Activity-Dependent Regulation of Calcium/Calmodulin-Dependent Protein Kinase II Localization

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Calcium/calmodulin-dependent protein kinase II (CaMKII) is a ubiquitous enzyme, present in essentially every tissue but most concentrated in brain. It is a multifunctional Ca^{2+} /calmodulin-dependent protein kinase capable of phosphorylating protein substrates, such as AMPA receptors, synapsin I, tyrosine hydroxylase, L-type Ca^{2+} channels, and MAP-2 (microtubule-associated protein 2), substrates that are found in essentially every intracellular compartment (Hudmon and Schulman, 2002; Lisman et al., 2002; Colbran and Brown, 2004). Proper function of the kinase requires that it access and selectively phosphorylate numerous substrates in diverse intracellular sites. This review will discuss the role of kinase localization in optimizing fidelity of substrate phosphorylation.

Importance of spatial localization to kinase action

Protein kinases, including CaMKII, can be pre-positioned near their substrates before a stimulus or undergo a spatial translocation to an anchoring protein only after a proper stimulus (Bayer and Schulman, 2001; Colbran, 2004). There are several possible consequences of spatial control of substrate and kinase (Bauman et al., 2004). First, localization of kinase near some of its substrates achieves a concentration of the kinase that speeds onset of response to stimulus and increases the rate of phosphorylation. The signaling complex may also include anchoring of a phosphoprotein phosphatase (Westphal et al., 1999). Second, translocation can increase the signal-to-noise ratio by keeping kinase away from its substrate under basal conditions. Third, pre-positioning of kinases on anchoring proteins increases their local concentrations, even under basal conditions. This may increase sensitivity to their second messengers and even enable significant activity under basal conditions (Rosenmund et al., 1994). Ca^{2+} acts in restricted cellular domains (Allbritton et al., 1992); thus, positioning of CaMKII relative to the point source of the Ca^{2+} signal should markedly affect its activation. Fourth, in addition to substrate and Ca^{2+} , the localization of kinase relative to phosphatases (Strack et al., 1997b) and calmodulin can greatly impact activation amplitude and kinetics.

Subcellular distribution

Intracellular distribution of CaMKII is dependent on the expressed isoforms(s) and represents a combination of stimulus-independent and -dependent localization. In brain, a significant fraction of the enzyme can be readily extracted in a soluble form, and the remainder is associated with the cytoskeleton, including the PSD (postsynaptic density) and membrane. The major PSD protein (mPSDp), constituting ~3% of its protein mass (and increasing rapidly after decapitation to 20% or more) was subsequently found to be CaMKII (Kennedy et al., 1983; Goldenring et al., 1984; Kelly et al., 1984).

CaMKII isoforms are highly homologous, but the variable domain can contain peptide segments ("inserts"), e.g., by alternative splicing, that promote distinct intracellular targeting that can involve anchoring proteins. In addition, kinase activation and/or autophosphorylation that expose interaction sites normally occluded by its autoinhibitory domain lead to stimulus-dependent translocation to other targets. Kinase isoforms with distinct targeting sequences can coassemble into the same holoenzyme, and thus localization and translocation are dependent on the isoform composition. The expression ratio of CaMKII isoforms is dynamic both during development and with cellular activity.

Nuclear targeting

Splice variants of the α and the δ gene (α_B - and δ_B -CaMKII) contain a nuclear localization signal (NLS) that directs each of these to the nucleus (Srinivasan et al., 1994; Brocke et al., 1995). Not surprisingly, CaMKII-mediated changes in gene expression require nuclear localization (Ramirez et al., 1997). A transcription factor, NeuroD, that regulates dendritic growth and morphology has been found recently to be phosphorylated by CaMKII in granule cells, but it is not known which kinase isoform is involved (Gaudilliere et al., 2004). α_B -CaMKII is abundant in brain but essentially restricted to the midbrain and diencephalon, regions that have approximately equal amounts of α - and α_B -CaMKII (Walaas et al., 1983; Brocke et al., 1995). Holoenzymes with primarily nuclear subunits are found almost exclusively in the nucleus and vice versa, whereas both nuclear and cytosolic localization is seen at equal expression of the two isoforms (Srinivasan et al., 1994).

