°C. Rinsed slides were then incubated with streptavidin CY3 (1:100; Sigma Chemical) for 30 min. Slides were washed three times 10 min in PBS and mounted using a solution of phenylaminediamine (0.1 mM) in 50% glycerol before examination with a Zeiss Axioplan microscope and a confocal scanning microscope (Wigle *et al.*, 1997). The negative control for immunofluorescence studies was C2C12 cells transfected with the empty pcDNA3 vector.

B.21 CaM-dependent Kinase Peptide Assay

The CaM-dependent protein kinase activity of recombinant GST-SOCK on glutathione agarose beads was measured using a CaM-dependent Protein Kinase II Assay Kit

(Upstate Biochtechnogy Inc., NY). The assay kit is designed to measure the phosphorylation of a specific substrate peptide, auto camtide II (KKALRRQETVDAL), a well-known peptide substrate of multifunctional CaM kinase II isoforms. The phosphorylation was performed according to the manufacturer's recommendations. Final kinase buffer concentrations were as follows: 10 mM MOP, pH 7.2, 12 mM β-glycerol phosphate, 0.5 mM sodium orthovanadate, 0.5 mM dithiothreitol, 0.5 mM $CaCl_2$, 250 μM auto camtide II, 0.4 μg CaM, 18 mM $MgCl_2$ and 125 μM ATP (10 μCi/vial), 10 μg GST-SOCK. Samples were incubated for 10 min at 30°C and the reaction was terminated with 20 μL ice-cold 40% trichloroacetic acid. Twenty microlitre aliquots were blotted onto P81 phosphocellulose squares that were washed three times 5 min in 0.75% phosphoric acid and once in acetone before quantitation by liquid scintillation counting. All reactions were performed in duplicate. The results were corrected for background counts obtained in the absence of added enzyme activity. Ten microlitre aliquots of the reaction were subjected to SDS-PAGE followed by autoradiography. The effect of src tyrosine kinase on SOCK was compared in a set of parallel studies where 10 units of recombinant human c-src kinase (Upstate Biotechnology #14-117), was preincubated for 15 min in the above assay. Control experiments were performed to ensure that the components of the c-src formulation buffer (25 mM HEPES, pH 7.0, 50% glycerol, 0.1% NP40, 1 mM DTT) had no effect on SOCK phosphorylation activity. Hck, lyn and fyn tyrosine kinases used in preliminary experiments to study the effect on SOCK in kinase assays were obtained from the laboratories of Dr. Andrew Wilks (Ludwig Institute, Australia) and the CaM-dependent protein kinase substrate used for the preliminary set of experiments was kindly provided by Dr. Bruce Kemp. The results represent the mean ± SEM

for four experiments.

B.22 Transfection and Expression of SOCK in P19 Embryonal Carcinoma Cells

The full length cDNA for SOCK was subcloned into the pPOP expression vector. P19 cells were transfected by the calcium phosphate method with three different plasmids: pPOP-SOCK; pgk-NeoR, coding for the neomycin resistance gene; B17, containing an intragenic segment of phosphoglycerate kinase -1 (pgk-1) shown to enhance expression of transfected genes in P19 cells (Chen and Okayama, 1987; Pratt *et al.*, 1990; McBurney *et al.*, 1994). Transfected cells were treated with G418 and stable clones were isolated and assayed for expression of SOCK by Northern (cDNA probe = proline-rich domain) and Western (RU16 = antibody) blot analysis. For Western blot analysis, cells grown in 60 mm tissue culture dishes were collected by scraping with a rubber policeman in RIPA solution (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) with protease inhibitors as previously described. The dislodged cells were sheared by repeated passes through a 23 gauge syringe needle and mixed with SDS-PAGE loading buffer. The polypeptides were then processed as described for immunoblotting with the RU16 antibody (1:1000) visualized with the alkaline phosphatase conjugated IgG colorimetric reaction

Control and SOCK-transfected P19 cells were induced to differentiate with dimethyl sulfoxide (DMSO) or retinoic acid as previously described (Rudnicki and McBurney, 1987). P19 cells were dispersed with trypsin and one million cells were transferred to a Petri dish containing 10 mL of α-MEM supplemented with 5% donor bovine serum and 5% fetal bovine serum. The culture medium was changed every 48 h until completion of the experiment. The

morphological appearance of the cells was recorded by black and white photography using a single lens reflex camera mounted on a light microscope.

B.23 Northen Blot Analysis of P19 Cultures

P19 cells were cultured as described previously (Pratt *et al.*, 1990). Total RNA from the cultures was isolated on the indicated days using the Trizol Reagent (Gibco/BRL) following the manufacturer's protocol. Aliquots of total RNA (20 μg/lane) were electrophoresed through formaldehyde agarose (1%) gels, blotted onto nylon membranes (MSI) and cross-linked under UV light. The cDNA probes (rat myogenin and actin) were prepared by random primer labelling with $\alpha[^{32}P]$CTP. Blots from control and SOCK-transfected cultures were prehybridized for 1 h at 42°C in hybridization solution and then incubated overnight at 42°C with the probes. The blots were washed at low (0.1% SDS, 1X SSC,25°C) or high stringency (0.1% SDS, 0.1XSSC, 65°C) as required to reduce background signal and were exposed to Kodak Biomax MR film at -70 °C with an intensifying screen. The P19 cells were kindly provided by Dr. Christine Pratt.

C. RESULTS

C.1 Biochemical Characterization of Calmodulin-Dependent Protein Kinases from Skeletal Muscle Membranes

Although calmodulin-dependent protein kinase activities have been shown to associate with skeletal muscle membranes, the identity of the polypeptides containing the kinase activities and the nature of their associations remain unknown. Consistent with the definition of a kinase, a polypeptide with calmodulin-dependent protein kinase enzymatic activity would necessarily contain binding sites for calmodulin (CaM) and ATP. It would also demonstrate the ability to phosphorylate both itself and other substrates in a Ca^{2+}/CaM-dependent manner.

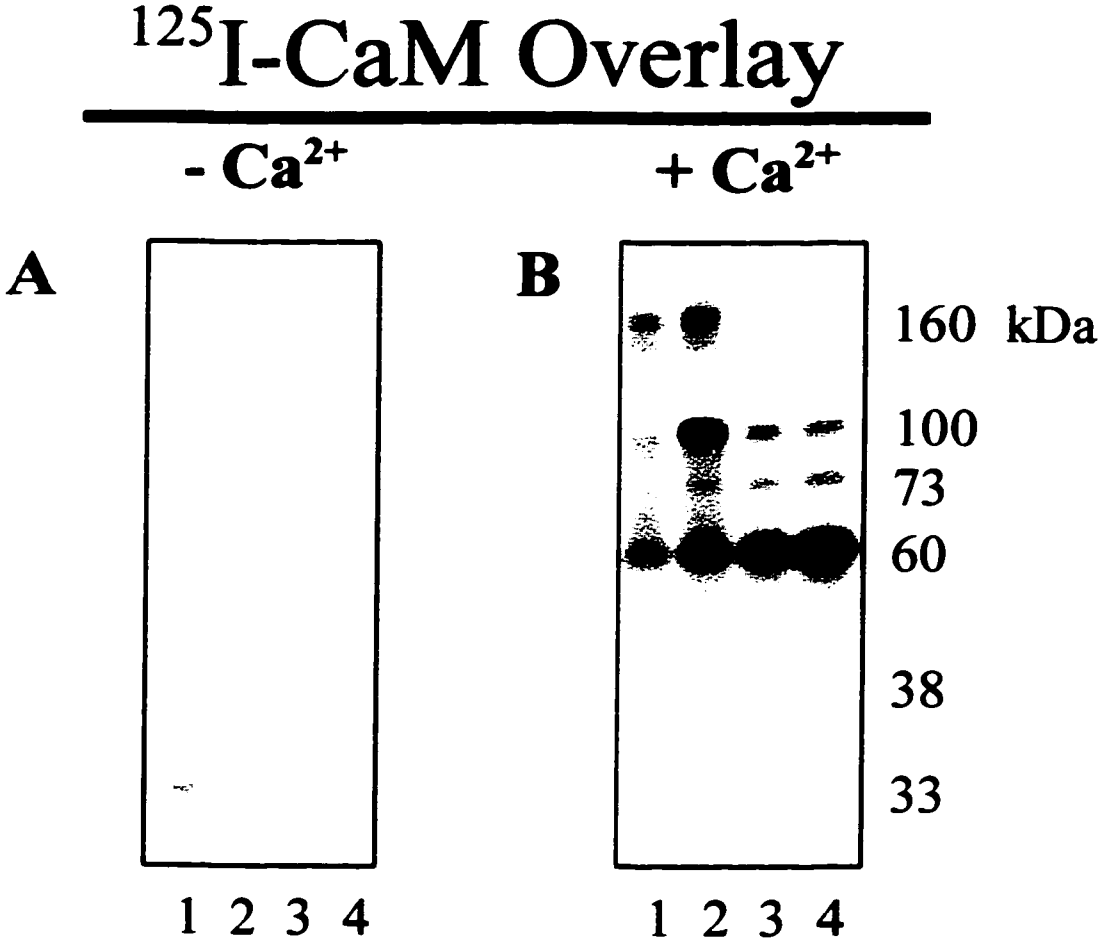
C.1.1 CaM-binding Proteins in Subcellular Fractions from Skeletal Muscle

In order to identify CaM kinases in muscle cells, the CaM receptors in skeletal muscle were investigated in binding assays. CaM-binding polypeptides were identified by incubating radioactive [^{125}I]-CaM with various subcellular fractions of rabbit skeletal muscle that were previously separated by-SDS-PAGE and transferred to nitrocellulose. Subcellular fractions were obtained by high-speed differential centrifugation of rabbit muscle homogenates that separated the denser material (membranes and organelles) from the cytosol (134 000 g supernatant). Repeated differential centrifugations were used to purify a crude microsomal fraction away from nuclei and mitochondria. The mixed membrane population of the crude microsomal fraction was further purified by discontinuous gradient centrifugation. Using sucrose as a gradient material, fractions enriched in plasma membrane (sarcolemma),

transverse tubules (invaginations of sarcolemma) and sarcoplasmic reticulum were isolated according to established protocols. Sarcoplasmic reticulum membranes could be further fractionated into light SR (enriched in longitudinal sections) or heavy SR (enriched in terminal cisternae regions). The identification of the isolated membrane fractions was confirmed using enzymatic markers and ligand binding assays according to established protocols. For example, the transverse tubules fractions were enriched in dihydropyridine-binding sites whereas the light SR and heavy SR fractions were enriched in Ca^{2+}ATPase activity and ryanodine-binding sites, respectively. The CaM-binding polypeptides in these subcellular fractions were visualized by autoradiography as shown in Fig. 4. In the presence of calcium (Fig. 4, panel B), a 60 kDa polypeptide, enriched in the heavy SR membrane preparations (lane 4), was the predominant CaM-binding protein. In addition, polypeptides of 38, 73, 100 and 160 kDa bound CaM in the presence of calcium. The 73 kDa CaM-binding polypeptide was enriched in the heavy SR membrane while the 38, 100 and 160 kDa polypeptides were enriched in the transverse-tubule fractions (Fig. 4, lane 2). The binding of CaM was calcium-dependent because in the presence of the calcium chelator, EGTA (Fig. 4, panel A), only a 33 kDa polypeptide present in the light membrane fraction (lane 1) was labelled by [^{125}I]-CaM whereas the 38, 60, 73, 100 and 160 kDa polypeptides were not. It is possible that additional CaM-binding polypeptides in skeletal muscle may not have been detected in this overlay experiment because their calmodulin-binding sites were irreversibly denatured in SDS-PAGE. It should be noted that the presence of a CaM-binding site alone is not sufficient to attribute CaM-dependent protein kinase activity to a polypeptide. A CaM-dependent kinase must also

Figure 4 Identification of [^{125}I]-CaM-Binding Proteins in Rabbit Skeletal Muscle

Skeletal muscle fractions were separated by SDS-PAGE and transferred to nitrocellulose: light membrane fraction (lane 1); t-tubules (lane 2); light SR (lane 3); heavy SR (lane 4). The nitrocellulose was incubated with [^{125}I]-CaM in the presence of (A) 2 mM EGTA or (B) 1 mM Ca^{2+}. The dried nitrocellulose membranes were exposed for autoradiography. Two hundred and fifty micrograms of protein were loaded per lane. The calmodulin-binding proteins are indicated on the right along with their molecular weights in kiloDaltons (kDa).


^{125}I-CaM Overlay
- Ca^{2+}
+ Ca^{2+}
A
B
160 kDa
100
73
60
38
33
1 2 3 4
1 2 3 4

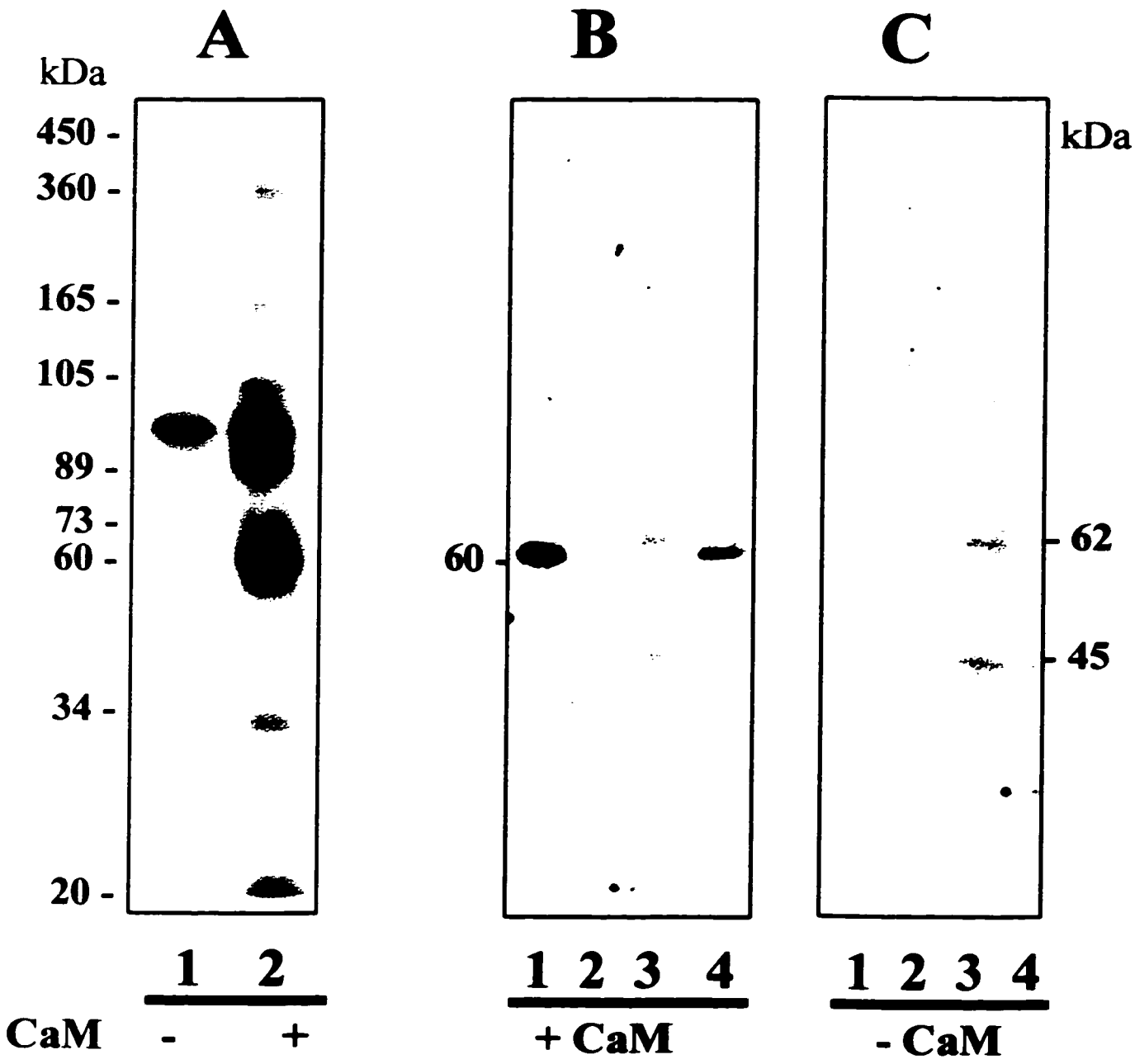
demonstrate the ability to phosphorylate itself and its various substrates in a CaM-dependent fashion.

C.1.2 Identification of the Polypeptides Exhibiting Autophosphorylation Activity in SR Membranes

The sarcoplasmic reticulum membrane-enriched fractions were selected to study CaM-binding polypeptides since the 60 and 73 kDa CaM-binding sites were significantly enriched in these fractions. The CaM-dependent phosphorylation activity associated with the sarcoplasmic reticulum of skeletal muscle was visualized by incubating SR membranes with radioactive ATP in the presence of kinase buffer. The phosphorylation reaction was dependent on magnesium (10 mM $MgCl_2$,) and required calcium (10-100 μM) and CaM (0.6 μM). Since CaM was known to associate with the sarcoplasmic reticulum, the SR-enriched membranes were first extracted with the calcium chelator EGTA to remove any endogenous CaM that associates with its receptors through calcium. SR membranes were incubated for 90 s and the incorporation of radioactivity from the gamma phosphate of ATP was visualized by autoradiography following SDS-PAGE. The results of a representative phosphorylation assay are shown in Fig. 5, panel A. SR membranes were phosphorylated with [γ-^{32}P]ATP in the absence (lane 1) or presence (lane 2) of 0.6 μM CaM. The major substrates of the CaM-dependent phosphorylation activity, as evidenced by amounts of ^{32}P incorporation, were polypeptides of 89, 60, 34 and 20 kDa. This data was consistent with the CaM-dependent phosphorylated polypeptides previously defined in skeletal muscle (Tuana and MacLennan, 1988). Polypeptides of 73, 105, 165, 360 and 450 kDa were also

Figure 5 **Identification of Autophosphorylation Activity of Sarcoplasmic Reticulum Proteins.**

Heavy sarcoplasmic reticulum-enriched membranes were extracted with EGTA to remove endogenous bound CaM and incubated in a phosphorylation assay as described in the methods. (A) Autoradiogram of the phosphorylation pattern of sarcoplasmic reticulum proteins by endogenous kinases with 50 μM [γ^{32}P]ATP in the absence (lane 1) and presence (lane 2) of 0.6 μM CaM. In (B) and (C), subcellular fractions of rabbit skeletal muscle were separated by SDS-PAGE, transferred to nitrocellulose and the polypeptides renatured as described: crude microsomes (lane 1); transverse-tubules (lane 2); light SR (lane 3); heavy SR (lane 4). After renaturation, autophosphorylation was assayed in the presence of either (B) Ca^{2+}/CaM or (C) Ca^{2+} alone. The nitrocellulose was then dried and exposed for autoradiography. One hundred micrograms of protein were loaded in each lane. The phosphorylated polypeptides are identified according to their interpolated molecular weight in kDa.


A
B
C
kDa
450 -
360 -
165 -
105 -
89 -
73 -
60 -
34 -
20 -
60 -
kDa
62
45
1 2
CaM - +
1 2 3 4
+ CaM
1 2 3 4
- CaM

phosphorylated in a CaM-dependent manner. Two proteins of 97 and 53 kDa were phosphorylated in the absence of CaM and may act as substrates for a CaM-independent kinase activity (lane 1).

Having identified not only the protein kinases activities residing in SR but also the substrates for CaM-dependent phosphorylation, it was necessary to determine which of these polypeptides possessed the ability to autophosphorylate in the presence of CaM. A protocol to renature proteins, first described by Celenza and Carlson (Celenza and Carlson, 1986), was used to identify the polypeptides containing protein kinase domains. This method allows the detection of autophosphorylation activity of protein kinases transferred to nitrocellulose after size-fractionation by SDS-PAGE. This technique was modified, as described in the Methods section, to renature membrane-associated proteins and to detect autophosphorylation activity in skeletal muscle membrane fractions. Nitrocellulose blots of various muscle membrane fractions renatured according to this method were incubated in a phosphorylation assay with [γ-^{32}P]ATP. The blots were washed with 1N KOH following the renaturation reaction to remove residual radioactivity resulting only from ATP-binding (base labile) rather than ATP-dependent phosphorylation activity (base stable). Representative autoradiograms are shown in Fig. 5, panels B and C. A 60 kDa polypeptide in the microsomal fraction incorporates the greatest quantity of ^{32}P in a Ca^{2+}/CaM-dependent manner (Fig. 5B, lane 1). The microsomal fraction comprises a mixture of membranes originating from the sarcolemma, transverse tubules, sarcoplasmic reticulum and other organelles. Upon fractionation of the microsomes, the 60 kDa autophosphorylated polypeptide was found to associate predominantly with membranes enriched in heavy SR (lane 4), although it was also present in the light SR-

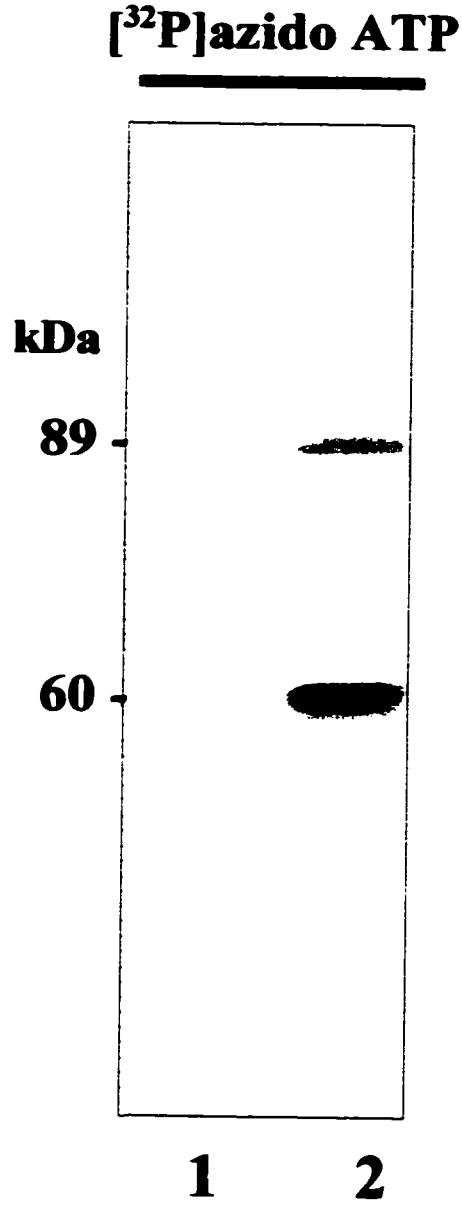
enriched fraction (lane 3). No autophosphorylation of the 60 kDa polypeptide was detected in the absence of CaM (Fig. 5C). Interestingly, CaM-independent autophosphorylation was noted on polypeptides of 62 and 45 kDa enriched in the light SR membranes (Figs 5B and 5C, lane 3). A similar autophosphorylation pattern for the 62 and 45 kDa polypeptides was observed in the presence of EGTA whereas the 60 kDa polypeptide did not show any autophosphorylation under these conditions. No autophosphorylation activity was detected in the t-tubule-enriched fraction (lane 2).

C.1.3 The 60 kDa Polypeptide Contains ATP and CaM-Binding Sites

Protein kinases are known to contain high affinity ATP-binding domains (Hunter, 1991). In an attempt to provide further evidence for the identity of the 60 kDa polypeptide as a CaM-dependent protein kinase, ATP-binding sites in sarcoplasmic reticulum polypeptides were identified by covalent labelling with [^{32}P]azido-ATP. Azido groups are compounds that are chemically inert. When exposed to ultraviolet light at wavelengths between 250-460 nm they are photolyzed into reactive compounds. The activated azido group reacts non-selectively to form a covalent bond with the protein thereby irreversibly tagging the polypeptide with its radioactive ATP ligand. To reduce non-specific background signal, our experiment was carried out on partially purified CaM-dependent protein complexes from sarcoplasmic reticulum. These kinase complexes were tightly associated with heavy SR-enriched membranes and required detergent solubilization prior to purification by CaM-affinity chromatography. The CaM kinase complexes were only eluted from the CaM-agarose column with EGTA, demonstrating the specificity of the interaction on the column. The

Figure 6 **Photoaffinity Labelling of the CaM-dependent Protein Kinase Complex with [^{32}P]azido-ATP.**

The CaM-dependent protein kinase complex of skeletal muscle was partially purified as described. Photolabelling was carried out using assay conditions similar to those used for the phosphorylation reaction, except that 10 nM [^{32}P]azido-ATP replaced [γ^{32}P]-ATP in the kinase buffer. The samples were subjected to SDS-PAGE and autoradiography. Labelling with [^{32}P]azido-ATP in the presence (lane 1) and absence (lane 2) of excess unlabelled ATP is shown. The position of the ATP-binding polypeptides is indicated on the left along with their molecular weight in kDa.


[32P]azido ATP
kDa
89
60
1
2

covalent attachment of [^{32}P]azido-ATP, in the presence (lane 1) and absence (lane 2) of excess unlabelled ATP, was visualized in these CaM kinase complexes. A representative autoradiogram is shown (Fig. 6). [^{32}P]azido-ATP was specifically bound to a 60 kDa polypeptide in the purified kinase preparation. Upon longer exposure of the autoradiogram, detectable amounts of ATP-binding could also be observed in a 89 kDa polypeptide. No incorporation of the label was detected in the absence of ultraviolet light. The 60 kDa polypeptide is the major CaM-binding polypeptide in the heavy SR-enriched membranes (Fig. 4). It has been shown that a 60 kDa polypeptide also contained the CaM-binding sites in the purified CaM kinase complex preparation (Tuana and MacLennan, 1988). In the same study, phosphopeptide mapping revealed that the 89 kDa polypeptide was a structurally distinct polypeptide which co-purified with the 60 kDa polypeptide on the CaM-affinity column.

Our results, showing that the 60 kDa polypeptide contained CaM- and ATP-binding sites, as well as its ability to undergo CaM-dependent autophosphorylation in renaturation assays on nitrocellulose, provide strong evidence that the 60 kDa polypeptide is a CaM-dependent protein kinase associated with SR membranes. Using the renaturation-overlay technique, we have also identified a Ca^{2+}/CaM-independent autophosphorylation activity associated with polypeptides of 62 and 45 kDa enriched in the light SR membranes. Since a Ca^{2+}-independent kinase activity that phosphorylates polypeptides of 62, 42 and 20 kDa in SR vesicles has been described (Campbell and Shamoo, 1980), this kinase activity may be attributable to the 62 and 45 kDa polypeptides of the light SR-enriched membranes. While the 60 and 73 kDa polypeptides were found to enrich in the heavy SR, we did not detect any autophosphorylation activity in the t-tubule-enriched membranes although previously

uncharacterized CaM-binding polypeptides of 160, 100, and 38 kDa were identified and found to enrich in these membranes.

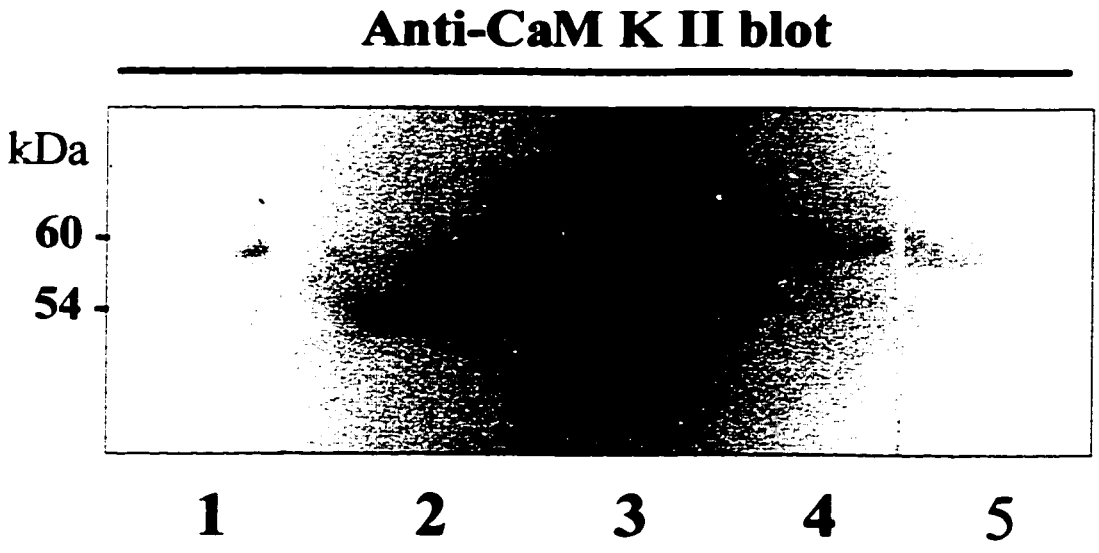
Although our data provide clear evidence for a 60 kDa polypeptide as a CaM-dependent protein kinase, we cannot rule out the existence of other polypeptides exhibiting CaM-dependent protein kinase activity. Additional CaM-binding and ATP-binding polypeptides, such as the 89 kDa polypeptide, were detected in the SR membrane and their enzyme activities may not be fully renatured under our assay conditions. Likewise, the detection of CaM and ATP-binding sites on a polypeptide do not necessarily signal the presence of CaM-dependent kinase activity.

C.1.4 The 60 kDa CaM Kinase of SR and the Soluble Multifunctional CaM kinase II

Tissue expression studies have previously detected the classical multifunctional CaM kinase II isoforms, first described in brain, to be present in skeletal muscle (Shenolikar *et al.*, 1986). The properties of the membrane-associated 60 kDa CaM kinase of the SR and the soluble multifunctional CaM kinase II isoforms were compared. The immunoreactivity of an affinity-purified polyclonal antibody (RU16) that recognizes conserved amino acid sequences of the multifunctional CaM kinase II isoforms was examined in Western blots of subcellular fractions from skeletal muscle (Fig. 7). The RU16 antibody was raised against a peptide derived from the amino acid sequence (a.a.444-468) of the CaM kinase II β isoform. This peptide sequence, TAFEPEALGNLVEGLDFHRFYFENL, is well conserved across isoforms of the multifunctional CaM kinase II family. Two immunoreactive polypeptides of 60 and 54 kDa were detected by the antibody in crude microsomal preparations from skeletal muscle

Figure 7 **Immunostaining of Rabbit Skeletal Muscle Fractions with Anti-(CaM kinase II) Antibody**

Skeletal muscle membrane fractions were size-fractionated by SDS-PAGE and transferred to nitrocellulose blots. Immunoreactive polypeptides were detected in crude microsomes (lane 1), supernatant from pyrophosphate wash (lane 2), pyrophosphate-washed heavy SR (lane 3), isolated CaM kinase complex (lane 4) and junctional face membrane (lane 5) using the anti-(CaM kinase II) at a dilution of 1:3000. Twenty-five micrograms of protein were loaded in each lane. The molecular weight of the immunoreactive polypeptides is indicated in kDa on the left.


