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Nuclear concentration and mitotic dispersion of the essential cell cycle protein, p13^{suc1}, examined in living cells

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ABSTRACT **Stamen hair cells of *Tradescantia virginiana* have been microinjected with p13^{suc1} labeled with carboxyfluorescein (CF) and studied throughout the division cycle in living cells by using the confocal laser scanning microscope. The protein, p13^{suc1}, is essential for the rapid inactivation of the key mitotic catalyst, p34^{cdc2} kinase, at anaphase and for completion of nuclear division. During interphase or prophase, CF-p13^{suc1} concentrates quickly (<2 min) in nuclei, reaching levels that are ≈2-fold greater than those in the cytoplasm. At nuclear envelope breakdown, CF-p13^{suc1} permeates throughout the entire spindle and nonspindle cytoplasm. The protein is excluded from the tightly condensed chromosomes but otherwise no regions accumulate or exclude the protein. It remains evenly distributed throughout metaphase, anaphase, and well into cytokinesis; however, during telophase CF-p13^{suc1} reconcentrates in the daughter nuclei.**

The protein, p13^{suc1}, is essential for the normal function of p34^{cdc2} kinase, which occupies a pivotal position in the control of cell division in eukaryotic organisms (1–4), including plants (5). Depletion of the *suc1* gene has shown that p13^{suc1} is necessary for the inactivation of p34^{cdc2} at anaphase and for completion of mitosis. Cells of fission yeast that lack p13^{suc1} become locked in mitosis and arrest with elongated mitotic spindles that are characteristic of anaphase or telophase (6). A requirement of p13^{suc1} for the inactivation of p34^{cdc2} after metaphase correlates with the evidence for physical association of p13^{suc1} with p34^{cdc2}, which has been widely demonstrated *in vitro* (7) and is indicated *in vivo* by the restoration of division in cells containing some mutant forms of p34^{cdc2}, when p13^{suc1} is expressed at higher levels (8, 9). Like other fundamental regulatory proteins, p13^{suc1} homologues are found within divergent taxa from yeast to humans (10) to flowering plants (11).

Given the interaction of p13^{suc1} with p34^{cdc2} and its role in the regulation of kinase activity, it becomes a matter of great interest where this protein is localized in the cell throughout division. There have been two immunofluorescent studies (12, 13) and both find that p13^{suc1} antibodies, in mammalian cells, stain the nucleus and the cytoplasm. During division, staining is evident within the mitotic apparatus and, of special note, some staining in HeLa cells is localized within the centrosome at the spindle poles (12), a feature that may require detergent extraction to be made evident. The p13^{suc1} staining patterns reported by the two studies, however, are not identical since in HeLa cells Bailly *et al.* (12) note a marked punctate vesicular staining of the interzone at anaphase/telophase, whereas in rat 208F fibroblasts Riabowol *et al.* (13) observe a much more even distribution.

We have been concerned that the standard immunofluorescent methods are not appropriate for spatially localizing p13^{suc1}, a largely soluble protein. Recent work by Melan and Sluder (14) provides convincing evidence that soluble proteins, even under the most favorable preparative immunofluorescent procedures, suffer redistribution when compared to the position of the same protein in a living cell. It is especially noteworthy that soluble proteins may nonspecifically accumulate in the centrosome (14), thus raising questions concerning the significance of those examples, including p13^{suc1} and p34^{cdc2}, which have been localized by immunofluorescent methods to the centrosome.

To avoid problems of protein redistribution, we have microinjected fluorescently tagged p13^{suc1} into living stamen hair cells of *Tradescantia* and determined its spatial localization throughout division by using the confocal laser scanning microscope. Our results show that p13^{suc1} is rapidly targeted to the nucleus. At nuclear envelope breakdown, the protein spills into the entire cell with no evident specific association to spindle elements. Finally, at the end of division, p13^{suc1} reconcentrates in the daughter nuclei.

