Regulatory Segments of Ca\(^{2+}\)/Calmodulin-dependent Protein Kinases

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Catalytic cores of skeletal and smooth muscle myosin light chain kinases and Ca\(^{2+}\)/calmodulin-dependent protein kinase II are regulated intrinsically by different regulatory segments containing autoinhibitory and calmodulin-binding sequences. The functional properties of these regulatory segments were examined in chimeric kinases containing either the catalytic core of skeletal muscle myosin light chain kinase or Ca\(^{2+}\)/calmodulin-dependent protein kinase II with different regulatory segments. Recognition of protein substrates by the catalytic core of skeletal muscle myosin light chain kinase was altered with the regulatory segment of protein kinase II but not with smooth muscle myosin light chain kinase. Similarly, the catalytic properties of the protein kinase II were altered with regulatory segments from either myosin light chain kinase. All chimeric kinases were dependent on Ca\(^{2+}\)/calmodulin for activity. The apparent Ca\(^{2+}\)/calmodulin activation constant was similarly low with all chimeras containing the skeletal muscle catalytic core. The activation constant was greater with chimeric kinases containing the catalytic core of Ca\(^{2+}\)/calmodulin-dependent protein kinase II with its endogenous or myosin light chain kinase regulatory segments. Thus, heterologous regulatory segments affect substrate recognition and kinase activity. Furthermore, the sensitivity to calmodulin activation is determined primarily by the respective catalytic cores, not the calmodulin-binding sequences.

It is well known that many protein kinases are maintained in an inhibited state by an autoinhibitory segment within the protein or by a distinct inhibitory subunit (1). The activation of Ca\(^{2+}\)/calmodulin-dependent protein kinases involves the binding of Ca\(^{2+}\)/calmodulin to a calmodulin-binding sequence in the regulatory segment that also contains the autoinhibitory sequence. Binding of Ca\(^{2+}\)/calmodulin removes the autoinhibitory sequence from the catalytic core, exposing the active site for protein substrate binding and phosphorylation (2–11). In the case of myosin light chain kinase and the CaMKII, the linker region between the catalytic core and calmodulin-binding sequence contributes to inhibition. However, the boundary between the autoinhibitory and calmodulin-binding sequences remains controversial.

Recently, the crystal structures of Ca\(^{2+}\)/calmodulin-dependent protein kinase I (12) and twitchin kinase (13, 14) presented structural insights into the autoinhibitory mechanism. The regulatory segments have extensive molecular contacts with residues on the surface of their catalytic cores, with binding energy provided by both electrostatic and hydrophobic interactions. Twitchin, a protein kinase related to myosin light chain kinases, does not have a calmodulin-binding sequence involved in regulation. However, the structure of Ca\(^{2+}\)/calmodulin-dependent protein kinase I shows multiple contacts between residues in the catalytic core and the calmodulin-binding sequence. It seems reasonable to assume a similar structural arrangement for myosin light chain kinases.

Regulatory segments of protein kinases may be involved in different functions. In the case of Ca\(^{2+}\)/calmodulin-dependent protein kinases, in addition to calmodulin binding and autoinhibition, the regulatory segment may affect enzyme stabilization (15, 16) or increase Ca\(^{2+}\)/calmodulin-independent activity through autophosphorylation (17). Dekker et al. (18) reported that protein kinase C-\(\alpha\) was not able to phosphorylate histone; however, after deletion or substitution of some residues in the pseudosubstrate region of the enzyme, histone became a good substrate. Exchange of all or part of the pseudosubstrate sequence between protein kinase C-\(\alpha\) and protein kinase C-\(\eta\) resulted in changed substrate selectivity. Fujise et al. (19) found that deletion of the regulatory domain in protein kinase C decreased efficiency of substrate peptide phosphorylation.

The apparent diverse functions of some protein kinase regulatory segments stimulated our investigation on two types of Ca\(^{2+}\)/calmodulin-dependent protein kinases. Questions are raised on the respective roles of the catalytic cores and calmodulin-binding sequences in determining sensitivity to activation by Ca\(^{2+}\)/calmodulin and whether putative interactions between a regulatory segment and a catalytic core affects substrate recognition. In the present approach, the functional properties of regulatory segments were investigated by expressing skeletal muscle myosin light chain kinase and CaMKII catalytic cores containing chimeric regulatory segments. The results show that heterologous regulatory domains had effects on phosphorylation properties of protein substrates. Surprisingly, the sensitivity to calmodulin activation was determined primarily by the respective catalytic cores, not the calmodulin-binding sequences.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Myosin Light Chain and Calmodulin—Recombinant human smooth and rabbit skeletal muscle myosin light chains were expressed and purified as described previ-
RESULTS