Anti-CaM K II blot
kDa
60
54
1
2
3
4
5

(Fig. 7, lane 1). To determine the nature of association of these immunoreactive polypeptides with the membrane, crude microsomal preparations were extracted either with high salt solutions or detergents to identify peripheral and integral proteins, respectively. The 54 kDa polypeptide could be selectively removed from the microsomal membranes with a pyrophosphate wash (lane 2) which is thought to release cytoskeletal and peripheral proteins from the membrane preparations. The 60 kDa polypeptide remained associated with the heavy SR-enriched membranes (lane 3) and more specifically to SR fractions enriched in junctional face membrane (lane 5). The junctional face membrane corresponds to the segment of SR that is in close apposition to the transverse tubule and contains a high density of Ca^{2+} release channels. The 60 kDa polypeptide was the major immunoreactive polypeptide in the CaM-affinity purified kinase preparation (lane 4). This immunoreactive polypeptide migrated in SDS-PAGE with identical mobility to the 60 kDa polypeptide which exhibits CaM-dependent autophosphorylation activity, and CaM- binding.

The 60 kDa CaM kinase of SR was different from the soluble multifunctional CaM kinase II in that it was membrane-associated and could only be solubilized with detergents. Affinity-purified polyclonal antibodies raised against the brain multifunctional CaM kinase II subunits cross-reacted with a 60 kDa polypeptide of SR and a 54 kDa polypeptide in skeletal muscle. The cross reactivity of the brain antibody was expected since it was raised against a peptide sequence that was highly conserved across different CaM-dependent protein kinase isoforms. Our results indicated a selective association of the 60 kDa CaM kinase with specific membrane populations within the muscle cell since a 54 kDa polypeptide recognized by the antibodies was readily dissociated from the microsomal membranes by a treatment known to

remove peripheral proteins (Mitchell *et al.*, 1983). In contrast, the 60 kDa CaM kinase was not only enriched in the heavy SR fractions but also detected in the junctional face membrane of SR. The junctional face represents the section of SR that is enriched in Ca^{2+}-release channels and plays an important role during excitation-contraction coupling.

The function of CaM kinases has been investigated with specific CaM inhibitors. By using a specific CaM kinase antagonist, Wang and Best (1992) provided strong evidence for the existence of a CaM kinase localized close to and responsible for gating the Ca^{2+}-release channel. This peptide inhibitor is derived from the inhibitory domain of the CaM kinase II polypeptide (Fig.8, panel A), hence it is referred to as CaM kinase II inhibitory fragment (290-309) (Payne *et al.*, 1988). In unstimulated cells, this autoinhibitory sequence maintains CaM-dependent protein kinases in an inactive state. This inhibition can be overcome by the binding of Ca^{2+}/CaM that induces a conformational change in the polypeptide. The sensitivity of muscle CaM-dependent protein kinases to this inhibitor of CaM functions was examined in view of comparing its response with other CaM kinases and determining its usefulness as an inhibitor of CaM-dependent phosphorylation in skeletal muscle. The effect of increasing concentrations of CaM-kinase II inhibitory fragment (290-309) on the CaM-dependent phosphorylation of SR proteins was investigated (Fig. 8, panel B inset). The CaM-dependent phosphorylation was inhibited by the CaM kinase II fragment and the inhibition of the CaM-dependent ^{32}P incorporation into the 450, 360, 165, 105, 89, 73, 45, 34 and 20 kDa polypeptides followed the same qualitative pattern as that of the 60 kDa polypeptide. The CaM-dependent phosphorylation of the 60 kDa phosphoprotein was

Figure 8 **Inhibition of the CaM-dependent Kinase Complex of SR by the CaM Kinase II Fragment (290-309).**

(A) Amino acid sequence of the CaM kinase II inhibitory fragment (290-309). Numbering of amino acid residues is based on the CaM kinase II α isoform.

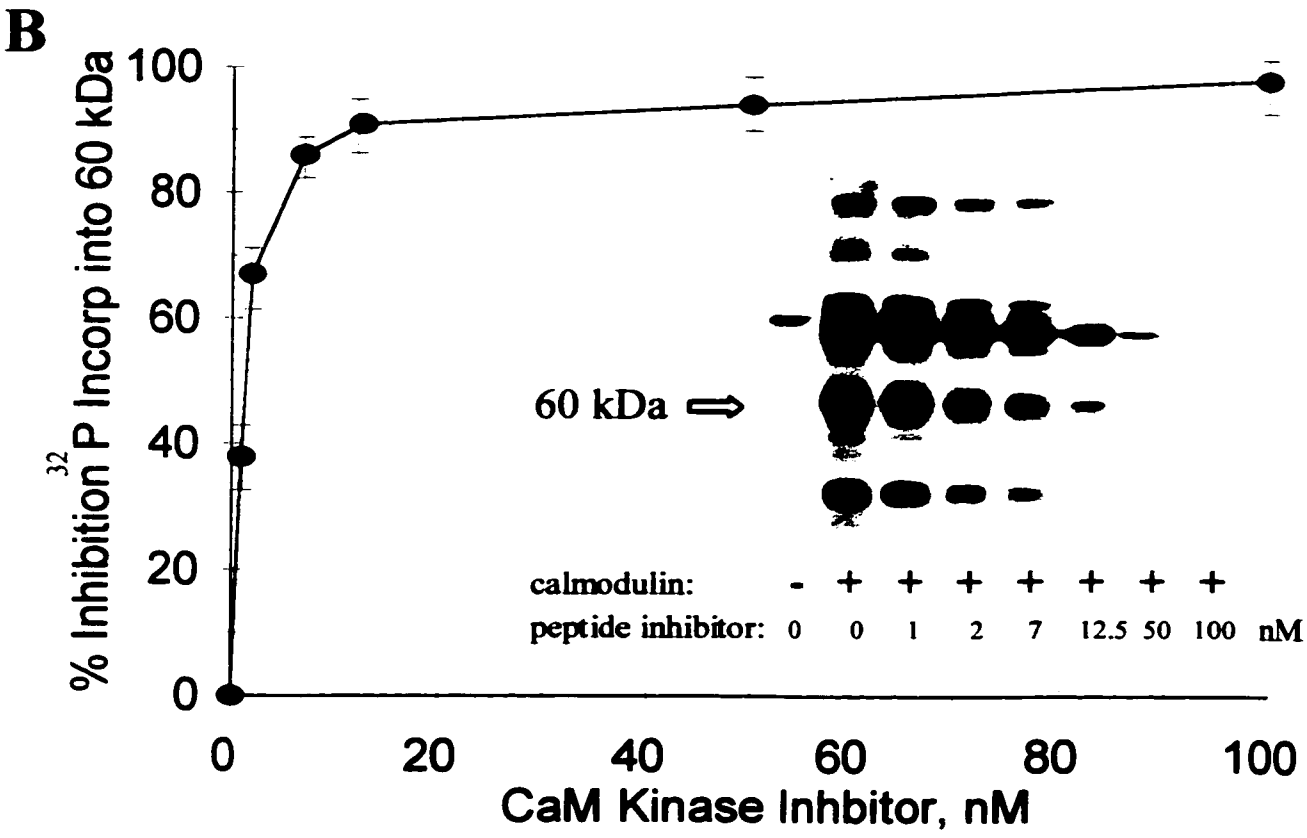
(B) SR membranes were phosphorylated (see inset) as described in the absence (lane 1) and presence (lanes 2-8) of 0.6 μM CaM with increasing concentrations of the CaM kinase inhibitor. The position of the 60 kDa phosphopeptide is indicated in the inset by an arrowhead on the left. The CaM-dependent phosphorylation of the 60 kDa polypeptide was identified by autoradiography and quantified by liquid scintillation. The inhibition of CaM-dependent ^{32}P incorporation into the 60 kDa polypeptide by the CaM kinase II fragment (290-309) was expressed as a percentage of the control values measured in the presence of CaM and absence of CaM kinase II fragment. The results represent the mean ± SEM for three experiments.

A

...LKKFNARRKLKGAILTTMLA...

Amino acid # 290 309

B



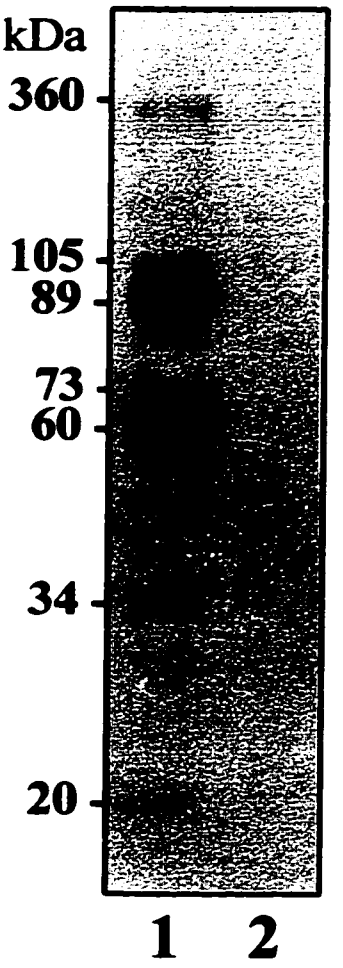
quantified by liquid scintillation counting of the radioactive polypeptides identified by autoradiography and cut from SDS gels. The inhibition of CaM-dependent ^{32}P incorporation into the 60 kDa polypeptide was plotted as a function of CaM kinase II inhibitor concentration (Fig. 8B). CaM-dependent incorporation of ^{32}P into the 60 kDa polypeptide was completely eliminated at inhibitor concentrations of 100 nM. The estimated IC_{50} for this inhibition was 2 nM, a value well below the previously reported IC_{50} values of 1 to 200 μM obtained for the brain multifunctional CaM kinases using CaM kinase II fragments of varying lengths (Colbran *et al.*, 1989).

C.1.5 Associations of the CaM-dependent Protein Kinases of SR

Molecular data shows that CaM-dependent protein kinases contain association domains at the C-terminus of the polypeptide. In the multifunctional CaM kinase II isoforms, these domains are responsible for the formation of complex quaternary structures between kinase molecules (Kanaseki *et al.*, 1991). To investigate the protein-protein interactions of the CaM kinases of the SR membrane, immunoprecipitation experiments were performed using the RU16 anti-(CaM kinase II) antibody. Fractions enriched in SR membranes were incubated with [γ-^{32}P] ATP under phosphorylating conditions and solubilized with the detergent NP40. The solubilized proteins were immunoprecipitated and size-fractionated by SDS-PAGE. As shown by autoradiography (Fig. 9), a number of phosphoproteins, including the 60 kDa polypeptide, were immunoprecipitated by the anti-(CaM kinase II) antiserum (lane 1). The other phosphoproteins that were co-immunoprecipitated included the 20, 34, 73, 89, 105 and 360 kDa phosphoproteins. No

Figure 9 **Immunoprecipitation of the SR CaM-dependent Protein Kinase Complex by the Anti-(CaM kinase II) Antibody.**

Skeletal muscle SR-enriched membranes were phosphorylated and solubilized with NP40 as described in the Methods section. The solubilized fractions were incubated with the anti-(CaM kinase II) antibody and the antibody complexes were isolated with Protein A-agarose. The proteins eluted from Protein A-agarose were subjected to SDS-PAGE and autoradiography. Immunoprecipitations with anti-(CaM kinase II) antibody (lane 1) and with serum from non-immunized rabbits (lane 2) are shown. The molecular weight of the immunoprecipitated phosphoproteins is indicated on the left.


kDa
360
105
89
73
60
34
20
1
2

phosphoproteins were immunoprecipitated by serum from non-immunized rabbits (lane 2).

The associations of these polypeptides were further explored using density centrifugation. Linear sucrose density centrifugation can separate polypeptide complexes according to size and density. The individual polypeptide components of the isolated complexes can then be visualized by subjecting gradient fractions to SDS-PAGE. CaM kinase complexes of SR obtained by CaM-affinity purification were phosphorylated in a CaM-dependent manner and were centrifuged on a 5-20% linear sucrose density gradient. Fractions were collected, size-fractionated by SDS-PAGE and visualized by autoradiography (Fig. 10). The complex of 89, 73, 60 and 34 kDa phosphoproteins was found to co-migrate toward the bottom of the sucrose gradient, providing further evidence of association among these polypeptides. The 34 kDa phosphoprotein was run off the gel shown in Fig. 10. The 34, 60 and 73 kDa phosphoproteins were only isolated as complexes with the 89 kDa phosphoprotein while some 89 kDa polypeptides did not display associations to other phosphoproteins. This data may indicate the presence of two distinct 89 kDa phosphoproteins or reflect the different endogenous states of associations of a 89 kDa phosphoprotein.

Polyclonal antibodies to the gel-purified 60 kDa kinase polypeptide were raised in guinea pigs in order to further characterize this SR-associated protein. Western blots were performed on the material isolated from the CaM-affinity column (Fig. 11). The anti-(60 kDa kinase polypeptide) serum cross-reacted with a single 60 kDa polypeptide (lane 2) whereas the other components of the SR CaM kinase complex were not immunoreactive. The anti-(60 kDa polypeptide) serum did not cross-react with the CaM kinase isolated from cardiac SR

Figure 10. Linear Sucrose Density Gradient Fractionation of CaM-Affinity Purified Kinase Complex of SR Membranes

Heavy SR-enriched membranes from rabbit skeletal muscle were solubilized with NP40. The solubilized fraction was affinity-purified by CaM affinity chromatography and eluted with EGTA. The resulting CaM-dependent protein kinase complex was phosphorylated in the presence of 0.6μM CaM and size-fractionated by centrifugation through a 5-20% linear sucrose density gradient. Fractions (0.5 mL) were collected, concentrated and subjected to SDS-PAGE and autoradiography.

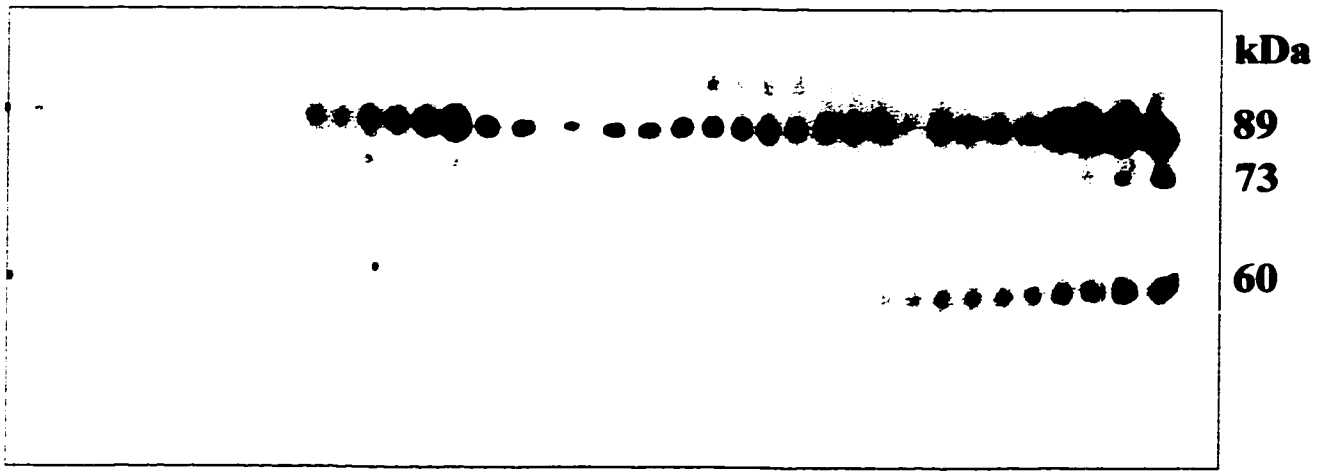
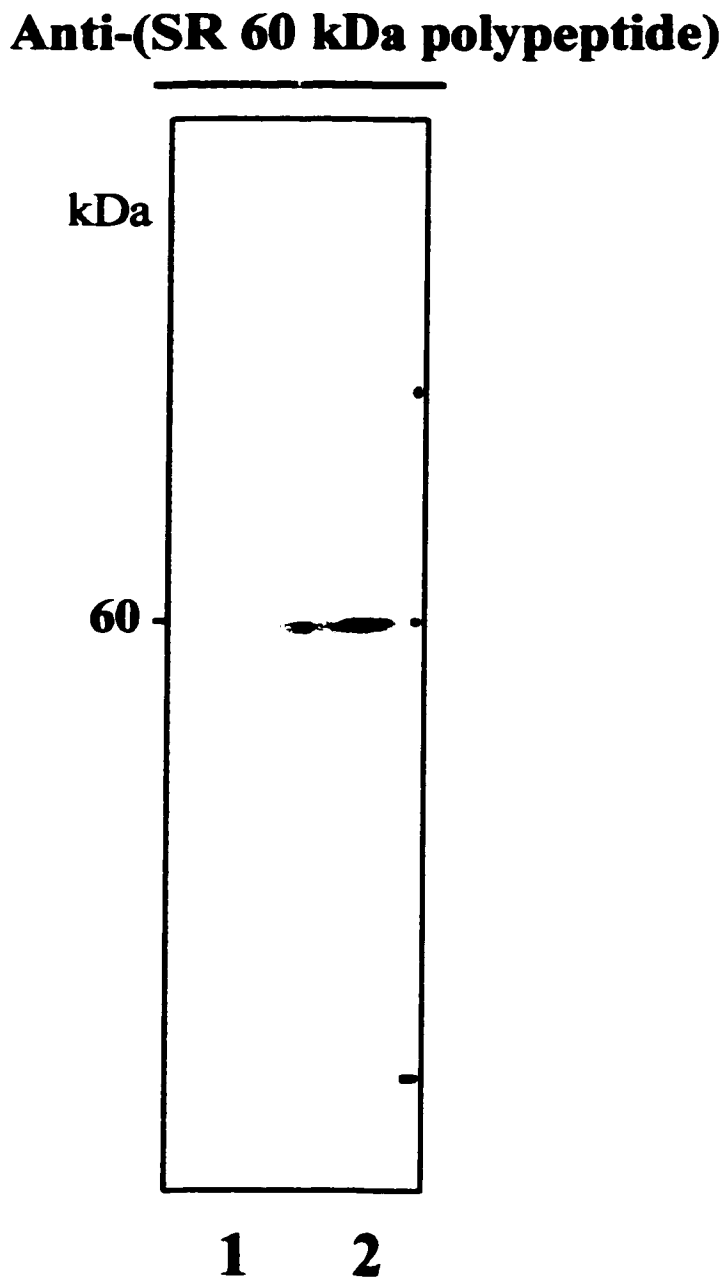

kDa
89
73
60
5%
Linear sucrose gradient
20%
Top
Bottom

Figure 11. Immunoreactivity of the Anti-60 kDa Serum with Components of the CaM Kinase Complex.

The 60 kDa kinase was isolated from a CaM-affinity column and gel purified. Polyclonal antibodies against the 60 kDa polypeptide were raised in guinea pigs as described in the Methods section. A Western blot was performed on the material isolated on the CaM affinity column from cardiac SR (lane 1) and skeletal muscle SR (lane 2). A 1 to 250 dilution of anti-(60 kDa polypeptide) serum was used and the reaction was developed with alkaline phosphatase-conjugated secondary antibody.


Anti-(SR 60 kDa polypeptide)
kDa
60
1
2

(lane 1) or the soluble multifunctional CaM kinase II from skeletal muscle and brain. The purified SR CaM kinase complex was phosphorylated as described above, treated with SDS and immunoprecipitated with the anti-(60 kDa polypeptide) serum. The 60 kDa phosphoprotein was immunoprecipitated by the anti-(SR 60 kDa polypeptide) serum from the CaM-dependent phosphorylation reaction (results not shown). None of the phosphoproteins associating with the 60 kDa polypeptide could be immunoprecipitated by the anti-(60 kDa polypeptide) because the antibody only recognized the SDS-denatured form of the 60 kDa polypeptide.

Anti-(60 kDa polypeptide) antibodies did not cross-react with the other phosphoproteins of SR suggesting that the 360, 105, 89, 34 and 20 kDa proteins do not share epitopes that are in common with the 60 kDa polypeptide. This is consistent with a previous study which showed that the 89, 60, 34 and 20 kDa proteins of the CaM kinase complex were structurally distinct polypeptides (Tuana and MacLennan, 1988). The anti-(60 kDa SR kinase) did not cross-react with a CaM kinase from cardiac SR or the subunits of the brain multifunctional CaM kinase II. Furthermore, the 60 kDa CaM kinase of SR was much more sensitive to an inhibitor of CaM kinase functions than the soluble brain CaM kinase II. These results suggest that the 60 kDa polypeptide of SR may be a distinct isoenzyme of the CaM-dependent protein kinase family.

C.1.6 The CaM Kinase Complex and Phosphorylation of the Ca^{2+} Release Channel

Since CaM-dependent phosphorylation has been implicated in the regulation of Ca^{2+} release from the SR, we sought to resolve whether the Ca^{2+} release channel was a substrate

for the CaM-dependent protein kinase of SR. The Ca^{2+} release channel was purified from skeletal muscle heavy SR using its ability to bind to the plant alkaloid, ryanodine. Briefly, the SR-enriched membranes were labelled with [^{3}H] ryanodine, solubilized with detergent, and size-fractionated on a linear sucrose density gradient. The radioactivity associated with the fractions collected from the gradient is shown in Fig. 12. The major peak of radioactivity detected near the top of the gradient appears to represent unbound [^{3}H] ryanodine. A second peak of radioactivity, fractions 17-23, was pooled and concentrated. The inset shows a representative silver-stained SDS gel of the pooled peak fractions (17-23) of radioactivity. The 450 kDa polypeptide visualized on the gel corresponds to the molecular weight of the Ca^{2+} release channel/ryanodine receptor.

A silver-stained gel of the purified ryanodine receptor and the CaM-dependent protein kinase complex of skeletal muscle SR used in the phosphorylation experiments is shown in Fig. 13, panel A. The purified ryanodine receptor migrated as a doublet of 450 and 425 kDa (panel A, lane 1) whereas the CaM kinase complex appeared as polypeptides of 89, 60 and 34 kDa (panel A, lane 2). The 20 kDa species was run off the gel. The ryanodine receptor was incubated with the CaM kinase in the presence of [γ^{32}P]ATP and phosphorylation buffer as described in the Methods. The resulting autoradiogram is shown in Fig. 13, panel B. Despite CaM-dependent incorporation of radiolabel into the 60 kDa CaM kinase (panel B, lane 2) and of its substrates (apparent upon longer exposure), no phosphorylation was detected at the level of the ryanodine receptor (panel B, lane 4) by using three different preparations of the purified ryanodine receptor and the CaM kinase. The phosphorylation of the ryanodine receptor was also investigated in its native membrane environment. The SR

Figure 12. Purification of the Ca^{2+} Release Channel from Skeletal Muscle SR

Sarcoplasmic reticulum membranes from rabbit skeletal muscle were labelled with [^{3}H] ryanodine, solubilized with detergent, and size-fractionated on a 5-20% linear sucrose density gradient. Fractions were collected in 1 ml increments and radioactivity was measured by liquid scintillation. Corrected values for [^{3}H] ryanodine binding are plotted versus individual fractions. The results represent a typical purification experiment. The inset panel shows a representative silver-stained SDS-PAGE gel of the pooled peak fractions of radioactivity.

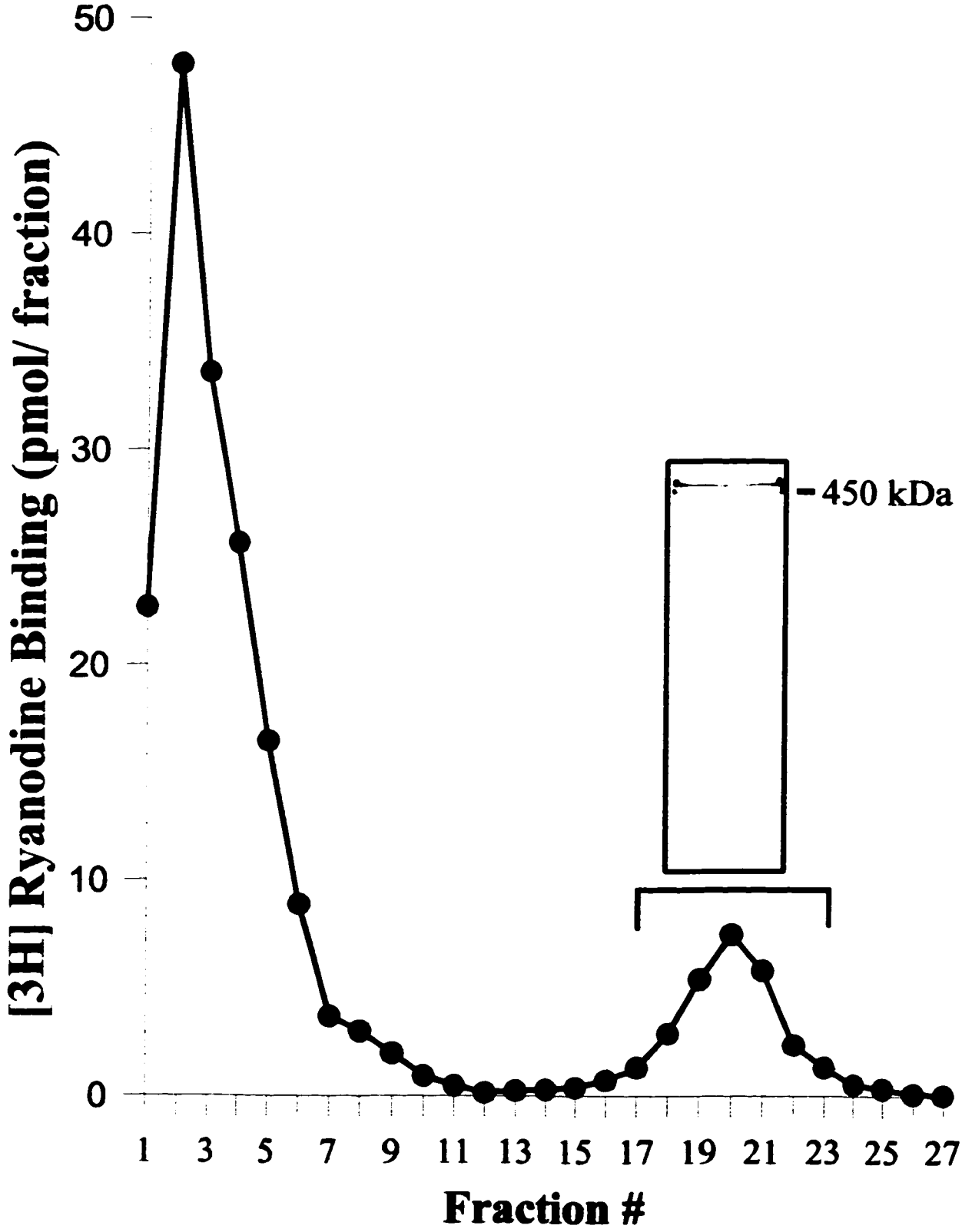
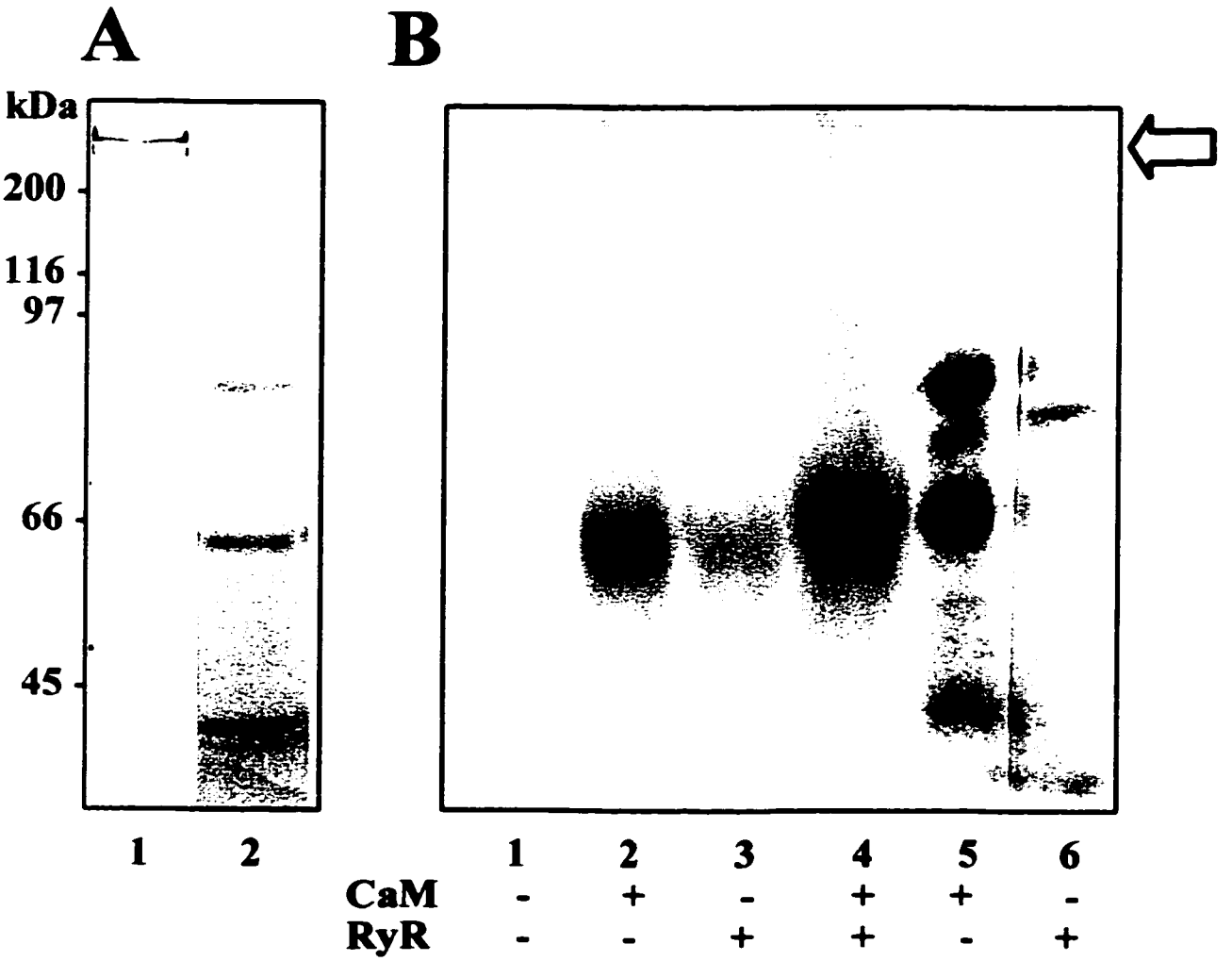

50
40
30
20
10
0
[3H] Ryanodine Binding (pmol/ fraction)
450 kDa
1
3
5
7
9
11
13
15
17
19
21
23
25
27
Fraction #

Figure 13 Reconstitution of CaM-dependent Protein Kinase and Ca^{2+} Release Channel of the SR.

