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Cdk5 and the mystery of synaptic vesicle endocytosis

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Regulation of endocytosis by protein phosphorylation and dephosphorylation is critical to synaptic vesicle recycling. Two groups have now identified the neuronal kinase Cdk5 (cyclin-dependent kinase 5) as an important regulator of this process. Robinson and coworkers recently demonstrated that Cdk5 is necessary for synaptic vesicle endocytosis (SVE) (Tan et al., 2003), whereas a new report in this issue claims that Cdk5 negatively regulates SVE (Tomizawa et al., 2003). Careful examination of the data reveals a model that helps resolve the apparently contradictory nature of these reports.

Since Bernard Katz's revolutionary studies in the 1950s revealed the quantal nature of neurotransmission (del Castillo and Katz, 1954), the synaptic vesicle life cycle has been shown to include exocytosis, endocytosis, and trafficking. After exocytosis and neurotransmitter release, the fused vesicles are retrieved via endocytosis and trafficked back to eventually become part of the readily releasable pool. Despite the equal importance of each of these processes to neurotransmission, knowledge of the molecular mechanisms and regulation of endocytosis and the trafficking of vesicles has lagged far behind that of exocytosis.

Synaptic vesicle endocytosis (SVE) is mediated by the structurally unrelated dephosphin family of phosphoproteins, which includes amphiphysin I and dynamin I. These proteins are constitutively phosphorylated and are dephosphorylated in response to excitation of the presynaptic terminal by the Ca^{2+}-dependent phosphatase calcineurin (Cousin and Robinson, 2001). Tan et al. (2003) have identified the first dephosphin kinase as Cdk5. Despite its name, Cdk5 is not cyclin dependent but requires either the neuronal cofactor p35 or its homologue p39 for activity (Dhavan and Tsai, 2001). In studies reported in this issue, Tomizawa et al. (2003) also identify Cdk5 as an endogenous dephosphin kinase. However, the findings of the two groups differ substantially, and the implications of their data for the role of Cdk5 in SVE appear to be in complete opposition.

One of the most obvious differences between the two reports is the site of Cdk5 phosphorylation of dynamin I. Tan et al. (2003) concluded that Ser774 and Ser778 are the physiologically relevant phosphorylation sites, whereas Tomizawa et al. (2003) ascribe this function to Thr780. Both groups identified the phosphorylation site(s) of dynamin I through mass spectrometric analysis and assigned physiologic relevance to their respective sites with immunoblots of brain tissue using phosphorylation state–specific antibodies. Pertinence to SVE was demonstrated with decreased levels of phospho-dynamin I upon stimulation of synaptosomes or hippocampal slices with KCl or electrical impulses. This effect was reversed by addition of calcineurin inhibitors.

Tomizawa et al. (2003) provide particularly convincing evidence that Cdk5 is responsible for phosphorylation of dynamin I at Thr780 by probing tissue from $p35^{-/-}$ mice with their phosphorylation state–specific antibody. Tan et al. (2003) employed a similar approach but relied on the use of the Cdk5 inhibitor roscovitine (100 μM) rather than a knockout system. Used at a 100 μM concentration, roscovitine may have exerted a number of other known and unknown effects. Tan et al. (2003) recognized these limitations and compensated by confirming their results in cultured neurons through the transfection of a dominant-negative form of GFP–Cdk5. All in all, the use of phosphorylation state–specific antibodies by both groups provides strong evidence that dynamin I is indeed phosphorylated by Cdk5 at all three identified sites and that these sites are physiologically relevant.

Other than the sites(s) of dynamin I phosphorylation by Cdk5, the reports differ regarding the effect of phosphorylation of dynamin I on the binding of amphiphysin I. Tan et al. (2003) reported no effect, whereas Tomizawa et al. (2003) used two different binding assays to demonstrate a decrease in binding to both whole-length amphiphysin I and its isolated SH3 domain upon phosphorylation of dynamin I. These seemingly contradictory results may not be so contradictory, for one must remember that the two groups are studying different phosphorylation sites on dynamin I. Alternatively, the discrepancy may result from the fact that Tan et al. (2003) used only functional domains of both proteins, whereas Tomizawa et al. (2003) used whole-length proteins in their binding assays.