Nuclear targeting is subject to inhibitory control by phosphorylation at either of two sites, one catalyzed by other kinases and the other by autophosphorylation. *In vitro*, both CaMKI and CaMKIV phosphorylate a serine residue immediately adjacent to

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the NLS of α_B -CaMKII (Ser³²²), resulting in block of nuclear targeting (Heist et al., 1998). The block is attributable to placement of a negatively charged phosphate that interferes with binding of an NLS receptor. Autophosphorylation at Thr²⁸⁶ (α_B -CaMKII) also blocks nuclear targeting (M. Srinivasan, K. Heist, and H. Schulman, unpublished observations), suggesting that nuclear kinase synthesized during periods of prolonged Ca^{2+} -linked stimulation would remain outside the nucleus.

Membrane targeting

The most unusual CaMKII isoforms is α KAP (α kinase anchoring protein), a catalytically deficient isoform generated by alternative splicing and an alternative promoter within the CaMKII coding region (Bayer et al., 1996). In place of the N-terminal kinase domain is a short hydrophobic sequence, followed by the association domain. The association domain enables it to coassemble into holoenzymes containing catalytically competent subunits, whereas the hydrophobic sequence targets α KAP and any coassembled subunits to membranes. In essence, it serves as a built-in anchoring protein for CaMKII. In skeletal muscle, α KAP targets catalytically competent holoenzymes to the endoplasmic reticulum, a site that may facilitate modulation of the ryanodine receptor (Bayer et al., 1998).

CaMKII is associated with synaptic vesicles and, through its catalytic domain, enhances binding of synapsin I to synaptic vesicles (Benfenati et al., 1992). Synapsin I, in turn, is associated with the actin cytoskeleton. Evidence suggests that synapsin I is tethered to synaptic vesicles via α -CaMKII, and thus this soluble kinase must either undergo a posttranslational modification that increases its interaction with the lipid bilayer or interact with a membrane protein.

Cytoskeletal targeting

Early subcellular fractionation identified significant cytoskeletal localization for CaMKII (Sahyoun et al., 1985) that was subsequently refined to include a specific interaction with actin that could be disrupted by Ca^{2+} /calmodulin (Ohta et al., 1986). *In vitro*, β -CaMKII, but not α -CaMKII, binds actin filaments, and the complex is dissociated by Ca^{2+} /CaM but not by either Ca^{2+} or CaM alone. This is consistent with dissociation of GFP (green fluorescent protein)- β -CaMKII from actin stress fibers in transfected cells during Ca^{2+} -linked stimulation (Shen and Meyer, 1999). An insert in the association domain present in β -CaMKII, but not in α -CaMKII, is responsible for targeting to F-actin (Shen et al., 1998; Fink et al., 2003). Binding activity is partially gained by chimeric α -CaMKII construct to which the β -specific insert was introduced. The crystal structure of the association domain of α -CaMKII has a large hydrophobic cavity with a positive electrostatic potential at its greatest depth that may be a binding site for CaMKII anchoring proteins, a site that may be modulated by isoforms-specific inserts (Hoelz et al., 2003).

GFP-CaMKII constructs have been applied to identify CaMKII localization to the cell cortex, dendritic branches, and filopodia-like branches in cultured neurons and to stress fibers in cell lines (Shen et al., 1998; Shen and Meyer, 1999). F-actin-bound β -CaMKII appears to function in motility of filopodia and neuritic branches and in formation of dendritic branches and synapses. A highly selective cell-permeable peptide inhibitor of CaMKII [antCnT (Chang et al., 1998; Fink et al., 2003)] and downregulation of β -CaMKII with siRNA reduced movement of neuritic branches and decreased arborization. An α/β -CaMKII chimera with partial actin binding produces a partial effect on the size of the dendritic arbor (Fink et al., 2003), and non-neuronal

isoforms with partial actin binding produced actin-rich neurite extensions (Caran et al., 2001). The findings are consistent with a need for actin localization for the kinase to affect neurite activity. Stimulation of glutamate receptors in mature neurons stabilizes dendritic arbors (Wu and Cline, 1998) and decreases actin-based spine dynamics (Fischer et al., 2000), perhaps by producing a net displacement of CaMKII away from actin.

