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In Vitro Fusion of Endocytic Vesicles Is Inhibited by Cyclin A-cdc2 Kinase

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Receptor-mediated endocytosis and recycling are inhibited in mitotic mammalian cells, and previous studies have shown that inhibition of endocytic vesicle fusion in vitro occurs via cyclin B-cdc2 kinase. To test for the ability of cyclin A-cdc2 kinase to inhibit endocytic vesicle fusion, we employed recombinant cyclin A proteins. Addition of cyclin A to interphase extracts activated a histone kinase and markedly reduced the efficiency of endocytic vesicle fusion. By a number of criteria, inhibition of fusion was shown to be due to the action of cyclin A, via the mitosis-specific cdc2 kinase, and not an indirect effect through cyclin B. Two-stage incubations were used to demonstrate that at least one target of cyclin A-cdc2 kinase is a cytosolic component of the fusion apparatus. Reconstitution experiments showed that this component was also modified in mitotic cytosols and was unaffected by N-ethyl maleimide treatment.

INTRODUCTION

Mitosis in mammalian cells is accompanied by profound changes in cellular architecture and function, including disassembly of the nuclear lamina and nuclear envelope breakdown (Gerace and Blobel, 1980), chromatin condensation (Gurley *et al.*, 1978), and assembly of the mitotic spindle (Robbins and Gonatas, 1964). The Golgi complex provides the most striking example of membrane redistribution, breaking down from a single copy organelle to thousands of vesicles via an intermediate of vesicle clusters (Lucocq *et al.*, 1987, 1989). There is also a general cessation of membrane traffic (Warren, 1985, 1993), including inhibition of the endocytic pathway as first described by Fawcett (1965). Both invagination of coated pits (Berlin *et al.*, 1978; Pypaert *et al.*, 1987) and recycling of receptors to the cell surface (Sager *et al.*, 1984; Warren *et al.*, 1984) are blocked. From these observations it can be inferred that fusion between endocytic vesicles is also inhibited in mitotic cells.

Analysis of the mechanism of inhibition of membrane transport during mitosis has been aided by recent advances in understanding the control of the cell cycle (Nurse, 1990). Entry of cells into mitosis is initiated by activation of cdc2 kinase, which requires binding of cyclin to the p34 catalytic subunit (Brizuela *et al.*, 1989; Pines and Hunter, 1989) and posttranslational modification (Dunphy and Newport, 1989; Gautier *et al.*, 1989; Gould and Nurse, 1989; Ducommun *et al.*, 1991; Gautier and Maller, 1991; Gould *et al.*, 1991; Norbury *et al.*, 1991). A- and B-type cyclins accumulate during interphase, rising to maximum levels at the G2-M transition (Evans *et al.*, 1983) when activation of cdc2 kinase occurs. Degradation of cyclins during metaphase (Murray *et al.*, 1989; Glotzer *et al.*, 1991) is followed by inactivation of the kinase. Cellular proteins phosphorylated by cdc2 kinase are dephosphorylated by phosphatases (Ohkura *et al.*, 1989) and the cell returns to the interphase state.

Although both cyclin A and cyclin B are required for the mitotic cycle (Draetta *et al.*, 1989; Lehner and O'Farrell, 1990; Pagano *et al.*, 1992), the precise role that each cyclin plays in mitosis-specific events has yet to be determined. Cyclin A and B accumulate during the cell cycle (Evans *et al.*, 1983; Pines and Hunter, 1989, 1991), and either cyclin mRNA can promote maturation when microinjected into stage VI *Xenopus* oocytes (Swenson *et al.*, 1986; Pines and Hunt, 1987) as

can either cyclin protein (Roy *et al.*, 1991). However, cyclins A and B do not appear to be functionally identical but rather carry out specific roles during mitosis (Draetta *et al.*, 1989; Lehner and O'Farrell, 1990; Whitfield *et al.*, 1990; Knoblich and Lehner, 1993). It is known that the kinetics of activation and degradation of cyclin A- and B-type kinases differ, with levels of cyclin A rising slightly earlier and falling before those of cyclin B (Luca and Ruderman, 1989; Minshull *et al.*, 1990; Whitfield *et al.*, 1990; Pines and Hunter, 1991). Moreover, there are recent reports that cyclin B, but not cyclin A, is able to signal cyclin destruction (Luca *et al.*, 1991; Lorca *et al.*, 1992), indicating, perhaps, that cyclin B-cdc2 kinase controls the release from mitosis. The cellular localization of cyclins A and B also differs. In mammalian cells cyclin A is present in the nucleus during S phase, but as the cell enters prophase, residual cyclin A is relocated to the cytoplasm where it remains during metaphase. In contrast, (presumably inactive) cyclin B is cytoplasmic during interphase and then concentrates around the mitotic spindle in metaphase (Pines and Hunter, 1991). Despite such evidence for distinct roles, the two kinases possess broadly overlapping substrate specificities in vitro, based on their ability to phosphorylate histones at similar sites (Minshull *et al.*, 1990).