Constructs of Wild-type, Truncated, and Chimeric Kinases—All of the wild-type, truncated, and chimeric kinases were constructed as shown in Fig. 1. tSkMLCK was made as de-

Fig. 1. Schematic representation of the primary structure of wild-type, truncated, and chimeric kinases. Regions representing SkMLCK are open; SmMLCK, black; CaMKII, slant hatched; and nNOS, straight hatched. The numbers represent the positions of residues in the respective enzymes. The dotted lines represent schematic deletions of sequences between the N terminus and the catalytic core.
The oligomerization of CaMKII depends upon the association domain located C-terminal of the catalytic core and calmodulin-binding sequence (24). Deletion of the association domain does not change the kinetic properties of the enzyme in vitro (32). In the present paper, tCaMKII was constructed by removing the association domain, and the activity assays showed that the $K_m$ value was 6 μM and that the $V_{\text{max}}$ value was 475 pmol of 32P incorporated/minute of kinase with smooth muscle regulatory light chain as a substrate. Although the skeletal muscle light chain could be phosphorylated, the rates were too low to accurately determine $K_m$ and $V_{\text{max}}$ values, similar to results obtained with tSkMLCK[CamKII] with skeletal muscle regulatory light chain. The $K_m$ value of chimeric tCaMKII[tSkMLCK] was increased 4-fold, whereas the $V_{\text{max}}$ value was decreased 2-fold. Similar results were obtained with tCaMKII[SmMLCK]. Kinetic values with skeletal muscle light chain as a substrate were undeterminable for all chimeras containing the tCaMKII catalytic core (Table I).

We also constructed tSkMLCK, tSmMLCK, and tCaMKII, in which their respective regulatory domains were deleted, keeping only the catalytic core sequences. In all cases insignificant kinase activities were detected (data not shown).

**Ca2\(^+\)/Calmodulin Activation Properties of Wild-type, Truncated, and Chimeric Kinases**—The Ca2\(^+\)/calmodulin activation properties were determined by performing assays at a high calmodulin concentration (1 μM) with a Ca2\(^+\)/EGTA buffer used to vary the free Ca2\(^+\) concentration and hence the Ca2\(^+\)/calmodulin concentration (10, 28). All of the truncated and chimeric kinases were dependent on Ca2\(^+\)/calmodulin for activity. The concentration of Ca2\(^+\)/calmodulin for half-maximal activation of myosin light chain kinases is about 1 μM (33). In comparison, wild-type and tSkMLCK had similar $K_{\text{CaM}}$ values with both skeletal and smooth muscle light chain substrates (Table II). The relative values for tSkMLCK[SmMLCK] and tSkMLCK[CamKII] were less but in the same range.

In contrast to myosin light chain kinase, tCaMKII has an apparent lower affinity for Ca2\(^+\)/calmodulin, with half-maximal activity attained between 25–100 μM (34). In this study, tCaMKII had a $K_{\text{CaM}}$ value of 50 μM relative to tSkMLCK (Table II). The chimeric tCaMKII[tSkMLCK] and tCaMKII[SmMLCK] also had relatively high $K_{\text{CaM}}$ values of 27 and 28 nM, respectively (Table II).

**Stability Studies on Chimeric SkMLCK**—The extent of structural identity among the regulatory segments of tSkMLCK, tSmMLCK, and tCaMKII is low (Fig. 2), and thus it could be expected that the stability properties of the chimeras would be significantly altered. Alterations in the structure of the catalytic core with different regulatory segments may lead to significant increases in susceptibility to digestion by proteases (5). Therefore, limited trypsin digestion was used to assess structural and activation properties of the chimeras (Fig. 4). tSkMLCK, tSkMLCK[SmMLCK], and tSkMLCK[CamKII] were resistant to trypsin digestion in the presence of EGTA. In the presence of Ca2\(^+\)/calmodulin, tSkMLCK and tSmMLCK were more sensitive to digestion by trypsin. However, the pattern of digestion of tSkMLCK[CamKII] was similar in the absence and presence of Ca2\(^+\)/calmodulin and different from tSmMLCK and tSkMLCK[SmMLCK].

When the trypsin digestion was performed in the presence of Ca2\(^+\)/calmodulin, the Ca2\(^+\)/calmodulin-dependent kinase activities of the chimeras decreased with increasing trypsin concentrations with partial conversion to Ca2\(^+\)/calmodulin-independent activities for tSkMLCK[CamKII], tSkMLCK[SmMLCK], and tSkMLCK[CamKII]. The decrease in activity for tSkMLCK[CamKII] associated with a modest reduction in the apparent mass of the chimera compared with tSkMLCK and tSkMLCK[SmMLCK] (Fig. 4).