The purified ryanodine receptor and the CaM kinase complex were separated by SDS-PAGE and the protein composition was revealed by silver staining (panel A, lanes 1 and 2 respectively). The purified ryanodine receptor and CaM kinase complex were reconstituted in a phosphorylation reaction and the phosphoprotein composition analyzed by SDS-PAGE and autoradiography. Panel B represents a typical autoradiogram of CaM- dependent phosphorylation of purified CaM kinase (lanes 1 and 2); purified ryanodine receptor plus kinase complex (lanes 3 and 4); heat denatured SR plus purified kinase complex (lane 5); purified ryanodine receptor plus purified protein kinase C (lane 6). The arrowhead indicates the phosphorylated ryanodine receptor.


A
B
kDa
200
116
97
66
45
1
2
1
2
3
4
5
6
CaM
- + - + + -
RyR
- - + + - +

membranes were heat-treated prior to phosphorylation to inactivate any endogenous kinase activity. In heat-inactivated preparations of the SR membrane, the purified kinase phosphorylated the 89, 73, 60, 34 and 20 kDa polypeptides, but not the ryanodine receptor (panel B, lane 5), although the ryanodine receptor was a substrate of purified protein kinase C (panel B, lane 6) that appears as an 80 kDa phosphoprotein. The phosphoprotein located close to the dye front (panel B, lane 6) may be a breakdown product of the Ca^{2+}- release channel or protein kinase C or a polypeptide that co-purifies with ryanodine receptor.

C.2 Molecular Characterization of a Novel CaM-Dependent Protein Kinase in Skeletal Muscle

The 60 kDa polypeptide of SR was initially proposed to be quite similar to the classical multifunctional CaM kinase II β isoform from brain. In the previous chapter, we showed that the 60 kDa polypeptide of the SR membrane was indeed a CaM-dependent protein kinase that phosphorylates and associates with several SR proteins but possesses some distinct characteristics in comparison to the multifunctional CaM kinase II β isoform from brain. Our evidence also suggested that other polypeptides, such as the 73 and 89 kDa polypeptides, were potential candidates for CaM-dependent protein kinase activity. The relatively low abundance and tight membrane association made it difficult to purify sufficient amounts of CaM-dependent protein kinases from muscle membranes. Furthermore, to date, no multifunctional CaM-dependent protein kinases from skeletal muscle had been characterized at the molecular level. Therefore, to further our understanding of this signalling system, we sought to characterize the CaM kinases of skeletal muscle using the techniques

Figure 14. Strategy for Cloning CaM-dependent Protein Kinases in Skeletal Muscle

A human skeletal muscle cDNA library was probed for CaM-dependent protein kinase sequences. Positive clones were detected using a 500 bp cDNA probe derived from the β isoform of the brain multifunctional CaM kinase II. The probe was generated by reverse transcriptase-directed PCR of rat brain mRNA using the primers (P1 and P2) which were specifically chosen to flank the area of greatest diversity in the CaM kinase family.

of molecular biology.

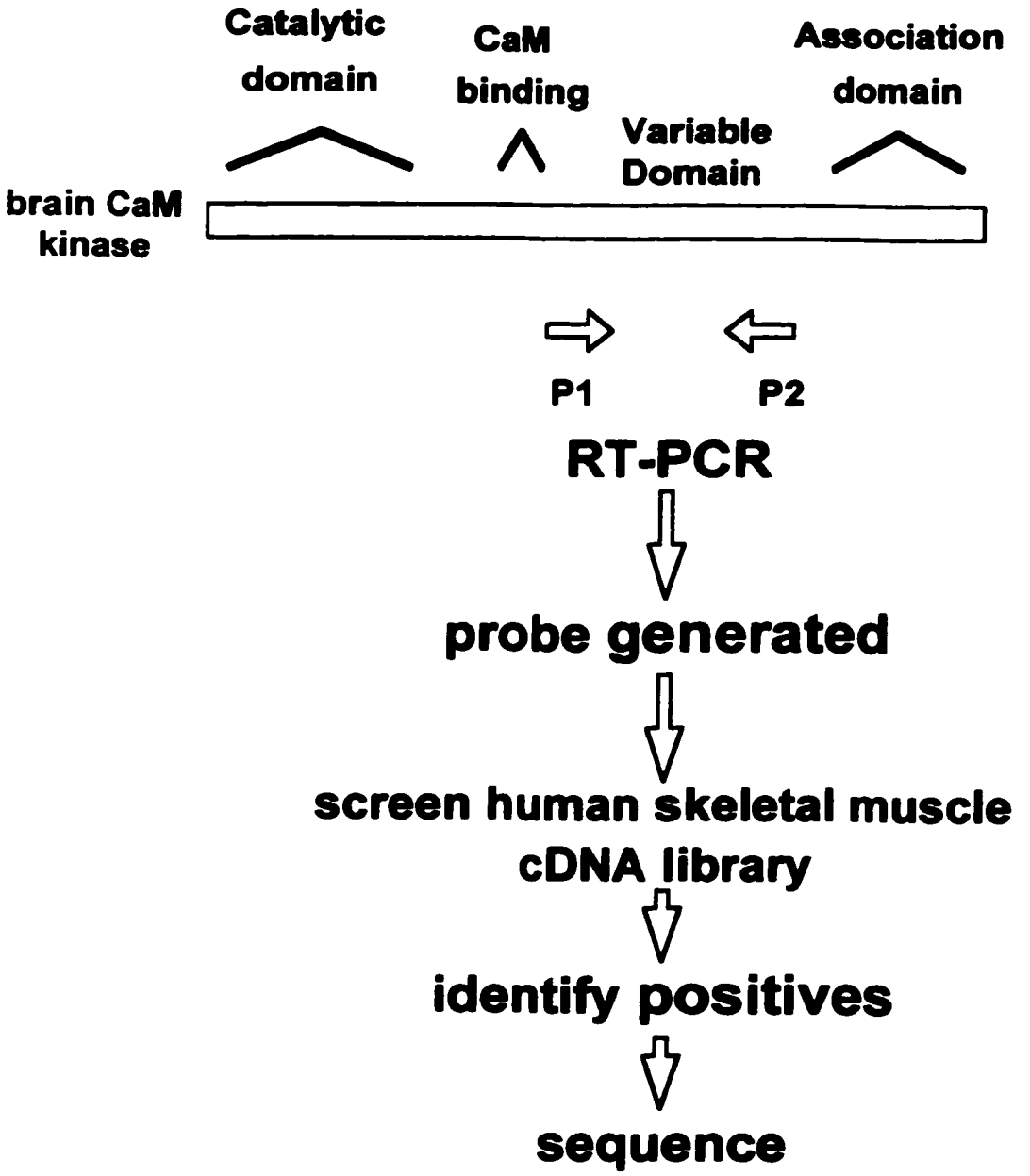
C.2.1 Primary Structure of a Novel β-CaM Kinase from Human Skeletal Muscle

Previous studies have led to the cloning of the cDNA of the multifunctional CaM kinase II β isoforms from brain and pancreas (Bennett and Kennedy, 1987; Urquidi and Ashcroft, 1995). To identify members of the CaM kinase family in skeletal muscle, a human skeletal muscle cDNA library was screened with a 500 bp cDNA probe. The probe was derived from the β isoform of the brain multifunctional CaM kinase II and was generated by reverse transcriptase-directed PCR of rat brain RNA. The primers were specifically chosen to flank the variable domain of the kinase, area of greatest diversity in the CaM kinase II family (Fig. 14). The screening of approximately one million clones yielded four clones, 7, 8, 16 and 18 cHM, that were plaque purified. Further screening of the library yielded two additional clones, 9cHM and 14 cHM, spanning an area of 1750 bp. Nucleotide sequence analysis indicated that all six cDNA clones contained putative open reading frames but did not contain a consensus start site. The rapid amplification of cDNA ends (RACE) was therefore used to obtain the 5' end of the clones. Three RACE products that were sequenced contained the 5' region of the mRNA which provided perfect overlap over 400 bp with the 5' end of the previously sequenced clones. A schematic representation of the overlapping clones and restriction endonuclease sites is shown in Fig. 15. The overlapping clones were sequenced on both strands and the composite nucleotide sequence of 2.2 kb is presented in Fig. 16. A continuous open reading frame was obtained by splicing the longest 5' RACE product (Race-3) to the longest cDNA clone containing the stop codon (9 cHM). The first methionine

Figure 15. Sequencing Strategy and Restriction Endonuclease Map for the cDNA Clones Encoding CaM kinase II

A linear composite map of the full length CaM kinase II cDNA is shown. The open bar indicates the coding region. The overlapping cDNA clones that were sequenced are shown below. The following restriction endonuclease sites are identified: Apa I (A), Hinc II (H), KspI (K), Pst I (P), Tth 111 I (T).

Strategy for cloning CaM kinase



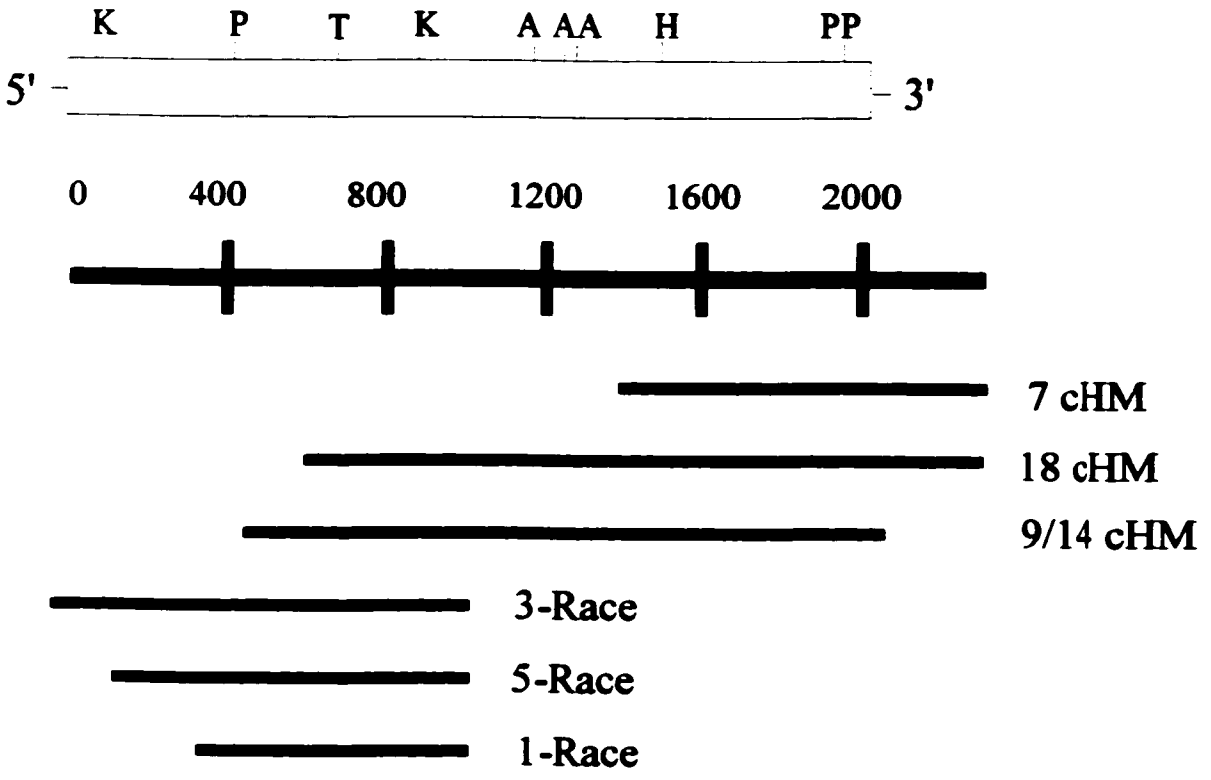

K
P
T
K
A AA
H
PP
5'
3'
0
400
800
1200
1600
2000
7 cHM
18 cHM
9/14 cHM
3-Race
5-Race
1-Race

Figure 16. Nucleotide and Deduced Amino Acid Sequence of Human Skeletal Muscle CaM Kinase II

The nucleotide sequence of human skeletal muscle CaM kinase II is shown with nucleotide positions indicated on the right. Numbering of the nucleotide sequence is positive after the initiation codon and is negative prior to the initiation codon. The stop codon is indicated by the symbol ">". The deduced amino acid sequence is shown below and is numbered on the left. Regions of interest are indicated as follows: ATP-binding site (***); calmodulin-binding site (»»»»), novel proline-rich region (underlined); peptide recognized by RU16 anti-(CaMK II) antibody (*italics*).

```
                                                                  CCGTG -71
    CCGCGAGCGGAGCCGGAGTCGCCGCCGCCCGAGCGCAGCCGAGCGCACGCCGAGCCCGTCCGCCGCGGCC  -1
    ATGGCCACCACGGTGACCTGCACCCGCTTCACCGACGAGTACCAGCTCTACGAGGATATTGGCAAGGGGG  70
1   M  A  T  T  V  T  C  T  R  F  T  D  E  Y  Q  L  Y  E  D  I  G  K  G  A
                                                                 **********
    CTTTCTCTGTGGTCCGACGCTGTGTCAAGCTCTGCACCGGCCATGAGTATGCAGCCAAGATCATCAACAC 140
25   F  S  V  V  R  R  C  V  K  L  C  T  G  H  E  Y  A  A  K  I  I  N  T
     ************************************************************
    CAAGAAGCTGTCAGCCAGAGATCACCAGAAGCTGGAGAGAGAGGCTCGGATCTGCCGCCTTCTGAAGCAT 215
48   K  K  L  S  A  R  D  H  Q  K  L  E  R  E  A  R  I  C  R  L  L  K  H
    TCCAACATCGTGCGTCTCCACGACAGCATCTCCGAGGAGGGCTTCCACTACCTGGTCTTCGATCTGGTCA 285
71  S  N  I  V  R  L  H  D  S  I  S  E  E  G  F  H  Y  L  V  F  D  L  V  T
    CTGGTGGGGAGCTCTTTGAAGACATTGTGGCGAGAGAGTACTACAGCGAGGCTGATGCCAGTCACTGTAT 355
95    G  G  E  L  F  E  D  I  V  A  R  E  Y  Y  S  E  A  D  A  S  H  C  I
    CCAGCAGATCCTGGAGGCCGTTCTCCATTGTCACCAAATGGGGGTCGTCCACAGAGACCTCAAGCCGGAG 425
118  Q  Q  I  L  E  A  V  L  H  C  H  Q  M  G  V  V  H  R  D  L  K  P  E
    AACCTGCTTCTGGCCAGCAAGTGCAAAGGGGCTGCAGTGAAGCTGGCAGACTTCGGCCTAGCTATCGAGG 495
141 N  L  L  L  A  S  K  C  K  G  A  A  V  K  L  A  D  F  G  L  A  I  E  V
    TGCAGGGGGACCAGCAGGCATGGTTTGGTTTCGCTGGCACACCAGGCTACCTGTCCCCTGAGGTCCTTCG 565
165   Q  G  D  Q  Q  A  W  F  G  F  A  G  T  P  G  Y  L  S  P  E  V  L  R
    CAAAGAGGCGTATGGCAAGCCTGTGGACATCTGGGCATGTGGGGTGATCCTGTACATCCTGCTCGTGGGC 635
188  K  E  A  Y  G  K  P  V  D  I  W  A  C  G  V  I  L  Y  I  L  L  V  G
    TACCCACCCTTCTGGGACGAGGACCAGCACAAGCTGTACCAGCAGATCAAGGCTGGTGCCTATGACTTCC 705
211 Y  P  P  F  W  D  E  D  Q  H  K  L  Y  Q  Q  I  K  A  G  A  Y  D  F  P
    CGTCCCCTGAGTGGGACACCGTCACTCCTGAAGCCAAAAACCTCATCAACCAGATGCTGACCATCAACCC 775
235   S  P  E  W  D  T  V  T  P  E  A  K  N  L  I  N  Q  M  L  T  I  N  P
    TGCCAAGCGCATCACAGCCCATGAGGCCCTGAAGCACCCGTGGGTCTGCCAACGCTCCACGGTAGCATCC 845
258  A  K  R  I  T  A  H  E  A  L  K  H  P  W  V  C  Q  R  S  T  V  A  S
    ATGATGCACAGACAGGAGACTGTGGAGTGTCTGAAAAAGTTCAATGCCAGGAGAAAGCTCAAGGGAGCCA 915
281 M  M  H  R  Q  E  T  V  E  C  L  K  K  F  N  A  R  R  K  L  K  G  A  I
                                                      »»»»»»»»»»»»»»»»»»»»»»»»»»»»»»»»
    TCCTCACCACCATGCTGGCCACACGGAATTTCTCAGTGGGCAGACAGACCACCGCTCCGGCCACAATGTC 985
305   L  T  T  M  L  A  T  R  N  F  S  V  G  R  Q  T  T  A  P  A  T  M  S
      »»»»»»»»»»»»»»»»»»»»»»»»»»»»»»»»
    CACCGCGGCCTCCGGCACCACCATGGGGCTGGTGGAACAAGCCAAGAGTTTACTCAACAAGAAAGCAGAT 1055
328  T  A  A  S  G  T  T  M  G  L  V  E  Q  A  K  S  L  L  N  K  K  A  D
    GGAGTCAAGCCCCAGACGAATAGCACCAAAAACAGTGCAGCCGCCACCAGCCCCAAAGGGACGCTTCCTC 1125
351 G  V  K  P  Q  T  N  S  T  K  N  S  A  A  A  T  S  P  K  G  T  L  P  P
    CTGCCGCCCTGGAGCCTCAAACCACCGTCATCCATAACCCAGTGGACGGGATTAAGGAGTCTTCTGACAG 1195
375   A  A  L  E  P  Q  T  T  V  I  H  N  P  V  D  G  I  K  E  S  S  D  S
    TGCCAATACCACCATAGAGGATGAAGACGCTAAAGCCCCCAGGGTCCCCGACATCCTGAGCTCAGTGAGG 1265
398  A  N  T  T  I  E  D  E  D  A  K  A  P  R  V  P  D  I  L  S  S  V  R
                                        ___________________________________
    AGGGGCTCGGGAGCCAGAAGCCGAGGGGCCCCTGCCTGCCCATCTCCGGCTCCCTTTAGCCCCCTGCCAG 1335
421 R  G  S  G  A  R  S  R  G  A  P  A  C  P  S  P  A  P  F  S  P  L  P  A
    ___________________________________________________________________
    CCCCATCCCCCAGGATCTCTGACATCCTGAACTCTGTGAGAAGGGGTTCAGGAACCCCAGAAGCCGAGGG 1405
445  P  S  P  R  I  S  D  I  L  N  S  V  R  R  G  S  G  T  P  E  A  E  G
    ___________________________________________________________________
    CCCCCTCTCAGCGGGGCCCCCGCCCTGCCTGTCTCCGGCTCTCCTAGGCCCCCTGTCCTCCCCGTCCCCC 1475
468  P  L  S  A  G  P  P  P  C  L  S  P  A  L  L  G  P  L  S  S  P  S  P
    ___________________________________________________________________
    AGGATCTCTGACATCCTGAACTCTGTGAGGAGGGGCTCAGGGACCCCAGAAGCCGAGGGCCCCTCGCCAG 1545
491 R  I  S  D  I  L  N  S  V  R  R  G  S  G  T  P  E  A  E  G  P  S  P  V
    ___________________________________________________________________
    TGGGGCCCCCGCCCTGCCCATCTCCGACTATCCCTGGCCCCCTGCCCACCCCATCCCGGAAGCAGGAGAT 1615
515  G  P  P  P  C  P  S  P  T  I  P  G  P  L  P  T  P  S  R  K  Q  E  I

    CATTAAGACCACGGAGCAGCTCATCGAGGCCGTCAACAACGGTGACTTTGAGGCCTACGCGAAAATCTGT 1685
538  I  K  T  T  E  Q  L  I  E  A  V  N  N  G  D  F  E  A  Y  A  K  I  C
    GACCCAGGGCTGACCTCGTTTGAGCCTGAAGCACTGGGCAACCTGGTTGAAGGGATGGACTTCCACAGAT 1775
561 D  P  G  L  T  S  F  E  P  E  A  L  G  N  L  V  E  G  M  D  F  H  R  F
    TCTACTTCGAGAACCTGCTGGCCAAGAACAGCAAGCCGATCCACACGACCATCCTGAACCCACACGTGCA 1825
585   Y  F  E  N  L  L  A  K  N  S  K  P  I  H  T  T  I  L  N  P  H  V  H

    CGTCATTGGAGAGGATGCCGCCTGCATCGCTTACATCCGGCTCACGCAGTACATTGACGGGCAGGGCCGG 1895
608  V  I  G  E  D  A  A  C  I  A  Y  I  R  L  T  Q  Y  I  D  G  Q  G  R
    CCCCGCACCAGCCAGTCTGAGGAGACCCGCGTGTGGCACCGCCGCGACGGCAAGTGGCAGAACGTGCACT 1965
631 P  R  T  S  Q  S  E  E  T  R  V  W  H  R  R  D  G  K  W  Q  N  V  H  F
    TCCACTGCTCGGGCGCGCCTGTGGCCCCGCTGCAGTGAAGAGCTGCGCCCTGGTTTCGCCGGACAGAGTT 2035
655   H  C  S  G  A  P  V  A  P  L  Q  <
    GGTGTTTGGAGCCCGACTGCCCTCGGGCACACGGCCTGCCTGTCGCATGTTTGTGTCTGCCTCGTTCCCT 2105
    CCCCTGGTGCCTGTGTCTGCAGAAAAACAAGACCAGATGTGATTTGTT                        2150
```

codon (ATG) was located 71 nucleotides into the sequence and conformed with the consensus sequences established for translation initiation sites (Kozak, 1991). A stop codon was present at nucleotide 2063. The nucleotide sequence homology search revealed a 90% identity with members of the CaM kinase II β isoform family. An open reading frame encoded a protein of 665 amino acid residues with a predicted molecular mass of 72,836 Da. The polypeptide was predicted to be slightly basic with a theoretical pI value of 8.06. The relative hydrophobicity of the amino acid sequence was examined according to the Kyte-Doolittle algorithm (Kyte and Doolittle, 1982). The Kyte-Doolittle plot generated from the deduced amino acid sequence suggested that the protein was predominantly hydrophilic with one area of increased hydrophobicity (Fig. 17). In fact, this peptide sequence (a.a. 195-215) was also selected as a putative membrane spanning sequence by transmembrane domain predictive algorithms (Hofmann and Stoffel, 1992). Several other protein motifs, as predicted by the PROSITE database, were noted within the novel polypeptide. The most striking feature of the molecule was a proline-rich region located at a.a. 410-532. Potential N-glycosylation sites were found starting at a.a. 313, 357 and 399. Numerous consensus phosphorylation sites were also detected. Most notable were the additional three cAMP-dependent protein kinase (a.a. 420, 457, 500) and six protein kinase C (a.a. 418, 446, 455, 489, 498, 532) sites found within the proline-rich domains. A potential tyrosine phosphorylation site was predicted to occur at a single site, RFTDEYQLY, located at the extreme N-terminus of the polypeptide (a.a. 9-17).

The predicted amino acid sequence of the novel clone also showed a number of characteristic motifs associated with the CaM kinase II family of proteins (Fig. 16). These

Figure 17. Kyte-Doolittle Analysis of Skeletal Muscle CaM Kinase II Sequence

The Kyte-Doolittle hydrophobicity plot for the predicted amino acid sequence of the human skeletal muscle CaM Kinase II was generated setting the window length at seven amino acids. The polypeptide displays mostly hydrophilic properties with the exception of one segment, amino acid residues 195-215, that displayed significant hydrophobicity as indicated by the marked positive deviation on the plot.

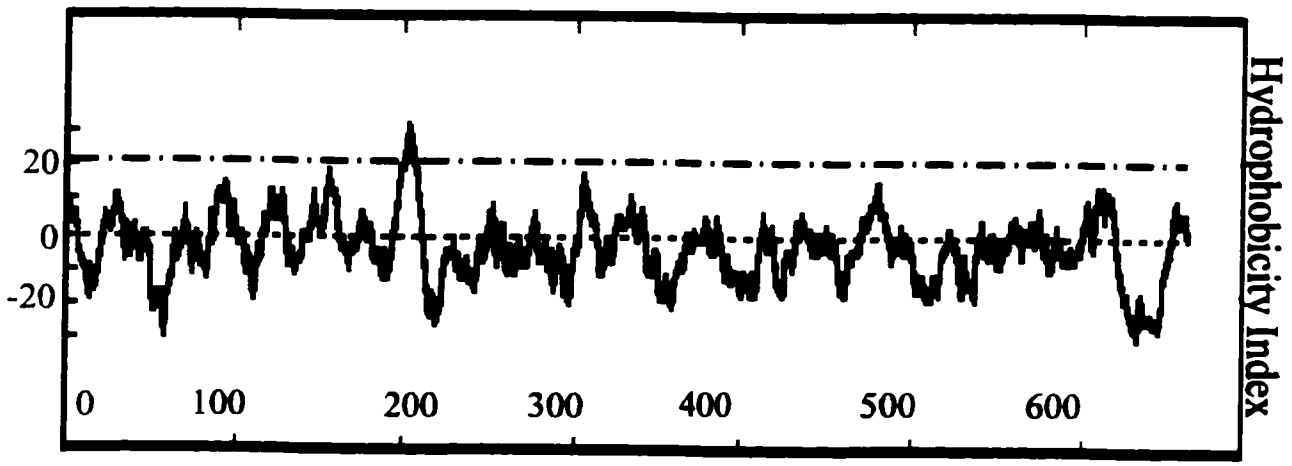
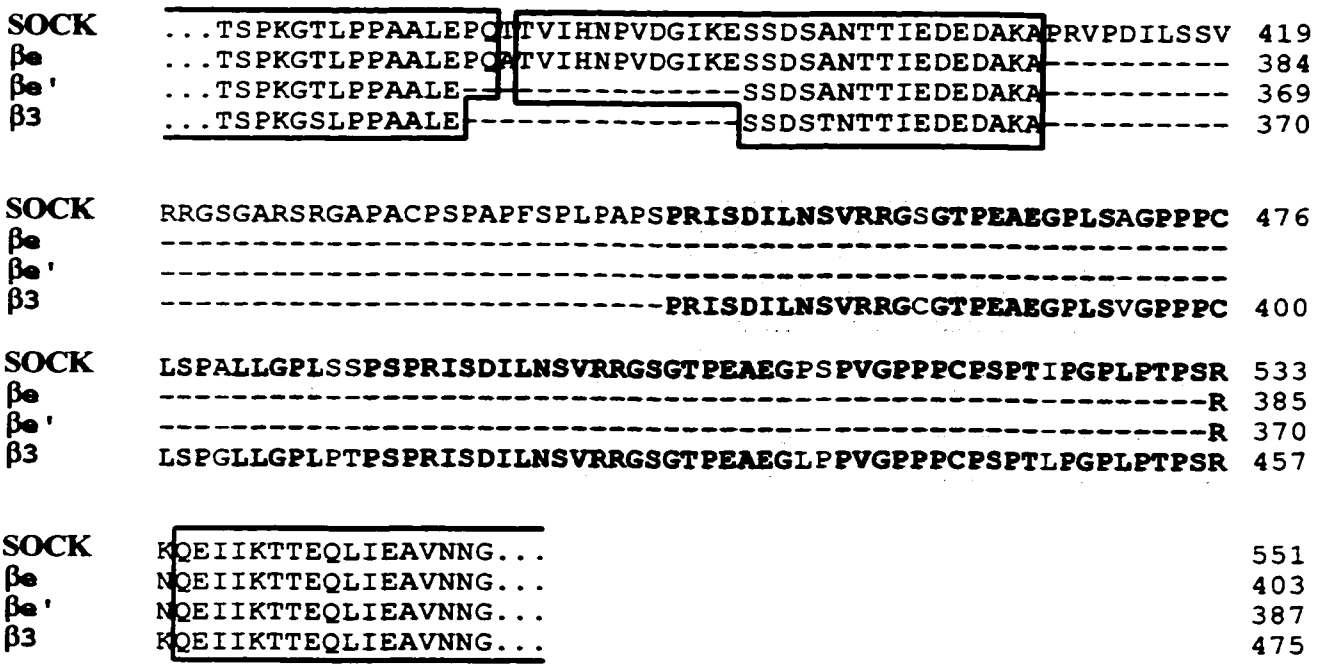

20
0
-20
0
100
200
300
400
500
600
Hydrophobicity Index

Figure 18. Comparison of the Amino Acid Sequence of the Variable and Association Domains of CaM kinase II β Isoforms

The amino acid alignment of CaM-dependent protein kinase II β isoforms from human tissue is shown. Amino acids are numbered on the right. Shared amino acid residues are boxed. Shaded areas indicate homologous amino acid residues within the proline-rich regions. Gaps are indicated by dashes. The polypeptides are labelled as follows: SOCK, novel cloned kinase in skeletal muscle; βe and βe', embryonal β isoforms; β3, isoform characterized in pancreas.