The difficulty of activating cyclin A- and cyclin B-type cdc2 kinases independently in mammalian cells has so far prevented analysis, in intact cells, of the role that each kinase plays in inhibition of the endocytic pathway. The availability of assays to measure endocytic events in vitro (Davey *et al.*, 1985; Smythe *et al.*, 1989; for reviews see Warren *et al.*, 1988; Gruenberg and Howell, 1989) offers an alternative approach to study this question. Recently, Tuomikoski *et al.* (1989) reported that the efficiency of endocytic vesicle fusion in vitro was reduced using cytosol from mitotic *Xenopus* eggs compared with interphase *Xenopus* cytosol. Furthermore, vesicle fusion was inhibited by addition of starfish cyclin B-cdc2 kinase to interphase cytosol. We have also demonstrated that vesicle fusion is supported poorly by cytosol prepared from mitotic mammalian cells, which contains levels of cyclin B and cdc2 kinase activity (assessed by histone kinase activity) severalfold higher than cytosol from interphase cells (Woodman *et al.*, 1992).

These observations indicate that vesicle fusion can be blocked in both mammalian and *Xenopus* extracts by the action of cyclin B-activated cdc2 kinase. In a more recent study using *Xenopus* extracts, kinase(s) activated by cyclin A did not inhibit fusion of mammalian endocytic vesicles (Thomas *et al.*, 1992). In contrast, we have employed recombinant proteins to produce mammalian cell extracts containing an activated cyclin A cdc2 kinase and demonstrate that the kinase inhibits endocytic vesicle fusion. Using these extracts, we show that a cytosolic component required for vesicle fusion is a target for cyclin A-cdc2 kinase action.

MATERIALS AND METHODS

Materials

All reagents, unless otherwise specified, were obtained from Sigma Chemical (St. Louis, MO) or BDH Chemicals (Poole, Dorset, U.K.). Microcystin-LR was bought from GIBCO (Paisley, Scotland) and stored in water at −20°C. Sheep anti-transferrin antiserum was obtained from the Scottish Antibody Production Unit (Carluke, Scotland). The kinase inhibitor staurosporine was bought from Calbiochem (San Diego, CA) and stored at −20°C as a 5-mM stock in dimethyl sulfoxide.

Radiolabeling

Human transferrin was radiolabeled with 125-iodine (Amersham International, Amersham, U.K.) to a specific activity of $\sim 10^7$ cpm/μg exactly as described (Woodman and Warren, 1991).

Cells

A431 cells were maintained in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum (FCS), 100 U/ml of both penicillin and streptomycin. Suspension HeLa cells were grown in Eagle's minimum essential medium (MEM) modified for suspension cultures (GIBCO) containing 5% (vol/vol) FCS, 100 U/ml of both penicillin and streptomycin, and nonessential amino acids. Mitotic suspension HeLa cells were prepared by growing cells in the presence of the microtubule inhibitor nocodazole as described previously (Woodman *et al.*, 1992). HeLa cells were synchronized in G1 phase by preparing mitotic HeLa cells from roller bottle cultures (Klevecz, 1975) and then allowing the cells to exit from mitosis in normal growth medium supplemented with 10 μg/ml cycloheximide (Collins and Warren, 1992). FT210 cells and the parent cell-line, FM3A, were maintained at 32°C as suspension cultures in RPMI 1640 medium supplemented with 10% (vol/vol) FCS and 100 U/ml of both penicillin and streptomycin.

Cyclin A Fusion Proteins

Protein A-cyclin A Fusion Proteins. A partial cDNA clone of bovine cyclin A, encoding all but the first 24 residues (compared with the sequence of human cyclin A), was isolated from a bovine lymphocyte cDNA library. The large *Sau*3AI fragment from this clone was inserted into the *Bam*HI site of a vector derived from pET3a encoding the IgG binding domain of protein A (isolated by polymerase chain reaction from pRIT2T [Pharmacia, Piscataway, NJ]). The resulting construct encoded the protein A domain at the N-terminus and cyclin A (minus the first 76 amino acids) at the C-terminus. Mutant *Acc*18 was constructed by deleting the amino acids 273–290 with a *Bal*31 deletion from a unique *Acc*I site.

Escherichia coli strain BL21 (DE3) was transformed with the constructs and synthesis of the fusion proteins and was induced by 0.1 mM isopropylthiogalactosidase (IPTG) at 25°C when the A600 of the culture reached 0.5. The fusion protein was isolated by the method of Solomon *et al.* (1990) with the following modifications. Bacteria were lysed by freeze-thawing and digesting with 2 mg/ml lysozyme in 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, 2 mM EDTA, 1 mM dithiothreitol (DTT), 0.25 mM phenylmethylsulfonyl fluoride (PMSF) at 0°C for 20 min followed by sonication. The extract was centrifuged at 60 000 rpm for 15 min in a TL100.2 rotor in a TL100 centrifuge (Beckman Instruments, Palo Alto, CA), and the supernatant was applied to an IgG-Sepharose column (Pharmacia), equilibrated in 150 mM NaCl, 5 mM DTT, 2 mM EDTA, 50 mM Tris-HCl, pH 8.0. The column was washed with this buffer, followed by 10 mM Na-*N*-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), pH 8.0, 3 mM DTT, and then eluted with 10 mM Na-HEPES, pH 8.0, 3 mM DTT containing 0.2 M lithium diiodo salicylate (Fluka, Buchs, Switzerland). The eluent was desalted into 10 mM Tris-HCl, pH 7.5, 1 mM DTT.