MATERIALS AND METHODS

Preparation of p13^{suc1} and Carboxyfluorescein (CF) Tagging. p13^{suc1} was obtained by overexpression in *Escherichia coli*, by using the strategy of Brizuela *et al.* (7), and was purified to homogeneity by chromatography on Sephadex G-75 and 2-phenyl-Sepharose, as described by John *et al.* (11). It was tagged as described for tubulin (15), by reaction for 1 hr at 25°C with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (Boehringer) at a dye-to-p13^{suc1} molar ratio of 40:1 in 54 mM $NaHCO_3$, pH 8.5/46% (vol/vol) dimethyl sulfoxide, freed of unbound dye by Sephadex G-25 (Pharmacia) chromatography in 0.1 M $NaHCO_3$ (pH 8.5) and a 10 × 300 mm column, and then dialyzed extensively against water at 4°C. As shown in Fig. 1*A*, the labeled protein is a single band of ≈13 kDa. Essentially all of the p13^{suc1} was recovered and its reaction in a protein dye-binding assay was unaltered; therefore, that assay was used to set protein concentrations for microinjection. The ratio of dye to protein was determined by absorbance at 496 nm, taking ε_{496} = 75,000 $cm^{-1} \cdot mol^{-1}$, and indicated an average of 0.6 dye molecule per p13^{suc1} molecule. Dialyzed protein was lyophilized and redissolved in 100 mM KCl for microinjection, usually at 0.15 mg/ml.

Test of CF-p13^{suc1} Protein Authenticity. Unmodified and fluorescently labeled p13^{suc1} were compared in their ability to bind to plant p34^{cdc2} and to elute it from beads containing covalently coupled p13^{suc1}. Beads coupled with p13^{suc1} at 8 mg/ml of gel were loaded with p34^{cdc2} protein that had been extracted and purified from meristem tissue of seedling wheat leaf as described (11). Aliquots (25 μl) of beads were washed twice with HDW [6 mM Na_2HPO_4/4 mM NaH_2PO_4/2 mM EDTA, pH 7.2/150 mM NaCl/1% Nonidet P-40/leupeptin (5 μg/ml)/0.1 mM sodium vanadate/50 mM NaF, pH 7.2] and then washed once in PMD (7.3 mM Na_2HPO_4/15 mM KH_2PO_4/136 mM NaCl/3 mM KCl/3 mM EDTA, pH 7.4).

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Abbreviation: CF, carboxyfluorescein.

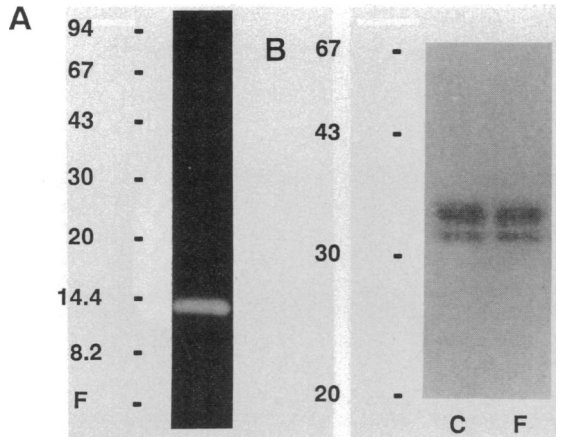

FIG. 1. (*A*) Fluorescently labeled p13^{suc1} separated by SDS/PAGE on a 16% gel. Size markers are as described (7) and absence of extraneous protein bands or fluorescence at the buffer front (F) indicates a single labeled protein uncontaminated by free dye. (*B*) Histone H1 phosphorylated by p34^{cdc2} kinase that was eluted from p13^{suc1} beads by control unmodified p13^{suc1} (lane C) or fluorescently labeled p13^{suc1} (lane F), each at 0.08 mg/ml. Beads containing covalently coupled p13^{suc1} were loaded with partially purified p34^{cdc2} from meristem tissue of seedling wheat leaf, as described (7). After two washes with 40 gel volumes of HDW containing 1% Nonidet P-40 and one wash with HMD, 25-μl portions of gel were suspended in 50 μl of HMD containing unmodified p13^{suc1} (0.08 mg/ml) or the same protein concentration of fluorescently labeled p13^{suc1}. Eluted p34^{cdc2}, which was recovered in the supernatant, was assayed by its phosphorylation of histone H1, under conditions that gave linear response to the amount of enzyme, as described (7).

Bound p34^{cdc2} kinase was eluted by suspension of the gel for 10 min of rotation at 4°C in 50 μl of HMD (25 mM Hepes/10 mM $MgCl_2$/1 mM dithiothreitol, pH 7.4) containing p13^{suc1} at 0.08 mg/ml, determined by the Coomassie dye binding assay. The same protein concentration was used for unmodified and fluorescently labeled p13^{suc1}. Eluted p34^{cdc2} was assayed for H1 histone kinase activity under conditions giving a linear response to amount of enzyme as described (11).

