buffer, uncorrected *p*H meter reading 6.1; 220 blocks of free induction decays were collected as 8192 data points (each represented the average of 800 transients). The recycling time for each accumulation was 1.16 seconds, and the total experiment time was 74 hours. The level of enrichment was determined by measuring the ^{13}C satellite peaks in the ^{1}H NMR spectrum of proline isolated from a protein hydrolyzate.
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Calpain II Involvement in Mitosis

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Mitotic spindle disassembly requires major structural alterations in the associated cytoskeletal proteins and mitosis is known to be associated with Ca^{2+}-sequestering phenomena and calcium transients. To examine the possible involvement of a ubiquitous Ca^{2+}-activated protease, calpain II, in the mitotic process, synchronized PtK_1 cells were monitored by immunofluorescence for the relocation of calpain II. The plasma membrane was the predominant location of calpain II in interphase. However, as mitosis progressed, calpain II relocated to (i) an association with mitotic chromosomes, (ii) a perinuclear location in anaphase, and (iii) a mid-body location in telophase. Microinjection of calpain II near the nucleus of a PtK_1 cell promoted the onset of metaphase. Injection of calpain II at late metaphase promoted a precocious disassembly of the mitotic spindle and the onset of anaphase. These data suggest that calpain II is involved in mitosis.

THE REDISTRIBUTION OF Ca^{2+} (*1–4*) and calcium-binding proteins (*5*, *6*) during the mitotic cycle has been well documented. Additionally, disassembly of the mitotic spindle (*7*, *8*) or microtubules in lysed cells (*9*) has been shown to be Ca^{2+}-dependent. In this study, I have examined the possibility that a Ca^{2+}-activated protease, calpain II (E.C. 3.4.22.17), may be involved directly or indirectly in the mediation of specific mitotic events by Ca^{2+}. In the first part of the study PtK_1 cells were synchronized by a double thymidine block and then monitored via immunofluorescence for the redistribution or translocation of calpain II (Fig. 1). At plating of the cells (prometaphase), calpain II appeared to be exclusively associated with the chromosomes (Fig. 1a). As mitosis proceeded, calpain II remained associated with the chromosomes during early and late anaphase (Fig. 1, b and c) but then was observed at a bilaterally symmetrical location at the periphery of the cells as well as in association with the cytoplasmic bridge during telophase or cytokinesis (Fig. 1d). An example of one daughter cell at the end of cytokinesis is shown with remaining mid-body (Fig. 1e). Finally, at interphase, calpain II had a predominantly plasma membrane association (Fig. 1f). Different stages of mitosis shown in Fig. 1 are representative of approximately 20 cells for each panel.

In order to better assess the dynamic nature of the calpain II involvement in mitosis, microinjection techniques were used to place rhodamine isothiocyanate–labeled calpain II (calpain II–RITC) at selected cellular locations and then monitor calpain II translocation and the time required for the completion of mitotic stages.

Calpain II–RITC injected near the nucleus of a cell in interphase (Fig. 2, a and b) promoted the onset of metaphase, and the labeled calpain II quickly became associated with the metaphase chromosomes (Fig. 2c). Onset of metaphase occurred approximately 5 minutes after calpain II–RITC injection at interphase. The same cells, shown as they progressed through anaphase displayed redistribution of calpain II–RITC from a predominantly chromosomal association (Fig. 2d) to a bilaterally symmetrical location near the reforming nucleus and association with the cytoplasmic bridge (Fig. 2, e and f). One daughter cell, shown 1 hour after calpain II–RITC injection, displayed calpain II–RITC staining associated exclusively with the plasma membrane (Fig. 2g). Cells injected at late metaphase display a more rapid than normal transition from metaphase to anaphase (approximately 1 to 1.5 minutes relative to a normal transition time for PtK cells of 16 minutes) and the injected calpain II–RITC appeared to condense at the middle of the cytoplasmic bridge (Fig. 2i), with eventual localization at the mid-body of daughter cells (Fig. 2j). Although the fluorescence pattern seen in Fig. 2j is not identical with that in Fig. 2, e and f, or Fig. 1c, this is a staining pattern frequently seen in cells that have been neither injected nor synchronized. Results obtained after microinjection of unlabeled calpain II paralleled those seen with calpain II–RITC. Control injections of similar volumes and protein loads of bovine serum albumin–conjugated RITC (BSA-RITC) did not appear to promote metaphase or anaphase.

Microinjection techniques were then used to examine the Ca^{2+} requirements of calpain

Table 1. Effect of interaction of Ca^{2+}, calpain II, and calpastatin on anaphase onset. Injection was made in synchronized cells shortly after plating; by inspection these cells were seen to be in metaphase. Twenty cells were injected for each treatment.