Maltose-binding Protein (MBP)-Cyclin A. The same partial cDNA clone of bovine cyclin A was excised as an *Eco*RI fragment and cloned in frame into the *Eco*RI site of pMAL-c-2 (New England Biolabs, Beverly, MA). *E. coli* strain BL21 (DE3) was transformed, and synthesis of the fusion protein was induced by 0.1 mM IPTG as above. After generation of an extract in lysis buffer, NaCl was added to a final concentration of 0.5 M and the extract was applied to a 2-ml amylose agarose column (New England Biolabs). The column was washed in 10 mM HEPES-NaOH, pH 7.0, containing 0.5 M NaCl, 3 mM DTT, and the construct was eluted in the same buffer containing 10 mM maltose. Peak fractions were combined and desalted into 50 mM KCl, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*,-tetraacetic acid, 50 mM HEPES, pH 7.2, 2 mM $MgCl_2$ (KEHM) buffer.

Membrane Preparations

Donor membrane preparations were made from suspension HeLa cells as follows. Cells (a 1-l culture of $\sim 5 \times 10^5$ cells per ml) were washed three times in phosphate-buffered saline and resuspended in 5 ml of binding medium (MEM containing 20 mM HEPES, pH 7.4, and 2 mg/ml bovine serum albumin [BSA]). The cells were warmed to 37°C and [^{125}I]transferrin was added to 10 μg/ml. After 5 min, the suspension was diluted to 50 ml with ice-cold binding medium, and the cells washed three times in HEPES buffer (HB; 140 mM sucrose, 70 mM potassium acetate, 20 mM HEPES, pH 7.2). The cell pellet was resuspended in 5 ml of HB supplemented with 1 mM DTT and protease inhibitors (1 μg/ml chymostatin, 1 μg/ml pepstatin A, 2 μg/ml E64, 1 μg/ml antipain, 40 μg/ml PMSF). Cells were homogenized and a membrane fraction prepared as described previously (Woodman and Warren, 1989). Acceptor preparations were made from A431 cells containing internalized anti-transferrin antibodies. Full details for the internalization of antibodies and preparation of a membrane fraction are provided elsewhere (Woodman and Warren, 1991).

Cytosol Preparations

HeLa cells ($\sim 5 \times 10^8$ cells in 1 l of growth medium) were harvested by centrifugation and washed three times in KEHM buffer. The final pellet was resuspended in 5 ml of KEHM buffer supplemented with 1 mM DTT and protease inhibitors. Cells were homogenized as for membrane preparations, and the extracts were centrifuged at 400 000 × *g* for 30 min in a TL100 centrifuge. Cytosol was frozen and stored in liquid nitrogen. The abilities of cytosols prepared from mitotic HeLa cells to support vesicle fusion were compared with those of cytosols prepared from parallel cultures of interphase cells.

Cytosols were prepared from parallel cultures of FM3A and FT210 cells grown at the permissive temperature (32°C) and harvested at $\sim 10^6$ cells per ml. Cytosols were made as for HeLa cytosols, except that protease inhibitors were omitted.

Fusion Assays

Assays for endocytic vesicle fusion were carried out as described (Woodman and Warren, 1989) with the following modifications. In a standard assay, cytosol (200 μg) was incubated with 4 μl of an ATP-regenerating cocktail and an appropriate volume of cyclin A fusion protein or control buffer. After 1 h at 37°C, samples were returned to ice and donor membranes (3 μl), unlabeled transferrin (2 μl of 2 mg/ml in water), and acceptor membranes (7 μl) were added, and the final volume was made to 40 μl with KEHM buffer. In some experiments, membrane fractions were preincubated separately for 1 h at 37°C with appropriate amounts of cytosol, cyclin A, and ATP cocktails before mixing. After a further 2 h at 37°C, samples were diluted in ice-cold immunoprecipitation buffer (IB), containing 0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, 5 mM $MgCl_2$, 1% (wt/vol) Triton X-100, 0.5% (wt/vol) sodium dodecyl sulfate, 1% (wt/vol) sodium deoxycholate, and 0.1% BSA, and processed exactly as described (Woodman and Warren, 1989). When experiments included protein A-cyclin A fusion proteins, nonimmune rabbit serum (1 μl) was added in 50 μl HB, and samples were incubated for 30 min at 4°C before addition of IB followed by 30 μl of *Staphylococcus aureus* cells. This additional step ensured that the soluble protein A did not interfere with the immunoprecipitation of [^{125}I]transferrin. Each experiment included a control sample, where the ATP-regenerating cocktail was replaced by an ATP-depleting cocktail of 50 mM glucose and 50 IU/ml hexokinase (4 μl).

Histone Kinase Assays

Cytosols were assayed for histone kinase activity as described earlier (Woodman *et al.*, 1992). Because cytosols activated by cyclin A contained an ATP-regenerating cocktail, it is difficult to express the incorporation of radiolabeled phosphate from [γ-^{32}P]ATP into histones as moles of phosphate per minute. Therefore, all incubations included an ATP-regenerating cocktail, and results were calculated as dpm [^{32}P]phosphate incorporated per μg cytosolic protein per minute.