SOCK ...TSPKGTLPPAALEPQTTVIHNPVDGIKESSDSANTTIEDEDAKAPRVPDILSSV 419
βe ...TSPKGTLPPAALEPQTTVIHNPVDGIKESSDSANTTIEDEDAKA---------- 384
βe' ...TSPKGTLPPAALE----------SSDSANTTIEDEDAKA---------- 369
β3 ...TSPKGSLPPAALE----------SSDSTNTTIEDEDAKA---------- 370
SOCK RRGSGARSRGAPACPSPAPFSPLPAPSPRISDILNSVRRGSGTPEAEGPLSAGPPPC 476
βe ----------
βe' ----------
β3 ----------PRISDILNSVRRGCGTPEAEGPLSVGPPPC 400
SOCK LSPALLGPLSSPSPRISDILNSVRRGSGTPEAEGPSPVGPPPCPSPTIPGPLPTPSR 533
βe ----------R 385
βe' ----------R 370
β3 LSPGLLGPLPTPSPRISDILNSVRRGSGTPEAEGLPPVGPPPCPSPTLPGPLPTPSR 457
SOCK KQEIIKTTEQLIEAVNNG... 551
βe NQEIIKTTEQLIEAVNNG... 403
βe' NQEIIKTTEQLIEAVNNG... 387
β3 KQEIIKTTEQLIEAVNNG... 475

included an ATP-binding site (amino acid 20-44), a serine/threonine kinase domain (a.a. 132-144), an autoinhibitory domain (a.a. 280-290), a calmodulin-binding site (a.a. 297-315) and associations domains (a.a.320-end). Computer-assisted sequence homology searches (BLAST) showed highest homology with the β isoforms of the CaM kinase II family, followed by other related isoforms of the CaM kinase II family. A computer-assisted (SEQAID) alignment of the predicted amino acid sequence with previously characterized human β CaM kinase II isoforms (Fig. 18) revealed that the predicted polypeptide contained a novel region of 122 amino acids that was highly enriched in proline residues (24%). Furthermore, the proline-rich region could be subdivided into three distinct motifs. The latter two motifs contained identical sequence over a span of 20 amino acids (a.a. 446-466 and 489-509) and shared some homology with the pancreatic CaM kinase II β3 isoform.

However, differences existed between the pancreas and novel β CaM kinase II isoform sequences. The β3 isoform of the pancreas lacked a stretch of 15 amino acids in the variable domain corresponding to the amino acids, PQTTVIHNPVDGIKE (a.a. 379-393), in the novel polypeptide. Other differences were noted, especially in terms of the putative proteolytic PEST sequences. Polypeptides enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) residues target proteins for rapid degradation by proteolysis (Rechsteiner and Rogers, 1996). An algorithm defining PEST sequences detected two stretches of amino acids in the pancreas β3 isoform (a.a. 359-383 and a.a. 439-471) but not the novel skeletal muscle isoform, that contain possible PEST sequences. The absence of PEST sequences in the novel muscle isoform could prove to be significant in terms of protection offered to the novel polypeptide in skeletal muscle.

Figure 19. Homology of SOCK Proline-rich Region with SH3-Binding Motifs

The proline-rich region of SOCK was compared to SH3-binding motifs in polypeptides containing the minimal consensus sequence P-X-X-P. The amino acid residues that are represented are indicated in parentheses and the species is identified in subscript: H, human; M, mouse.

SOCK ($426\text{-}436_H$)	R	S	R	G	A	**P**	A	C	**P**	S	P
SOCK ($512\text{-}522_H$)	S	P	V	G	P	**P**	P	C	**P**	S	P
SOS1 ($1148\text{-}1156_H$)			P	V	P	**P**	P	V	**P**	P	R
Shc p52 ($299\text{-}307_H$)	Q	M	L	P	P	**P**	P	C	**P**		
AFAP-110 ($77\text{-}87_H$)	P	D	N	G	P	**P**	P	L	**P**	T	S
CDC42 GAP ($252\text{-}261_H$)	P	K	P	M	P	**P**	R	P	**P**	L	
hnRNP K ($308\text{-}316_H$)			P	L	P	**P**	P	P	**P**	P	R
p62 ($294\text{-}302_M$)			A	P	P	**P**	P	P	V	P	R
Paxillin ($42\text{-}50_H$)			A	V	P	**P**	P	V	**P**	P	P
Consensus						**P**	X	X	**P**		

The three proline-rich motifs of the novel polypeptide were compared with motifs known to bind src homology 3 (SH3) domains via their proline-rich structures, also named SH3-binding domains. The novel polypeptide contained three sequences that incorporated the minimal consensus sequence (P-X-X-P) required for SH3 binding (Fig. 19), where P represents proline and X represents any amino acid. The motif localized between amino acid residues 512-522 most closely resembled the Shc p52 SH3 domain. Because these proline-rich regions were reminiscent of other proline-rich polypeptides such as SOS, this novel CaM kinase polypeptide was named SOCK, an acronym for Son of CaM Kinase.

C.2.2 Genomic Organization of SOCK

The majority of differences between the α, β, γ and δ isoforms of the CaM kinase II family reside in the variable domain (Tobimatsu and Fujisawa, 1989). The presence of three previously uncharacterized proline-rich regions, inserted next to the variable domain of the cloned CaM kinase, raised the possibility that SOCK could arise from alternative splicing of the CaMKII β gene. The partial genomic organization of the mouse β CaMKII gene has been previously elucidated (Karls *et al.*, 1992) and described a number of exons, including those proposed to flank the novel proline-rich regions. To search for the genomic organization of the proline-containing exons, it was first necessary to obtain the cDNA sequence of SOCK from mouse muscle. Surprisingly, the sequence of the muscle CaMKII β isoform has not been reported.

To identify members of the CaM kinase family in mouse, a mouse skeletal muscle cDNA library was screened with the same cDNA probe as previously described. The

Figure 20. Nucleotide and Predicted Amino Acid Sequence of Mouse SOCK.

Nucleotide sequence of mouse skeletal muscle SOCK with final nucleotide and amino acid positions indicated on the bottom right. The deduced amino acid sequence is shown below the nucleotide sequence. Regions of interest are indicated as follows: ATP-binding site (***); calmodulin-binding site (»»»»), proline-rich region (underlined); peptide recognized by RU16 anti-(CaMK II) antibody (*italics*). The stop codon is indicated by the symbol ">".

```
ACGCCGAGCCCAATCGCCACCGCCATGGCCACCACGGTGACCTGCACCCGTTTCACCGACGAGTACCAGC
                          M  A  T  T  V  T  C  T  R  F  T  D  E  Y  Q  L

TATACGAGGAGATTGGCAAGGGGGCTTTCTCTGTGGTCCGACGCTGTGTCAAGCTCTGTACCGGCCATGA
  Y  E  E  I  G  K  G  A  F  S  V  V  R  R  C  V  K  L  C  T  G  H  E
           ********************************************************

GTATGCAGCCAAGATCATTAATACCAAGAAGCTGTCGGCCAGAGATCACCAGAAACTGGAGAGAGAGGCT
 Y  A  A  K  I  I  N  T  K  K  L  S  A  R  D  H  Q  K  L  E  R  E  A
**********

CGGATCTGCCGCCTGCTGAAGCATTCCAACATTGTACGCCTCCATGACAGCATCTCTGAAGAGGGCTTCC
R  I  C  R  L  L  K  H  S  N  I  V  R  L  H  D  S  I  S  E  E  G  F  H

ACTACCTGGTCTTCGACCTGGTCACTGGTGGGGAGCTCTTTGAAGACATCGTGGCAAGAGAGTACTACAG
  Y  L  V  F  D  L  V  T  G  G  E  L  F  E  D  I  V  A  R  E  Y  Y  S

TGAAGCTGATGCCAGTCACTGCATCCAGCAGATCCTGGAAGCTGTTCTCCATTGTCACCAAATGGGGGTC
 E  A  D  A  S  H  C  I  Q  Q  I  L  E  A  V  L  H  C  H  Q  M  G  V

GTCCACAGAGACCTCAAGCCTGAGAACCTGCTTCTGGCCAGCAAATGCAAAGGCGCCGCAGTGAAGCTGG
V  H  R  D  L  K  P  E  N  L  L  L  A  S  K  C  K  G  A  A  V  K  L  A

CAGACTTCGGCCTGGCCATCGAGGTTCAGGGAGACCAGCAGGCATGGTTTGGATTTGCGGGAACGCCAGG
  D  F  G  L  A  I  E  V  Q  G  D  Q  Q  A  W  F  G  F  A  G  T  P  G

CTACCTGTCTCCCGAGGTCCTTCGGAAGGAGGCCTACGGCAAACCTGTGGACATCTGGGCATGTGGGGTG
 Y  L  S  P  E  V  L  R  K  E  A  Y  G  K  P  V  D  I  W  A  C  G  V

ATCCTGTATATCCTGCTGGTGGGCTACCCACCTTTCTGGGATGAGGACCAACACAAGCTGTACCAGCAGA
I  L  Y  I  L  L  V  G  Y  P  P  F  W  D  E  D  Q  H  K  L  Y  Q  Q  I

TCAAGGCTGGGGCGTATGATTTCCCATCCCCTGAGTGGGACACCGTTACTCCTGAAGCCAAAAACCTCAT
  K  A  G  A  Y  D  F  P  S  P  E  W  D  T  V  T  P  E  A  K  N  L  I

CAACCAGATGCTGACCATCAACCCTGCCAAGCGCATCACGGCCCATGAGGCCCTGAAGCACCCATGGGTC
 N  Q  M  L  T  I  N  P  A  K  R  I  T  A  H  E  A  L  K  H  P  W  V

TGCCAACGTTCCACCGTGGCCTCTATGATGCACAGACAGGAGACTGTGGAATGTCTGAAGAAGTTCAATG
C  Q  R  S  T  V  A  S  M  M  H  R  Q  E  T  V  E  C  L  K  K  F  N  A

CAAGGAGGAAGCTCAAGGGAGCCATCCTCACCACTATGCTGGCCACACGGAATTTCTCAGTGGGCAGACA
  R  R  K  L  K  G  A  I  L  T  T  M  L  A  T  R  N  F  S  V  G  R  Q
>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>

GACCACCGCTCCGGCCACAATGTCCACCGCGGCCTCCGGCACCACCATGGGGCTGGTGGAACAAGCCAAG
 T  T  A  P  A  T  M  S  T  A  A  S  G  T  T  M  G  L  V  E  Q  A  K

AGTTTACTCAACAAGAAAGCAGATGGAGTCAAGCCCCAGACAAACAGCACCAAAAACAGCTCGGCCATCA
S  L  L  N  K  K  A  D  G  V  K  P  Q  T  N  S  T  K  N  S  S  A  I  T

CCAGCCCCAAAGGATCTCTCCCTCCTGCTGCCCTGGAGCCTCAAACCACCGTTATCCATAACCCAGTGGA
  S  P  K  G  S  L  P  P  A  A  L  E  P  Q  T  T  V  I  H  N  P  V  D

CGGGATTAAGGAATCTTCCGACAGCACCAACACAACCATAGAGGATGAAGATGCCAAAGCCCCCAGGGTC
  G  I  K  E  S  S  D  S  T  N  T  T  I  E  D  E  D  A  K  A  P  R  V

CCTGATGTCCTGAGCTTGGTGAGGAGGGCCTCAGGAGCCCCCGAAGCCGAGGGCCCCCTCTCCTGCCAAT
P  D  V  L  S  L  V  R  R  A  S  G  A  P  E  A  E  G  P  L  S  C  Q  S

CTCCAGTTCCCATTAGCCCCCTTCCAACCCCGTCCCCCAGGATCTCTGACATCCTGAACTCAGTGAGAAG
  P  V  P  I  S  P  L  P  T  P  S  P  R  I  S  D  I  L  N  S  V  R  R

GGGCTGTGGAACCCCAGAAGCCGAGGGACCCCTATCAGTGGGGCCCCCACCCTGCTTGTCTCCGGGTCTC
  G  C  G  T  P  E  A  E  G  P  L  S  V  G  P  P  P  C  L  S  P  G  L

CTAGGCCCCCTGCCCACCCCGTCCCCCAGGATCTCTGACATCCTCAACTCAGTGAGGCGGGGCTCAGGGA
L  G  P  L  P  T  P  S  P  R  I  S  D  I  L  N  S  V  R  R  G  S  G  T

CCCCAGAAGCTGAGGGCCTCCCACCAGTGGGCCCCCCACCCTGCCCATCTCCGACTCTCCCTGGCCCCTT
  P  E  A  E  G  L  P  P  V  G  P  P  P  C  P  S  P  T  L  P  G  P  L

GCCCACCCCATCCCGGAAGCAGGAAATCATCAAGACCACAGAGCAGCTCATTGAGGCCGTCAACAATGGG
P  T  P  S  R  K  Q  E  I  I  K  T  T  E  Q  L  I  E  A  V  N  N  G

GACTTTGAGGCCTATGCGAAAATCTGTGACCCAGGCCTGACCTCATTTGAGCCTGAAGCTCTGGGCAACC
D  F  E  A  Y  A  K  I  C  D  P  G  L  T  S  F  E  P  E  A  L  G  N  L

TGGTCGAAGGGATGGATTTCCACAGATTCTACTTTGAGAACCTGCTGGCCAAGAACAGCAAGCCGATCCA
  V  E  G  M  D  F  H  R  F  Y  F  E  N  L  L  A  K  N  S  K  P  I  H

CACCACCATCCTGAACCCGCACGTGCACGTCATTGGCGAGGATGCGGCATGCATCGCCTACATCCGCCTC
 T  T  I  L  N  P  H  V  H  V  I  G  E  D  A  A  C  I  A  Y  I  R  L

ACACAGTACATCGATGGCCAGGGCAGACCCCGTACCAGCCAGTCCGAAGAGACCCGTGTGTGGCACCGCC
T  Q  Y  I  D  G  Q  G  R  P  R  T  S  Q  S  E  E  T  R  V  W  H  R  R

GCGACGGCAAGTGGCAGAATGTACATTTCCACTGCTCGGGCGCTCCAGTGGCCCCGCTGCAGTGAGAGCT   1998
  D  G  K  W  Q  N  V  H  F  H  C  S  G  A  P  V  A  P  L  Q  <     666

GCGCCTGGTTTCGCCGGACAGAGTTGGTGTTTGGAGCCCAGCCGCCCTCGGGCGCACGGCCTGCCTGTCG
CATGTTTGTGTCTGCCTCGTCCCCTCCCCTGGTGCCTGTGTCTGCAGAAAAACAAGACCAGATGTGATTT
GTTTTTAAAAATAAGATGATGACAACGACAACCACACGCAAACCAACAAACAAAATTTTGGCATCGGATG
AAATGGAAAACAAGCAAACAAACAAAAACCTAAAGAAAGGAAAAAGCTGTAAAAATCTGGCATTTGTGGG
GCCACCCCACCCCCAAGCTCCACTGTGTCTGTTGTGCTCCTGGCTTTGGGGACCCTCAGGGCGTGAA...
```

Figure 21. Comparison of Predicted Amino Acid Sequences of Human versus Mouse Skeletal Muscle SOCK

Computer-assisted alignment algorithms were used to generate an amino acid alignment of novel SOCK isoforms from human and mouse skeletal muscle. Amino acids are numbered on the right. Gaps are indicated by dashes. The amino acid sequences are identified as follows: HM-SOCK, human skeletal muscle SOCK; MM-SOCK, mouse skeletal muscle SOCK. The two sequences demonstrate extensive sequence homology with some noted exceptions.

```
                                                                  MM-SOCK      70
MATTVTCTRFTDEYQLYEEIGKGAFSVVRRCVKLCTGHEYAAKIINTKKLSARDHQKLEREARICRLLKH
:::::::::::::::::: :::::::::::::::::::::::::::::::::::::::::::::::::::::
MATTVTCTRFTDEYQLYEDIGKGAFSVVRRCVKLCTGHEYAAKIINTKKLSARDHQKLEREARICRLLKH
                                                                  HM-SOCK      70

                                                                  MM-SOCK     140
SNIVRLHDSISEEGFHYLVFDLVTGGELFEDIVAREYYSEADASHCIQQILEAVLHCHQMGVVHRDLKPE
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SNIVRLHDSISEEGFHYLVFDLVTGGELFEDIVAREYYSEADASHCIQQILEAVLHCHQMGVVHRDLKPE
                                                                  HM-SOCK     140

                                                                  MM-SOCK     210
NLLLASKCKGAAVKLADFGLAIEVQGDQQAWFGFAGTPGYLSPEVLRKEAYGKPVDIWACGVILYILLVG
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NLLLASKCKGAAVKLADFGLAIEVQGDQQAWFGFAGTPGYLSPEVLRKEAYGKPVDIWACGVILYILLVG
                                                                  HM-SOCK     210

                                                                  MM-SOCK     280
YPPFWDEDQHKLYQQIKAGAYDFPSPEWDTVTPEAKNLINQMLTINPAKRITAHEALKHPWVCQRSTVAS
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
YPPFWDEDQHKLYQQIKAGAYDFPSPEWDTVTPEAKNLINQMLTINPAKRITAHEALKHPWVCQRSTVAS
                                                                  HM-SOCK     280

                                                                  MM-SOCK     350
MMHRQETVECLKKFNARRKLKGAILTTMLATRNFSVGRQTTAPATMSTAASGTTMGLVEQAKSLLNKKAD
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
MMHRQETVECLKKFNARRKLKGAILTTMLATRNFSVGRQTTAPATMSTAASGTTMGLVEQAKSLLNKKAD
                                                                  HM-SOCK     350

                                                                  MM-SOCK     419
GVKPQTNSTKNSSAITSPKGSLPPAALEPQTTVIHNPVDGIKESSDSTNTTIEDEDAKAPRVPDVLSLVR
:::::::::::: : ::::: ::::::::::::::::::::::::::: ::::::::::::::: :: ::
GVKPQTNSTKNSAAATSPKGTLPPAALEPQTTVIHNPVDGIKESSDSANTTIEDEDAKAPRVPDILSSVR
                                                                  HM-SOCK     419

                                                                  MM-SOCK     484
RASGAPEAEGPLSCQSPVPISPLPTPSPRISDILNSVRRGCGTPEAEGPLSVGPPPCLSPGLLGPLPTPS
:::::    :   : :: : :::: :::::::::::::: :::::::::: ::::::::: :::::   ::
RGSGARSR-GAPACPSPAPFSPLPAPSPRISDILNSVRRGSGTPEAEGPLSAGPPPCLSPALLGPLSSPS
                                                                  HM-SOCK     483

                                                                  MM-SOCK     551
PRISDILNSVRRGSGTPEAEGLPPVGPPPCPSPTLPGPLPTPSRKQEIIKTTEQLIEAVNNGDFEAYAKI
:::::::::::::::::::::    ::::::::: ::::::::::::::::::::::::::::::::::::
PRISDILNSVRRGSGTPEAEGPLSAGPPPCPSPTIPGPLPTPSRKQEIIKTTEQLIEAVNNGDFEAYAKI
                                                                  HM-SOCK     550

                                                                  MM-SOCK     621
CDPGLTSFEPEALGNLVEGMDFHRFYFENLLAKNSKPIHTTILNPHVHVIGEDAACIAYIRLTQYIDGQG
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
CDPGLTSFEPEALGNLVEGMDFHRFYFENLLAKNSKPIHTTILNPHVHVIGEDAACIAYIRLTQYIDGQG
                                                                  HM-SOCK     620

                                                                  MM-SOCK     666
RPRTSQSEETRVWHRRDGKWQNVHFHCSGAPVAPLQ
::::::::::::::::::::::::::::::::::::
RPRTSQSEETRVWHRRDGKWQNVHFHCSGAPVAPLQ
                                                                  HM-SOCK     665
```

screening of the library yielded many positives including one full length clone, referred to as 11D, which contained the novel proline-rich sequence. A second positive clone, 10 MM, was much less abundant and did not contain the sequence encoding the proline-rich domains. Both clones were sequenced completely. The 4.5 kb nucleotide sequence of the 11D clone contained a continuous open reading frame of 1998 nucleotides shown in Fig. 20. The resulting open reading frame encoded a protein of 666 amino acid residues with a predicted molecular mass of 72,926 Da. The predicted amino acid sequence of the mouse clone 11D showed the expected characteristics associated with the β isoforms of the CaM kinase II family of proteins including an ATP-binding site, a serine/threonine kinase domain, an autoinhibitory domain, a calmodulin-binding motif and associations domains. The predicted amino acid sequence of the mouse clone showed highest homology to the cloned human SOCK isoform (Fig. 21) with which it shares the novel proline-rich regions. Comparison to the SOCK isoform from human revealed some species specific differences between the SOCK polypeptides. Some single amino acid substitutions (such as a.a.19 D=>E, a.a. 363 A=>S, a.a. 365 A=>I, a.a. 398 A for T) were noted but did not target amino acids with known assigned functions. However, major sequence differences were within the proline-rich regions of the mouse clone which contained two additional amino acids that disrupted the homology in the region. Overall homology between the polypeptides for the two species was 95%.

The second mouse skeletal muscle clone, 10 MM, contained a nucleotide sequence with an open reading frame of 1626 nucleotides coding for a polypeptide of 542 amino acids in length with a predicted molecular mass of 60,482 Da. A computer-assisted sequence homology search found complete homology with the classical β isoform of the CaM kinase

Figure 22. Comparison of the Predicted Amino Acid Sequences of SOCK and Classical β CaM Kinase II Isoform in Mouse Skeletal Muscle

Amino acid alignment of SOCK and the multifunctional CaM kinase II β isoform in mouse skeletal muscle. Amino acids are numbered on the right. Gaps are indicated by dashes. MM-CaMKIIβ, mouse skeletal muscle multifunctional CaM kinase II β isoform (10 MM clone); MM-SOCK, mouse skeletal muscle SOCK (11D clone).

```
                                                                MM-CaMKII β    70
MATTVTCTRFTDEYQLYEEIGKGAFSVVRRCVKLCTGHEYAAKIINTKKLSARDHQKLEREARICRLLKH
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
MATTVTCTRFTDEYQLYEEIGKGAFSVVRRCVKLCTGHEYAAKIINTKKLSARDHQKLEREARICRLLKH
                                                                MM-SOCK        70

                                                                MM-CaMKII β   140
SNIVRLHDSISEEGFHYLVFDLVTGGELFEDIVAREYYSEADASHCIQQILEAVLHCHQMGVVHRDLKPE
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
SNIVRLHDSISEEGFHYLVFDLVTGGELFEDIVAREYYSEADASHCIQQILEAVLHCHQMGVVHRDLKPE
                                                                MM-SOCK       140

                                                                MM-CaMKII β   210
NLLLASKCKGAAVKLADFGLAIEVQGDQQAWFGFAGTPGYLSPEVLRKEAYGKPVDIWACGVILYILLVG
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NLLLASKCKGAAVKLADFGLAIEVQGDQQAWFGFAGTPGYLSPEVLRKEAYGKPVDIWACGVILYILLVG
                                                                MM-SOCK       210

                                                                MM-CaMKII β   280
YPPFWDEDQHKLYQQIKAGAYDFPSPEWDTVTPEAKNLINQMLTINPAKRITAHEALKHPWVCQRSTVAS
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
YPPFWDEDQHKLYQQIKAGAYDFPSPEWDTVTPEAKNLINQMLTINPAKRITAHEALKHPWVCQRSTVAS
                                                                MM-SOCK       280

                                                                MM-CaMKII β   350
MMHRQETVECLKKFNARRKLKGAILTTMLATRNFSVGRQTTAPATMSTAASGTTMGLVEQAKSLLNKKAD
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
MMHRQETVECLKKFNARRKLKGAILTTMLATRNFSVGRQTTAPATMSTAASGTTMGLVEQAKSLLNKKAD
                                                                MM-SOCK       350

                                                                MM-CaMKII β   409
GVKPQTNSTKNSSAITSPKGSLPPAALEPQTTVIHNPVDGIKESSDSTNTTIEDEDAKA-----------
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GVKPQTNSTKNSSAITSPKGSLPPAALEPQTTVIHNPVDGIKESSDSTNTTIEDEDAKAPRVPDVLSLVR
                                                                MM-SOCK       420

                                                                MM-CaMKII β   409
-------------------------------------------------------------------------

RASGAPEAEGPLSCQSPVPISPLPTPSPRISDILNSVRRGCGTPEAEGPLSVGPPPCLSPGLLGPLPTPS
                                                                MM-SOCK       490

                                                                MM-CaMKII β   436
-------------------------------------------RKQEIIKTTEQLIEAVNNGDFEAYAKI
                                           ::::::::::::::::::::::::::::
PRISDILNSVRRGSGTPEAEGLPPVGPPPCPSPTLPGPLPTPSRKQEIIKTTEQLIEAVNNGDFEAYAKI
                                                                MM-SOCK       560

                                                                MM-CaMKII β   506
CDPGLTSFEPEALGNLVEGMDFHRFYFENLLAKNSKPIHTTILNPHVHVIGEDAACIAYIRLTQYIDGQG
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
CDPGLTSFEPEALGNLVEGMDFHRFYFENLLAKNSKPIHTTILNPHVHVIGEDAACIAYIRLTQYIDGQG
                                                                MM-SOCK       630

                                                                MM-CaMKII β   542
RPRTSQSEETRVWHRRDGKWQNVHFHCSGAPVAPLQ
::::::::::::::::::::::::::::::::::::
RPRTSQSEETRVWHRRDGKWQNVHFHCSGAPVAPLQ
                                                                MM-SOCK       666
```

II family. A comparison of the predicted amino acid sequence of mouse SOCK and 10 MM revealed that these two sequences were identical except for the three novel proline-rich regions found exclusively in SOCK (Fig. 22). Both SOCK and classical β CaM kinases contain a hydrophobic stretch of amino acids (a.a. 195-215 in SOCK) that was also selected as a putative membrane spanning sequence. Despite being common to all previously characterized β CaM kinase II isoforms, this region has never generated much interest as a candidate for membrane interactions. Both isoforms also contained a tyrosine phosphorylation consensus sequence that was found in the amino acid sequence of the β isoforms of the multifunctional CaM kinase II family (a.a. 9-17).

Having cloned the SOCK and classical β isoform cDNA sequences from mouse skeletal muscle, we sought to determine whether the SOCK polypeptide might arise from alternative splicing of the classical β isoform. Using the SOCK sequence coding for the novel proline-rich region as a probe, a mouse genomic library was screened. DNA from positive phage were sequenced after subcloning into pTZ19 and intron boundaries were identified by divergence from the cDNA sequence and detection of consensus donor and acceptor splicing sequences for intron/exon boundaries (Karls *et al.*, 1992). A summary of the identified exons and intervening introns is shown in Table 2. It appears that three exons of the proline-rich domains were localized 5' to the sequence of the first exon of the association domain. The novel exons were contained in a genomic fragment of 4.5 kb. Each intron began with the nucleotides GT and ended with AG, thereby obeying the GT/AG rule of splice-sequences (Mount *et al.*, 1992). The first additional exon consisted of 114 nucleotides and was localized 3' to the last identified exon of the variable domain. The second and third additional exons

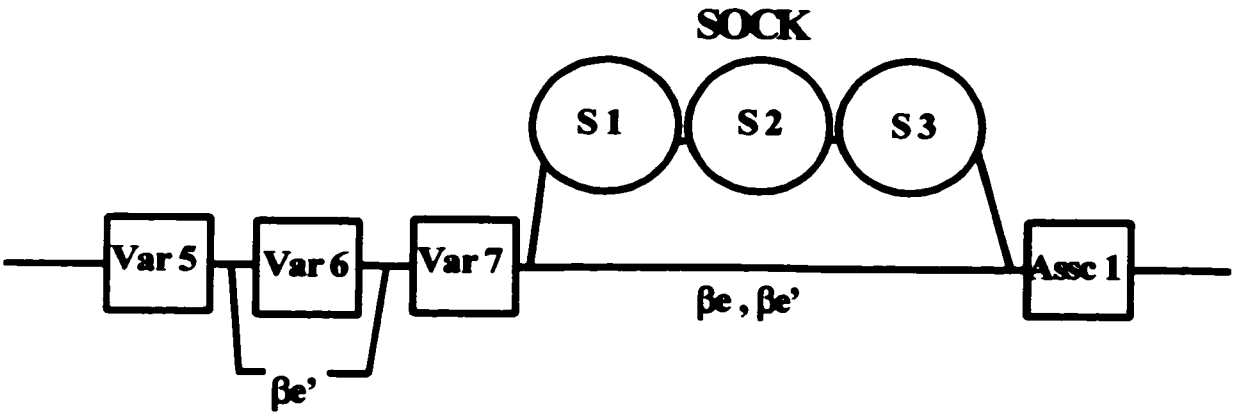
Table 2. Characteristics of the Exon-Intron Junctions of the Variable Domain of the SOCK Gene

The identified exon-intron junctions of the proline-rich domain of the SOCK gene are shown along with 5' splice donor and 3' splice acceptor sites. The consensus sequences (Cons) established for these boundaries is shown at the bottom of the table. The size of each exon is shown in base pairs. Variable domain (Var), SOCK proline-rich domain (S) and association domain (Assc) are numbered in order of appearance in the genomic organization.

Exon	Size (bp)	5' Splice Donor	3' Splice Acceptor	Exon
			ccctctat**ag**GAGCCTCA	Var 6
Var 6	45	GGGATTAAG**gt**actgcc	gtctcctt**ag**GAATCTTC	Var 7
Var 7	49	GATGCCAAAG**gt**acttca	cctgcctc**ag**CCCCCAGG	S 1
S 1	114	CCAACCCCGT**gt**aagtag	tccgcccc**ag**CCCCCAGG	S 2
S 2	130	CCCACCCCGT**gt**aagtag	tctgcccc**ag**CCCCCAGG	S 3
S 3	126	CCCACCCCAT**gt**aagtag	taccctgc**ag**CCCGGAAG	Assc
Cons		.....EXON|**gt**...	.....**ag**|EXON...	

Figure 23. Schematic View of the Proposed Alternative Splicing of β CaM-dependent Protein Kinase II Gene

Various exons of the human CaM kinase II β isoforms are represented as boxes and circles. Lines represent the intervening intron sequences. Var, variable domain exon; S, novel proline-rich exon in SOCK; Assc, association domain exon. Numbers label consecutive exons in a given domain.


SOCK
S1
S2
S3
Var 5
Var 6
Var 7
Assc 1
βe , βe'
βe'

contained nucleotide sequences of 130 and 126 bp respectively. Together, these three exons correspond to and encode the three distinct proline-rich motifs identified in the predicted amino acid sequence. The deduced amino acid sequence obtained from the genomic sequence shows 100% identity with the one derived from the cDNA sequence. The discovery of these three additional exons lend credence to the proposed mechanism of alternative splicing for the generation of multiple β isoforms (Fig. 23).

