Citron Kinase is a Regulator of Mitosis and Neurogenic Cytokinesis in the Neocortical Ventricular Zone

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Successful cell division in neural progenitors in the neocortical ventricular zone (VZ), as in all dividing cells, depends critically upon coordinating chromosome segregation during mitosis with cytokinesis. This coordination further suggests that common molecular regulators may link events in mitosis with those in cytokinesis. Recent genetic evidence indicates that cytokinesis in CNS neuronal progenitors, but not in most other cell types of the body, requires the function of citron kinase. In neocortex, citron kinase is most critical for neurogenic cytokinesis. In citron kinase null mutants, a large proportion of neuronal cells within neocortex are binucleate; however, very few glial cells are binucleate. In addition, confocal time-lapse imaging of mitoses at the VZ surface shows that citron kinase is also necessary for phases of the cell cycle just prior to cytokinesis. Deficits in mitosis seen in mutants indicate aberrant mitotic spindle function, and like deficits in cytokinesis, occur in some but not all cells at the VZ surface. Citron kinase is therefore an essential multifunctional regulator of cell divisions in the VZ, and may serve to coordinate chromosome segregation with cytokinesis in neuronal precursors.

Introduction

The position, movement and morphology of neuronal progenitors as they progress through mitosis and cytokinesis at the ventricular zone (VZ) surface have been well described. In contrast, the specific molecular mechanisms that control events during cell division in the VZ are not well understood. While many mechanisms that regulate neurogenic cell divisions are likely to be the same as mechanisms that regulate division in other cell types, recent genetic evidence indicates that some molecular requirements of cytokinesis are specific to neural progenitors. In both rat (flathead mutant) and mouse mutants, absence of citron kinase has been shown to lead to cytokinesis failure and subsequent apoptosis in many neuronal precursors in the CNS, but not in cells in all other organ systems investigated to date, including the peripheral nervous system (Di Cunto et al., 2000; Sarkisian et al., 2002). In addition, not all neurogenic cytokineses in the CNS absolutely require citron kinase: ~50% of all neurons in citron kinase mutants have single normally sized nuclei and the remaining are binucleate. This suggests a surprising degree of molecular heterogeneity for molecular control of cell division during neurogenesis in different cell types within the body.

Citron kinase was initially identified in a screen for genes with similarity to the kinase domain of Rho-activated kinases (ROCK and ROK kinases), downstream effectors of Rho family GTPases (Di Cunto et al., 1998; Madaule et al., 1998). In a functional test, C-terminal truncation mutants of citron kinase were shown to cause erratic cytokinetic behaviors and cytokinesis failure when overexpressed in Hela-cells (Madaule et al., 2000). These gain-of-function experiments suggested that citron kinase is essential to stabilizing the position and ensuring normal function of the cytokinesis furrow and midbody. More recent analyses of a citron kinase null mutant mouse (Di Cunto et al., 2000) and of the flathead mutation in rat (Cogswell et al., 1998; Sarkisian et al., 2002) further confirmed that citron kinase is essential to cytokinesis in vivo, and further showed that this requirement is specific for CNS precursors.

The expression pattern of citron kinase through embryonic and perinatal development suggests that it is essential for many embryonic and postnatally generated neurons but not for postnatally generated glia. Citron kinase message and protein is expressed across the VZ; however, by postnatal day 1 citron kinase expression is absent in the forebrain but still present in the cerebellum throughout the period of granule cell neuronogenesis (Di Cunto et al., 2000; Sarkisian et al., 2002). Similarly, immunocytochemistry and Western blot analyses show that citron kinase is not expressed in the sub-ventricular zone in postnatal cortex (unpublished observation).

Considering the relative specificity of citron kinase to neurogenic cell divisions, it becomes important to define the signaling systems that engage citron kinase during neurogenesis. Based both on amino acid sequence and functional studies, citron kinase protein has multiple functional domains that can interact with other signaling and structural proteins (Di Cunto et al., 1998; Madaule et al., 1998; Zhang et al., 1999; Eda et al., 2001). The amino terminal kinase domain shows significant homology to ROCK kinases and phosphorylates regulatory myosin light chain (RMLC) (Matsumura et al., 2001). The C-terminal contains many protein interaction domains including a coiled-coil domain, Rho binding domain, PDZ-binding domain, zinc finger domain, and putative SH3-binding domain (Madaule et al., 1998). In addition to multiple protein interaction domains, citron kinase moves to different cellular compartments through mitosis (Eda et al., 2001). Citron kinase moves from the cytoplasm to the spindle midzone, and then is transported in telophase to the cytokinesis furrow in a Rho GTPase dependent fashion. This dynamic localization through the cell cycle and multi-domain structure of citron kinase suggest that it may have multiple functions during neurogenic cell divisions.