Phosphatase activity was calculated by measuring the ability of cytosol to release [^{32}P]phosphate from histones prelabeled with cdc2 kinase. cdc2 kinase was activated by mixing MBP-cyclin A (5 μg) with cytosol (1 mg) and 1 mM ATP for 30 min at 37°C. cdc2 kinase was immobilized by incubating for 1 h at room temperature with 50 μl protein A-Sepharose (Pharmacia) precoated with a monoclonal antibody to cdc2. After washing three times in KEHM buffer, the beads were incubated with 300 μl histones (5 mg/ml) and 0.2 mM [γ-32P]ATP. After 30 min at 37°C, the sepharose beads were pelleted to remove active kinase, and the incubation was desalted into KEHM to remove ATP. Samples were incubated with cytosol at 37°C and then spotted onto P81 paper. Release of [^{32}P]phosphate was calculated by subtraction.

Antibodies

Western blot analysis of cytosol preparations for cyclin B was performed as described (Woodman *et al.*, 1992). Immunoprecipitation of histone kinases from cyclin-activated extracts followed the methods described by Hamaguchi *et al.* (1992). Cyclin-activated or control cytosol (200 μg) in KEHM buffer (200 μl) was incubated with anti-cyclin A or anti-cyclin B sera (1 μl) for 2 h at 4°C. Samples were diluted to 1 ml in KEHM containing 0.2 M NaCl and 0.1% Triton X-100 and then transferred to tubes containing 20 μl protein A-Sepharose pellets equilibrated in the same buffer. After incubating for a further 1 h at 4°C on a rotator, samples were washed twice in the same buffer and then resuspended in 25 μl of a histone kinase assay mix and assayed as described above. After the histone kinase assay, samples were spun for 30 s in a microfuge to pellet the protein A-Sepharose and 15 μl of the supernatant was spotted onto P81 paper.

Protein Assays

Protein concentrations were determined by the method of Bradford (1976).

RESULTS

Cyclin A Inhibits Endocytic Vesicle Fusion

Endocytic vesicle fusion was measured as described previously. Vesicles containing [^{125}I]transferrin were mixed with those containing anti-transferrin antibodies. The radiolabeled immune complex formed upon vesicle fusion was isolated on *S. aureus* cells after solubilizing the membranes in detergent. Fusion required cytosol and Mg-ATP (Woodman and Warren, 1988); a background signal obtained from incubations including an ATP-depleting cocktail, in all cases less than 5% of the maximum signal, was subtracted from values to yield

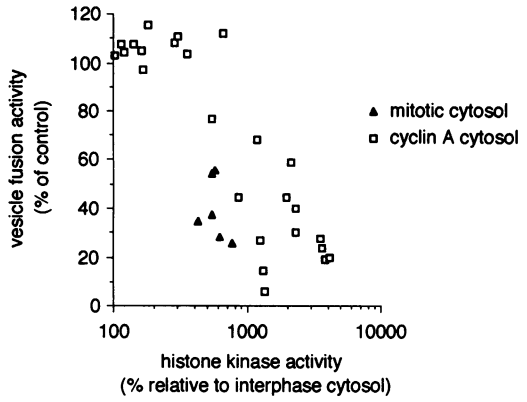

Figure 1. Cyclin A inhibits endocytic vesicle fusion in vitro. Interphase cytosol (200 μg) was preincubated with various quantities of MBP-cyclin A for 1 h at 37°C, and then membrane fractions were added and vesicle fusion activity was measured. Parallel samples were assayed for histone kinase activity after preincubation of cytosol. For comparison, vesicle fusion activity and histone kinase activity were measured using cytosols prepared from mitotic cells without preincubation of cytosol. Vesicle fusion and histone kinase activities for each mitotic cytosol are expressed relative to values for parallel interphase cytosols. Values are means of duplicate determinations.

an ATP-dependent immunoprecipitation of radiolabeled transferrin.

Substitution of cytosol fractions from cells from unsynchronized (interphase) cultures with cytosol prepared from mitotic cells decreased the efficiency of vesicle fusion (Fig. 1) as described in an earlier study (Woodman *et al.*, 1992). The fall in fusion activity correlated with the elevation of levels of cyclin B protein (see Fig. 6A) and histone kinase activity, which we ascribe to cyclin B-cdc2 kinase. It is likely that the measurement of histone kinase activity underestimated the true extent of cyclin B-cdc2 kinase activation in mitotic cells, because the increase in cyclin B levels appeared greater than the increase in kinase activity. Furthermore, histone kinase activities fall significantly during the course of fusion incubations (see Woodman *et al.*, 1992).

To investigate whether cyclin A-cdc2 kinase might also regulate vesicle fusion, we added a recombinant fusion protein between MBP and the complete sequence excepting the first 24 amino acids of bovine cyclin A (MBP-cyclin A) to incubations containing interphase cytosol. This produced a rapid, stable, and titratable activation of histone kinase activity. Maximal kinase activity (requiring ~1 μg MBP-cyclin A) appeared severalfold higher than that measured from extracts of mitotic cells and was accompanied by marked inhibition of endocytic vesicle fusion. In general, titers of MBP-cyclin A that inhibited vesicle fusion to levels supported by mitotic cytosols produced histone kinase measurements that were two to three times greater. Maximal inhibition at each concentration of MBP-cyclin A required preincubation of MBP-cyclin A with cytosol and ATP before addition of the membrane fractions to the incubation. In a typical experiment preincubation of the cytosol fraction for 60 min with MBP-cyclin A increased the extent of inhibition from 30 to 72%.