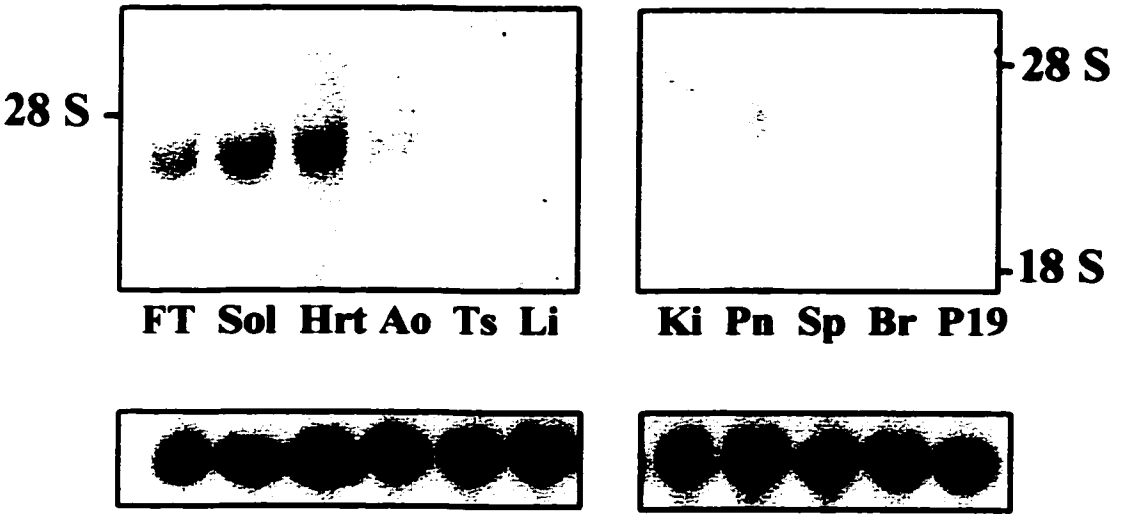
C.2.3 Expression of SOCK is Restricted to Various Muscle Types

Given the findings that SOCK contained potential SH3-binding domains and appeared to be generated via alternative splicing of the β CaM kinase gene, it was important to determine its tissue distribution, which might in turn provide some clues as to SOCK's potential function in cells.

The tissue pattern of expression of SOCK was examined by analysing RNA from different tissues using a cDNA probe encoding for the novel proline-rich region of SOCK. Northern blot analysis revealed a RNA transcript of 4.8 kb in skeletal muscle (fast twitch and slow twitch), aorta and heart (Fig. 24). The RNA transcript of SOCK in muscle is easily localized as it runs immediately below the 28S ribosomal RNA. No transcript could be detected in the liver, testes, whole brain, kidney, spleen and P19 embryonal carcinoma cells. The expression of GAPDH was used as an internal control for RNA loading. It is noteworthy that the 4.8 kb transcript detected by Northern blot analysis is considerably larger than the 2.2 kb cDNA that was isolated and sequenced. This discrepancy can be explained by the truncation of the 3' untranslated region as evidenced by the absence of a consensus sequence

Figure 24 Tissue Distribution of SOCK

The distribution of SOCK in a variety of tissues was analyzed by Northern blot using a radiolabelled probe generated from the novel proline-rich domain. RNA was obtained from the following tissues: FT, fast twitch skeletal muscle; Sol, soleus; Hrt, heart; Ao, aorta; Ts, testes; Li, liver; Ki, kidney; Pn, pancreas, Sp, spleen; Br, brain; P19, embryonal carcinoma cells. A 4.8 kb transcript was detected exclusively in muscle tissues. The mobility of the 18S and 28S is indicated on the right. The bottom panel represents expression of GAPDH as a control for RNA loading.


28 S
28 S
18 S
FT Sol Hrt Ao Ts Li
Ki Pn Sp Br P19

for the polyadenylation signal AATAAA. A reasonable explanation can be provided for the absence of any clones extending to the 3' end in the cDNA library. A long series of adenosine nucleotides is located a few hundred base pairs downstream of SOCK's termination codon. If this polyadenosine segment served as a substrate for the oligo-dT nucleotides used to generate the cDNA library, the resulting reverse transcription would have produced SOCK cDNAs truncated at the 3' end. A similar phenomena was observed by Bennett and Kennedy (1987) upon cloning the β CaM kinase II isoform cDNA from rat brain.

To identify the potential polypeptide encoded by the mRNA transcript, we investigated the expression of CaM kinase in a variety of tissues by Western blot analysis using an anti-peptide antibody raised to a peptide highly conserved in SOCK and designed to recognize CaM kinase II isoforms. To verify that this antibody could indeed recognize the SOCK polypeptide, the immunoreactivity of the antibody was tested against fusion protein constructs in Western blot analysis. Full-length SOCK was subcloned into the carboxyl end of the coding sequence of *Shistosoma manosi* glutathione-S-transferase (GST) of the pGEX3xΔB vector (Fig. 29, panel A). Bacterial expression of the plasmid-encoded fusion protein was induced by IPTG. The fusion protein was purified by sonication of bacteria and affinity chromatography of the lysate on glutathione-agarose beads. The high stability of the GST-substrate complex allows for the use of immobilized glutathione to affinity-purify the fusion protein. A GST-SOCK fusion protein of 100 kDa, the predicted molecular weight of the fusion protein, was specifically eluted from the glutathione agarose beads. The anti-CaMKII antibody (RU16) revealed a 100 kDa immunoreactive polypeptide with identical mobility on SDS-PAGE that was produced following induction of expression (Fig25, panelA)

Figure 25. Immunoreactivity of SOCK to an Anti-(CaMKII) Antibody

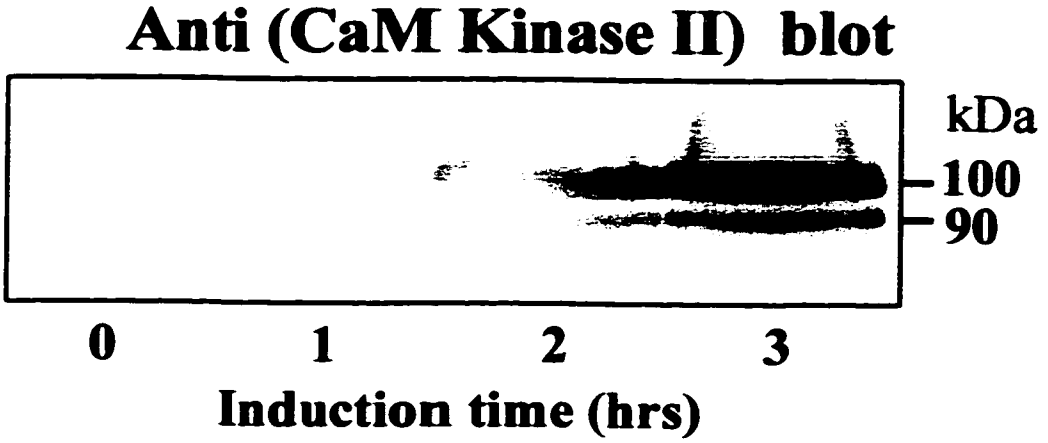
The immunoreactivity of SOCK to the RU16 anti-(CaMKII) antibody was investigated using the GST-SOCK fusion protein.

(A): GST-SOCK fusion proteins were induced with IPTG and purified at one hour intervals. The affinity-purified bacterial extracts were subjected to SDS-PAGE and transferred to nitrocellulose.

(B): An aliquot of affinity-purified GST-SOCK was subjected to SDS-PAGE and transferred to nitrocellulose (lane 1). The remaining sample was incubated with Factor Xa protease at room temperature. Samples of released (cleaved) material were collected at various intervals and prepared for Western blot analysis.

Western blotting was performed using the RU16 affinity-purified anti-(CaMKII) antibody at a dilution of 1 to 3000. The molecular weight of the detected immunoreactive polypeptides is indicated in kDa.

A


Anti (CaM Kinase II) blot
kDa
100
90
0
1
2
3
Induction time (hrs)

B

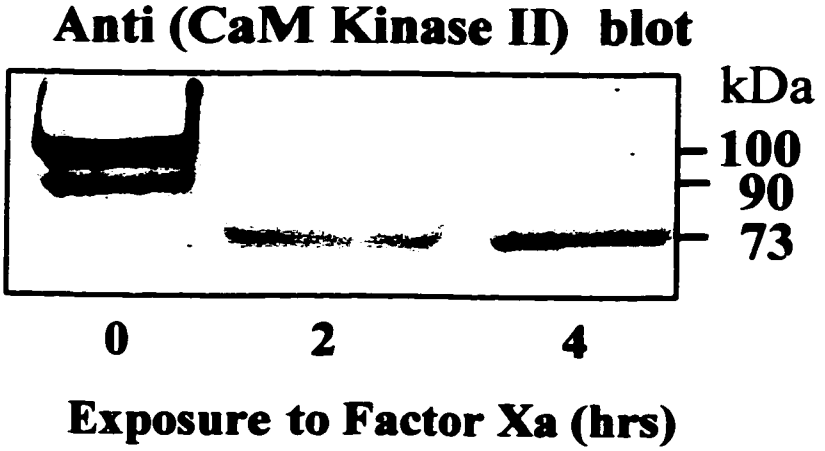
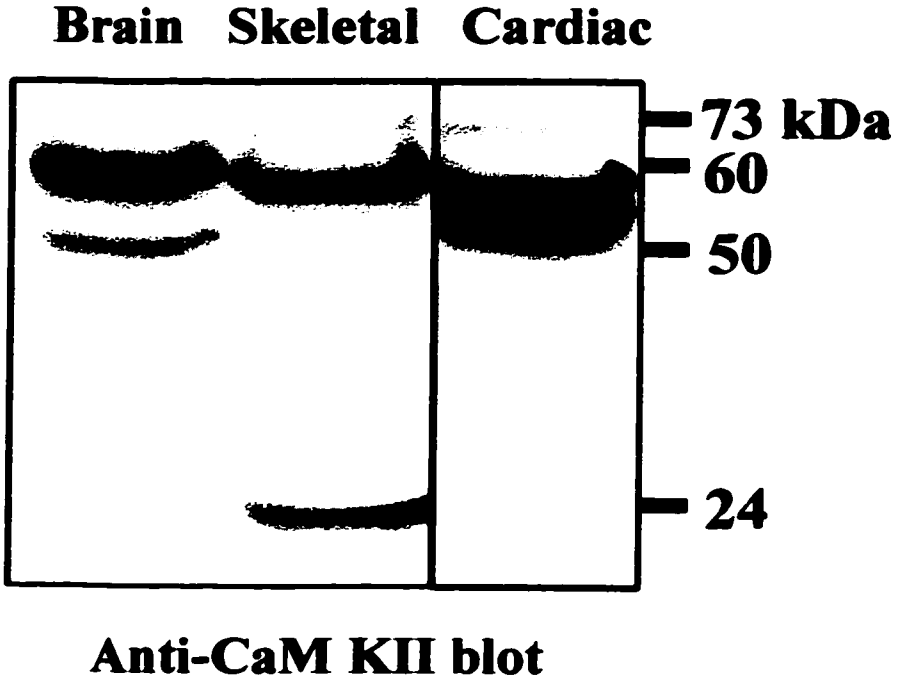

Anti (CaM Kinase II) blot
kDa
100
90
73
0
2
4
Exposure to Factor Xa (hrs)

Figure 26. Immunostaining of Various Rabbit Tissue Microsomal and Subcellular Fractions with Anti-(CaM kinase II) Antibody

(A) Microsomal fractions isolated from rabbit brain, skeletal muscle and heart were subjected to SDS-PAGE and transferred to nitrocellulose. Western blotting was performed using the RU16 affinity-purified anti-(CaMKII) antibody at a dilution of 1 to 5000. Each lane contained two hundred and fifty micrograms of protein. The molecular weight of the immunoreactive polypeptides is shown in kDa. A 73 kDa immunoreactive polypeptide was detected in skeletal and cardiac muscle.

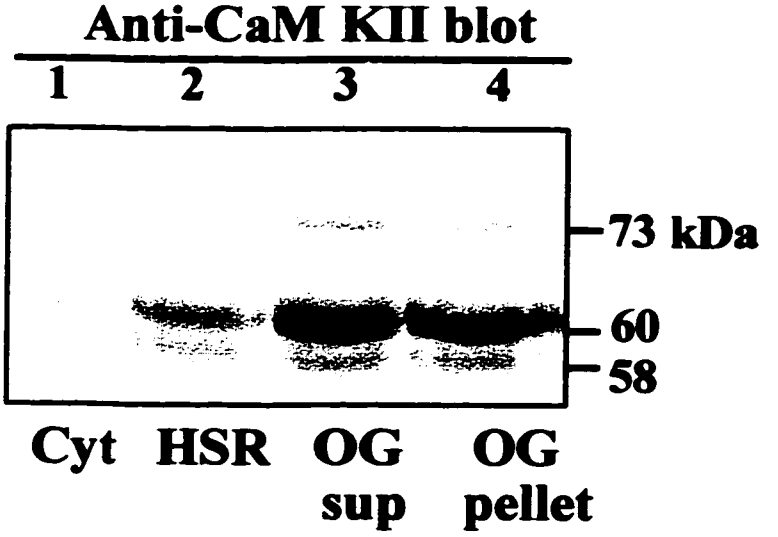
(B) Rabbit skeletal muscle homogenates were separated into cytosol (cyt) and heavy SR membranes (HSR) by differential centrifugation. Heavy SR-enriched membranes were extracted with 1% octyl glucoside and centrifuged to obtain supernatant (OG sup) and insoluble pellet (OG pellet) fractions. A 73 kDa immunoreactive polypeptide was solubilized from the membranes by octyl glucoside.

A



Anti-CaM KII blot

B



A second 90 kDa immunoreactive polypeptide may have resulted from limited proteolysis of the fusion protein during the purification scheme or may represent a frameshift during the production of the polypeptide. The identity of the 100 kDa immunoreactive polypeptide was further confirmed by limited proteolysis of the GST-fusion protein with Factor Xa. A Factor Xa site was engineered into the vector at the fusion point between the GST and SOCK polypeptides. Following proteolytic release of SOCK from the GST-moiety by Factor Xa, the immunoreactive polypeptide shifted to a molecular weight of 73 kDa, corresponding to the predicted molecular weight of the expressed SOCK polypeptide alone (Fig. 25, panel B).

Having confirmed the utility of the RU16 anti-(CaMKII) antibody in detecting the SOCK polypeptide, the tissue distribution of CaMKII isoforms was then examined using the antibody described above. Several abundant polypeptides of 60, 54 and 24 kDa were detected in skeletal muscle whereas immunoreactive polypeptides of 50 and 54 kDa were detected in brain and heart respectively (Fig. 26). Although these polypeptides have been previously characterized, their relationship to classical isoforms of CaMKII (50, 54, 60 kDa) remains to be investigated. The 24 kDa immunoreactive polypeptide most likely corresponded to a novel polypeptide proposed to be a kinase anchoring protein (Bayer *et al.*, 1996). Interestingly, a 73 kDa immunoreactive polypeptide matching the predicted molecular weight of SOCK was detected in skeletal muscle (Fig. 26) and to a lesser extent in heart but not in brain homogenates. The similarity in the tissue distribution with the mRNA transcript, the immunodetection of the expressed SOCK fusion protein by this antibody, and the concurrence of the predicted and observed molecular weights of the immunoreactive polypeptide provide convincing evidence for the identification of the 73 kDa immunoreactive

polypeptide as SOCK. Collectively, this data would suggest that the detectable levels of SOCK expression are limited to various muscle types.

The detection of a 73 kDa immunoreactive polypeptide was, at first, somewhat intriguing. Although a 73 kDa polypeptide exhibiting CaM-binding and CaM-dependent phosphorylation was detected in skeletal muscle membranes, no 73 kDa immunoreactive polypeptide had been visualized. At first, the slight differences in amino acid sequence between SOCK and the CaMKII antigen peptide (92% homology) were proposed to explain the lack of immunoreactivity of the antibody towards SOCK. We soon realized, however, that SOCK was present at much lower concentrations in the membranes, as compared to the 60 kDa immunoreactive polypeptide, and that higher concentrations of proteins needed to be loaded onto SDS-PAGE gels to detect the 73 kDa polypeptide successfully.

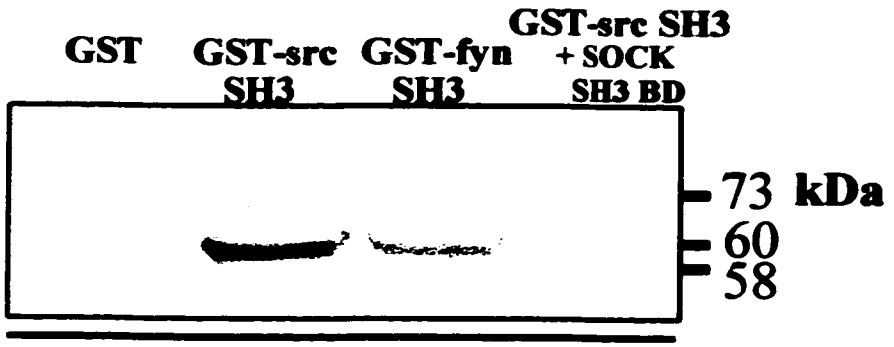
The subcellular distribution of SOCK in skeletal muscle was further investigated by Western blot analysis. Rabbit skeletal muscle was fractionated into cytosol and heavy SR-enriched membranes. Following SDS-PAGE and electroblotting, CaM-dependent protein kinases were visualized with the RU16 anti-(CaMKII) antibody. A faint 60 kDa immunoreactive polypeptide was detected in cytosol (Fig. 26, panel B) whereas immunoreactive polypeptides of 73, 60 and 58 kDa were observed in heavy SR-enriched membranes. The 73 kDa immunoreactive polypeptide was not removed from SR membranes by extractions with EGTA or KCl indicating that it was tightly associated with the membrane. Solubilization of the SR membranes with a solution of 1% octyl glucoside (OG) resulted in the enrichment of 73, 60 and 58 kDa immunoreactive polypeptides although a significant signal was still recovered in the insoluble pellet. These subcellular distribution studies indicate

Figure 27. Binding of SOCK to SH3 Domains of src and fyn Tyrosine Kinases

(A): Interactions between GST-SH3 domains of src and fyn with polypeptides solubilized from rabbit skeletal muscle membranes were investigated. Western blotting was performed using the RU16 anti-(CaMKII) antibody at a dilution of 1 in 1000 in TBS. The molecular weight of the immunoreactive polypeptides is indicated in kDa on the right. The binding of immunoreactive polypeptides was greatly diminished in the presence of the SOCK proline-rich binding domain.

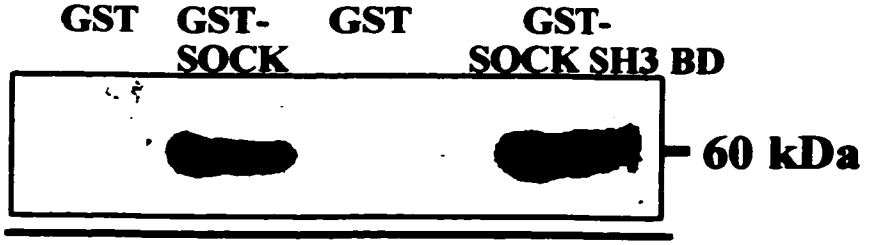
(B): The binding of src in skeletal muscle cytosol to immobilized GST-fusion proteins of SOCK or SOCK proline-rich domains is shown. A 60 kDa immunoreactive polypeptide was detected with the anti-c-src antibody used at a dilution of 1 in 1000.

A



Anti-CaM Kinase II blot

B



Anti-src blot

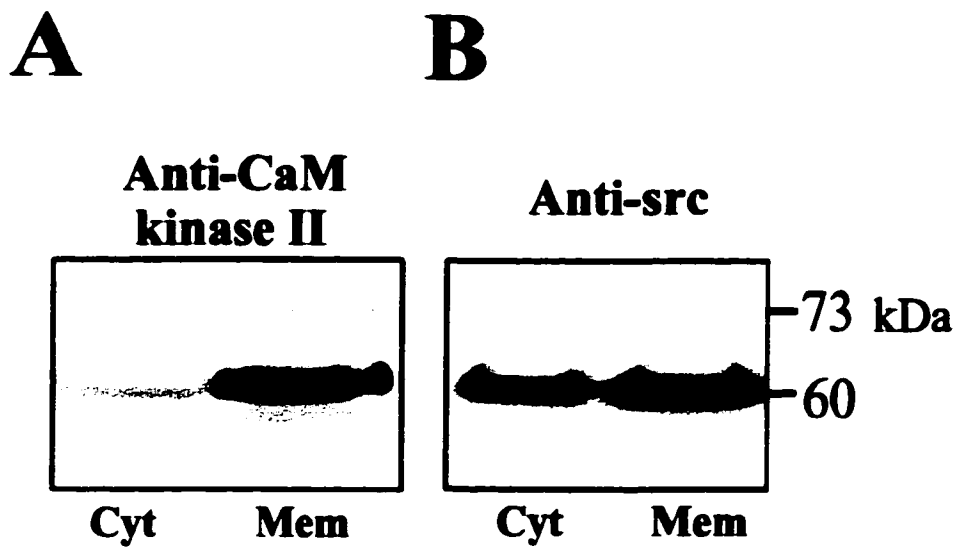
that SOCK is tightly associated with the membrane and that the large scale purification of the 73 kDa polypeptide will be hindered by its low abundance and limited detergent solubility.

C.2.4 SOCK Binds SH3 Domains of Src

A notable feature arising from the elucidation of the amino acid sequence of SOCK was the presence of three novel proline-rich domains within the structure of a CaM-dependent protein kinase. Many proline-rich domains have demonstrated the ability to bind to SH3 motifs (Mayer and Eck, 1995). To explore the binding characteristics of SOCK's proline-rich region further, we examined the potential associations of this motif with various SH3 domains in an in vitro assay. Recombinant GST-fusion proteins corresponding to the SH3 domains of src and fyn were expressed and immobilized on glutathione-agarose beads. GST-srcSH3 and GST-fyn SH3 were incubated with detergent-solubilized rabbit skeletal muscle membranes to test their ability to bind to SOCK. The polypeptides binding to the fusion-protein matrices were separated by SDS- PAGE. The presence of SOCK was detected by Western blot analysis with the anti-(CaMKII) antibody (Fig. 27, panel A). Immunoreactive polypeptides of 73, 60 and 58 kDa bound specifically to the GST-src SH3 constructs (lane 2) and not to immobilized GST alone (lane 1). The SH3 domain of src binds the immunoreactive polypeptides quite effectively while fyn showed association to a lesser extent (lane 3). The binding of SOCK , as well as the 60 kDa and 58 kDa polypeptides, to GST-srcSH3 was greatly reduced in the presence of a competing peptide corresponding to the proline-rich domain of SOCK (a.a. 410-532), demonstrating the specificity of the interaction (lane 4) and indicating that this region was responsible, at least in part, for the

Figure 28. Subcellular Distribution of SOCK and src Tyrosine Kinase in Skeletal Muscle.

Immunostaining was performed on rabbit skeletal muscle cytosol (cyt)and membrane (mem) fractions with anti-CaM kinase II (panel A) and anti-src (panel B). Western blot analysis was performed using a dilution of 1 in 1000 for both primary antibodies. Each lane contained one hundred and fifty micrograms of protein. The molecular weight of the immunoreactive polypeptides is indicated in kDa.


A
B
Anti-CaM kinase II
Anti-src
73 kDa
60
Cyt
Mem
Cyt
Mem

observed interaction.

To determine whether immobilized SOCK would be equally effective in binding endogenous src, skeletal muscle cytosol was incubated with GST-SOCK and GST-$SOCK_{410-532}$, a construct containing only the proline-rich domains of SOCK (a.a. 410-532). Polypeptides binding to the fusion proteins were separated in SDS-PAGE as above and analyzed by Western blot with the anti-c-src antibody (Fig. 27, panel B). A single 60 kDa immunoreactive polypeptide, corresponding to src, was affinity-purified by the SOCK and $SOCK_{410-532}$ fusion proteins (lanes 2 and 4). No immunoreactive polypeptides were purified by the GST matrix alone (lanes 1 and 3).

C.2.5 Co-distribution of SOCK and src in Muscle Membranes.

Given the potential for interactions between SOCK and src tyrosine kinase, we next investigated the distribution of these two kinases in skeletal muscle fractions. Skeletal muscle cytosol and membrane fractions were analyzed by Western blot with antibodies directed against either src or CaM-dependent protein kinases (Fig. 28). Immunoreactive polypeptides of 60 kDa detected by the src antibody in both cytosol and membrane fractions, when corrected for protein loading, appeared to be equally distributed in both fractions (Fig. 28, panel B). The anti-(CaM kinase II) antibody detected a very faint signal for the 60/58 kDa immunoreactive polypeptides in the cytosol while immunoreactive polypeptides of 73 kDa, corresponding to SOCK, as well as 60 and 58 kDa were observed in the skeletal muscle membrane fractions (Fig. 28, panel A).

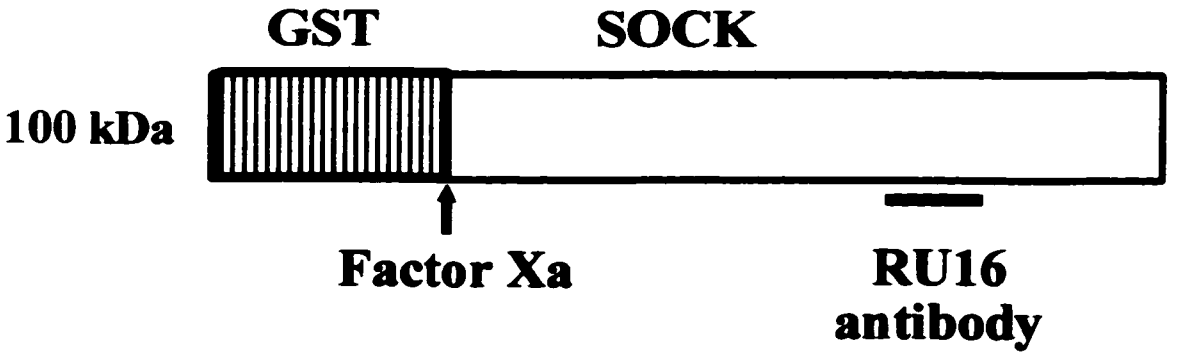
A number of skeletal muscle cell culture systems exist and can be used to study muscle

Figure 29 Schematic Representation of SOCK Fusion Proteins.

Full length SOCK restriction fragments were subcloned to the carboxyl side of the protein or peptide tags. (A) Glutathione-S-transferase (GST) has a predicted molecular mass of 26 kDa. The 100 kDa GST-SOCK fusion protein construct was expressed, purified and used in immunoreactivity, phosphorylation and association experiments.

(B) The myc-tagged SOCK construct was expressed in C2C12 cells. It has a predicted molecular weight of 76 kDa and was utilized in subcellular localization studies.

A


GST
SOCK
100 kDa
Factor Xa
RU16
antibody

B

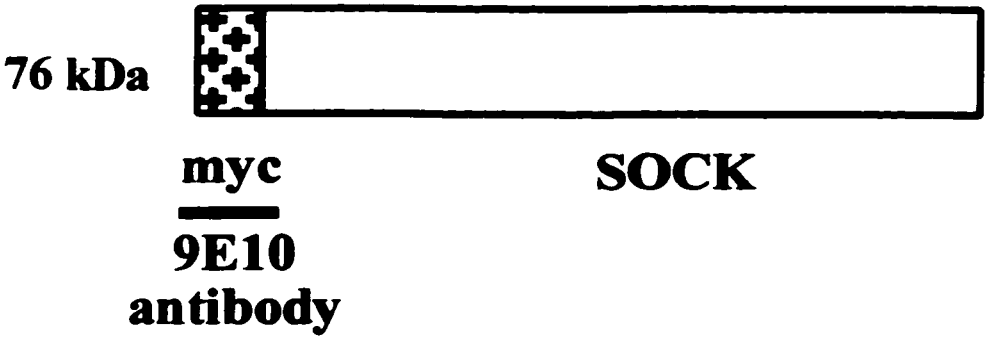
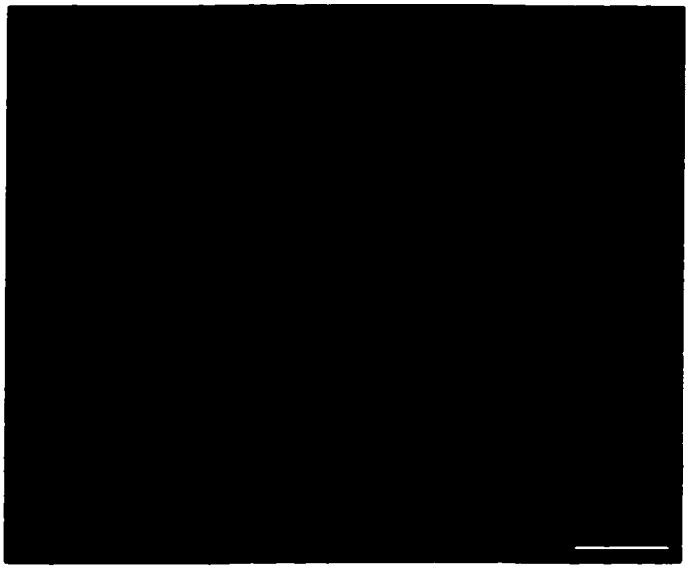

76 kDa
myc
SOCK
9E10
antibody

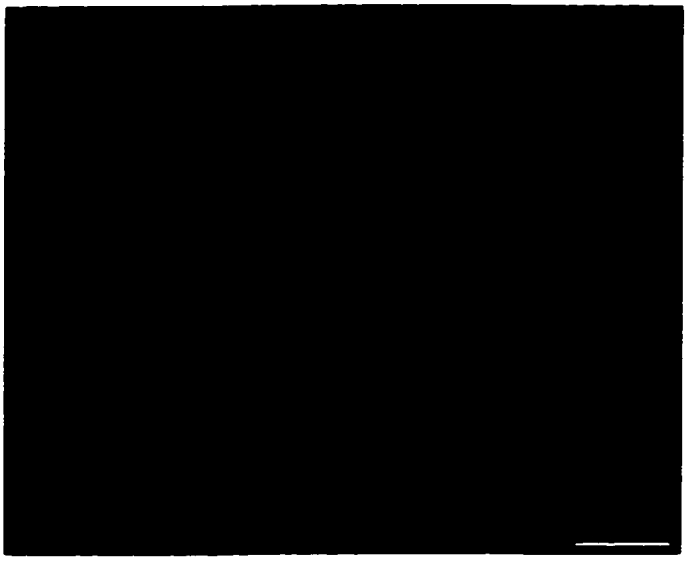
Figure 30 Subcellular Distribution of SOCK in C2C12 Skeletal Muscle Cells

Undifferentiated C2C12 cells were transfected with myc-tagged SOCK. The cells were grown on coverslips, fixed and double stained for src tyrosine kinase (panel A) and for the myc-tagged SOCK (panel B). The src signal appeared as green fluorescence and the myc-tagged SOCK appeared as red. The merged images of panels A and B resulted in a yellow signal indicating areas where src and-myc-tagged SOCK shared localizations within the cell (panel C). The scale bar represents 15 μm.