Whereas α -CaMKII has been associated with potentiation of synaptic strength (Lisman et al., 2002), the β -isoform may have a specialized role in promoting fine dendritic branching and filopodial movement and the establishment of new synaptic contacts. This was demonstrated by upregulation and downregulation of β -CaMKII, using transfection of β -CaMKII or siRNA, respectively, and finding corresponding increases or decreases in synapse number (Fink et al., 2003).

CaMKII in brain consists of holoenzymes composed of α - and β -CaMKII subunits as well as homomultimers of only one isoforms type, and these differ in catalytic and regulatory function (Brocke et al., 1999) and in targeting. The expression ratio of CaMKII isoforms changes during development (Miller and Kennedy, 1985; Wu and Cline, 1998; Brocke et al., 1999) as well as in response to synaptic activity (Thiagarajan et al., 2002). α -CaMKII mRNA is selectively found in dendrites, relative to β -CaMKII, and is subject to local synthesis and regulation after synaptic activity (Ouyang et al., 1999; Steward and Schuman, 2001; Atkins et al., 2004). High synaptic activity shifts the α/β isoform ratio toward α during increased activity and toward β after periods of decreased activity (Thiagarajan et al., 2002). Shifts in the α/β isoform ratio would be expected to alter cytosolic versus cytoskeletal targeting of CaMKII, thus leading to functional consequences, e.g., greater involvement in arborization and synapse formation versus modulation of unitary synaptic strength.

Dynamic activity-dependent translocation

There are a number of CaMKII interacting proteins at the synapse, making it difficult to directly link macroscopic changes in CaMKII localization and translocation with specific targeting molecules. It was first shown that treatment of hippocampal slices under conditions that activate CaMKII approximately doubles the amount of CaMKII associated with the PSD (Strack et al., 1997a). This redistribution of the kinase appears to be a dynamic process that has been visualized in cultured hippocampal neurons and brain slices stimulated with glutamate or depolarization with high K^+ (Shen and Meyer, 1999; Dosemeci et al., 2001). Thin section electron microscopy reveals a fivefold increase in CaMKII associated with the cytoplasmic face of PSDs after depolarization of hippocampal cultures that may primarily account for a doubling of PSD density (Dosemeci et al., 2001). Glutamate treatment produced a similar effect and is relatively fast and transient. The level of CaMKII associated with PSDs at a given time may be a combination of such transiently associated kinase and a subpopulation of kinase that is retained after translocation and that may encode the history of correlated synaptic activity.

Transfection of cultured neurons with GFP-CaMKII constructs described above provided a complementary approach to track CaMKII translocation in real time and enable a better mechanistic understanding of its targeting (Shen and Meyer, 1999; Shen et al., 2000; Fink et al., 2003). Stimulation of NMDA receptors produced a rapid (20 sec) and reversible redistribution of kinase from the actin cytoskeleton and cytosol to synaptic sites likely to include PSDs. This is consistent with dissociation of F-actin sequestered kinase by Ca^{2+} /calmodulin (Shen and

Meyer, 1999). Translocation of GFP–CaMKII is not a pathophysiological effect of strong neuronal stimulation; repeated sensory stimulation in a defined zebrafish neuronal pathway resulted in reproducible and reversible translocation of the kinase to the PSD (Gleason et al., 2003).

This approach was elegantly extended by Shen et al. (2000) who used electrophoretic puffs of glutamate to simulate synaptic activity to identify both short- and long-term resident times of kinase at synaptic sites. Autophosphorylation-defective kinase (T286A) translocated from the cytosol/cytoskeleton to synaptic sites with short resident times, whereas wild-type kinase displayed long resident times of several minutes. In contrast, autophosphorylation of Thr^{305/306}, which occurs after dissociation of calmodulin from autonomously active enzyme, facilitates dissociation from synaptic sites (Shen et al., 2000; Elgersma et al., 2002). Interestingly, PSDs from a mouse model of Angelman's syndrome, in which there is a higher steady-state level of phosphorylated Thr^{305/306}, are deficient in CaMKII (Weeber et al., 2003).