Materials and Methods

Mutant and wild-type embryos were harvested from timed pregnant animals anesthetized with ketamine/xylazine. Embryos were removed and brains were dissected in warmed culture media (DMEM, high glucose, 25 mM HEPES, 20% FCS, 20 ng/ml bFGF). Explants ~2 × 2 mm were removed from dorsal medial cortex. Explants were then placed VZ surface down on cover slips previously coated with collagen and poly-D-lysine, and the explants were held in place by nylon netting. Explants were then stained with syto 11 (5 μm) for 5 min. We found that maintaining the explants in warmed media, never in iced solutions, through dissection and putting the VZ surface against a collagen/poly-D-lysine substrate prevents the G2/M block that has been observed in previous explant experiments and removes the requirement to let explants recover for 2–4 h before the cell cycle resumes. In fact, we found that with these methods we were able to image multiple VZ divisions,

with no apparent disruption, in wild-type embryos within 10 min of removing the embryo, or as quickly as we are able to stain and begin imaging. For imaging, explants were moved to a sealed and heated imaging chamber (34°C, Warner Instruments) and time lapse images were made for 3–4 h on a Leica Spectrum confocal system. Image stacks were taken every 7.5 min, a stack of 10 images through 10–20 µM of the plane tangential to the VZ surface. Time lapse movies were analyzed by Image J after acquisition. We genotyped embryos either by amplifying exon 1 of the *CitK* gene and then cut with *Ban*II, a restriction site that is missing in the flathead mutant *CitK* gene, or by identifying E17 embryo brains that had a 25–30% decrease in cerebrum length. In *post hoc* PCR tests, phenotyping based on brain size at E17 was found to be predictive of a homozygous flathead genotype every time. Immunocytochemistry procedures on postnatal animals (Fig. 1) were similar to those described previously (Sarkisian *et al.*, 2001, 2002). All protocols were approved by the University of Connecticut IACUC (assurance no. A3124-01).

Results

Citron Kinase Affects Neurogenic Cytokineses
Previous reports have shown that citron kinase null mutants have binucleate neurons throughout the CNS. We have used multiple neuronal and glial cell markers to determine the proportions of different types of neurons and glia that are binucleate. As shown in Figure 1, neuronal markers label binucleate neurons that are positive for GABA, and several other markers including NeuN, Pyramidal cell marker (Swant), and doubleortin (not shown). In a previous study we also showed that many cells that label with interneuron markers are also binucleate. In contrast to the neuronal markers, glial markers, GFAP and NG2, a marker for oligodendrocyte precursors, do not label significant numbers of binucleate cells. In the flathead mutant rat from P0–P21, while nearly 50% of cells that are positive for neuronal markers are binucleate, only 3% of GFAP+ (*n* = 500 cells, four animals) and 0% of NG2+ cells are binucleate (out of 700 cells, in four animals). Therefore, citron kinase appears to be essential for normal completion of cell divisions that produce neurons, but not for most of the cell divisions that produce glial cells. This phenotype is also consistent with the temporal expression pattern of citron kinase, which is expressed in prenatal periods in the neocortex but not postnatally when the majority of gliogenic cell divisions in cortex occur.