The patterns of tSkMLCK[SmNOS] digestion in the presence of Ca2\(^+\)/calmodulin or EGTA were similar to the patterns of those that were boiled before digestion (data not shown). Additionally, tSkMLCK[SmNOS] showed no kinase activity, even...
after limited digestion (data not shown). Thus, this chimera may not fold correctly for activity.

Thermal instability was also used to assess structural perturbations in chimeric tSkMLCKs. The decrease in sensitivity to trypsin digestion in tSkMLCK[CamKII] in the presence of Ca\(^{2+}\)/calmodulin could be due to the loss of a proteolytic cleavage site in the chimera. Incubation of tSkMLCK, tSkMLCK[CamKII], and tSkMLCK[SmMLCK] at 46 °C in the presence of EGTA resulted in similar time-dependent losses of kinase activities (Fig. 5). Immunoblots show that the loss of activities was most likely due to thermal denaturation, because there was no evidence of proteolysis (Fig. 5).

**DISCUSSION**

Myosin light chain kinases and Ca\(^{2+}\)/calmodulin-dependent protein kinase II have similar but distinct biochemical properties. Myosin light chain kinase is a dedicated protein kinase with high substrate specificity toward myosin regulatory light chain (35), whereas Ca\(^{2+}\)/calmodulin-dependent protein kinase II is a multifunctional protein kinase with diverse substrate recognition (24, 36–38). Proteolysis studies supported the hypothesis that both types of kinases contain an autoinhibitory sequence in the C-terminal region close to the catalytic core (3, 39–47). The current structural model is that the kinases are inactive in the absence of Ca\(^{2+}\)/calmodulin due to an autoinhibitory sequence folding back onto the catalytic core and blocking substrate binding. This type of regulation is referred to as intrasteric regulation (48).

The recently determined structure of twitchin kinase provides some insight into how an autoinhibitory segment may bind to the catalytic core (13, 14). The autoinhibitory segment has multiple interactions with the catalytic core of enzyme, with 47 hydrogen bonds and as many as 351 van der Waal contacts. The structure of Ca\(^{2+}\)/calmodulin-dependent protein kinase I (12) shows that the position of its autoinhibitory sequence differs dramatically from twitchin kinase. The twitchin autoinhibitory sequence enters the active site in the cleft between the two lobes of the catalytic core passing over the activation loop of the enzyme. The autoinhibitory and calmodulin-binding sequences of Ca\(^{2+}\)/calmodulin-dependent protein kinase I also traverse the surface of the large lobe of the catalytic core, but they turn in an opposite direction and avoid the activation loop entirely by traversing the small lobe. However, like twitchin kinase, the regulatory segment of Ca\(^{2+}\)/calmodulin-dependent protein kinase I has multiple contacts with the catalytic core.

In the present work, we constructed chimeric protein kinases with heterologous regulatory segments and tested their autoinhibitory function. The activity of all chimeric kinases containing kinase regulatory segments were Ca\(^{2+}\)/calmodulin-dependent, with no significant activity in the presence of EGTA. Thus, the heterologous regulatory segments had a similar autoinhibitory function that was reversed by Ca\(^{2+}\)/calmodulin. However, there was some apparent specificity because the chimera tSkMLCK[nNOS] was not active in the presence or absence of Ca\(^{2+}\)/calmodulin, even though it bound calmodulin. The calmodulin-binding sequence of neuronal nitric oxide synthase contains basic residues at its N terminus in a sequence arrangement similar to the putative pseudosubstrate sequence in the calmodulin-binding sequence of myosin light chain kinases (2, 49). This structure alone does not provide sufficient stability to the catalytic core, which is consistent with the hypothesis that the linker region between the catalytic core and the calmodulin-binding sequence is important in autoinhibition and stability of the catalytic core (11). The other heterologous regulatory segments, however, appear to provide similar structural stability, as revealed by limited proteolysis and thermal denaturation measurements. Faux et al. (15) found that a 61-kDa tryptic fragment of SmMLCK in which the regulatory segment was truncated underwent rapid inactivation. However, a synthetic peptide with a similar primary structure of the autoinhibitory sequence protected the 61-kDa fragment from thermal inactivation. Kennelly et al. (50) found calmodulin binding to skeletal muscle myosin light chain kinase resulted in a time- and temperature-dependent inactivation in the absence of substrates. Separation of the components of the inactive complex yielded functional calmodulin but catalytically inert kinase. Similarly, Ishida and Fujisawa (16) removed the autoinhibitory sequence of Ca\(^{2+}\)/calmodulin-dependent protein kinase II and