Vesicle fusion was also inhibited by inclusion of a fusion protein between the IgG-binding domain of protein A and the C-terminal 356 residues of bovine cyclin A (pA-cyclin A) (Table 1). Pretreatment of pA-cyclin A at 95°C or with elastase abolished the ability of the fusion protein both to activate histone kinase and to inhibit vesicle fusion. Inclusion of an equal amount of protein A, or addition of the pA-cyclin A fusion protein just before the immunoprecipitation step, did not affect the fusion signal.

Cyclin A Inhibits Vesicle Fusion via cdc2 Kinase

We ascribe the inhibition of endocytic vesicle fusion by cyclin A–containing fusion proteins to activation of a histone kinase. Two lines of evidence support this. First, similar concentrations of the protein kinase inhibitor staurosporine blocked both the histone kinase activity and the inhibition of vesicle fusion elicited by MBP-cyclin A (Fig. 2). Second, a fusion protein between protein A and a deletion mutant of cyclin A (*Acc*18), which is unable to bind to and activate cdc2 kinase (Kobayashi *et al.*, 1992), was compared with the wild-type fusion protein. *Acc*18 cyclin A failed to block endocytic vesicle fusion (Fig. 3) in a staurosporine-sensitive manner, whereas the same amount of wild-type pA-cyclin A reduced vesicle fusion by over 80% of the control value.

These data confirm that cyclin A inhibits vesicle fusion via the activation of a protein kinase. Recent evidence, however, has shown that cyclin A activates both the

Table 1. Inhibition of fusion requires active cyclin protein

Addition	ATP-dependent immunoprecipitation of [125-I] transferrin (cpm)	Histone kinase activity (relative)
None	622	1.00
Cyclin A	94	9.00
Heat-treated cyclin A	602	1.16
Elastase-treated cyclin A	500	0.93
Inactivated elastase	520	n.d.
Protein A	602	n.d.
Cyclin A (added at end of incubation)	611	n.d.

Interphase cytosol (100 μg) was preincubated with ATP and the following additions: 0.2 μg protein A—cyclin A; 0.2 μg protein A—cyclin A pretreated for 15 min at 95°C; 0.2 μg protein A—cyclin A pretreated for 15 min at 37°C with 0.4 μg elastase and then quenched with 0.1 μg PMSF; 0.4 μg elastase quenched with 0.1 μg PMSF; 0.2 μg protein A; 0.2 μg protein A—cyclin A added after the 2-h incubation with membranes but before processing to measure fusion activity. In a parallel experiment, histone kinase activity was measured after pretreating cytosols as above. Results are means of duplicates.

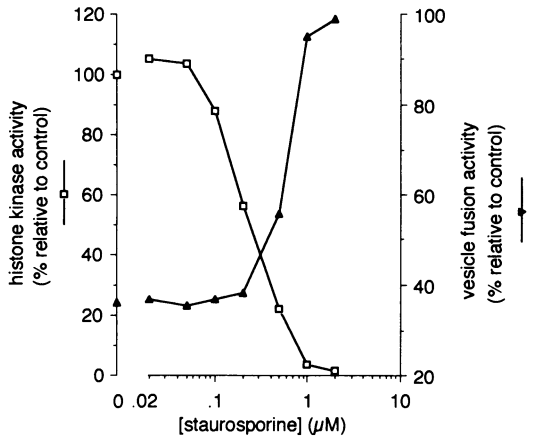

Figure 2. Inhibition by cyclin A requires a histone kinase activity. Cytosol (200 μg) was preincubated with 1 μg MBP-cyclin A containing the indicated concentrations of staurosporine. After 1 h, parallel samples were assayed for vesicle fusion activity and histone kinase activity in the same final concentrations of staurosporine. One hundred percent vesicle fusion activity indicates activity without either MBP-cyclin A or staurosporine. Values are means of two experiments, each performed in duplicate.

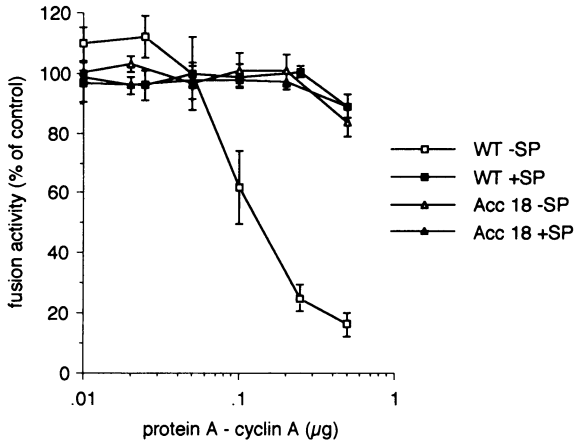

Figure 3. A mutant cyclin A does not inhibit vesicle fusion. Vesicle fusion activity was assayed using the indicated amounts of either wild-type pA-cyclin A (WT) or a deletion mutant of pA-cyclin A (*Acc*18) in the presence or absence of 10 μM staurosporine (SP). One hundred percent vesicle fusion activity indicates activity without either pA-cyclin A or staurosporine. Values are means ± SEM from three experiments, each performed in duplicate.