Cell Culture and Microinjection. Stamen hair cells of *Tradescantia* were isolated from young buds and immobilized in 1% low-temperature-gelling agarose (Sigma, type VII) containing 0.02% Triton X-100 (15). When solidified, the preparation was flooded with a buffer medium (5 mM Hepes/5 mM KCl/0.1 mM $CaCl_2$, pH 7.0). Microinjection was performed by the method of Zhang *et al.* (15). Briefly, CF-p13^{suc1} in 100 mM KCl was hydraulically forced through a micropipette into the cell until the introduced volume caused a moderate bulging of the end wall. From previous work, we have determined that this is equal to ≈1% of the cell volume (15). In addition to p13^{suc1}, we injected other macromolecules for control purposes including bovine serum albumin, assembly-incompetent tubulin, calmodulin, and 10-kDa dextran.

Microscopy. The cells were examined with a confocal microscope (Bio-Rad MRC 600, attached to a Zeiss Axiovert microscope; 40×, n.a. 1.3 objective lens). Care was taken to keep chromophore bleaching to a minimum by inserting neutral-density filters (1–0.1% transmittance) to attenuate the laser beam and by using the highest gain setting on the photomultiplier tube to record the faint signals. Images, derived from 10 scans (Kalman averaging), were contrast-enhanced and photographed on Ilford Pan-f 35-mm film directly from a high-resolution video screen. Quantitative fluorescence analysis was carried out on the raw images by using the COMOS program.

RESULTS

Protein Binding Affinity of CF-p13^{suc1}. To investigate whether fluorescent labeling had modified the protein binding properties of p13^{suc1}, its affinity for the p34^{cdc2} kinase was investigated. Binding of soluble p13^{suc1} to p34^{cdc2} kinase can be detected by its ability to elute the enzyme from beads containing covalently coupled p13^{suc1} (7). A subsaturating concentration (0.08 mg/ml) of unmodified or fluorescently labeled p13^{suc1} was tested and ≈20% of the p34^{cdc2} that could be eluted by unmodified p13^{suc1} at 1 mg/ml was eluted (Fig. 1*B*), therefore indicating that the affinity of p13^{suc1} for p34^{cdc2} was not appreciably altered by covalent linkage with CF.

Localization of CF-p13^{suc1} in Living Cells. When CF-p13^{suc1} was microinjected into nondividing stamen hair cells, it quickly (<2 min) accumulated in the nucleus (Fig. 2 *a–d*). Cells injected at various stages of interphase, from post telophase through to prophase, revealed that CF-p13^{suc1} accumulation occurred at all times and thus seemed to be dependent only on the presence of an intact nuclear envelope. Within the nucleus, the protein filled the spaces between the chromatin (Fig. 2*d*); nucleoli also excluded CF-p13^{suc1} (Fig. 2*c*).

To quantitatively determine the distribution of p13^{suc1}, fluorescence measurements were made over the nucleus and selected areas of the cytoplasm, being careful to avoid vacuoles. Background values were obtained from the region adjacent to the cell and were subtracted from the nuclear and cytoplasmic values. The resulting ratio revealed that nuclei contain 1.91 ± 0.41 (mean ± SD, n = 18 cells) times the fluorescence of the cytoplasm.

When nuclear envelope breakdown occurred, marking the onset of prometaphase, CF-p13^{suc1} spilled out of the nucleus and quickly diffused to all parts of the cell uniformly (Fig. 2 *e–h*). With the exception of the chromosomes and vacuoles, both of which were evident as dark regions against a much lighter background, there was no structure that stood out either because it accumulated or excluded CF-p13^{suc1} (Fig. 2 *f–h*). The uniform labeling pattern remained through metaphase, anaphase, and well into cytokinesis (Fig. 3 *a–f*). Although the phragmoplast may have appeared brighter than the rest of the cytoplasm (Fig. 3*g*), this was due to its greater accessible volume resulting from the exclusion of organelles by the palisade of microtubules (15) and not because of an accumulation of p13^{suc1}; space-filling molecules such as bovine serum albumin yielded a similar image (Fig. 4*g*).