Citron Kinase Is Essential for Mitosis
In cell lines, citron kinase has been shown to initially localize to the spindle midzone during anaphase and then move to the cytokinesis furrow and midbody after activation by Rho GTPase (Eda *et al.*, 2001). To date, the analysis of citron kinase mutants has indicated an essential role during cytokinesis, and a potential role for citron kinase during mitosis when citron kinase is present at the spindle midzone has not been previously examined. To test the hypothesis that citron kinase plays a role during mitosis we compared time-lapse images from E17 and E15 VZ explants from wild-type and homozygous mutant flathead embryos (fh/fh). Images were taken form four wild-type and four mutant embryos at E17 and two wild-type and mutant embryos at E15 in a plane tangential to the surface of the VZ. This explant preparation enabled us to image multiple mitoses in a single plane without cell behavior being affected by cut edges of a slice. The majority of metaphase plates rotate in a plane parallel to the plane of the image, and ~5% were seen to rotate in a plane perpendicular to the plane of the VZ surface in this preparation. Metaphase plates stopped rotating just prior to entering anaphase, and chromosome separation was completed most typically within 15 min (two frames) after cessation of
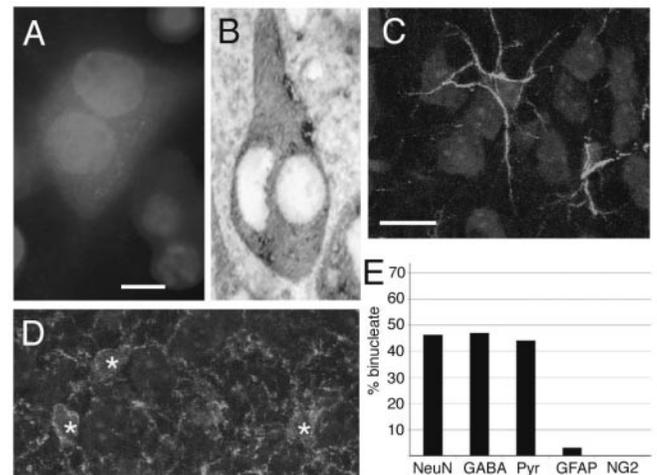

Figure 1. Cytokinesis defects in flathead mutants are prevalent in neuronal but not glial cell types. Examples of binucleate neurons labeled with GABA antibodies (*A*) or an antibody to pyramidal cells anti-Pyr (swant) (*B*) present in the neocortex of flathead mutants. In contrast, markers for glial cell types GFAP (*C*) and NG2 (*D*) show that very few, if any, cells are binucleate in flathead mutants. Calculating the percentage of binucleate cells (*E*) that are positive for different neuronal and glial markers indicates that while ~50% of cells that label with neuronal markers in the flathead mutant are binucleate, only a small percentage of GFAP+ cells (3%) are binucleate and no NG2+ cells are binucleate in mutants. These results indicate that citron kinase function is specific for cell divisions that produce neurons.

metaphase plate rotation. In wild-type explants, metaphase plates divided symmetrically through the length, and were never observed to separate incompletely or asymmetrically. In addition, while some anaphases appeared to be delayed after the formation of a metaphase plate, no metaphase plates in wild-type were observed not to enter anaphase within a 2 h imaging session.

In contrast to images of mitoses in wild-type, time lapse imaging of mitotic behavior of cells at the VZ surface of flathead mutants indicated many disrupted mitoses (Fig. 2, movie in supplementary data). Metaphase plates formed normally in most mitotic cells in fh/fh; however, ~50% of cells in flathead either did not progress into anaphase or showed disrupted patterns of chromosome segregation during anaphase. As shown in Figure 2, these abnormalities could be grouped generally into two different categories. Approximately 20% of mitoses in mutants were either blocked or greatly slowed in progression from metaphase to anaphase (Fig. 2*b*). These cells formed mitotic plates but over at least 2 h of imaging never progressed into anaphase as assessed by chromosome separation. At the edge of the metaphase plates in these cells there were often stray chromosomes that were seen to move in and out of the metaphase plate. Approximately 20% of mitoses in mutant at E15 and 30% at E17 showed disruptions in anaphase that were characterized as aberrant because of altered or failed chromosome separation (Fig. 2*c–e*). Since chromosome separation primarily requires the normal function of the spindle apparatus, we interpret the patterns of disrupted mitoses in the flathead mutant as disruptions in spindle function. Normal spindle function during cell division at the VZ surface is therefore dependent upon citron kinase.