mitotic cdc2 kinase and the related cdk2 kinase that is required for transition of cells into S phase (Fang and Newport, 1991; Pagano *et al.*, 1992). To establish beyond doubt that inhibition was mediated via the mitotic kinase, we took advantage of the recent identification of a murine cell-line (FT210) with a temperature-sensitive lesion in cdc2 kinase activity (Th'ng *et al.*, 1990; Hamaguchi *et al.*, 1992). Decline of cdc2, but not cdk2, activity occurs rapidly both in vivo and in vitro upon shifting from 32 to 39°C (Th'ng *et al.*, 1990). We therefore examined whether vesicle fusion incubations employing cytosols from FT210 cells were refractory to inhibition by cyclin A at the restrictive temperature. A compromise of 37°C was used as the restrictive temperature for this study, because both vesicle fusion activity and the ability of cyclin A to inhibit fusion were reduced significantly at 39°C in wild-type (FM3A) and in HeLa extracts. However, even at 37°C, the ability of cyclin A to inhibit vesicle fusion using extracts from FT210 cells compared with those from the parent FM3A cells was reduced markedly (Fig. 4). Inhibition by cyclin A in FT210 extracts did not reach the level found in FM3A extracts even at the permissive temperature, consistent with previous reports that levels of cdc2 protein in FT210 cells are reduced compared with those in wild-type cells even at the permissive temperature (Th'ng *et al.*, 1990).

Our previous study (Woodman *et al.*, 1992) and those of others (Tuomikoski *et al.*, 1989; Thomas *et al.*, 1992) have indicated that endocytic vesicle fusion is blocked by cyclin B-cdc2 kinase. The results presented above demonstrate that cyclin A also inhibits fusion via cdc2 kinase. However, extracts from unsynchronized cell cultures contain some inactive cyclin B-cdc2, and it remained formally possible that inhibition of fusion by cyclin A could be accounted for by subsequent activation of endogenous cyclin B-cdc2 rather than by the direct action of cyclin A. Indeed, some evidence suggests that cyclin A-cdc2 kinase might activate cyclin B-cdc2 kinase via cdc25 protein in vivo (Devault *et al.*, 1992; Lee *et al.*, 1992). Two lines of argument preclude such an interpretation of our results. First, we found no evidence that cyclin A could activate a cyclin B-associated, or other, downstream histone kinase activity to any significant extent in these extracts. In a typical experiment,

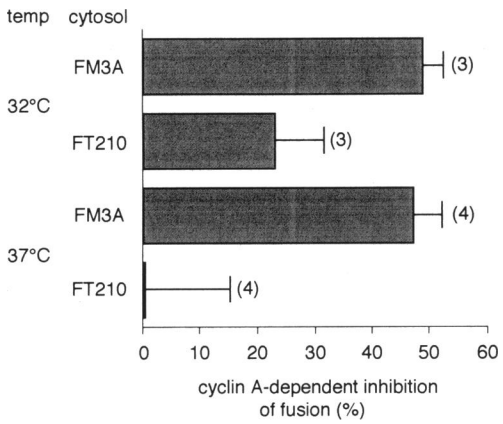

Figure 4. Inhibition by cyclin A requires active cdc2 protein. Cytosols (200 μg) prepared from FT210 or FM3A cells were incubated with or without MBP-cyclin A (2 μg) for 1 h at the indicated temperature, and then vesicle fusion was assayed as above and cyclin-dependent inhibition of fusion calculated. Values are means ± SEM from the indicated number of experiments, each performed in duplicate.

shown in Figure 5, MBP-cyclin A was added to interphase cytosol at sufficient concentration to cause a 550% increase in histone kinase activity. Kinase activity immunoprecipitated with an anti-cyclin A antiserum rose by 490%. In contrast, activity precipitated with an anti-cyclin B antiserum rose by only 90%, whereas that precipitated with a nonspecific serum rose by 50%. In support of this data, we also observed that when previously activated pA-cyclin A-cdc2 kinase was immobilized on IgG Sepharose beads and subsequently incubated with interphase cytosol in the presence of an ATP-regenerating cocktail, ~95% of the histone kinase activity present in the incubation could be reisolated with the IgG Sepharose beads. Therefore, virtually all the histone kinase activity in these incubations remained associated with cyclin A.

Second, if cyclin A inhibited vesicle fusion via activation of cyclin B-cdc2 kinase, one would expect that the extent of inhibition by cyclin A would depend on the concentration of cyclin B present in the cytosol preparation. As stated previously, cytosols prepared from nonsynchronized populations of cells contain low but significant amounts of cyclin B. However, because cyclin B is destroyed as cells progress through mitosis (Evans *et al.*, 1983; Murray *et al.*, 1989; Pines and Hunter, 1989), levels of cyclin B protein in cytosols prepared from cells synchronized in G1 phase should contain far less cyclin B than cytosols from normal interphase cells. Western blot analysis of cyclin B levels in mitotic cytosols, interphase cytosols, and cytosols from cells chased into G1 in the presence of cycloheximide shows that this was the case (Figure 6A). Despite the reduction of cyclin B levels in G1 cytosol by ≥80% compared with interphase cytosol, the titer and extent of inhibition of vesicle fusion by MBP-cyclin A remained unaltered (Figure 6B).