During cytokinesis, CF-p13^{suc1} was sequestered in the reforming nuclei (Fig. 3 *h–l*), in a process of reaccumulation, which was slower than that occurring at interphase and prophase. When the cell plate had extended to the two side walls, the daughter nuclei and cytoplasm had similar amounts of CF-p13^{suc1} (Fig. 3*h*); however, during the next 40–50 min, CF-p13^{suc1} reached levels in the daughter nuclei that were ≈2 times that in the surrounding cytoplasm (Fig. 3*l*).

Effects of p13^{suc1} on Cell Division. During the course of these studies, we have monitored the effects of CF-p13^{suc1} on the course of cell division. Given a 1% volume delivery of CF-p13^{suc1} at 0.15 mg/ml, we estimate that the cellular concentration of the protein may have been increased as much as 4-fold during microinjection. Although injected cells tended to progress through mitosis more slowly than uninjected controls, these anomalies did not appear to be specific for CF-p13^{suc1}, since other proteins, such as tubulin (15) and bovine serum albumin (P.K.H., unpublished observations), can show the same effect. For example, the cell depicted in Fig. 4 had been injected in prometaphase and took 40 min to complete metaphase transit (i.e., nuclear envelope breakdown to anaphase onset) and an additional 28 min to initiate a cell plate. Control values on uninjected cells are 32 and 19 min, respectively, for these two events (16).

Localization of Nonspecific Macromolecules. Although it is well known that only some proteins are targeted to the nucleus, we have nevertheless directly tested this question using a variety of nonspecific macromolecules. Our results