Discussion
The phenotypes of the flathead rat and the citron kinase mutant mouse indicate that citron kinase is required for mitosis and
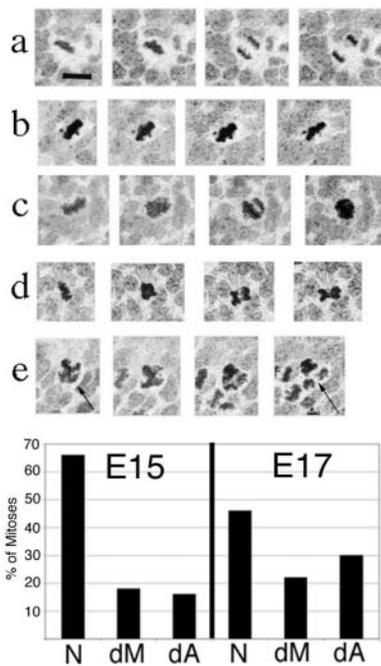
Figure 2. Time-lapse of disrupted mitoses at the neocortical VZ surface in mutant embryos (E15 and E17). Disrupted and/or delayed metaphase and anaphase in flathead mutants were apparent in ~50% of mitoses imaged at the VZ surface over 2–4 h imaging sessions. Time lapse sequences for five representative cells imaged at the VZ surface of dorsal neocortex explanted from an E17 flathead embryo (*a–e*). Frames were taken every 15 min for *a, c, d* and *e*, and every 45 min for *b*. (*a*) An example of a normal mitosis (N), (*b*) An example of a disrupted or delayed metaphase–anaphase transition (dM). (*c–e*) Examples of disrupted anaphase (dA) all imaged from the VZ surface of a flathead mutant at E17. The bar graph shows a quantification of differently classified disrupted (dM, dA) and normal (N) mitoses in VZ explants from flathead mutants at E15 and E17. At both ages, similar percentages of mitoses showed disruptions in metaphase and anaphase (over 500 mitoses were imaged and classified for each age). Similar analysis of non-mutant embryos revealed no disruptions in mitoses. Scale bar = 6 µm.

cytokinesis in VZ precursors. The disruptions in mitoses are diverse in flathead cells, and such disruptions suggest aberrant function of the spindle apparatus and in normal restructuring of cytoskeletal elements necessary for both normal chromosome segregation and function of the cytokinesis furrow. The dual function of citron kinase is consistent with previous protein expression studies in cell lines. Citron kinase is localized to the cytoplasm in interphase, accumulates at the spindle midzone in anaphase and then moves to the cytokinesis furrow in telophase (Eda *et al.*, 2001). One possible common target for citron kinase at both the spindle midzone and cytokinesis furrow is RMLC. RMLC is phosphorylated at serine 19 by citron kinase (Matsumura *et al.*, 2001) and this phosphorylated form has been shown to appear at the spindle midzone in anaphase and then at the cytokinesis furrow (Komatsu *et al.*, 2000). In addition, a mutation of MLC at serine 19, a site phosphorylated by citron kinase, causes defects in both mitosis and cytokinesis in cell lines that are similar to that seen in flathead VZ cells (Komatsu *et al.*, 2000). Together this evidence suggests that citron kinase phosphorylates RMLC at both the spindle midzone to enable appropriate chromosome separation and then at the cytokinesis furrow to enable cytokinesis. The transport of citron kinase from the midzone to the furrow may serve to coordinate chromosome separation with cytokinesis. This movement appears to be triggered by activated Rho binding to citron kinase at the

midzone (Eda *et al.*, 2001). Rho activation may therefore play a role in coordinating the timing of mitosis and cytokinesis in neuronal progenitors, and such timing may be important to the determination of timing and rates of cell production from the VZ surface.

It is not currently known why loss of citron kinase affects only a subset of progenitors. ROCK kinases are present within VZ cells and are the most likely candidates for compensating for citron kinase function (Di Cunto *et al.*, 2000). Alternatively, cell division in some progenitors may be independent of Rho and associated kinases. For example, inhibition of Rho has been shown to disrupt cytokinesis of weekly adherent cells in culture but not strongly adherent cells, suggesting that Rho may not be essential for division of cells in certain functional states (O'Connell *et al.*, 1999).

Why do different progenitors require different cell division mechanisms? One possibility is that different fates are generated preferentially from different types of cell divisions. Such a suggestion has been previously made for cortex (Adams, 1996; O'Connell *et al.*, 1999; Miyata *et al.*, 2001; Noctor *et al.*, 2001) and in *Drosophila*, orientation of division has been shown to determine neurogenic fates (Knoblich *et al.*, 1995; Lin and Schagat, 1997). We have not observed a quantitative difference in the orientation of cell divisions in flathead, however (unpublished observation), so it seems unlikely that different cytokinesis mechanisms revealed by analysis of the flathead mutant are related to different division planes. Nevertheless, some cell types are disproportionately affected in flathead mutants. Upper layer pyramidal neurons, for example, are nearly missing in flathead and similarly cortical inhibitory interneurons are severely depleted in all layers (Roberts *et al.*, 2000; Sarkisian *et al.*, 2001). This suggests that there may be different mechanisms for mitoses that generate different cell types in cortex. By determining the function of different proteins in the signaling pathway associated with citron kinase it may be possible to determine a more detailed relationship between mechanisms of cell division and the generation of different neocortical cell types.

Notes

Supplementary Material

Supplementary material can be found at: http:\\www.cercor.oupjournals.org

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