[Ca^{2+}] (μM)	Calpain II (1 mg/ml)	Calpastatin* (1 mg/ml)	Onset of anaphase, time in minutes ($\bar{X}$ ± SE)
0.1	−	−	14 ± 1.3
1.0	−	−	3 ± 0.9
5.0	−	−	5 ± 1.3
10.0	−	−	15 ± 1.5
0.1	+	−	2 ± 0.5
1.0	+	−	3 ± 0.6
5.0	+	−	3 ± 0.7
10.0	+	−	11 ± 0.8
0.1	−	+	15 ± 1.7
1.0	+	+	†
5.0	+	+	†
10.0	−	+	17 ± 1.2
Buffer alone	−	−	14 ± 1.8

*Homogenous preparation of calpastatin (170 kD) from porcine skeletal muscle was used in these studies. Purification and biochemical characterization of porcine skeletal calpastatin has been described (*30*). †Cells did not progress to anaphase.

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II and the interaction of Ca^{2+} and the endogenous inhibitor of calpain II, calpastatin, in the regulation of calpain II (Table 1). As described by Izant (*8*), effective Ca^{2+} values for the induction of anaphase ranged between 1 and 10 μM; however, in the presence of calpain II, lower Ca^{2+} levels (0.1 μM) appeared to be effective in terms of promoting anaphase. The presence of the inhibitor, either in combination with calpain II and 0.1 μM Ca^{2+} or with 5.0 μM Ca^{2+} alone, was effective in blocking the onset of anaphase. Not only did calpain II injection at metaphase shorten the metaphase-anaphase transition, but the transition from prophase to interphase was also substantially shortened by calpain II injection at prophase. With cells injected in prophase, the prophase-interphase phase transition takes approximately 30 minutes, 1/2 to 1/3 the time required for a normal transition. The lack of unambiguous markers for the phases of mitosis contributes to the imprecision of the times recorded for each phase after prophase microinjection. It was apparent, however, that all the observed phases of mitosis were reduced. Cells in G_1 or S were not examined in this study.

Thus, it would appear that calpain II can function at considerably lower Ca^{2+} levels than the millimolar Ca^{2+} levels required for in vitro activation (*10*). A number of explanations for this phenomenon are possible. First, by co-injecting calpain II and Ca^{2+}, the injected calpain II may now be in excess relative to levels of an endogenous inhibitor and, therefore, more likely to be active. Additionally, the locational control that may be normally exerted on calpain II could be obviated by the injection of unassociated calpain II. The redistribution or relocation

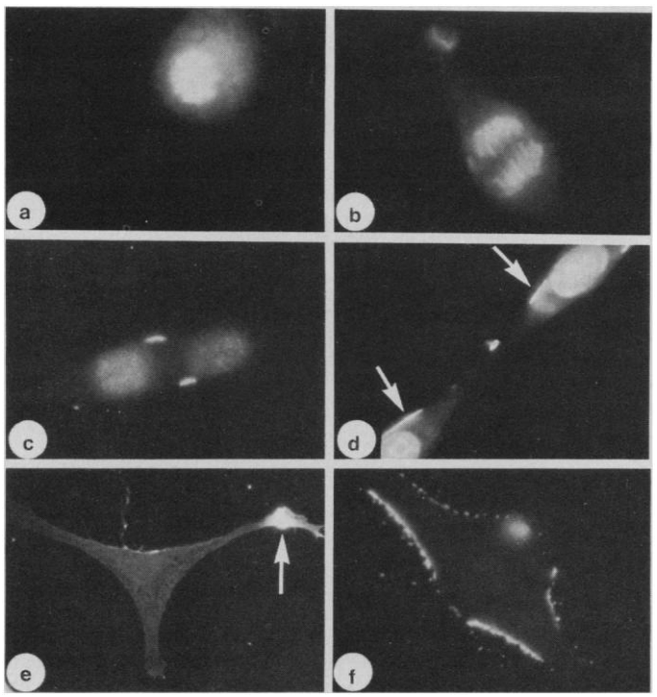

Fig. 1. Synchronized PtK_1 cells were seeded onto glass cover slips and were observed at the following time intervals (all times given will be 120 minutes after plating): (**a**) prometaphase, 20; (**b**) early anaphase, 25; (**c**) anaphase, 28; (**d**) telophase, 30; (**e**) end of cytokinesis, 40; and (**f**) interphase, 75 (×769). Arrows in (d) denote the bilaterally symmetrical staining; arrow in (e) indicates the location of the stained mid-body. Cells were originally plated in Hamm's F-12 medium with 10% fetal bovine serum. Twenty-four hours after plating, cells were synchronized by the thymidine double-block method (*26*), mechanically shaken off the culture dishes, and reseeded onto glass cover slips. Specificity of antibodies to calpain II and immunofluorescence protocols have been described (*27*). A Zeiss Photomicroscope III equipped with a 63× phase planachromat objective was used for photography.