A



B



C

cells at various stages of development. The C2C12 cell line represents a population of mouse myoblast cells capable of differentiating in culture to form myotubes (Yaffe and Saxel, 1977). They have been used extensively to study how gene expression affect muscle growth and development in vitro (McMahon *et al.*, 1994).

The distribution of SOCK and src kinases was further investigated using C2C12 undifferentiated skeletal muscle cells. The use of the RU16 anti-(CaMKII) antibody was not favourable as it would have resulted in the detection of all CaM kinase related polypeptides in C2C12 cells. Since difficulties arose in the production of specific antibodies against the novel proline-rich region of SOCK, a fusion protein of SOCK, tagged with the myc epitope, was generated for use in subcellular localization studies. The use of epitope tagging constructs has greatly facilitated the study of novel polypeptides against which no antibodies are available. The myc epitope, consisting of the fragment 408-439 of the oncogene product c-myc, has been widely used in purification, immunoprecipitation and subcellular localization studies. A schematic representation of the myc-tagged SOCK and GST-SOCK fusion proteins is shown in Fig. 29. A full length SOCK restriction fragment was subcloned at the carboxyl end of the myc epitope tag vector that was transfected into C2C12 cells. Using a well characterized anti-myc epitope monoclonal antibody (9E10), an immunofluorescent signal for tagged SOCK was detected in approximately twenty percent of C2C12 cells (Fig. 30, panel B). Myc-tagged SOCK was detected using the fluorophore CY3 which emits a red fluorescence signal. Confocal laser microscopy revealed a predominantly cytosolic distribution of myc-tagged SOCK with more intense staining in the perinuclear compartment and at the edge of membrane lamella extending onto the coverslip surface. Very little signal

was detected in the nucleus. Src kinase, as detected by the anti-c-src antibody, was present in all C2C12 cells (Fig. 30, panel A) and appeared to be widely distributed within each cell. The src tyrosine kinase distribution was revealed using fluoroisothiocyanate (FITC) conjugated antibodies which emit a green fluorescent signal. Double immunofluorescence staining techniques have been used to visualize common areas of immunostaining for two or more polypeptides within cells or tissue. C2C12 cells stained to detect both myc-tagged SOCK and src kinase were analysed. Confocal laser microscopy revealed that some subcellular compartments, particularly the perinuclear area, appear to contain both SOCK and src tyrosine kinases (Fig. 30, panel C). No immunofluorescent signal was detected in C2C12 cells transfected with the empty vector.

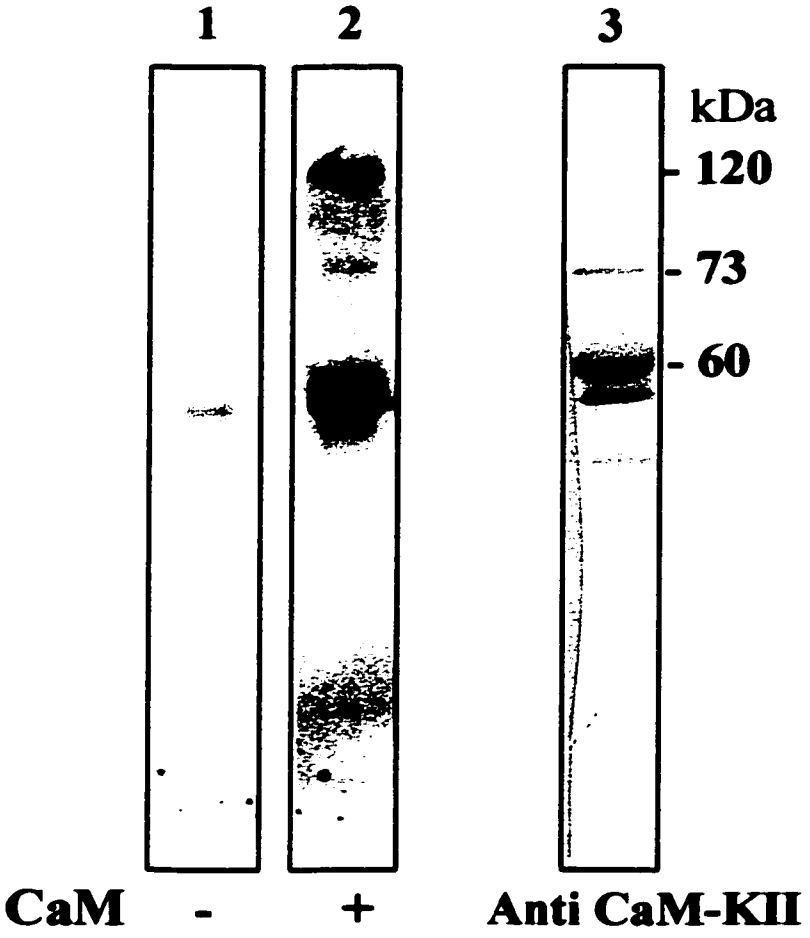
The likelihood of SOCK-src tyrosine kinase interaction occurring in the cell was further substantiated by the localization of SOCK and src to similar cellular compartments within muscle cells. These two molecules appear to share some co-localization within the membrane/particulate fraction of skeletal muscle. Although the β isoforms of CaM kinase II are not thought to be integral membrane proteins, β CaM kinases have been shown to associate with specific membrane structures in skeletal muscle cells (Campbell and MacLennan, 1982). Our data would suggest that this membrane interaction might occur, at least in part, through interactions with src tyrosine kinase, whose interactions with membranous structures are regulated by myristoylation (Brown and Cooper, 1996).

C.2.6 Phosphorylation Activity of SOCK

The interaction between SH3 modules and their target proline-rich domains has been

Figure 31 Identification of Autophosphorylation Activity in SOCK.

Crude microsomal membranes prepared from rabbit skeletal muscle were subjected to SDS-PAGE, transferred to nitrocellulose and allowed to renature as described. Following renaturation, autophosphorylation was assayed in the presence of either Ca^{2+} alone (lane 1) or Ca^{2+}/CaM (lane 2). The nitrocellulose was then dried and exposed for autoradiography. A set of nitrocellulose blots prepared under parallel conditions were analysed by Western blot using the anti-(CaMKII) antibody at a dilution of 1 in 3000. Three hundred micrograms of protein were loaded in each lane. Polypeptides of 60, 73 and 120 kDa demonstrated CaM-dependent autophosphorylation activity.


1
2
3
kDa
120
73
60
CaM
-
+
Anti CaM-KII

proposed to direct polypeptides to various subcellular compartments, facilitate enzyme-substrate encounters and potentially regulate enzyme activities (Cohen *et al.*, 1995). To elucidate possible functions of SOCK, its phosphorylation activity was further characterized. Refinements to the previously described renaturation assay warranted new attempts at defining novel CaM-dependent autophosphorylation activity in rabbit skeletal muscle membranes. Polypeptides were separated by SDS-PAGE without boiling the samples prior to loading them on to the gel. They were then transferred to nitrocellulose, renatured overnight at 4°C and incubated with kinase buffer in the presence of radiolabelled ATP. The results shown in Fig. 31 indicate that polypeptides of 60, 73 and 120 kDa demonstrated enhanced autophosphorylation activity (Fig. 31, lane 2) in the presence of exogenous calmodulin. Low levels of CaM-independent autophosphorylation activity were also detected in a 60 kDa polypeptide (Fig. 31, lane 1). The observed CaM-independent autophosphorylation activity of the 60 kDa polypeptide has been shown to occur in CaM kinases following phosphorylation of a threonine residue in the regulatory domain. The residual CaM-independent activity associated with the 60 kDa polypeptide could originate from endogenous phosphorylation of the regulatory site of the 60 kDa polypeptide prior to its extraction from the membrane. Immunoreactive polypeptides of 60 and 73 kDa, with mobilities corresponding to the autophosphorylated polypeptides, as well as polypeptides of 50 and 58 kDa were detected by Western blot analysis using an anti-(CaMKII) antibody (Fig. 31, lanes 2&3). Improvements to the kinase renaturation methodology led to the identification of a 73 kDa polypeptide that could also undergo autophosphorylation in a Ca^{2+}/CaM-dependent manner (Fig. 31). The improved autophosphorylation activity into the

60 kDa polypeptide was apparent under these conditions (Fig. 5 versus Fig. 31). The 120 kDa polypeptide demonstrating autophosphorylation activity may have resulted from the formation of 60 kDa polypeptide dimers that could not be entirely dissociated under the mild electrophoresis conditions used (ie. samples were neither boiled nor reduced with DTT before loading) or may represent a novel CaM-dependent kinase activity. A strong case can now be made to characterize the 73 kDa polypeptide, in addition to the 60 kDa polypeptide, as a CaM-dependent protein kinase of muscle membranes. In summary, a 73 kDa polypeptide was shown to bind Ca^{2+}/calmodulin, to be phosphorylated in a CaM-dependent manner, to contain an immunoreactive peptide highly conserved within CaM kinase and finally to demonstrate CaM-dependent autophosphorylation activity in a renaturation assay.

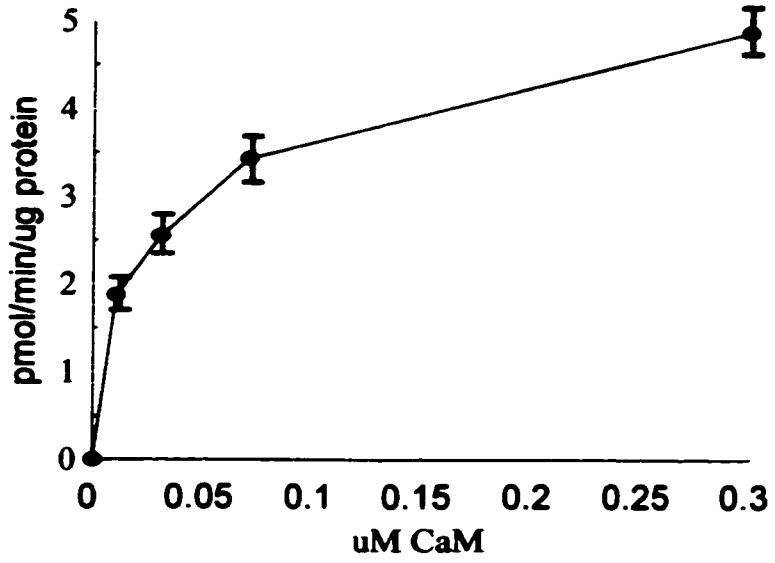
To further characterize the phosphorylation activity of SOCK, GST-SOCK fusion protein was immobilized on glutathione beads and was tested for its ability to phosphorylate Auto Camtide-2 (KKALRRQETVDAL), a well known peptide substrate of multifunctional CaM kinase II isoforms. The phosphorylated peptide substrate was separated from the [^{32}P]ATP in the assay using P81 phosphocellulose paper that was quantitated by liquid scintillation counting. Radioactivity incorporated into the substrate peptide was measured in the presence of increasing concentrations of CaM and the results are presented in Fig. 32, panel A. SOCK phosphorylated the peptide substrate with a maximal CaM-dependent phosphorylation activity of 4.86 pmol/min/(μg protein) observed at a concentration of 300 nM CaM. The Kd(CaM) for the GST-SOCK peptide assay was estimated at 10 nM. Autophosphorylation of the purified GST-SOCK polypeptide was also investigated by subjecting aliquots taken during the peptide assay to-SDS-PAGE and autoradiography

Figure 32 Autophosphorylation of the GST-SOCK Fusion Protein and Phosphorylation of a CaM kinase II Substrate

(A): GST-SOCK fusion protein was immobilized on glutathione beads and was tested for its ability to phosphorylate auto camtide-2, a peptide substrate of the multifunctional CaM kinase II family. Radioactivity incorporated into the substrate peptide in the presence of increasing concentrations of CaM was measured by binding the phosphorylated peptide to phosphocellulose and determining the incorporated radioactivity by liquid scintillation counting. ^{32}P radioactivity incorporated into the SOCK polypeptide increased in a CaM-dependent manner.

(B): Affinity-purified GST-SOCK was autophosphorylated in the presence of increasing concentrations of exogenous CaM. Aliquots of GST-SOCK were subjected to SDS-PAGE and a 100 kDa phosphoprotein was visualized by autoradiography.

A


5
4
3
2
1
0
pmol/min/ug protein
0
0.05
0.1
0.15
0.2
0.25
0.3
uM CaM

B

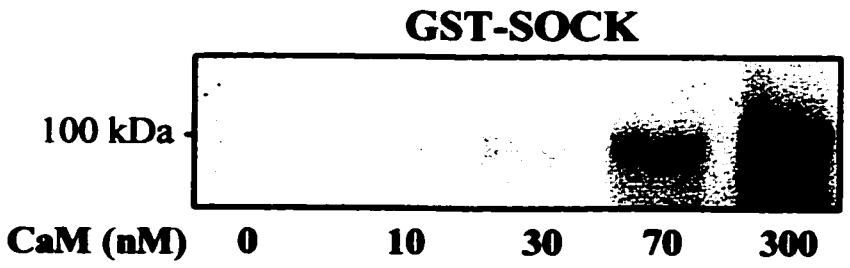
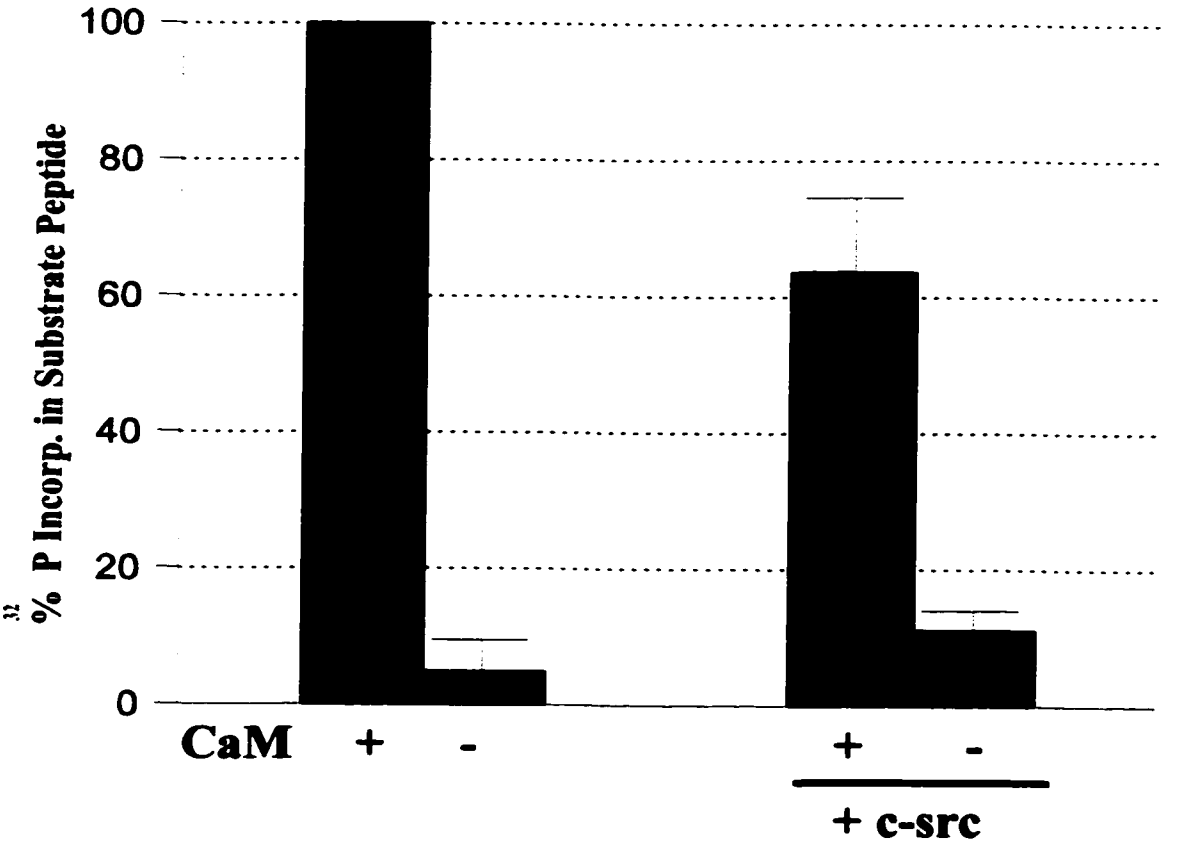

GST-SOCK
100 kDa
CaM (nM)
0
10
30
70
300

Figure 33 Effect of src Tyrosine Kinase on SOCK Phosphorylation Activity

Purified human recombinant c-src tyrosine kinase (10 units) was incubated with GST-SOCK in a CaM-dependent protein kinase peptide assay. Aliquots were blotted on phosphocellulose paper and counted by liquid scintillation. Assays were performed in duplicate and the results were corrected for assays containing no SOCK enzyme (background control). The results represent the mean ± SEM for four experiments. The results indicate that src tyrosine kinase inhibited CaM-dependent SOCK phosphorylation activity.


100
80
60
40
20
0
% ^{32}P Incorp. in Substrate Peptide
CaM
+
-
+
-
+ c-src

(Fig. 32, panel B). A single 100 kDa polypeptide corresponding to the predicted molecular weight of the GST-SOCK fusion protein could be detected to undergo autophosphorylation in a CaM-dependent manner. The GST-fusion protein provided an efficient method to generate sufficient quantities of catalytically active SOCK that was otherwise extracted in low abundance from the rabbit skeletal muscle membrane preparations.

In light of the data showing potential interactions between SOCK and SH3 domains of src tyrosine kinase, we investigated whether SOCK-SH3 domain interactions could influence the phosphorylation activity of SOCK in a peptide assay. Incorporation of radioactivity in the substrate peptide was measured in the presence and absence of purified recombinant c-src tyrosine kinase (Fig. 33). c-Src tyrosine kinase contributed to a mean decrease of 35 ± 10% in the CaM-dependent phosphorylation activity of SOCK in four independent experiments. No distinguishable changes were observed in the levels of CaM-independent activity. Decreases of CaM-dependent phosphorylation activity of the order of 10-20% were also observed in preliminary studies using hck and lyn tyrosine kinases (B.S. Tuana and A. Wilks, personal communication). Together, these data provide biochemical evidence for the interaction of SOCK and src kinase in skeletal muscle. In vitro experiments showed that the proline-rich region of SOCK can bind src tyrosine kinase and that src kinase can in turn interact with SOCK. Both kinases shared subcellular distribution as shown by the immunofluorescence and cell fractionation studies. Finally, at least under in vitro conditions, purified src can inhibit the activity of SOCK.

C.3 Expression of SOCK in a Pluripotent Cell System

Studies on the earliest commitment stages of muscle development can be undertaken with the aid of pluripotent cell systems such as the P19 embryonal carcinoma cell line. P19 embryonal carcinoma cells provide a flexible system with which to investigate a broad range of questions pertaining to tissue development. The P19 cell line originated from a primary teratocarcinoma induced by grafting a 7.5 day old mouse embryo onto the kidney capsule of an adult mouse (McBurney *et al.*, 1982). Upon treatment with dimethyl sulfoxide (DMSO), these pluripotent embryonal cells undergo differentiation to form derivatives of mesodermal tissue such as cardiac and skeletal muscle (Rudnicki *et al.*, 1990; Edwards *et al.*, 1983) whereas treatment with retinoic acid can induce differentiation along the neuroectodermal pathway (Edwards and McBurney, 1983; Pratt *et al.*, 1990). The mechanism of action of DMSO-dependent differentiation has been suggested to occur via a transient increase in intracellular calcium concentrations. Furthermore, CaM-dependent protein kinase activity is exquisitely sensitive to changes in intracellular calcium and has demonstrated effects on the differentiation of cells. Tyrosine kinases, such as src, have also been shown to play an important role in the growth and differentiation of not only embryonal cells but differentiated tissue as well. Since SOCK is a CaM-dependent protein kinase which is apparently structured to mediate interactions with the tyrosine kinase family, it was especially important to investigate possible roles of SOCK in cell growth and differentiation.

P19 cells provide an excellent system to examine the effects of SOCK overexpression in the early stages of muscle cell differentiation. Preliminary studies were undertaken to

Figure 34 Identification of Positive Clones Over-expressing SOCK in P19 Cells

(A) P19 embryonal carcinoma cells were transfected with an expression plasmid carrying either anti-sense (lane 1)or sense (lane 2&3) SOCK cDNA. Positive clones were identified by Northern blot analysis using a probe encoding the novel proline-rich domain of SOCK. A 1.8 kb transcript corresponding to the full-length SOCK was detected in sense-transfected clones. (B) The expression of the SOCK polypeptide was detected by Western blot analysis using the RU16 anti-(CaM KII) antibody. Immunoreactive polypeptides of 60,90 and 170 kDa were detected in all P19 cells. Clones expressing SOCK contained an additional 73 kDa immunoreactive polypeptide.

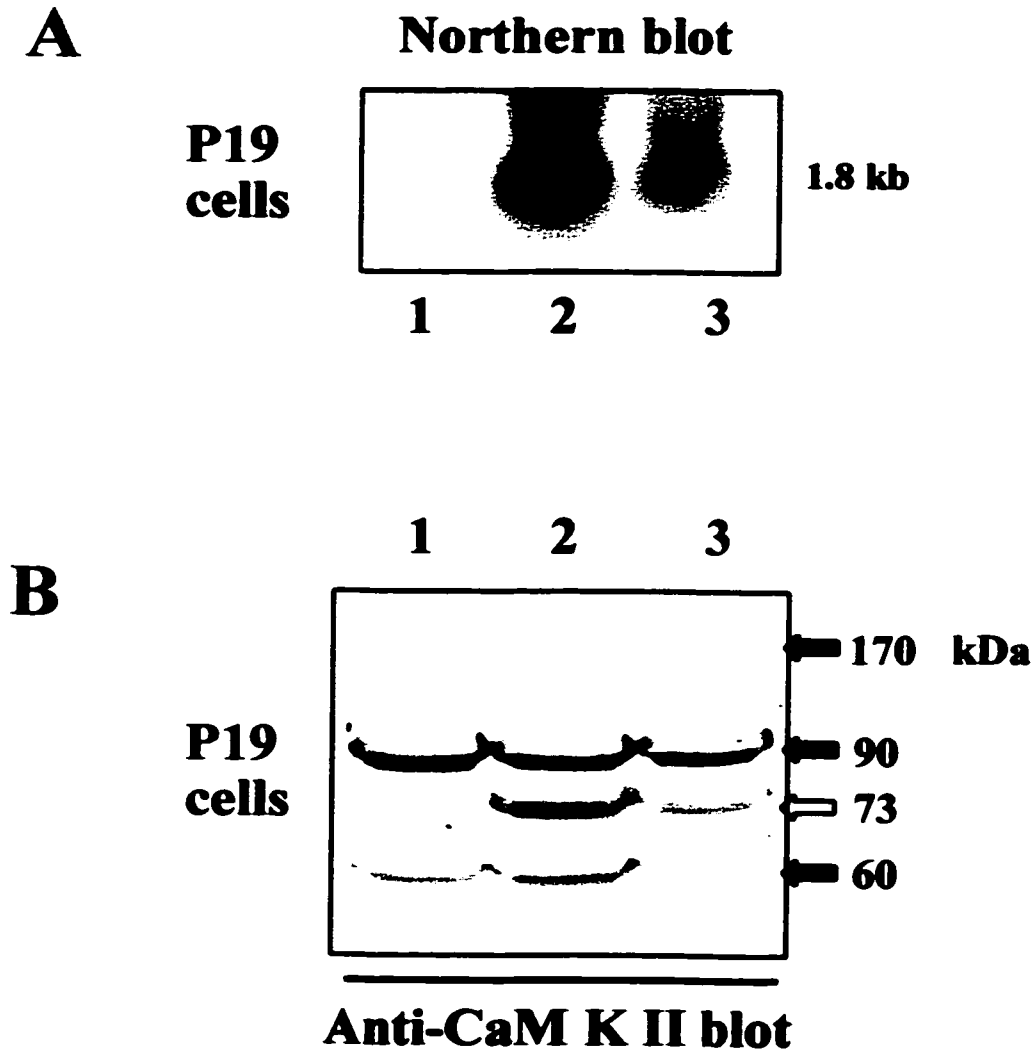
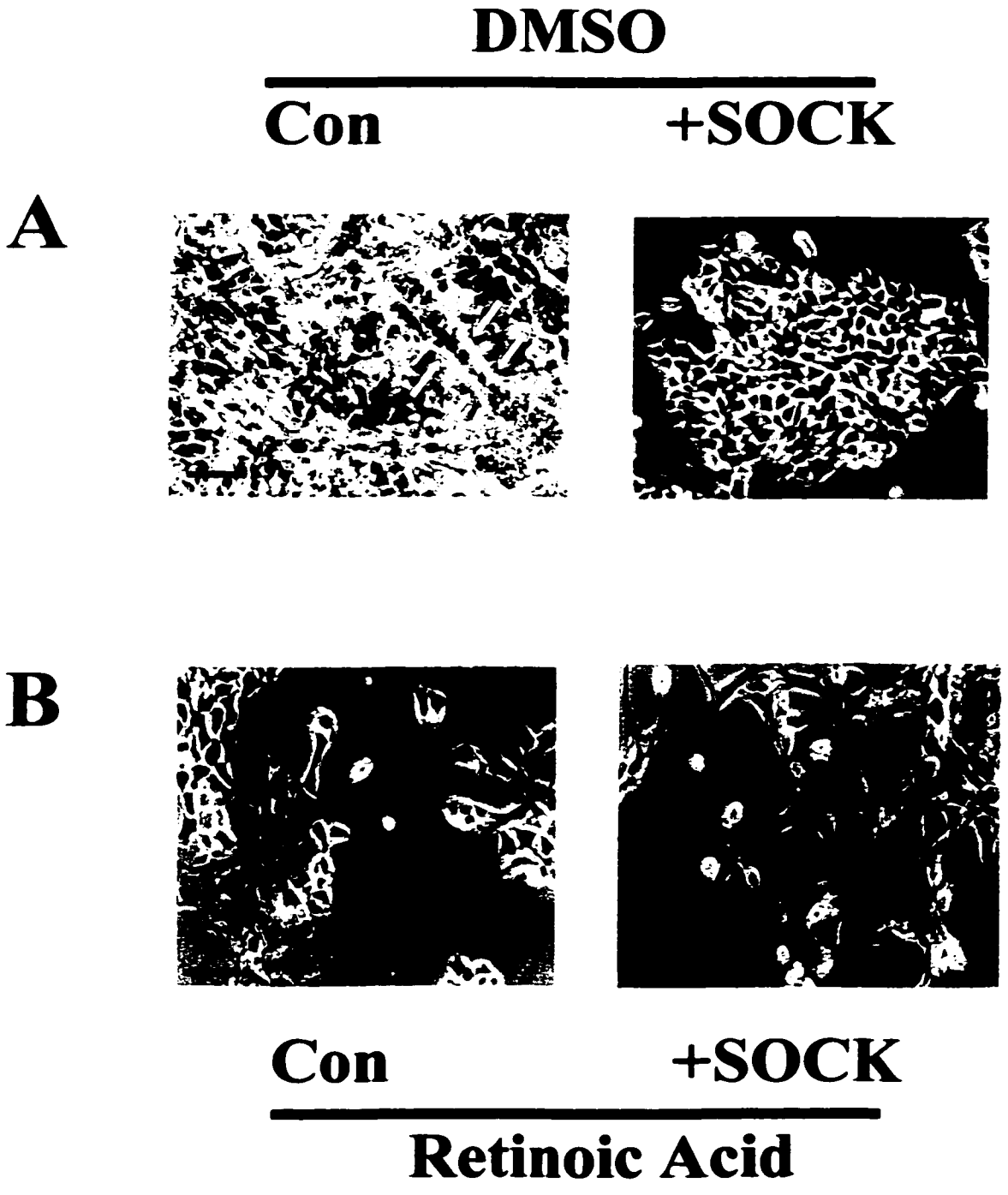

A
Northern blot
P19
cells
1.8 kb
1
2
3
B
1
2
3
170 kDa
P19
cells
90
73
60
Anti-CaM K II blot

Figure 35 Over-expression of SOCK in DMSO and Retinoic Acid Treated P19 Cells

The panels show phase contrast fields of control and SOCK-transfected P19 cells treated with dimethyl sulfoxide (DMSO) or retinoic acid (panel A and B respectively). The bar represents 50 μm. Arrowheads in panel A (control cells) indicate the position of a contracting loci of cells.

Effect of SOCK on P19 Cell Differentiation



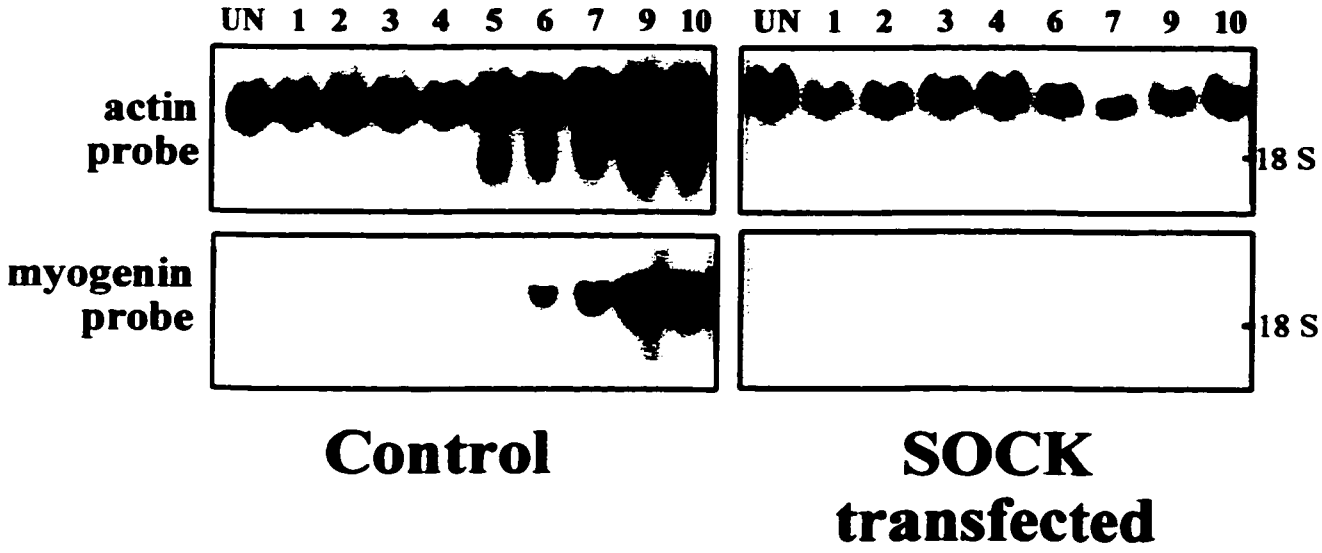
investigate endogenous levels of SOCK expression in the cell line. P19 cells were found to contain no detectable levels of SOCK transcripts or polypeptide. P19 mouse embryonal carcinoma cells were then transfected with the expression plasmid carrying either sense or anti-sense SOCK transcript and stable transfectants were selected in G418. Several clones were isolated which expressed both the SOCK transcript and the 73 kDa polypeptide as shown by Northern and Western blot analysis (Fig. 34, panel A). Using the RU16 antibody, raised against the conserved sequence of CaM kinases, immunoreactive polypeptides of 170, 90 and 60 kDa were detected in P19 cell homogenates. Clones that were successfully transfected and expressed sufficient levels of SOCK contained an additional 73 kDa immunoreactive polypeptide corresponding to the SOCK polypeptide (Fig. 34, panel B lanes 2&3). Transfection of P19 clones with anti-sense SOCK did not affect levels of the 60, 90, and 170 kDa endogenous CaM kinase related polypeptides. The transfected P19 cells were treated with retinoic acid to promote differentiation into neuroectoderm or with DMSO to promote mesoderm formation. SOCK-transfected colonies and control colonies treated with retinoic acid both differentiated normally along the neuroectoderm pathway as evidenced by the formation of long thin outgrowth resembling dendrites (Fig. 35). In contrast, SOCK-transfected colonies treated with DMSO did not respond to the differentiation signal as measured by morphological changes and changes in muscle-specific genes. The SOCK transfected clones did not display the typical morphology of mesoderm and were indistinguishable from undifferentiated cells as opposed to cells grown under control conditions that fused to form loci of contracting cells (Fig. 35, arrowhead denotes myotube). These loci contracted at a rate between ten and thirty contractions per minute. The

contraction was completely inhibited by incubating the cells in a medium containing 1 μM nifedipine, an antagonist of the L-type voltage-sensitive calcium channel that plays a role in the process of excitation-contraction coupling in both skeletal and cardiac muscle. The sensitivity of the observed contracting cells to the voltage-sensitive calcium channel blocker nifedipine provides further evidence for the differentiation of the DMSO-treated P19 control cells into mesoderm. It should be noted that concentrations of DMSO used on these cells is not toxic and the observed changes therefore result from differentiation and not selection of a small pool of already differentiated cells.