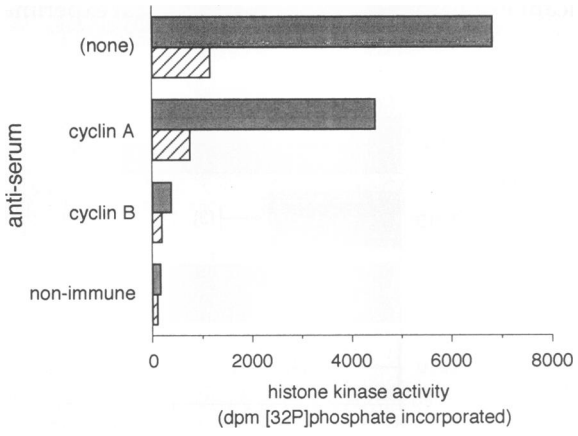

Figure 5. Cyclin A does not activate cyclin B-cdc2 kinase. Cytosol (200 μg) was incubated for 30 min at 37°C with (shaded bars) or without (hatched bars) MBP-cyclin A (0.2 μg). Samples were mixed with anti-cyclin A, anti-cyclin B, or nonimmune sera, and histone kinase activities were immunoprecipitated onto protein A-sepharose. Parallel samples (5 μg) of control or cyclin A-activated cytosols that were not treated for immunoprecipitation (none) were also assayed for histone kinase activity. Results are means of duplicate determinations of histone-dependent incorporation of [^{32}P]phosphate (dpm).

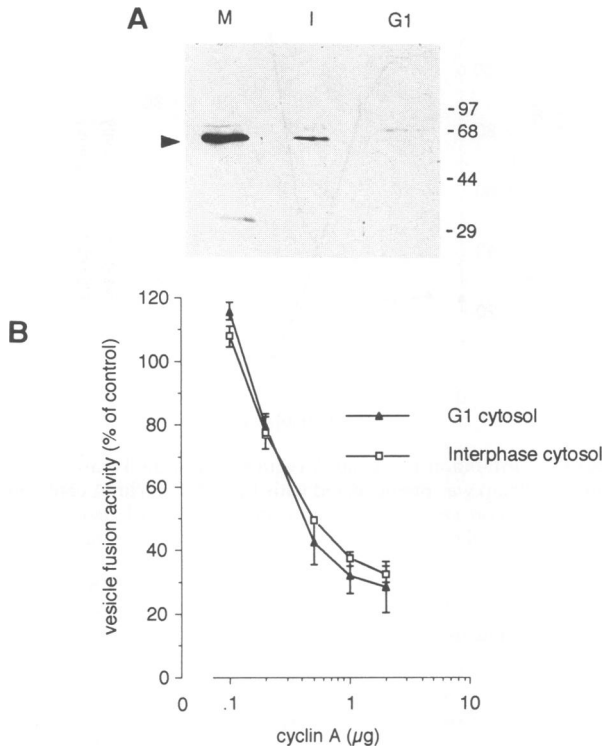

Figure 6. Cyclin A inhibits vesicle fusion in G1 phase cytosols. (A) Cytosols (25 μg) prepared from mitotic cells (M), nonsynchronized cells (I), and G1 cells (G1) were analyzed by immunoblotting for levels of cyclin B protein. Cyclin B is indicated by the arrow. (B) MBP-cyclin A was titrated against endocytic vesicle fusion activity using cytosols from G1 or nonsynchronized cells. Values are the means from three experiments, each performed in duplicate.

Preincubation of Cytosol with Active cdc2 Kinase Inhibits Fusion

Maximal inhibition of fusion demands preincubation of the cytosol fraction with cyclin A, suggesting that the kinase may modify cytosolic component(s) of the fusion apparatus. To explore this possibility further we used two-stage incubations in which membranes were added to the incubation mix after inhibition of cdc2 kinase by staurosporine. As shown in Figure 7A (*i* versus *ii*), staurosporine effectively blocked the ability of MBP-cyclin A to inhibit vesicle fusion when added at the start of the incubation. In contrast, inhibition of fusion was only partially reversed when staurosporine was added to samples after cytosol had been preincubated with cyclin for 2 h, despite ablation of histone kinase activity (Figure 7A, *iv*). Addition of staurosporine halfway through the preincubation restored activity to an intermediate level

(Figure 7A, *iii*). These findings are most simply explained by the cdc2-dependent phosphorylation of a cytosolic component of the fusion apparatus. Subsequent inhibition of the kinase would allow partial dephosphorylation of its substrate by endogenous cytosolic protein phosphatases. This explanation was supported by addition of the specific phosphatase inhibitor microcystin-leucine arginine (LR) (MacKintosh *et al.*, 1990) at the same time as staurosporine. When both were added at the end of the preincubation, or indeed halfway through, vesicle fusion was now inhibited almost in full (Figure 7A, *v–vii*). A delay in addition of microcystin-LR for 60 min after addition of staurosporine allowed substantial recovery of fusion activity (Figure 7A, *viii*). Staurosporine alone, or in combination with microcystin-LR, did not affect vesicle fusion in control-treated samples in a significant or systematic fashion (see Figures 2 and 7B).