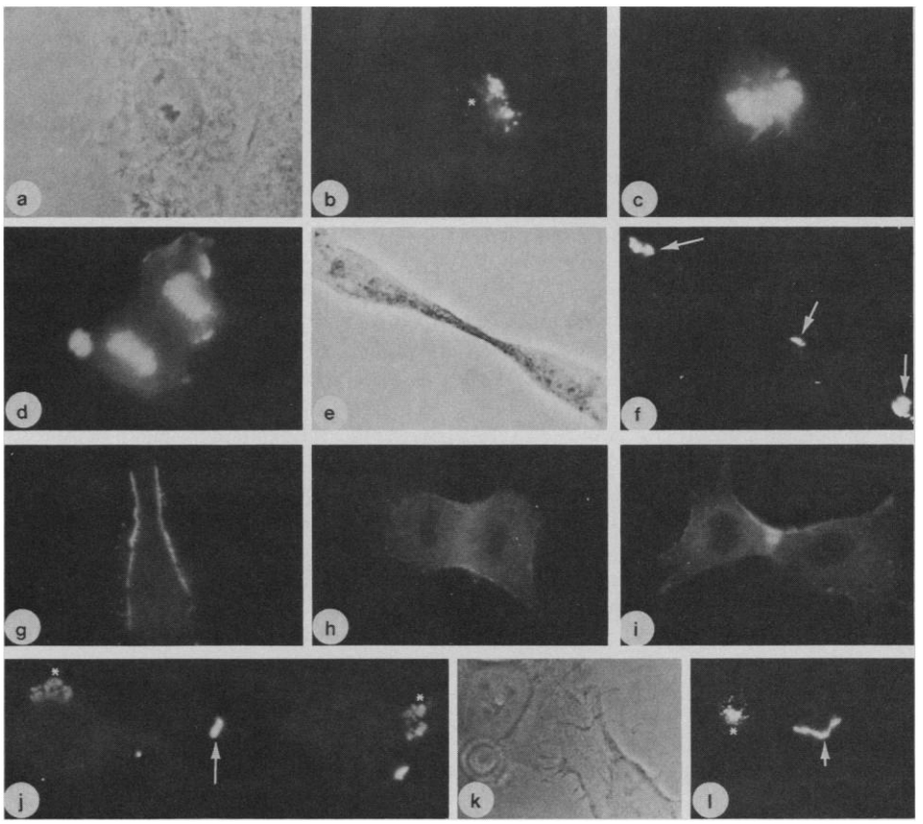

Fig. 2. PtK_1 cells microinjected with calpain II–RITC at various stages in the mitotic cycle. A prophase cell injected with calpain II–RITC near the nucleus (*) 2 minutes after injection [(**a**), phase; (**b**), fluorescence]. The same cell was examined at the following times (minutes) after injection: (**c**) 5; (**d**) 7; (e) 15, phase; and (**f**) 15, fluorescence. Arrows (f) indicate bilaterally symmetrical staining near the reforming nucleus and cytoplasmic bridge association. A daughter cell 30 minutes after injection is shown in (**g**). A cell injected at late metaphase (**h**); the same cell, 2 minutes after injection (**I**); and daughter cells 30 minutes after injection (**j**). In (j), the mid-body is indicated by an arrow and the bilaterally symmetrical staining near the nucleus by a (*). A common staining pattern for calpain II for cells in interphase is seen in (**k**), phase and (**l**), fluorescence. Intense calpain II staining is associated with residual mid-body (l, arrow) and punctuate staining is apparent over the nucleus (*). PtK_1 cells were maintained and processed for microinjection essentially as described (*8*). Both calpain II and BSA were labeled with RITC as described (*28*). Caseinolytic or myofibril assay (*10*) was used to document that calpain II activity was unchanged by RITC labeling. Calpain II–RITC and BSA-RITC were placed in the buffer system containing 0.1 μM $CaCl_2$ described by Izant (*8*). The direct needle microinjection technique (*29*) was the method of injection used. Volumes of injected calpain II–RITC or BSA-RITC [measured by the size of the transition vesicle formed during microinjection (*7*)] ranged from 1.0×10^{-13} to 3.0×10^{-13} liters per cell, a volume equal approximately to 1 to 30% of the cell volume (*25*). Protein concentrations used varied from 0.1 to 1 mg/ml.

of calpain II supports the idea that location of calpain II may be critical to the mode of calpain II function in terms of access to substrate or mode of activation or deactivation.