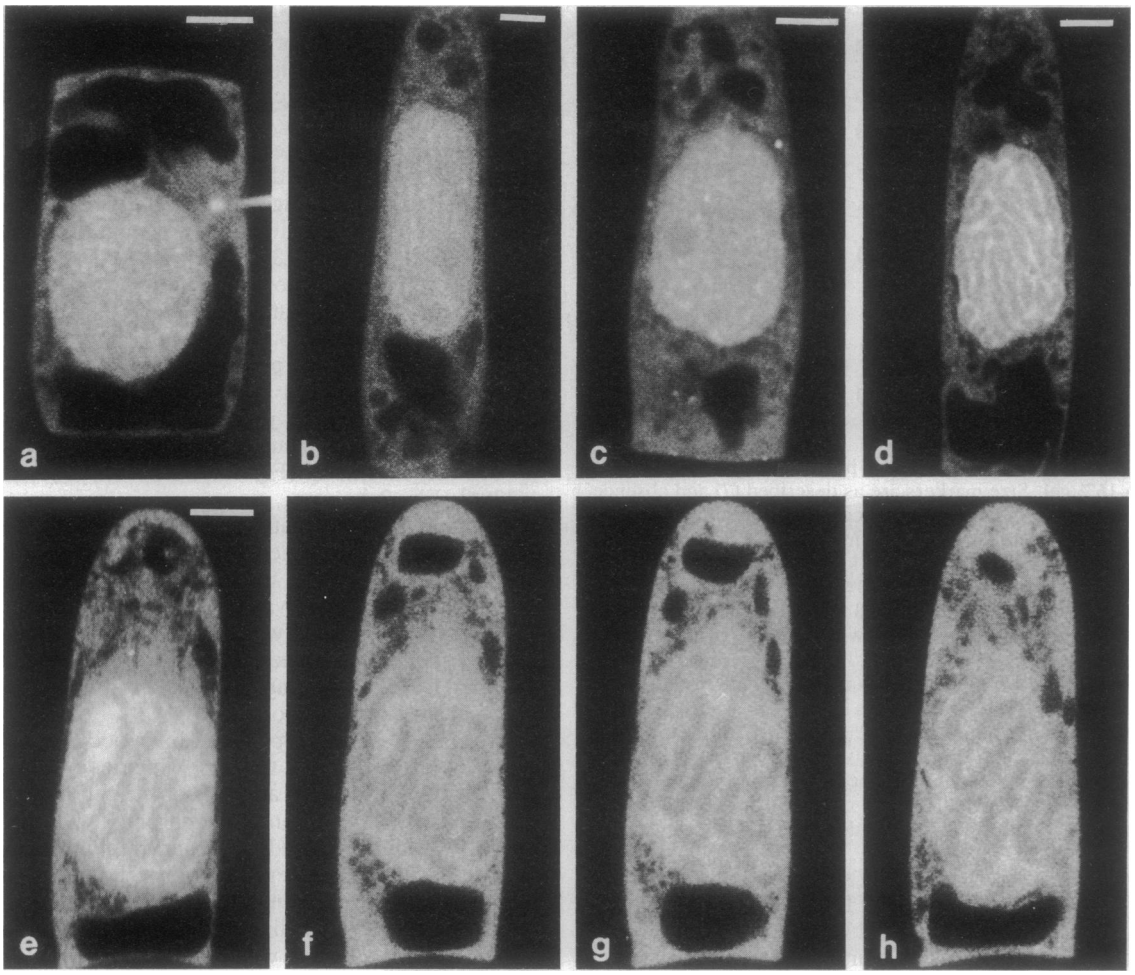

FIG. 2. (*a–d*) Interphase and prophase cells injected with CF-p13^{suc1}. In all instances the p13^{suc1} accumulates in the nucleus. (*a*) There is a bright spot at the end of the injection pipette that is a nonspecific aggregate of p13^{suc1}. Within the nucleus, CF-p13^{suc1} is excluded by the nucleolus (*c*) and condensing chromatin (*d*). (Bar = 10 μm.) (*e–h*) Transition from prophase to metaphase. In prophase CF-p13^{suc1} concentrates in the nucleus (*e*); however, when the nuclear envelope bursts (*f*), the protein immediately spreads throughout the entire spindle and nonspindle cytoplasm (*g* and *h*). At metaphase (*h*), there are no regions or localities of CF-p13^{suc1} accumulation or exclusion. Times, relative to nuclear envelope breakdown 0:00:00 (hr:min:sec), are as follows: *e*, −0:04:30; *f*, 0:00:00; *g*, 0:05:41; *h*, 0:10:12. (Bar = 10 μm.)

revealed that low molecular mass probes, comparable to p13^{suc1}, such as calmodulin (17 kDa; Fig. 4*a*) and fluorescent dextran (10 kDa; Fig. 4*b*), appear to uniformly equilibrate between the nucleus and cytoplasm. In contrast certain large macromolecules, for example, bovine serum albumin (67 kDa; Fig. 4*c*) and assembly-incompetent tubulin dimers (100 kDa; Fig. 4*d*), brightly labeled the cytoplasm but generally did not enter the nucleus. If nuclear envelope breakdown occurred, then the entire cell became fluorescent (Fig. 4*e*). Subsequently during cytokinesis, in cells injected with bovine serum albumin (Fig. 4 *f–j*), when the daughter nuclei reformed, they acquired some fluorescence, presumably from the nonspecific entrapment of protein. However, even here the cytoplasm remained brighter than the nuclei, indicating that the protein had been excluded somewhat from the daughter nuclei (Fig. 4*i*).

DISCUSSION

CF-p13^{suc1} partitions quickly into the nuclei of living nondividing cells, approaching levels 2 times that in the cytoplasm. In the nucleus the protein occupies the space between the condensing chromatin and nucleoli and does not appear to be tightly bound to intranuclear structures, since once the envelope bursts CF-p13^{suc1} quickly spreads throughout the entire cytoplasmic and spindle compartment but without any apparent association with microtubules or other spindle structures. Finally, the results demonstrate that during cytokinesis CF-p13^{suc1} becomes reconcentrated in the daughter nuclei. Since the nuclear/cytoplasmic ratio is identical when p13^{suc1} is introduced at interphase or after its reaccumulation at cytokinesis, we infer that the protein freely equilibrates between compartments when injected into interphase cells. Thus the breakdown and reassembly of the nuclear envelope in the presence of CF-p13^{suc1} does not allow the protein access to compartments or associations that previously might have been stably occupied by endogenous p13^{suc1}.

That the CF-p13^{suc1} is functionally competent is borne out by its ability to bind wheat p34^{cdc2} with an affinity similar to that of nonfluorescent p13^{suc1} (Fig. 1*B*). Since it has been reported that p13^{suc1} when overexpressed delays or blocks division (17–19), there is a question as to why similar defects have not been observed in this study. As noted in *Results*, the observed increase in mitotic transit time in injected cells does not appear to be specific for p13^{suc1} since other injected proteins, including tubulin and bovine serum albumin, routinely show this effect, and thus it is probably due to cell wounding. The lack of a specific effect by p13^{suc1} may stem from an insufficient amount, which is less than that required to produce cytological abnormalities. Although we have increased the cellular p13^{suc1} as much as 4-fold through microinjection, this is considerably less than the ≈100-fold increase that can be generated through overexpression (17, 18). In *Xenopus* extracts, an ≈10-fold