Another key difference between the two reports is the in vivo phosphorylation of amphiphysin I by Cdk5. Tan et al. (2003) asserted that Cdk5's phosphorylation of amphiphysin I is strictly an in vitro occurrence, whereas Tomizawa et al. (2003) identified five in vitro and in vivo Cdk5

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Abbreviation used in this paper: SVE, synaptic vesicle endocytosis.

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phosphorylation sites. Tan et al. (2003) performed a quadruple pull-down of dephosphins from ^{32}P-labeled synaptosomes with protein domain affinity resins containing portions of the SVE proteins α-adaptin and endophilin I. Since roscovitine did not block the increase in phospho-amphiphysin I during the repolarization stage after KCl stimulation, they concluded that amphiphysin I is not phosphorylated by Cdk5 in vivo. Alternative explanations could exist if amphiphysin I has multiple Cdk5 phosphorylation sites, as Tomizawa et al. (2003) suggest. If amphiphysin I has multiple phosphorylation sites and the dephosphorylation of any one of these sites enhances binding to the α-adaptin and endophilin I domains, the results may be confounded. In other words, even if roscovitine decreases the level of phospho-amphiphysin I, this pull-down assay may detect the same amount of phosphoprotein due to the change in binding affinity.

In a different approach, Tomizawa et al. (2003) demonstrate mobility shifts upon treatment with a calcineurin inhibitor in wild-type mice but not p35$^{-/-}$ mice, presumably due to the in vivo phosphorylation of amphiphysin I by Cdk5. While this report and two others (Rosales et al., 2000; Floyd et al., 2001) clearly demonstrate the in vitro phosphorylation of amphiphysin I by Cdk5, more definitive proof of the physiological significance of this reaction in vivo must await future studies that employ phosphorylation state–specific antibodies.

Up until this point, the role of Cdk5 in SVE has only been discussed on the molecular level in terms of what it phosphorylates where. What really put these two papers at odds are the fundamentally different observations concerning the effects of Cdk5 inhibition on SVE. Tan et al. (2003) performed a styryl dye study in which nerve terminals were stimulated with KCl to undergo two rounds of endocytosis. Despite the lack of effect on the first round of endocytosis, treatment with 100 μM roscovitine significantly inhibited the second round, and this corresponded with dramatic synaptic vesicle depletion by electron microscopic analysis. Furthermore, transfection of cultured neurons with a dominant-negative form of Cdk5 resulted in a 50% decrease in styryl dye uptake. Together, these studies suggest that Cdk5 is required for repetitive rounds of SVE.

In striking contrast, Tomizawa et al. (2003) performed essentially the same styryl dye study in a primary culture of hippocampal neurons and report seemingly opposite results. Upon treatment with either 10 μM roscovitine or another Cdk5 inhibitor, olomoucine, they found an increase in styryl dye puncta size and number after electrical stimulation. Treatment of primary hippocampal neurons from p35$^{-/-}$ mice with olomoucine did not produce any changes, suggesting that the effect of olomoucine was Cdk5 specific. In a cell-free assay, they observed massive formation of small vesicles from liposomes when incubated with dynamin I and amphiphysin I, but only in the dephosphorylated forms. Furthermore, phosphorylation inhibited the ability of dynamin I and amphiphysin I to copolymerize into ring structures, a step believed to be necessary in the final pinching off of synaptic vesicles. The physiological significance of these in vitro systems is difficult to ascertain when viewed in isolation. However, taken in the context of the styryl dye study, these in vitro systems make the hypothesis that Cdk5 suppresses SVE very attractive.