**Table I**

| Kinase               | Skeletal muscle light chain | Smooth muscle light chain |
|----------------------|-----------------------------|---------------------------|
|                      | \(K_m\) \(\mu M\) pmol/min/pmol | \(V_{\text{max}}\) pmol/min/pmol | \(V_{\text{max}}/K_m\) pmol/min/pmol | \(K_m\) \(\mu M\) pmol/min/pmol | \(V_{\text{max}}\) pmol/min/pmol | \(V_{\text{max}}/K_m\) pmol/min/pmol |
| Wild-type SmMLCK   | 4.8 ± 1.1                   | 2070                      | 431                          | 4.3 ± 1.1                     | 1890                      | 439                          |
| tSmMLCK             | 2.9 ± 0.4                   | 1710                      | 589                          | 2.7 ± 0.4                     | 1189                      | 440                          |
| tSmMLCK[CamKII]     | 6.8 ± 1.6                   | UD                        | 314                          | 34.5 ± 4.7                    | 2157                      | 62                           |
| tSmMLCK[SmMLCK]    | UD                          | 2173                      | 5.1 ± 0.1                    | 6.0 ± 2.4                     | 475                       | 79                           |
| tCaMKII             | UD                          | UD                        | 25.3 ± 1.7                   | 8                           |
| tCaMKII[SkMLCK]    | UD                          | UD                        | 50.9 ± 3.7                   | 4                           |
| tCaMKII[SmMLCK]    | UD                          | UD                        | 204                          | 4                           |
found that the enzyme was more labile than the original enzyme. It has been reported that the regulatory segment affects substrate recognition. Dekker et al. (18, 51) observed that the pseudosubstrate site mediated the low histone kinase activity of wild-type PKC-α. Deletion of the pseudosubstrate region generates a cofactor-independent kinase that has high histone kinase activity, but deletion of the regulatory segment decreases the phosphorylation rate for a peptide substrate. Fujise et al. (19) exchanged the autoinhibitory pseudosubstrate sequence between PKC-α and PKC-β, which changed substrate recognition properties. Our results show that regulatory segments of Ca²⁺/calmodulin-dependent protein kinases may also affect substrate recognition and hence may be a more general mode of regulation than previously recognized. Chimeric tSkMLCK[CaMKII] had a 7-fold decrease and undeterminable $V_{max}:K_m$ values for smooth and skeletal muscle light chains, respectively. Hence, the heterologous regulatory segment affected the recognition of the physiological substrate, the skeletal muscle light chain.

The substrate determinants for smooth and skeletal muscle light chains are different (33, 49). An Arg at the P-3 position relative to the phosphorylatable serine (P-0 position) is important for substrate recognition for the smooth muscle light chain. This residue is replaced with Glu in the skeletal muscle light chain. The Ca²⁺/calmodulin-dependent protein kinase II requires a basic residue at the P-3 position, which is why it readily phosphorylates smooth but not skeletal muscle light chain. tCaMKII[SkMLCK] and tCaMKII[SmMLCK] had 10–20-fold lower $V_{max}:K_m$ values for smooth muscle regulatory light chain. Thus, the heterologous regulatory segments in the chimeric kinases function differently in terms of affecting catalytic activity. The Ca²⁺/calmodulin-dependent protein kinase II requires a basic residue at the P-3 position, which is why it readily phosphorylates smooth but not skeletal muscle light chain. The regulatory segment, along with the complexed calmodulin, affects the structure of the catalytic core and hence kinase activity, or 2) the regulatory segment with bound calmodulin in the chimeric kinase sterically modifies substrate binding.

Recently, Krueger et al. (52) used small-angle x-ray and neutron scattering with contrast variation to obtain the first structural view of calmodulin complexed to a functional enzyme, tSkMLCK (52). The results show that calmodulin was
The mechanism by which calmodulin activates enzymes is intriguing. The crystal structure of Ca\(^{2+}\)/calmodulin shows a dumbbell-shaped molecule with two globular regions connected by an extended central helix (53). The structure of calmodulin bound to peptides of the regulatory segments of myosin light chain kinases and CaMKII shows that the extended structure collapses to a cis orientation, and the central helix is disrupted by a long flexible loop (54–56). Mutations, truncations, and chimeras of calmodulin reveal that different target proteins need different critical residues or domains of calmodulin to activate (57–59). Furthermore, N-terminal residues of calmodulin (Anthony Persechini), CaMKII (Howard Schulman), and neuronal nitric oxide synthase (Solomon Snyder), Purified rat brain CaMKII was kindly provided by Harold Singer. We also thank Phyllis Foley for preparation of the manuscript and figures.

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