Translocation to NMDA receptors

The level of CaMKII in the PSD can affect long-term potentiation and hippocampal-dependent learning (Elgersma et al., 2002), making it important to understand its translocation and associ-

ation with the PSD. The NMDA receptor and several other synaptic proteins likely contribute to this synaptic localization. A number of CaMKII targets have been identified at the synapse (for review, see Bayer and Schulman, 2001; Colbran, 2004). Among the interesting interactions in addition to the NMDA receptor are with densin-180, which is selective for α - over β -CaMKII, enhanced by autophosphorylation, and can form a ternary complex with α -actinin (Walikonis et al., 2001). Myosin-V, an unconventional myosin, interacts with CaMKII in an orientation that allows myosin-bound calmodulin to activate the kinase and for the kinase to phosphorylate myosin-V at a site that reduces its interaction with organelles and thus inhibits organelle transport (Costa et al., 1999). An additional cytoskeletal-associated protein interacting with CaMKII is synGAP (synaptic ras-GTPase-activating protein) (Li et al., 2001; Oh et al., 2004), which may couple Ca^{2+} and MAP kinase signaling pathways.

Targeting to NMDA receptors is responsible for some of the Ca^{2+} -linked translocation of CaMKII to synaptic sites, which is attributable to both stimulated dissociation from actin and stimulated exposure of high-affinity sites for the NMDA receptor. Different NMDA receptor subunits have been implicated as CaMKII targets using different methods and kinetics of binding. Early studies probed synaptic proteins resolved by SDS gels with [³²P]Thr²⁸⁶-labeled CaMKII to identify a number of CaMKII-

binding proteins, with the most prominent being 140 and 190 kDa proteins (McNeill and Colbran, 1995). Activity-dependent interaction of CaMKII with NR2B (and NR1) was first demonstrated in hippocampal slices (Leonard et al., 1999). The kinase and the NMDA receptor are coimmunoprecipitated from neuronal preparations (Gardoni et al., 1998; Strack and Colbran, 1998; Leonard et al., 1999). The 190 kDa constituent(s) likely include both NR2B and NR2A subunits of the NMDA receptor. Strack and Colbran (1998) applied the gel overlay method to find strong interactions of autophosphorylated CaMKII with the cytosolic C-terminal end of NR2B (amino acids 1260–1309) and only weak interaction with that of NR1 or NR2A. Strong interactions with both NR2A (amino acids 1349–1464) and NR2B were found using both gel overlay and chemical cross-linking and required autophosphorylated kinase (Gardoni et al., 1998, 1999). High-affinity binding to NR1 similarly required autophosphorylation and was localized to the region near binding of calmodulin and α -actinin (Leonard et al., 2002).

Bayer et al. (2001) found two NR2B binding sites on its C-terminal end, one that required Ca^{2+} /calmodulin but not autophosphorylation (distal site within residues 1259–1310) and a second that only bound Thr²⁸⁶-autophosphorylated kinase (proximal site within residues 839–1120). Interestingly, NR2B is not a passive participant in the Ca^{2+} /calmodulin-stimulated binding to the distal site. This NR2B binding locks the kinase in a persis-

Figure 1. Basis for stimulus-dependent translocation of CaMKII. Schematic representation of target binding site (T site) and substrate binding site (S site) that are blocked by the autoinhibitory gate but are exposed by the displacement of the gate by Ca^{2+} /calmodulin to allow binding to targets such as the distal site of NR2B. Further displacement of the gate after autophosphorylation is necessary for proper binding of other targets, such as L-type Ca^{2+} channel (LTCC). After dissociation of Ca^{2+} /calmodulin, the former interaction produces an autonomously active kinase, whereas the latter deactivates but remains Ca^{2+} /calmodulin responsive.

tently active state even after dissociation of calmodulin, similar to the effect of Thr²⁸⁶ phosphorylation (Bayer et al., 2001). A remarkably parallel set of requirements for binding and autonomous activity resulting from targeting was made with the C-terminal site of the *Drosophila* Eag potassium channel (Sun et al., 2004). Interaction with NR2B also decreases autophosphorylation at Thr^{305/306}, which may further increase resident time on the PSD (Shen et al., 2000; Elgersma et al., 2002).