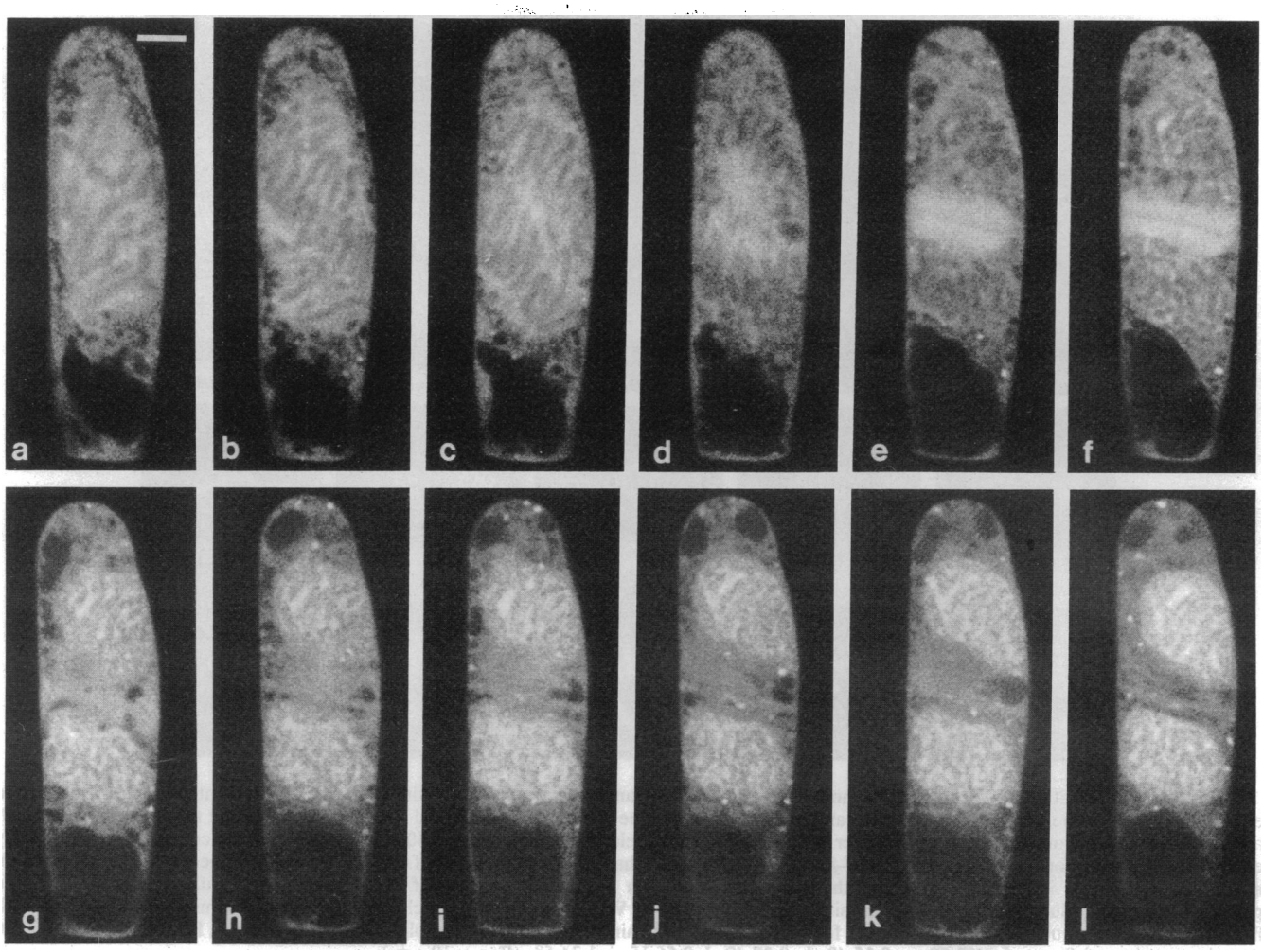

FIG. 3. (*a–l*) Transition from metaphase to the completion of cytokinesis and return to interphase. During metaphase (*a*) and anaphase (*b–d*), there is a uniform distribution of CF-p13^{suc1} throughout the cytoplasm; however, at cytokinesis, there is an apparent accumulation of CF-p13^{suc1} in the phragmoplast (*g*). This is due simply to the exclusion of organelles by the palisade of microtubules, thus creating a greater accessible volume for the CF-p13^{suc1} within the phragmoplast. The phragmoplast microtubules break down when the cell plate reaches the side walls and at this stage there is a uniform accumulation of CF-p13^{suc1} between reforming daughter nuclei and their surrounding cytoplasm (*h*). Subsequently, the nuclei progressively accumulate CF-p13^{suc1} (*i–l*), approaching a 2-fold concentration difference (*l*). Elapsed time, relative to midmetaphase 0:00:00 (hr:sec:min), is as follows: (note, the onset of anaphase was at 0:11:00): *a*, 0:00:00; *b*, 0:14:10; *c*, 0:24:17; *d*, 0:33:35; *e*, 0:48:55; *f*, 0:54:25; *g*, 1:13:04; *h*, 1:23:41; *i*, 1:33:40; *j*, 1:43:10; *k*, 1:53:18; *l*, 2:13:40. (Bar = 10 μm.)

increase in p13^{suc1} was necessary to inhibit tyrosine dephosphorylation and p34^{cdc2} activation (20).

The spatial localization of CF-p13^{suc1} throughout the cell cycle provides information that contributes to our understanding of its function and to that of p34^{cdc2} and, possibly, the cdc25 protein. There is direct genetic evidence that these three proteins have essential interactions during mitosis. The cdc25 protein is expected to overlap in distribution with p34^{cdc2}, at least during prophase when cdc25 phosphatase activity is necessary for dephosphorylation and activation of p34^{cdc2} kinase (21). Direct evidence for the association of the phosphatase with p34^{cdc2}–cyclin B complex is seen in the presence of all three proteins in immunoprecipitates obtained with antibodies against p34^{cdc2} or cdc25 (22, 23). A looser association is formed between p13^{suc1} and p34^{cdc2} that