Clearly, the nature of the interrelationship between calpain II and calpastatin is not yet understood. For instance, we do not yet know whether the mechanism by which calpastatin controls calpain II relies more heavily on (i) a physical association that necessitates physical proximity [sarcolemmal association of calpastatin and calpain II in striated muscle (*11*, *12*)] or whether (ii) the Ca^{2+} microenvironment may be more important in modulating the calpain II–calpastatin interaction. Injected calpain I, though inhibited during in vitro interactions with calpastatin, does not relocate during mitosis and does not appear to affect the progress of specific mitotic states (*13*). This result provides additional support for the theory that relocation of calpain II may be critical to how the protease functions in mitosis.

Insight into mechanisms by which the Ca^{2+} requirement of calpain II may be modulated has come from calpain activation studies in smooth muscle (*14*). The amount of Ca^{2+} required for the autolysis of half of a total amount of calpain II ($K_{0.5}$) can be reduced from 680 μM to 87 μM if phosphatidylinositol or diacylglyerol are added. However, these Ca^{2+} levels are still substantially greater than those reported at metaphase-anaphase transition (0.5 to 0.8 μM) (*4*) for PtK_1 cells. Additional work needs to address the potential involvement of Ca^{2+}-binding proteins in combination with other factors in the modulation of this protease.

Although one of the original reasons for the use of microinjection was to facilitate the identifications of cellular substrates, there may be an array of substrates for calpain II. Hydrolysis of specific cytoskeletal proteins (*15*, *16*) as well as microtubule-associated proteins (MAPs) (*17*–*19*) by calpain II has been well documented. Calpain II–promoted hydrolysis of MAP 1 or MAP 2 could result in the disassembly of the microtubules of the mitotic spindle. Moreover, the selective hydrolysis of filamin and other actin-binding proteins (*20*, *21*), as well as hydrolysis of intermediate filament components (*22*), has major implications for any of the mitotic processes dependent on these cytoskeletal components. The possible role that calpain II may play in association with the nucleus, nuclear membrane, or chromosomes is somewhat more speculative. Calpain II has been implicated in the Ca^{2+}-mediated intracellular processing of various receptors that become associated with the nucleus (*23*, *24*), as well as in the cytoskeletal-directed condensation of chromosomes (*25*).

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Identification and Characterization of a Neuron-Specific Nuclear Antigen in *Drosophila*

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An antigen found only in neuronal nuclei of *Drosophila melanogaster* is revealed by staining with a monoclonal antibody (Mab44C11). This antigen appears early in development, before neurons show any other signs of antigenic or morphologic differentiation, and persists throughout development. This nuclear staining permits reliable detection of neurons in developmental studies of wild-type and mutant flies. Protein immunoblot analyses and immune precipitation experiments show that the neuronal nuclear antigen is a 50-kilodalton polypeptide.

THE DIFFERENCE BETWEEN NEURONS and other types of cells remains a major question in developmental neurobiology. In *Drosophila*, this problem can be studied by two complementary approaches. (i) From mutational studies, one can identify genes that are important in neuronal determination (*1*–*3*). (ii) With neuron-specific antibodies, one can identify molecular differences between neurons and other cell types. Although a number of antibodies have been found to stain subsets of cells of the nervous system (*4*), only one antiserum, raised against horseradish peroxidase (HRP), was previously known to stain all *Drosophila* neurons [antibodies to HRP bind to carbohydrate epitopes on the neuronal membrane (*5*–*7*)]. Here we describe a monoclonal antibody (Mab44C11) that exclusively stains neuronal nuclei in *Drosophila*. We also present preliminary biochemical characterizations of this neuron-specific nuclear antigen.

The antibody Mab44C11 was found as an immunoglobulin M (IgM)–secreting clone from a hybridoma fusion with HRP as an immunogen; however, it does not recognize HRP. At all stages of *Drosophila* development that have been examined, Mab44C11 stains neuronal nuclei. In the central nervous system (CNS), nuclei of neurons but not neuroblasts are stained. Although it appears that all neurons are labeled, because of the high density of cells we could not be certain that every neuron in the CNS is stained. In the embryonic peripheral nervous system (PNS), on the other hand, the number and position of all neurons has been determined (*8*). Examination of many embryos revealed

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