To investigate the role of SOCK in the blockade of differentiation, the transfected and control colonies were tested for the expression of muscle-specific genes such as myogenin and cardiac actin (Fig. 36). Myogenin appeared in control cells at day 5 whereas no expression was detected in SOCK-transfected P19 cells. Similarly, using a probe that hybridizes mRNA encoding all actin isoforms, the 2.1 kb non-muscle and the 1.7 kb cardiac and skeletal muscle actin transcripts were detected. The 1.7 kb muscle actin transcripts first appeared on day 5 in the control cells but not in SOCK-transfected cells. The level of cytoskeletal actin mRNA remained constant during the period examined and the level of these mRNAs was used as a control for the amount of RNA loaded in each lane. These observations suggest a role for SOCK in regulating mesoderm formation induced by DMSO. It should be restated that DMSO can cause a transient increase in intracellular calcium concentration. It is therefore possible that SOCK is activated by this calcium signal and affects differentiation by interacting with a downstream SH3 domain containing proteins able to regulate stem cell differentiation into mesoderm.

Figure 36 Muscle- Specific Gene Expression in SOCK-transfected P19 cells Treated with DMSO

Total RNA isolated from untreated (UN) and treated P19 cells at the indicated intervals (days) was analyzed by Northern blot hybridization using actin and myogenin probes. The actin probe hybridized to both cytoskeletal (2.1 kb transcript) and striated muscle (1.7 kb transcript) forms of actin. The cytoskeletal transcript was expressed at constant levels in all cells and served as an internal control for RNA loading. The expression of cardiac/skeletal actin and myogenin mRNA was detected only in control cells starting at day 5. No signal was detected for these muscle-specific genes in SOCK-transfected clones. The location of the 18S ribosomal RNA is indicated on the right.


UN 1 2 3 4 5 6 7 9 10
UN 1 2 3 4 6 7 9 10
actin probe
18 S
myogenin probe
18 S
Control
SOCK transfected

D. DISCUSSION

Our understanding of muscle function has taken great strides from the earliest descriptions of its gross anatomy and morphology to the molecular deciphering of its complex contractile apparatus. Many of the proteins involved in muscle contraction are capable of detecting, regulating or responding to changes in intracellular calcium. An important polypeptide in the detection of calcium signals is the calcium-binding polypeptide calmodulin (CaM). Upon its activation by rising intracellular calcium levels, CaM can stimulate a number of downstream signalling effectors including the multifunctional Ca^{2+}/CaM-dependent protein kinase family (Braun and Schulman, 1995).

There is growing evidence to suggest that CaM-dependent protein kinase phosphorylation plays an important role in the regulation of excitation-contraction coupling in muscle cells. Previous studies have shown that a CaM kinase activity was associated with the sarcolemmal and sarcoplasmic reticulum (SR) membranes of cardiac and skeletal muscle (Tuana and MacLennan, 1988; Tuana *et al.*, 1987; Campbell and MacLennan, 1982; Kim and Ikemoto, 1986; Witcher *et al.*, 1991). As these membrane systems are critical regulators of cellular Ca^{2+} levels, the objective of this study was to pursue the biochemical and molecular characterization of the protein kinases implicated in the regulation of muscle membrane function. The biochemical characterization of muscle membranes led to the identification of at least two polypeptides, of 60 and 73 kDa, as Ca^{2+}/CaM-dependent protein kinases of muscle membrane systems. Although these enzymes showed some notable similarities to previously characterized CaM kinase II isoforms, striking differences hinted at the existence

of unique variations in their polypeptide structure and function (Leddy *et al.*, 1993). The molecular characterization of muscle CaM kinases led to the definition of these differences and to the identification of a novel CaM kinase isoform, which was named SOCK. Structural and functional features of the SOCK polypeptide implied a role in the regulation of Ca^{2+} transport in muscle cells. Furthermore, this kinase may serve to integrate intracellular calcium and tyrosine kinase signalling pathways and, as such, serve roles in mesoderm induction and differentiation.

The initial biochemical characterization of the CaM kinase activity in skeletal muscle focussed on the identification of CaM-binding polypeptides. Various subcellular muscle fractions, enriched in particular membrane systems such as sarcoplasmic reticulum, were obtained by differential centrifugation of homogenized skeletal muscle. The identity of each subcellular membrane fraction was confirmed on the basis of enrichment of characteristic markers for each type of membrane, such as the binding of dihydropyridine receptors for t-tubules and ryanodine receptors for SR (Chu *et al.*, 1988). CaM-binding sites were identified on polypeptides of 38, 60, 73, 100 and 160 kDa in skeletal muscle membrane fractions. The predominant CaM-binding polypeptide in muscle was a 60 kDa polypeptide that associated with membrane fractions enriched in heavy SR. It was also notable that a 73 kDa CaM-binding polypeptide was also found to enrich in these fractions.

A CaM-dependent protein kinase activity was shown to associate with SR-enriched membranes of skeletal muscle and to phosphorylate polypeptides of 450, 360, 165, 105, 89, 73, 60, 34 and 20 kDa in the presence of Ca^{2+} and CaM. These results are consistent with those reported previously (Campbell and MacLennan, 1982; Chiesi and Carafoli, 1983; Tuana

and MacLennan, 1984; Seiler *et al.*, 1984). The 89 and 165 kDa phosphoproteins may represent triadin and histidine rich Ca^{2+}-binding proteins respectively, two polypeptides of the SR that have been identified as substrates of the skeletal muscle CaM kinases (Damiani *et al.*, 1995). In addition, polypeptides of 97 and 53 kDa were found to be substrates of a Ca^{2+}/CaM-independent kinase activity. Of the phosphorylated polypeptides, the 60 and 73 kDa polypeptides were shown to harbour the Ca^{2+}/CaM-dependent protein kinase catalytic domains that are responsible, in part, for the Ca^{2+}/CaM kinase activity of the SR. It is notable that the 62 and 45 kDa polypeptides represent other protein kinases that do not exhibit CaM kinase activity.

The 60 kDa polypeptide from skeletal muscle that associated with the heavy SR-enriched membranes was identified as the major CaM-dependent protein kinase that undergoes CaM-dependent autophosphorylation. The 60 kDa polypeptide was shown to contain not only a CaM-binding site but also an ATP-binding site as visualized by cross-linking experiments using an azido derivative of ATP. An 89 kDa polypeptide was also found to bind ATP. The 89 kDa polypeptide was also phosphorylated in a Ca^{2+}/CaM-dependent manner. The ability of the 60 kDa polypeptide to bind both CaM and ATP, as well as undergo CaM-dependent autophosphorylation in renaturation assays on nitrocellulose, defines three key aspects required to designate a polypeptide as containing CaM-dependent protein kinase activity. However, whether the 89 kDa polypeptide is a CaM kinase still remains to be fully investigated.

A Ca^{2+}/CaM-independent autophosphorylation activity associated with polypeptides of 62 and 45 kDa was found to enrich in the light SR membrane fraction. A Ca^{2+}-independent

kinase activity that phosphorylates polypeptides of 62, 42 and 20 kDa has been previously described in SR vesicles (Campbell and Shamoo, 1980) and this Ca^{2+}-independent kinase activity may be attributable to the 62 and 45 kDa polypeptides of the light SR-enriched fractions. The Ca^{2+}-independent autophosphorylation activity of the 62 and 45 kDa polypeptides found in the light SR could play a potential role in the regulation of Ca^{2+} transport systems since a high density of Ca^{2+}-ATPase polypeptides are localized to this subcellular membrane fraction. While no autophosphorylation activity was renatured in the t-tubule-enriched membranes, novel Ca^{2+}/CaM-binding polypeptides of 160, 100, 73 and 38 kDa were identified in these membranes fractions. The 160 kDa CaM-binding polypeptide may represent the calmodulin-sensitive enzyme nitric oxide synthase, nNOS, which is found to associate with membranes in neuronal tissue and skeletal muscle (Brenman *et al.*, 1995). A previous study, using photoaffinity cross-linking rather than the calmodulin overlay method, identified a 60 kDa polypeptide as the major calmodulin receptor in SR as well as other CaM-binding polypeptides of 148, 125, 41, 31 and 23 kDa (Vale, 1988). Of these polypeptides, the 148 and 125 kDa polypeptides were proposed to represent subunits of phosphorylase kinase. Additional CaM-binding and ATP-binding polypeptides detected in the muscle membranes (38, 89 and 100 kDa polypeptides) may represent kinases whose enzymatic activities were not fully renatured under our assay conditions. A 33 kDa polypeptide, which bound CaM in the absence of calcium, was localized in the light membrane fraction. While we do not know the nature of this polypeptide, the 33 kDa CaM-binding polypeptide may play a role in sequestering free CaM to the regions of junctional face membrane under resting conditions (low [Ca^{2+}]). This would be in analogy to the way neuromodulin is thought to

serve as a sink for CaM in neuronal membranes. Neuromodulin is a CaM-binding protein that has been implicated in axonal elongation, regeneration, synaptic plasticity and neurotransmitter release (Liu and Storm, 1990). The distribution of the 33 kDa CaM-binding protein could therefore impart a further level of complexity to the calcium signal in muscle by targeting the CaM molecules to certain areas within the cell.

The characteristics of the CaM kinase activity of SR were compared with those of the classical CaM kinase II isoforms. The 60 kDa CaM kinase of SR was shown to have different membranous interactions as compared with the multifunctional CaM kinase II. The 60 kDa was tightly associated with membranes and could only be solubilized with detergents whereas the multifunctional CaM kinase II isoforms from brain are mostly soluble (Schulman and Hanson, 1993). Affinity-purified polyclonal antibodies raised against the brain multifunctional CaM kinase II β subunit cross-reacted with a 60, 73 and 54 kDa polypeptide of SR skeletal muscle. Such cross-reactivity was anticipated because the antibodies were raised against a peptide sequence that is highly conserved in different CaM-dependent protein kinase isoforms (Tobimatsu and Fujisawa, 1989).

The interactions of the 60 kDa polypeptide with the cellular membranes were further characterized by Western blot analysis using an anti-(CaM kinase II) antibody. A 60 kDa immunoreactive polypeptide displayed selective association with specific membrane populations of muscle whereas a 54 kDa immunoreactive polypeptide was readily dissociated from the microsomal membranes by a procedure known to remove peripheral proteins (Mitchell *et al.*, 1983). In contrast, the 60 kDa immunoreactive polypeptide was not extracted with high salt buffers that disrupt the association between peripheral proteins and

cell membranes. Rather, it was concentrated in the heavy SR-enriched fractions and in particular the junctional face membrane of the terminal cisternae which are highly enriched in the 450 kDa polypeptides representing the Ca^{2+} release channels (Costello *et al.*, 1988). The close association of the 60 kDa CaM kinase with membrane structures intimately involved with excitation-contraction coupling supported the postulated involvement of CaM kinases in the regulation of muscle contraction.

Immunoprecipitation studies of solubilized, phosphorylated SR membranes with the anti-(CaM kinase II) revealed that the 60 kDa polypeptide associated with phosphoproteins of 360, 105, 89,73, 34 and 20 kDa. Partially purified CaM kinase complexes were obtained from phosphorylated and solubilized SR membranes that were affinity purified by CaM-agarose chromatography. The eluted CaM kinase complex was subjected to sucrose density centrifugation to separate associated phosphoprotein complexes from single polypeptides. The 89, 73, 60, 34 and 20 kDa phosphoproteins co-sedimented on the gradient indicating that they formed some type of associative complex. Antibodies raised against the gel-purified 60 kDa polypeptide recognized this polypeptide in Western blots, but not the other phosphoproteins suggesting that these proteins do not share the epitopes detected in the 60 kDa polypeptide. This result is consistent with previous phosphoprotein mapping studies reporting that the 89, 60, 34 and 20 kDa phosphoproteins of the partially purified CaM kinase complex were structurally distinct polypeptides (Tuana and MacLennan, 1988). Unlike the soluble multifunctional CaM-dependent protein kinases that appear to form multimeric complexes of ten to twelve α and β subunits (Woodgett *et al.*, 1983; Schulman and Lou, 1989; Kanaseki *et al.*, 1991), the 60 kDa CaM-dependent protein kinase of SR appears to

form heterogenous complexes of polypeptides.

The sensitivity of muscle CaM-dependent protein kinases to the calmodulin antagonist CaM kinase II inhibitory fragment (a.a.290-309) was examined in view of comparing its response to other CaM kinases and determining its usefulness as an inhibitor of CaM-dependent phosphorylation in skeletal muscle. The estimated IC_{50} for the inhibition of the 60 kDa CaM kinase of SR was approximately 2 nM, a value almost two hundred fold more sensitive than the average inhibition reported for the multifunctional CaM kinases (Colbran *et al.*, 1989). Variations in the reported phosphorylation assay conditions and differences in the amino acid composition of the inhibitor fragment may account for the different IC_{50} values. The analysis of physiological functions of the multifunctional CaM kinase family has been limited in the past by the lack of specific inhibitors of CaM kinase activity (calmidazolium, W7, trifluoperazine). The design of more specific inhibitors, such as KN62 and KN93, is defining more clearly the roles of this growing family of protein kinases (Hidaka and Yokokura, 1996).

In view of the unique biochemical features of CaM kinases in muscle cells, molecular cloning technology was utilized to further characterize these important molecules. By screening cDNA libraries from human skeletal muscle and mouse skeletal muscle with a β CaM kinase cDNA probe, two cDNAs were cloned. One of the cDNAs represented the classical β CaM kinase encoding a polypeptide of 60 kDa while the second cDNA encoded a polypeptide of 73 kDa representing SOCK.

Computer-assisted sequence homology searches for the amino acid sequence deduced from the cDNA encoding the 60 kDa polypeptide indicated 100% identity with the β CaM

kinase II isoform previously cloned from mouse brain (Karls *et al.*, 1992). The predicted amino acid sequence revealed a number of characteristic CaM kinase motifs such as an ATP-binding site (a.a. 20-44), a serine/threonine domain (a.a. 132-144), an autoinhibitory domain (a.a. 280-290), a calmodulin-binding site (a.a. 297-315) and associations domains (a.a. 320-end) (Hanson and Schulman, 1992). This cDNA may therefore represent the membrane-associated 60 kDa CaM kinase found in muscle although no probable transmembrane sequences were identified by Kyte-Doolittle hydrophobicity analysis (Kyte and Doolittle, 1982).

The second cloned cDNA represented the 73 kDa polypeptide SOCK. The cloned human and mouse SOCK sequences revealed a 95% identity between the polypeptides indicating a high degree of conservation across species. Comparison of the deduced amino acid sequence from the cloned human and mouse cDNA with a database of protein motif consensus sites (PROSITE database) indicated the presence of consensus sequences also found in the 60 kDa polypeptide such as putative ATP- and CaM-binding motifs as well as serine/threonine kinase, regulatory and association domains (Colbran *et al.*, 1989). Of particular interest were the predicted phosphorylation sites, most notably a potential tyrosine phosphorylation site, RFTDEYQLY (a.a. 9-17), located at the extreme N-terminus of the polypeptide (Pearson and Kemp, 1991). This sequence is only present in the polypeptide sequence of CaM kinase II β isoforms and may represent a potential site of convergence between Ca^{2+}/CaM and tyrosine signalling pathways. Interestingly, no studies to date have investigated the potential tyrosine phosphorylation of this site. Although the SOCK cDNA showed a great deal of identity to the multifunctional CaM kinase II isoforms, it also

contained a 366 bp insert of novel sequence in the variable domain. The highest homology was observed with the CaM-dependent protein kinase β subunit nucleotide sequence cloned from mouse brain (Genbank Accession # X63615). The mouse brain β CaM kinase II isoform had 77% nucleotide identity with the human SOCK cDNA sequence, increasing to 92% identity in regions outside the novel 369 bp insert (Karls *et al.*, 1992). The nucleotide sequence of 2220 nucleotides contained an open reading frame sequence that encoded a polypeptide of 665 amino acids. Other than the region corresponding to the 369 bp insert, comparison of the deduced amino acid sequence with the 60 kDa polypeptide revealed only a few amino acid differences. Conserved changes observed in the SOCK polypeptide include amino acid residue 19 E=>D, 365 A=>I and 371 S=>T. Perhaps more significant in terms of functions, two potentially reactive amino acid residues, serine and threonine, were replaced by alanine residues in the SOCK polypeptide (a.a. 363 S=>A and 398 T=>A). None of the substitutions involved amino acid residues assigned to known functions within the polypeptide. Additionally, three cAMP-dependent protein kinase and six protein kinase C putative phosphorylation sites were found within the novel proline-rich motif, suggesting that it could possibly contain important regulatory sequences (Pearson and Kemp, 1991). Much like the 60 kDa polypeptide and other CaM kinase isoforms, SOCK displayed a predominantly hydrophilic profile with a single area of increased hydrophobicity (a.a. 195-215) that is unlikely to span the membrane bilayer. The 369 bp insert encoded a stretch of 123 amino acids largely enriched in proline residues inserted between the variable and association regions of the enzyme. The proline-rich regions contain the putative PXXP motifs known to represent SH3 -binding domains at a.a.410, 431, 438 and 516. Because these

proline-rich regions were characteristic in other SH3-binding polypeptides such as Son Of Sevenless (Nimnual *et al.*, 1998), this novel CaM kinase polypeptide was named SOCK, an acronym for Son of CaM Kinase.

The predicted molecular weight of SOCK from the deduced amino acid sequence was 72836 Da. An expression plasmid carrying the full length SOCK cDNA generated a polypeptide that migrated with an apparent molecular weight of 73 kDa in SDS-PAGE. The expressed 73 kDa polypeptide was recognized by a peptide specific anti-(CaM kinase II) antibody and exhibited Ca^{2+}/CaM-dependent autophosphorylation. Furthermore the expressed SOCK also phosphorylated Auto Camtide-2, a well recognized substrate of the multifunctional CaMKII family of enzymes. The Kd (CaM) for the phosphorylation of the peptide reaction was estimated at 10 nM, a value within the range of previously characterized Kd(CaM) values (Colbran *et al.*, 1989) for multifunctional CaM-dependent protein kinases (0.010 to 0.5 μM). A broader range of calmodulin concentrations would have been required to calculate a more precise Kd value. Comparing our results with other studies is difficult as the calculated Kd(CaM) are highly dependent on calcium levels and assay conditions. However, it was noted that the expressed SOCK polypeptide might be less active towards exogenous peptide substrates and demonstrated slightly decreased levels of autophosphorylation when compared with endogenous kinases and may therefore require interactions with other polypeptides or additional processing steps within the mammalian cell to acquire its maximal activity.

Computer-assisted homology searches revealed that the deduced amino acid of SOCK shows highest homology with other β CaMKII isoforms. At least five members of the β

CaMKII isoform family have been identified: the classical β and β' isoforms predominantly studied in brain tissue (Bennett and Kennedy, 1987; Karls *et al.*, 1992), their embryonal counterparts, βe and β'e (Brocke *et al.*, 1995), and the β3 isoform cloned from rat pancreas (Urquidi and Ashcroft, 1995). A single β CaMKII gene has been identified and localized to chromosome 11 in mouse (Karls *et al.*, 1992; Danciger *et al.*, 1992). The various β isoforms are believed to result from the alternative splicing of this single gene in the variable domain (Urquidi and Ashcroft, 1995). The detection of previously uncharacterized proline-rich regions, inserted within the variable domain of SOCK, raised the interesting possibility that SOCK was generated by alternative splicing of the CaMKII β gene. The partial genomic structure of the mouse β CaMKII gene has been previously elucidated (Karls *et al.*, 1992) and has identified a number of exons flanking the variable domain. In order to determine how SOCK was derived, we investigated the genomic structure of the β CaMKII gene in mouse to define the potential splicing pattern used to generate SOCK.

The genomic organization of SOCK revealed that the proline-rich domains were encoded by three distinct exons. The exon/intron boundaries were identified by divergence from the cDNA sequence and by detection of consensus sequences for intron splicing. Each intron was flanked by the consensus sequence "GT....AG" flanking the extremities of intronic sequences (Mount *et al.*, 1992). These exons were localized at the junction between the variable domain and association domain, an area of the greatest diversity among CaMKII isoforms (Tobimatsu and Fujisawa, 1989). The first SOCK exon (S1) consisted of 114 nucleotides and was localized 3' to the last identified exon of the variable domain. The second and third SOCK exons (S2 & S3) contained nucleotide sequences of 130 and 126 bp

respectively. The discovery of these three exons lend credence to the proposed mechanism of alternative splicing for the generation of multiple β isoforms. The genomic organization of SOCK suggests that alternative SOCK-like β isoforms may be generated from alternative splicing of the CaM KII β gene. . For example, a polypeptide containing only the S1 exon of SOCK would have a predicted molecular weight of 64 kDa and retain a potential proline-rich domain containing the putative SH3-binding motif.

The physiological importance of multiple isoforms of α, β, γ and δ CaM kinases is extremely significant. The variable domain that houses the area of greatest diversity resides between the regulatory calmodulin-binding domain and the association domains. Changes occurring in the variable region are thought to potentially target the subcellular localization and thus allow the enrichment of kinase near selected substrates (Yoshimura and Yamauchi, 1997; Leddy *et al.*, 1993; Chu *et al.*, 1990). Alterations in the CaM kinase variation domain may also alter the calmodulin-affinity of the enzyme or affect its ability to assemble into large multimeric complexes. It is generally thought that the α and β/β' CaM KII isoforms are predominantly associated with neuronal tissues whereas the γ and δ CaMKII isoforms are responsible for most of the multifunctional CaM kinase activity in other peripheral tissues (Tobimatsu and Fujisawa, 1989). Unlike the other β CaMKII isoforms, the tissue distribution of the SOCK 4.8 kb transcript, as detected by a probe generated from the novel sequence of the proline-rich domain, seems exclusive to muscle tissues. Our findings of high levels of expression of SOCK in fast and slow twitch skeletal, cardiac and aortic smooth muscle tissues, suggest a specialized role for β isoforms, more specifically SOCK, in the regulation of calcium signalling in muscle.

An antibody (RU16) raised against a conserved peptide in β CaMKII isoforms recognized the expressed SOCK polypeptide (Leddy *et al.*, 1993). This peptide sequence was well conserved in the predicted amino acid sequence of SOCK, exhibiting 92% identity. The anti-peptide antibody recognized a 73 kDa polypeptide, corresponding to the apparent molecular weight of SOCK, in microsomal fractions from both skeletal and heart muscle. Furthermore, a 73 kDa polypeptide was shown to bind CaM and autophosphorylate in a Ca^{2+}/CaM-dependent manner in skeletal muscle membranes. This 73 kDa polypeptide in muscle membranes most likely represents SOCK. This conclusion is further supported by the observations that SH3 domains could specifically bind a 73 kDa polypeptide from muscle membrane extracts that was recognized by the anti-(CaMKII) and that this interaction was inhibited by the proline-rich domain of SOCK.

The anti-(CaMKII) antibody also recognized polypeptides of approximately 60, 58 and 24 kDa in muscle membrane fractions. The 60 and 58 kDa immunoreactive polypeptides were the major antigens and may represent the classical β CaM kinase II isoforms reported to associate with muscle membrane (Tobimatsu and Fujisawa, 1989; Leddy *et al.*, 1993). The 24 kDa polypeptide may represent a degradation product or the kinase anchoring protein (KAP) that retains the amino acid sequence against which the antibody was raised. The KAPs have been proposed to integrate into the membrane via a novel highly hydrophobic stretch of 25 amino acids that is absent in other CaM kinase II isoforms. Some CaM kinase isoforms may be anchored to membrane structures through interactions with KAPs via their association domains (Bayer *et al.*, 1996; Sugai *et al.*, 1996). It is notable that in addition to the 73 kDa SOCK, the anti-(CaMKII) immunoreactive polypeptides may represent SOCK-related

products or additional β CaMKII isoforms of muscle membranes. These smaller SOCK-related polypeptides could be generated through the alternative splicing of only one of the three exons encoding the proline-rich motifs domains that associate with the src SH3 domains. A 120 kDa polypeptide in muscle membranes exhibiting a Ca^{2+}/CaM kinase activity as evidenced by the renaturation/autophosphorylation assay may represent another CaMKII family member or result from the dimerization of the 60 kDa polypeptide under the renaturation conditions. In this regard, the basic structural and molecular features of the various types of CaM kinases expressed in muscle remain to be fully defined.

The C-terminus of SOCK was characterized by a novel peptide stretch of 123 amino acids that was enriched in proline residues and contained a consensus PXXP motif that is believed to characterize SH3-binding domains (Rickles *et al.*, 1994). Accordingly, expressed SOCK was found to associate specifically with SH3 domains via the novel C-terminal peptide. The proline-enriched region of SOCK shares some similarity with the pancreatic β3 isoform (Urquidi and Ashcroft, 1995). However, there exist significant sequence differences between the β3 and SOCK sequences. In the variable domain common to all β isoforms, the β3 polypeptide lacks a stretch of 15 amino acids which has been shown to be encoded a single exon (Tombes and Krystal, 1997). The differences in the amino acid sequence extend into the proline-rich domain. The proline-rich domain of the β3 isoform is composed of two repetitive motifs while the SOCK polypeptide is composed of three motifs. The first motif in SOCK contains entirely novel sequence and the latter two consist of a repetitive motif. In these repetitive sequences, 21% of the amino acids differ between the SOCK and β3 isoforms. A single SH3-binding PXXP motif is observed in the repetitive sequences of both β3 and

SOCK while three additional PXXP motifs are found with the novel first proline-rich motif of SOCK. The presence of these three potential SH3-binding motifs suggests an important role for SOCK in SH3-mediated protein-protein interactions. Analysis of the susceptibility of SOCK and β3 to proteolysis, especially in terms of PEST sequences, revealed additional differences between these β isoforms. PEST motifs are peptide sequences enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) residues that target proteins for rapid destruction by proteolysis (Rechsteiner and Rogers, 1996). PEST motifs were detected in two stretches of amino acids (a.a. 359-383 and a.a. 439-471) in the pancreas β3 isoform, but not the skeletal muscle isoform. The absence of predicted PEST sequences in skeletal muscle may provide SOCK with some degree of additional protection toward proteolysis in comparison to the pancreas β3 isoform.

Many polypeptide domains enriched in proline residues have been shown to bind src homology 3 (SH3) domains (Ren *et al.*, 1993). First identified as conserved motifs in tyrosine kinases, SH3 domains have since been described in a variety of proteins and modulate signal transduction through protein-protein interactions (Pawson, 1994). Random phage display peptide libraries have identified the consensus sequence RPLPXXP as a potential SH3-binding motif (Cheadle *et al.*, 1994; Rickles *et al.*, 1994). However, other factors such as the presence of a polyproline type II helix and stabilization from distant amino acids also appear to contribute to SH3-binding specificity (Pawson, 1995). It appears that the more general consensus motif of P-X-X-P is still the best predictor of potential binding to SH3 domains until the contributions of distant flanking sequences are better understood. The interaction between SH3 domains and their target proline-rich domains has been proposed to localize

polypeptides to subcellular compartments, to facilitate enzyme-substrate encounters and to potentially regulate enzyme activities (Pawson and Scott, 1997; Cohen *et al.*, 1995). The ability of SOCK and more specifically the proline-rich peptide to bind src from muscle extracts suggests that these two kinases may interact with each other. The subcellular distribution of SOCK and src suggests that these kinases coexist in similar cellular compartments, namely the membrane fractions in muscle. The likelihood of SOCK-src interactions occurring in vivo was further implied by the partial co-localization of expressed SOCK and src in undifferentiated C2C12 mouse myoblast cells. The subcellular localization of SOCK was investigated using epitope tagged SOCK to circumvent the difficulties that arose in the production of antibodies against the novel proline-rich region of SOCK. Furthermore, the use of the RU16 anti-(CaMKII) antibody would have resulted in the detection of all CaM kinase II related polypeptides in C2C12 cells. SOCK expression was more intense in the perinuclear area of cells and at the borders of some membrane projections in direct contact with the culture dish surface while src staining was more diffuse throughout the cell. Neither SOCK nor src appeared to be localized to the nucleus of the cells. The merging of SOCK and src immunofluorescence patterns highlighted areas of potential colocalization, especially in the area immediately surrounding the nucleus. However, the colocalization immunofluorescence data must be interpreted cautiously as neither polypeptide was visualized in distinct, circumscribed areas of the cell and the colocalization of polypeptides over diffuse areas is less informative. Despite SOCK's almost exclusive association with muscle membranes in fractionation studies, the immunofluorescence studies demonstrated widespread distribution of SOCK throughout the cell. The overexpression of

myc-tagged SOCK may therefore have resulted in the additional subcellular targeting of SOCK beyond its normal pattern of distribution, especially if its localization is directed by saturable numbers of anchoring sites (Pawson and Scott, 1997).