does not survive *in vitro* during washing of immunoprecipitates but the distribution of p13^{suc1} is expected to overlap with p34^{cdc2} at least during anaphase when its presence is essential for inactivation of p34^{cdc2} and completion of mitosis (6). The physical association of p13^{suc1} with p34^{cdc2} is indicated by the usefulness of p13^{suc1} as an affinity reagent *in vitro* (7) (Fig. 1*B*) and its complementation of cdc2 mutants (8, 9). It is also possible that p13^{suc1} and cdc25 interact since p13^{suc1} can inhibit *in vitro* the dephosphorylation of p34^{cdc2}–cyclin B complex by a human cdc25 homologue (23). It is interesting, therefore, that there is remarkably close agreement between the localization pattern described herein for p13^{suc1} and that recently observed using immunofluorescence methods for the cdc25 protein (24) in fibroblasts. The correspondence between these two proteins supports the possibility of regulatory interactions.

The structural localization data for p13^{suc1} and p34^{cdc2} do not show such close correspondence. For example, in the stamen hair, we have not observed the punctate vesicular staining pattern seen for both p13^{suc1} and p34^{cdc2} in the interzone of HeLa cells in anaphase and telophase (12), nor have we ever noted a centrosomal (12, 25, 26) or kinetochore fiber localization (25). Our results on the localization of p13^{suc1} also differ substantially from those using indirect immunofluorescence to localize p34^{cdc2} in dividing root tip cells of *Allium* (27). Although a diffuse distribution is seen for p34^{cdc2} during mitosis, earlier when the nuclear envelope is intact, the protein appears much more concentrated in the cytoplasm than in the nucleus, an observation that is virtually the inverse from that which we report herein for p13^{suc1}. Of particular note Mineyuki *et al.* (27) report a distinct accumulation of p34^{cdc2} in the preprophase band of microtubules. We have not seen a comparable localization for p13^{suc1} in living preprophase and prophase cells of *Tradescantia*. In attempt-