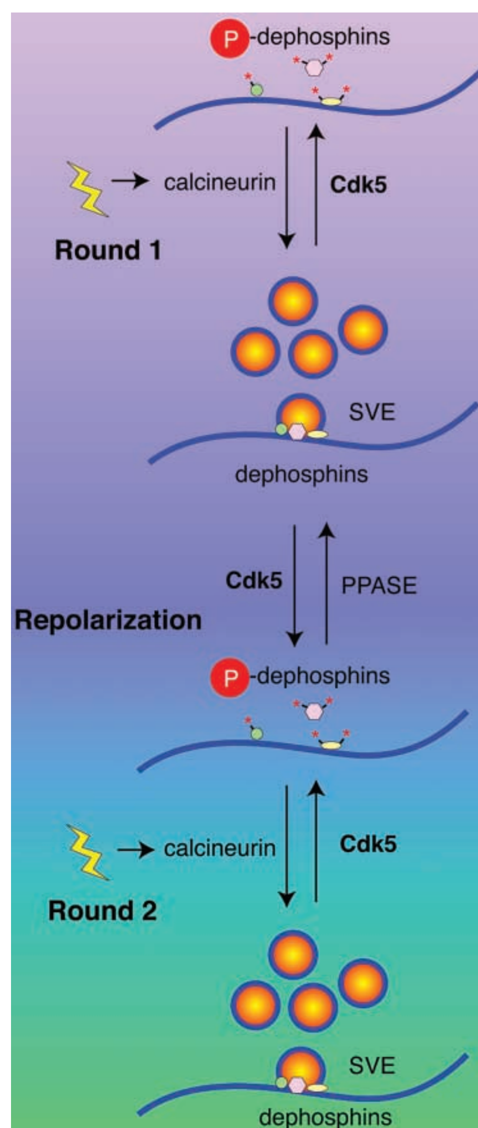

Figure 1. **Schematic diagram depicting the dephosphin life cycle.** Stimulation activates calcineurin-dependent dephosphorylation and results in SVE. During repolarization, Cdk5 rephosphorylates dephosphins for subsequent rounds of SVE. Inhibition of Cdk5 would enhance the first, but suppress the second, round of endocytosis.

So which is it? Is Cdk5 necessary for SVE or does Cdk5 suppress it? Only one of these hypotheses can be correct, right? Perhaps not. The key could lie in recognition of the fact that Tan et al. (2003) studied two rounds of endocytosis, whereas Tomizawa et al. (2003) studied one. Consider the following model (Fig. 1). The dephosphins exist in an equilibrium between their phosphorylated and unphosphorylated forms with Cdk5 and calcineurin mediating the interconversion. Tomizawa et al. (2003) shift this equilibrium in favor of the active, or unphosphorylated, form by using Cdk5 inhibitors. The result is that endocytosis is enhanced for the first round of endocytosis. But what about the next round? Without Cdk5, the dephosphins cannot be rephos-

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phorylated in preparation for the subsequent round of endocytosis. In effect, they are stuck in an unphosphorylated but inactive state, because they have already been "used" once. Endocytosis fails after the first round, resulting in the depletion of synaptic vesicles observed by Tan et al. (2003). Further support for this model can be gained by closer inspection of the Tan et al. (2003) paper, particularly the portions involving roscovitine treatment with one round of endocytosis. The bar graph depicting a 34% decrease in retrieval efficiency for the second round of SVE actually also depicts an ~12% increase for the first round. The set of electron micrographs revealing a dramatic reduction in the number of vesicles for the second round of SVE also reveals an apparent increase in the number of large-sized vesicles for the first round.

In conclusion, processes as complicated and as mysterious as SVE cannot be adequately summarized with global statements such as "Cdk5 is necessary for SVE" or "Cdk5 inhibits SVE." Cdk5 may do both; it may inhibit the first round of endocytosis but be necessary for the second round. Thus, one kinase can be necessary for a process and yet at the same time also suppress it.

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