Although the β CaM kinase II isoforms are not thought to be integral membrane proteins, the β CaM kinases associated with specific membranes in muscle and can only be solubilized by detergents (Tuana and MacLennan, 1988). All β isoforms of the CaM KII family contain a stretch of hydrophobic amino acid residues that could serve to anchor the polypeptide near hydrophobic structures. It is unlikely however that this hydrophobic segment adopts a transmembrane configuration as the lipid bilayer would create an impenetrable physical barrier between the catalytic and regulatory domains of the kinase. The targeting of CaM kinases to distinct subcellular sites may also be achieved by targeting of mRNA followed by localized translation as evidenced by the distribution of α and β CaMKII isoforms in the dendrites and cell bodies of neurons respectively (Mayford *et al.*, 1996). The association of SOCK with membranes suggests that the possibility that SOCK could be targeted to subcellular structures, a feature often associated with signalling molecules. In view of the properties of SOCK, it is possible that its membrane associations might occur through SH3 domains of src whose interactions with the membranes can be regulated by myristoylation (Brown and Cooper, 1996). Additional targeting of CaM kinases to the membrane may also occur via the oligomerization of CaM kinase isoforms through their association domains, which is a characteristic feature of the CaMKII family (Kanaseki *et al.*, 1991; Bennett *et al.*, 1983; Srinivasan *et al.*, 1994). The self-association of CaM kinase molecules has been shown to be influenced by pH and ATP concentrations in vivo and may

provide an additional mechanism for the regulation of catalytic properties of the enzyme (Hudmon *et al.*, 1996). Interactions of CaM kinases with specific kinase anchoring proteins (KAPs) are also proposed to occur through these association domains and could regulate the localization of CaM kinases in a manner analogous to AKAPs for cAMP-dependent protein kinase and protein kinase C (Bayer *et al.*, 1996). Such a targeting mechanism would leave SOCK's SH3-binding domain available for the formation of SH3-mediated signalling complexes.

An important physiological role mediated by CaM-dependent protein kinases is the modulation of the activity of numerous ion channels (Levitan, 1994; Dolphin, 1991; Catterall, 1991). CaM-dependent protein kinases have been implicated in regulating glutamate receptors (McGlade-McCulloh *et al.*, 1993), Cl^- channels (Nishimoto *et al.*, 1991), enhancing DHP sensitive Ca^{2+} current in smooth muscle (McCarron *et al.*, 1992), potentiating calcium transients (Meyer *et al.*, 1992) and increasing the open probability of the Ca^{2+} release channel of cardiac SR (Witcher *et al.*, 1991). In considering a physiological function of the CaM kinases of skeletal muscle, its specific association with membranes, such as the sarcoplasmic reticulum, sarcolemma and transverse-tubules, argues for an important role in excitation-contraction coupling and intracellular Ca^{2+} signalling since both the Ca^{2+} release channel and voltage-sensitive Ca^{2+} channels would be in close vicinity (Block *et al.*, 1988). Wang and Best (1992) identified a reversible phosphorylation event which was involved in the inactivation of the Ca^{2+} release channel current in its native lipid environment and inhibitable by the CaM kinase II fragment (273-302). Our results offer further evidence for the possible involvement of the 60 kDa or 73 kDa CaM kinases, since these were potently inhibited by the

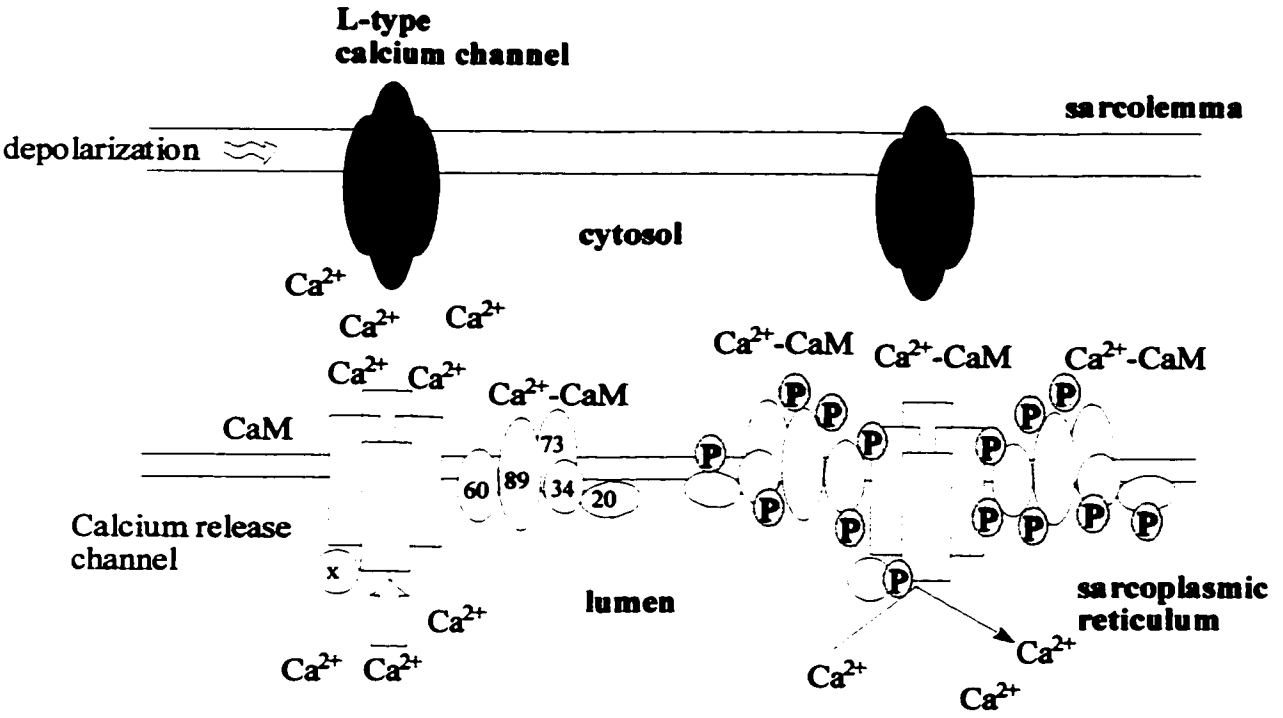
CaM kinase II inhibitory fragment that mimics the calmodulin-binding domain of the enzyme and inhibits its activity (Payne *et al.*, 1988). We have also observed significant levels of CaM-dependent phosphorylation of a 450 kDa polypeptide in rabbit skeletal muscle SR membranes. Some doubt exists as to the exact identity of this high molecular weight phosphoprotein in skeletal muscle (Seiler *et al.*, 1984) which is either the ryanodine receptor/Ca^{2+} release channel (Chu *et al.*, 1990) or an unrelated polypeptide (Witcher *et al.*, 1991). Both the cardiac and brain ryanodine receptor isoforms are substrates of the exogenously added brain CaM kinase II and phosphorylation occurs at a mutually conserved site, serine 2809 (Witcher *et al.*, 1991; Witcher *et al.*, 1992). Although the corresponding phosphorylation site is present in the skeletal muscle ryanodine receptor (Zorzato *et al.*, 1990), serine 2843, the surrounding amino acid residues, which are critical determinants of substrate specificity, are significantly different such that this skeletal muscle ryanodine receptor is not a substrate for CaM kinase II from brain. In addition, the inability to detect significant levels of CaM-dependent phosphorylation of the skeletal muscle ryanodine receptor may have been attributable to the use of the brain CaM kinase II that may be different in substrate specificity and time-course from the endogenous SR CaM kinase activity (Tuana and MacLennan, 1988). We sought to resolve whether the Ca^{2+} release channel was a substrate for the CaM-dependent protein kinase of SR. In reconstitution experiments of the purified ryanodine receptor and CaM kinase of SR, we were unable to detect CaM-dependent phosphorylation of the Ca^{2+} release channel, although it served as a substrate for protein kinase C (Takasago *et al.*, 1991). Furthermore in heat-treated SR membranes, where the endogenous CaM kinase activity was inactivated, the Ca^{2+} release channel did not serve as a substrate for the SR CaM

kinases. Since the ryanodine receptor was not phosphorylated by the partially purified CaM kinases, we propose a mechanism in which the phosphorylation of CaM kinase and its substrates in skeletal muscle SR could lead to the observed inactivation of the calcium release current reported previously (Wang and Best, 1992). A schematic representation of the proposed model for the regulation of Ca^{2+} release by CaM kinases is shown in Fig. 37. Following depolarization of the plasma membrane, rising cytoplasmic Ca^{2+} levels activate CaM which stimulates the 60 and 73 kDa CaM-dependent protein kinases. We propose that the resulting CaM-dependent phosphorylation of associated substrates leads to the inactivation of the Ca^{2+} release channel either through direct steric hindrance or through the accumulation of negative charges near the mouth of the channel.

The protein-protein interactions required for the proposed steric hindrance mechanism are supported by the estimated stoichiometric ratio of 1:1 to 1:2 between the 60 kDa CaM kinase and the Ca^{2+} release channel (Meissner, 1974; Campbell and MacLennan, 1982; Lai *et al.*, 1988) and the CaM-dependent phosphorylation activity of 1 to 2.5 nmol phosphate incorporation/(mg kinase) is comparable to the activity of other SR kinases (Orr *et al.*, 1991). The steric hindrance of the calcium release channel by the 360, 105, 89, 73, 60, 34 and 20 kDa phosphoproteins of the CaM kinase complex could occur in a manner that is qualitatively similar to the phosphorylation-dependent interaction that occurs between phospholamban and the Ca^{2+}-ATPase in cardiac SR (Fujii *et al.*, 1989; James *et al.*, 1989; Tada *et al.*, 1988).

Figure 37 Proposed Model for Involvement of CaM Kinase of SR in the Regulation of the Ca^{2+}-Release Channel

Following depolarization of the plasma membrane, cytoplasmic Ca^{2+} concentrations rise in response to the opening of the Ca^{2+} release channels of SR. The released Ca^{2+} activates CaM that in turn stimulates the 60 and 73 kDa CaM-dependent protein kinases. These CaM kinases undergo autophosphorylation and phosphorylate a number of associated substrates. For purposes of clarity, only the 89, 34 and 20 kDa substrates are shown. We propose that the inactivation of the Ca^{2+} release channel results either from the accumulation of negative charges near the mouth of the channel or from steric hindrance provided by one or many phosphorylated substrates.


L-type
calcium channel
sarcolemma
depolarization
cytosol
Ca2+
Ca2+
Ca2+
Ca2+
Ca2+
Ca2+-CaM
Ca2+-CaM
Ca2+-CaM
Ca2+-CaM
CaM
73
60
89
34
20
P
Calcium release
channel
x
lumen
sarcoplasmic
reticulum
Ca2+
Ca2+
Ca2+
Ca2+
Ca2+

Although a substrate for CaM kinases, the Ca^{2+} ATPase is not regulated by direct phosphorylation but rather through phosphorylation-dependent protein-protein interactions with phospholamban (Toyofuku *et al.*, 1994). The activity of the Ca^{2+} ATPase is normally inhibited by phospholamban. Following phosphorylation of phospholamban, the inhibition of the calcium pump is removed (Lytton and MacLennan, 1991). The role of CaM kinase isoforms in the phosphorylation of phospholamban and the resulting acceleration in cardiac relaxation has recently been confirmed in studies using phospholamban knockout mice (Li *et al.*, 1998). The CaM kinases of the SR may be operating in a similar fashion, with phosphorylation of the 60 or 73 kDa polypeptides leading to the inhibition of the Ca^{2+} release channel through direct, reversible protein-protein interactions.

Alternatively, the ryanodine receptor could be sensitive to changes in surface charge (Shoshan-Barmatz, 1988; Gechtman *et al.*, 1991) generated by the CaM-dependent phosphorylation of closely associated CaM kinase substrates. The dephosphorylation events required to reset the system could be generated from a phosphatase activity which is targeted to the SR (Horl and Heilmeyer, Jr. 1978; Campbell and MacLennan, 1982; Chiesi and Carafoli, 1983; Wegener and Jones, 1984; Hubbard *et al.*, 1990). In this regard, the Ca^{2+}-activated K^+ channel was shown to be modulated in a similar fashion by a membrane-associated kinase/phosphatase regulatory complex (Chung *et al.*, 1991). Having identified both a CaM-dependent kinase and Ca^{2+}/CaM-independent kinase of skeletal muscle SR, further studies will now be required to define the precise mechanisms by which these membrane-associated kinases may regulate SR function. Since the completion of this aspect of our study, a comprehensive electrophysiological study by Hain *et al.* (1994) clearly

indicated the need for further detailed analysis of the CaM kinases of the skeletal muscle. Their results demonstrated profound modulation of the skeletal muscle calcium release channel activity by CaM-dependent protein kinases, despite the fact that the channel does not appear to be a substrate for CaM kinases. Exceptionally, exogenous brain and endogenous skeletal muscle CaM kinases had differing results of channel activity. It was proposed that such a level of complexity for the regulation of the calcium release could be explained by the putative presence of multiple phosphorylation sites on the channel which could be differentially phosphorylated according to the subcellular localization of the kinases. It would therefore be of particular importance to investigate domains within CaM kinases that could regulate not only its localization but also its action on the substrate.

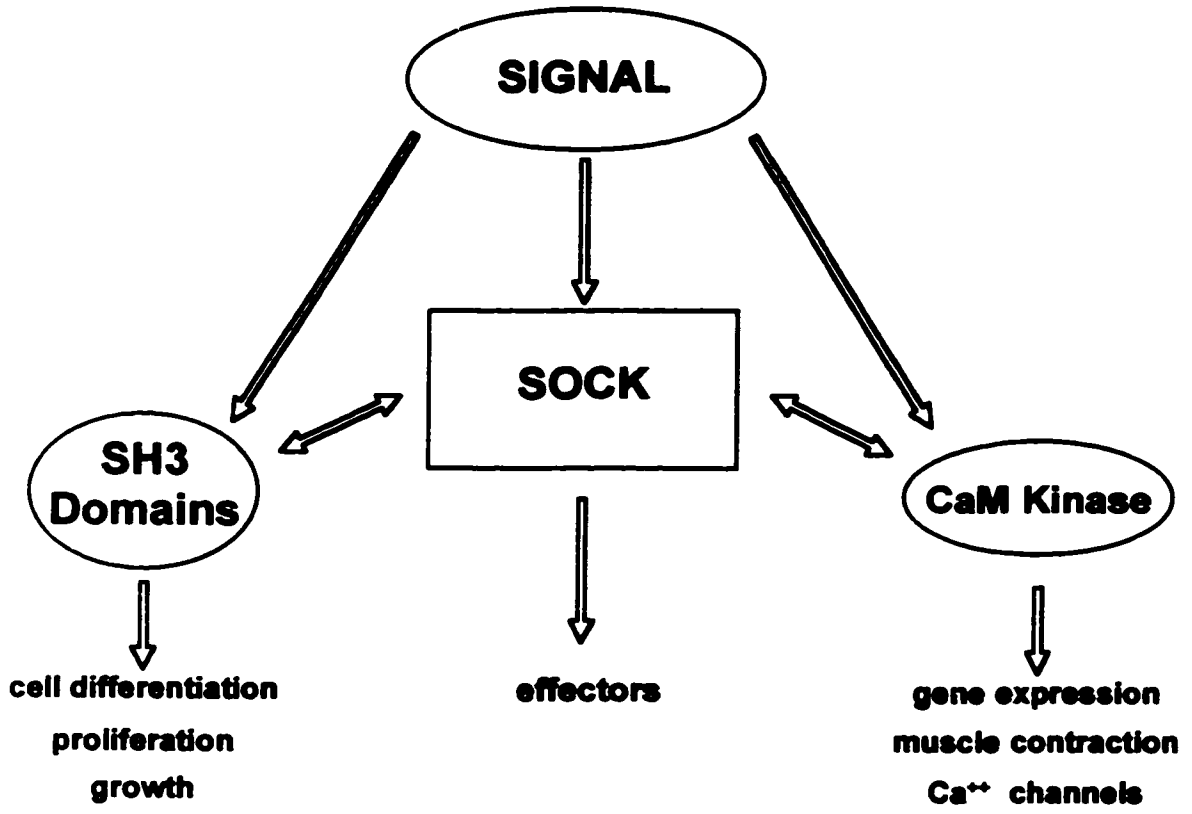
The activity of CaM kinases in muscle is not limited to the sarcoplasmic reticulum membranes. Clearly, the biochemical data indicates that both the 60 and 73 kDa CaM kinase are also localized to other membranes such as the sarcolemma and transverse tubules. These polypeptides may therefore be responsible for the CaM kinase activity known to regulate the L-type Ca^{2+} channels in smooth muscle and the Ca^{2+}-ATPase in cardiac muscle. The spatial distribution of the 60 and 73 kDa polypeptides to the membrane systems highly enriched in Ca^{2+} regulatory elements suggests an important role for these CaM kinases in the process of excitation-contraction coupling and intracellular calcium signalling.

While we do not know the precise physiological role of SOCK, it would appear that this molecule is uniquely structured and could function to integrate Ca^{2+}/CaM and tyrosine kinase signalling at the level of its SH3-binding domains. In view of the finding that, at least in vitro, SOCK-src interaction lead to changes in SOCK activity, it is possible that src may

regulate not only subcellular distribution but also phosphorylation of SOCK substrates. Further phosphoamino acid analysis of SOCK, as well as calmodulin, will be required to determine if the observed inhibition of SOCK activity is mediated through a tyrosine kinase phosphorylation event or solely through src-SOCK protein interactions. SOCK may thus serve to integrate the tyrosine kinase signalling components to the Ca^{2+}/CaM regulated cellular functions. A schematic representation of the potential roles of SOCK in mediating interactions between SH3 domains and CaM kinase activities is shown in Fig. 38. Through the incorporation of a SH3-binding module, the SOCK polypeptide gains the ability, beyond the classical CaM-dependent protein kinase activities, to interact with the tyrosine kinase signalling pathway. The modulation of SOCK activity by src may provide certain explanations for the apparent integration of calcium and tyrosine signalling pathways in a number of systems. For example, there is emerging evidence that tyrosine kinases, such as src, can regulate smooth muscle contraction by modulation of the voltage-dependent Ca^{2+} channels (Hu *et al.*, 1998). Since CaM kinases are also known to regulate these Ca^{2+} channels, it is possible that protein-protein interactions between src and SOCK recruit the kinases to the membrane and provide a integration point for the regulation Ca^{2+} channel activity by these two pathways. SOCK and src interactions may also provide the basis for convergence of the adrenergic Gi and Gq-mediated pathways, where recent findings have suggested that CaM kinases may provide the missing link in the calcium-dependent regulation of tyrosine phosphorylation by G-protein coupled receptors (Della Rocca *et al.*, 1997). It will be of great interest to further characterize SOCK's intracellular interactions to pinpoint its role in integrating these two important regulatory pathways.

Figure 38. Schematic Representation of the Potential Involvement of SOCK as a Mediator between SH3 Domain-containing Polypeptides and CaM Kinase Pathways

The interaction of SOCK with SH3-binding polypeptides provides an integration point between the calcium and tyrosine kinase signalling pathway. SOCK could therefore be instrumental in the cross-talk between CaM kinase regulated activities, such as gene expression, muscle contraction and regulation of calcium channels, and tyrosine kinase regulated activities such as cell differentiation, proliferation and growth.


SIGNAL
SOCK
SH3 Domains
CaM Kinase
cell differentiation
proliferation
growth
effectors
gene expression
muscle contraction
Ca++ channels

SOCK may serve to integrate calcium signals to the tyrosine kinase pathway via the proline-rich domains. This interaction could integrate a calcium/calmodulin-dependent component to pathways regulated by src tyrosine kinases such as ras, mitotic functions and, of particular interest in skeletal muscle, cytoskeletal reorganization and sarcomeric architecture (Erpel and Courtneidge, 1995; Castellani *et al.*, 1995). However, because of the flexible nature of SH3 interactions, one cannot discount the possibility that src-SOCK interactions are limited to certain tissues or subcellular compartments. In fact, src has been shown to bind to other proteins such focal adhesion kinases and Sam68 (Thomas *et al.*, 1998; Taylor *et al.*, 1995). Src-SOCK interactions within the cytosolic compartment might therefore be competed out by other SH3-binding proteins or proline-rich substrates.

The multiple convergent and divergent signalling pathways in a cell make defining the steps in a novel signal transduction pathway rather difficult. This is especially true for SOCK which assembles elements of both the calcium and tyrosine kinase dependent pathways. Both of these signalling pathways play important roles in the regulation of cell growth and differentiation. For example, src tyrosine kinase has been implicated in neuronal differentiation, migration of vascular smooth muscle and mitogenic signalling from platelet-derived growth factor and epidermal growth factor (Erpel and Courtneidge, 1995; Mureebe *et al.*, 1997; Broome and Hunter, 1996) whereas CaM-dependent protein kinase activity can affect growth, morphology and differentiation of PC12 cells and stabilizes dendritic arbor structure as an activity-dependent mediator of neuronal maturation (Masse and Kelly, 1997; Wu *et al.*, 1996). The ectopic expression of SOCK in the P19 embryonal carcinoma cell line specifically affected differentiation into mesoderm but not neuroectoderm. While no

detectable levels of SOCK transcript were observed by Northern blot analysis of undifferentiated P19 cells with a probe generated from the novel-proline rich domain of SOCK, Western blot analysis with the anti-(CaM kinase II) detected multiple immunoreactive polypeptides of 170, 90 and 60 kDa indicating the presence of CaM kinase II isoforms in these cells. Although no immunoreactive polypeptides correspond to the predicted molecular weight of SOCK (73 kDa), these immunoreactive polypeptides may represent uncharacterized and related isoforms of SOCK expressed in embryonal tissues. Both control and SOCK-transfected P19 clones, treated with retinoic acid to promote differentiation into neuroectoderm, appeared to differentiate normally along the neuroectodermal pathway as evidenced by the formation of long thin outgrowth resembling neuronal dendrites (McBurney, 1993).

In contrast, SOCK-transfected colonies treated with DMSO to promote mesoderm formation did not respond to the differentiation signal as measured by morphological changes and changes in the expression of muscle-specific genes. The SOCK-transfected P19 cells did not adopt a mesodermal-like morphology and were indistinguishable from undifferentiated P19 cells in contrast to control cells that fused to form loci of contracting cells. Transfection of P19 clones with anti-sense SOCK did not affect levels of the 60, 90, and 170 kDa endogenous CaM kinase related polypeptides or the differentiation capacity of these cells.

To investigate the role of SOCK in the blockade of differentiation along the mesodermal pathway, the transfected and control P19 colonies were analysed for the expression of muscle-specific transcripts, myogenin and cardiac and skeletal muscle actin. Myogenin is a basic-helix-loop-helix protein that together with other myogenic regulators can

bind to DNA and activate the transcription of many muscle-specific genes. Myogenin is expressed in myoblasts and is required for efficient muscle formation (Olson *et al.*, 1991). In P19 cells, cardiac and skeletal actin gene transcription has been shown to precede morphological differentiation into contracting myotubes. Myogenin appeared in control colonies at day 5 whereas no expression was detected in SOCK-transfected P19 cells thus confirming the morphological observations. Similarly, cardiac and skeletal muscle actin transcripts appeared to be expressed on day 5 in the control cells but not in SOCK-transfected cells. This data is in agreement with previous studies showing the appearance of cardiac actin, skeletal actin and ventricular myosin light chain mRNA expression on day 6 in DMSO-treated P19 cells (Rudnicki *et al.*, 1990). These observations suggest that SOCK can regulate the differentiation of P19 cells induced by DMSO. DMSO has been shown to cause a transient increase in intracellular calcium concentration (Morley and Whitfield, 1993). It is possible that SOCK is activated by this calcium signal, through calcium/CaM, and affects differentiation by interacting with a downstream effector, such as SH3 domain-containing proteins or myogenic factors, capable of regulating stem cell differentiation into mesoderm. The myogenic regulators, MyoD and myf5, appear to activate the determination program of precursor cells and initiate differentiation along the mesodermal pathway whereas myogenin is required for terminal differentiation and myoblast fusion (Megeney *et al.*, 1996; Megeney and Rudnicki, 1995). Since SOCK-transfected P19 cells did not appear to differentiate, it is possible that SOCK action is mediated through the inhibition of MyoD and/or myf5 activity. However, at this point, the identity of these downstream effectors is mere speculation.

Overexpression of a protein tyrosine phosphatase in P19 embryonal carcinoma cells

caused them to differentiate into neuronal instead of endodermal or mesodermal phenotypes (den Hertog *et al.*, 1993). This overexpression would likely result in decreased levels of tyrosine phosphorylation within the cell. SOCK activation may imitate this effect by activating tyrosine phosphatases or alternatively, inhibiting tyrosine kinases through its SH3-binding domains. Although src tyrosine kinase was shown to inhibit SOCK activity, further investigations will be required to determine the effects of SOCK on tyrosine kinase activity. The cytoskeleton of striated muscle is organized into stable sarcomeric structures that undergo complex remodelling either in response to exercise or injury. The activation of src tyrosine kinase in differentiated myotubes induces major changes in cell architecture involving the disassembly of contractile filaments from the highly structured sarcomere (Castellani *et al.*, 1995; Castellani *et al.*, 1996). SOCK may be involved with src in the complex regulatory processes to allow muscle to undergo such dynamic internal remodelling while maintaining a differentiated state. It will be of great interest to explore the characteristics of SOCK's interactions further to determine whether these result in altered enzymatic activities, phosphorylation states or subcellular localizations of this important novel CaM kinase II isoform.

Further examination of the biochemical and molecular properties of SOCK will require the generation of a specific antibody to the novel proline-rich domain. Such antibodies would allow a more detailed investigation into the subcellular localization of the kinase and the better characterization of its interactions in vivo. The results of the ectopic expression of SOCK in P19 cells clearly point out the need for in-depth developmental studies as the next focus for the study of SOCK expression. The careful analysis of these results might suggest

looking at the endogenous levels of SOCK in the early stages of somite cell determination and myoblast differentiation. The mechanisms that regulate SOCK activity in the cell most likely form an elaborate network of intracellular signalling systems. The multiple convergent pathways and multiple isoforms within pathways will make defining the steps involved in SOCK signal transduction difficult. In this regard, the generation of a SOCK-null transgenic mouse could greatly assist the characterization of SOCK function. The ultimate hope remains that the knowledge gained from these intricate intracellular regulatory systems may lead to well-targeted and designed interventions for the treatment of muscular disease since intracellular calcium is a major determinant of muscle function and dysfunction.

APPENDIX A.

LIST OF PUBLICATIONS

A.1 BOOK CHAPTER :

Leddy, J.J. and Tuana, B.S.: Preparation of Cardiac Sarcoplasmic Reticulum, in *Biochemical Techniques in the Heart*, 1996, J.H. McNeill, ed., CRC Press, Chapter 1.

A.2 PEER-REVIEWED PAPERS :

Leddy, J.J., Salih, M., Wigle, J.T., and Tuana, B.S.: Alternative Splicing of the β-CaMKII Gene Generates a Polypeptide That Binds SH3 Domains: Integrating Calcium and Tyrosine Kinase Signaling at the Variable Domains of the Multifunctional CaM Kinase. Submitted *J Biol Chem* (1999)

Leddy, J.J., Salih, M., and Tuana, B.S.: Molecular Cloning and Genomic Organization of the CaMKII β_4 Isoform (SOCK): a Novel CaM-Dependent Protein Kinase that Prevents Cells From Differentiating into Mesoderm. Manuscript in preparation (1999)

Meng, H., **Leddy, J.J.**, Frank, J., Holland, P. and Tuana, B.S.: The Association of Cardiac Dystrophin with Myofibrils/Z-disc Regions in Cardiac Muscle Suggests a Novel Role in the Contractile Apparatus. *J Biol Chem* (1996) **271**: 12364-12371.

Ray, A.R., Kyselovic, J., **Leddy, J.J.**, Wigle, J.T., Jasmin, B.J. and Tuana, B.S.: Regulation of Dihydropyridine and Ryanodine Receptor Gene Expression in Skeletal Muscle. Role of Nerve, Protein Kinase C and cAMP Pathways. *J Biol Chem* (1995) **270**: 25837-25844.

Kyselovic, J., **Leddy, J.J.**, Ray, A., Wigle, J. and Tuana, B.S.: Temporal Differences in the Induction of Dihydropyridine Receptor Subunits and Ryanodine Receptors during Skeletal Muscle Development. *J Biol Chem* (1994) **269**: 21770-21777.

Leddy, J.J., Murphy, B.J., Yi, Q., Doucet, J.-P., Pratt, C. and Tuana, B.S.: A 60 kDa Calmodulin-Binding Polypeptide of Skeletal Muscle Sarcoplasmic Reticulum is a Calmodulin-Dependent Protein Kinase that Associates with and Phosphorylates Several Membrane Proteins. *Biochem J* (1993) **295**: 849-856.

Surewicz, W.K., **Leddy, J.J.** and Mantsch, H.H.: Structure, Stability and Receptor Interaction of Cholera Toxin As Studied by Fourier-Transform Infrared Spectroscopy. *Biochemistry* (1990), **29**: 8106-8111.

A.3 ABSTRACTS :

Kyselovic, J., **Leddy, J.J.**, Ray, A., Wigle, J. and Tuana, B.S.: Differential Expression of the Dihydropyridine Receptor and Ryanodine Receptor During Skeletal Muscle Development. Molecular Biology of Muscle Development, Keystone Symposia 1994, Snowbird, Utah, USA.

Leddy, J.J. and Tuana, B.S.: The 60 kDa Calmodulin-Binding Polypeptide of Skeletal Muscle Sarcoplasmic Reticulum is a Calmodulin-Dependent Protein Kinase that Associates with and Phosphorylates Several Substrates Not Including the Ryanodine Receptor. Canadian Federation of Biological Societies 1993, Windsor, Ontario.

Leddy, J.J., Doucet, J.-P. and Tuana, B.S.: Characterization of the CaM-Dependent Protein Kinase System from Rabbit Skeletal Muscle Sarcoplasmic Reticulum. Eighth International Conference on Second Messengers and Phosphoproteins, 1992, Glasgow, Scotland.

Leddy, J.J., Doucet, J.-P. and Tuana, B.S.: Characterization of the 60 kDa Polypeptide Component of the CaM-Dependent Protein Kinase of Sarcoplasmic Reticulum. Canadian Federation of Biological Societies 1991, Kingston , Ontario.

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