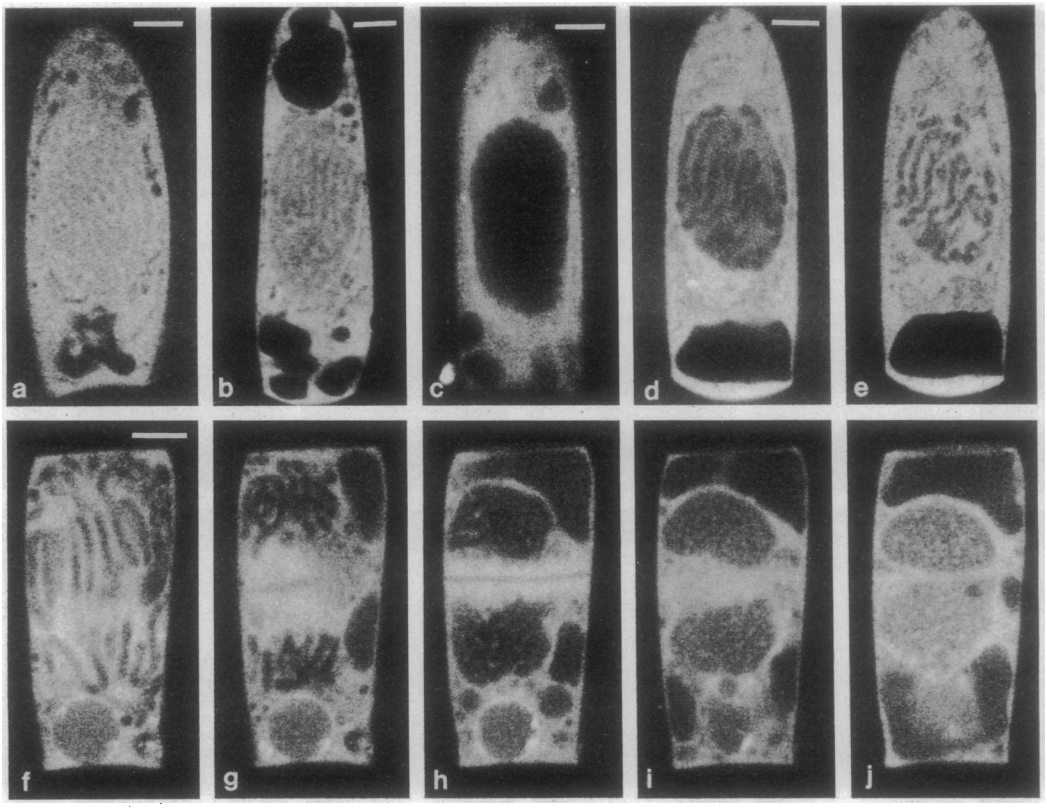

FIG. 4. (*a–e*) Injection of various macromolecular probes into stamen hair cells. Calmodulin (*a*) and fluorescent dextran (*b*) appear to equilibrate between the cytoplasmic and nuclear compartments. Bovine serum albumin (*c*) and assembly-incompetent tubulin (*d*), in contrast, are largely excluded from the nucleus. However, when the nuclear envelope bursts, 2 min and 40 sec later, assembly-incompetent tubulin freely enters the nuclear region (*e*). (Bar = 10 μm.) (*f–j*) Transition from anaphase to the completion of cytokinesis in a cell injected with bovine serum albumin. The protein is spread throughout the cytoplasm, although during early stages of cytokinesis it accumulates somewhat in the phragmoplast, again due to the greater accessible volume (*g* and *h*). When the daughter nuclei reform, some albumin becomes entrapped; however, much is excluded as indicated by the fact that the nuclei remain darker than the cytoplasm (*h*). Elapsed time, from first image 0:00:00 (hr:min:sec), is as follows: *f*, 0:00:00; *g*, 0:15:40; *h*, 0:30:49; *i*, 0:56:15; *j*, 1:34:58. (Bar = 10 μm.)

ing to resolve these various localization patterns, we again note the work of Melan and Sluder (14) showing that soluble proteins may suffer substantial redistribution after the preparative steps necessary for immunofluorescent microscopy. We argue, therefore, that technical, rather than biological, factors confuse our current understanding of the spatial distribution of these key cell cycle proteins, and we emphasize the need for observations on living cells.

In conclusion, we show that $p13^{suc1}$ behaves as a soluble nuclear-targeted protein. The fact that only a small fraction (5%) of $p34^{cdc2}$ may have $p13^{suc1}$ bound at any time (7) complicates inferences made from looking only at the pool of $p13^{suc1}$. Nevertheless, the absence of distinct regions of accumulation of $p13^{suc1}$ within the mitotic apparatus argues against the presence of particular sites of localization, a conclusion that may extend to $p34^{cdc2}$ in the living cell.

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