Generation of an autonomous enzyme by autophosphorylation of Thr²⁸⁶ and by NR2B binding at the distal site may involve essentially the same mechanism. One can view the autoinhibitory domain as a gate consisting of a pseudo-substrate segment that blocks binding of substrates to the S site, as well as a pseudo-target segment that blocks access to its target proteins (T site) (Hudmon and Schulman, 2002) (Fig. 1). Binding of Ca²⁺/calmodulin opens the gate and allows substrates to bind (kinase activation) and enables interactions with some targets, e.g., distal NR2B site (Bayer et al., 2001) and *Drosophila* Eag K channel (Sun et al., 2004) (stimulus-dependent translocation). Autophosphorylated Thr²⁸⁶ is positioned like a “wedge” in the inhibitory gate that further displaces the gate (Singla et al., 2001) and enables interactions with additional targeting proteins [e.g., proximal NR2B site, NR1, and L- and N-type Ca²⁺ channels (A. Hudmon, H. Schulman, J. Kim, P. Safa, J. M. Maltez, D. A. Nunziato, R. W. Tsien, and G. S. Pitt, unpublished observations)]. Indeed, the distal binding site of NR2B and the *Drosophila* Eag (Sun et al., 2004) show some homology with the region of the autoinhibitory domain near Thr²⁸⁶ (Bayer et al., 2001; Griffith, 2004). Autophosphorylation of Thr²⁸⁶ generates an autonomous kinase by disabling the inhibitory gate; the phosphorylated Thr functions as a wedge that keeps the gate and its pseudo-substrate sequence from blocking access to substrates (Yang and Schulman, 1999). Similarly, introducing a segment of NR2B or Eag across the T site (without blocking the S site) serves as a wedge that blocks the inhibitory gate from occluding substrate binding at the S site, and thus the kinase remains active (Bayer et al., 2001; Sun et al., 2004). A special feature of this form of autonomous activity is that it cannot be reversed by phosphatase activity. Interaction with NR2B may therefore serve to sensitize the kinase to brief Ca²⁺ spikes and prolong its active state.

The contribution of translocation and anchoring to NR2B to local phosphorylation has been demonstrated recently in a recombinant system (J. Tsui, M. Inagaki, and H. Schulman, unpublished observations). Ca²⁺-stimulated translocation to membrane-bound NR2B by wild-type kinase was followed by efficient phosphorylation at the membrane, whereas a translocation-defective kinase mutant neither translocated to NR2B nor phosphorylated substrate at the membrane, despite the fact that a cytosolic form of the substrate was well phosphorylated. This supports a critical role of CaMKII translocation and concentration at specific compartments, despite the fact that the kinase is generally abundant throughout the cell.

Future perspectives

Textbook descriptions of protein kinases and phosphatases encourage a simplistic view with on–off-like shifts in phosphorylation states. In contrast, CaMKII, among other kinases, exhibits a more baroque regulation. CaMKII exhibits a Ca²⁺ spike frequency-dependent activation, for example. Binding of Ca²⁺/calmodulin does more than simply activate kinase activity toward substrates but also exposes sites for target proteins, leading to a translocation of CaMKII. In some cases, e.g., one site on NR2B and *Drosophila* Eag, anchoring of the kinase locks it in an active

conformation that does not deactivate after dissipation of the Ca²⁺ signal, a form of molecular memory. Autophosphorylation achieved during appropriate stimulation produces a prolonged active state as well as translocation to other targets. We now need to evaluate the consequences of translocation to various targets in intact systems and ultimately understand how response specificity from extracellular stimulus to intracellular phosphorylation of specific substrates is achieved and how neurons use the repertoire of signaling molecules to achieve synaptic plasticity and other important neuronal functions. It is likely that genetic and proteomic approaches will have an increasingly important function in understanding signal transduction by protein phosphorylation and dephosphorylation.

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