Similar to interphase cytosols treated with cyclin A, mitotic cytosols remained deficient in vesicle fusion activity even after inhibition of cdc2 kinase. Inclusion of staurosporine and microcystin-LR did not restore fusion activity (Figure 7B), even though histone kinase activity was reduced by 96%. In contrast to cyclin A-activated cytosol, addition of staurosporine alone did not lead to any restoration of fusion activity.

Inhibition of Fusion Is Reversed by Addition of Untreated Cytosol

We have shown that cyclin A-cdc2 kinase inhibits vesicle fusion even when its activity is removed before addition of the membrane fractions. The simplest interpretation is that components of the fusion apparatus found in the cytosol fraction are modified by cyclin A-cdc2 kinase. Addition of both kinase inhibitor and phosphatase inhibitor "freezes" the phosphorylation state of these components. Hence, fusion activity should be restored after addition of the inhibitors by supplementing the incubation with untreated cytosol. This prediction is confirmed in the experiment illustrated in Figure 8A. Fusion was inhibited by 65% when staurosporine and microcystin-LR were added after preincubation of cytosol, compared with when they were added at the start of the incubation. Subsequent addition of increasing amounts of control-treated cytosol to the incubation increased fusion activity, so that addition of an equal amount of cytosol restored activity to >100% of the control value. This contrasted with the limited increase in fusion activity when the incubation was supplemented with MBP-cyclin A–treated cytosol.

Incubations contained a large excess of microcystin-LR to ensure that the restorative activity of added cytosol was not due to added phosphatase activity. This was confirmed by measuring the ability of cytosol to remove phosphate from histones prelabeled by cyclin A-cdc2 kinase. Phosphatase activity present in 200 or 400 μg of cytosol was almost completely blocked by the concentration of microcystin-LR (4 μM) employed in this study (Figure 8B).

The experiments described above demonstrate that a cytosolic component of the fusion apparatus is a substrate for cdc2 kinase. However, it is possible that mem-

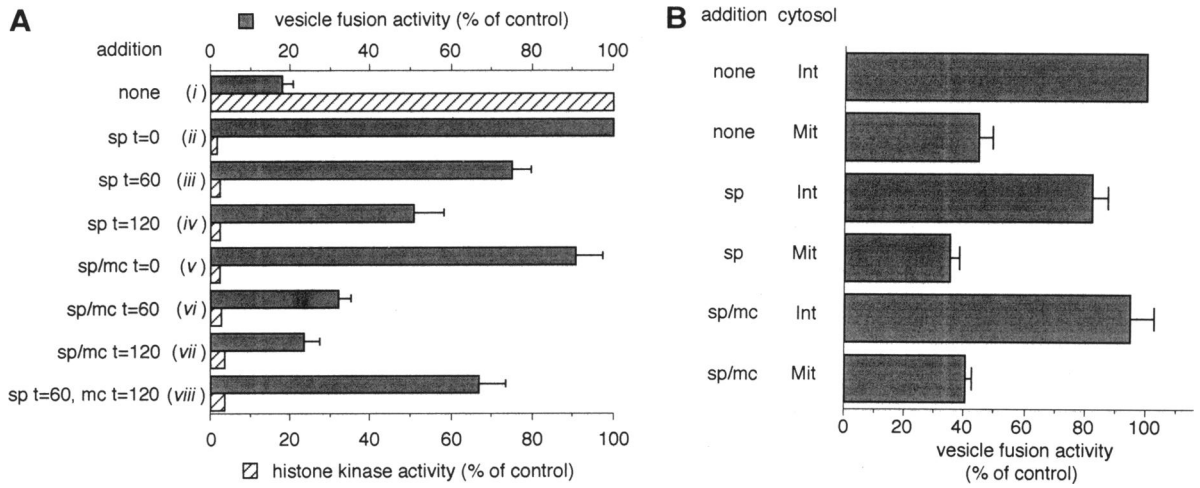

Figure 7. Preincubation of cytosol leads to staurosporine-insensitive inhibition. (A) Cytosol (200 μg) was preincubated for 120 min at 37°C with 1 μg MBP-cyclin A, and then parallel samples were assayed for vesicle fusion and histone kinase activities. At the indicated times (in minutes) during the preincubation, staurosporine (sp) and microcystin-LR (mc) were added as shown (final concentrations 10 and 4 μM, respectively). Values are means of two experiments (histone kinase) or three experiments ± SEM (fusion), all performed in duplicate. Histone kinase 100% indicates the activity of cytosol activated by cyclin A. Fusion 100% indicates the activity when staurosporine was added at t = 0. (B) Mitotic and interphase cytosols (200 μg) were assayed for vesicle fusion activities. Incubations contained combinations of staurosporine (sp; 10 μM) and microcystin-LR (mc; 4 μM) as indicated. Values are means ± SEM of three experiments, each performed in duplicate (100% = interphase cytosol without inhibitors).

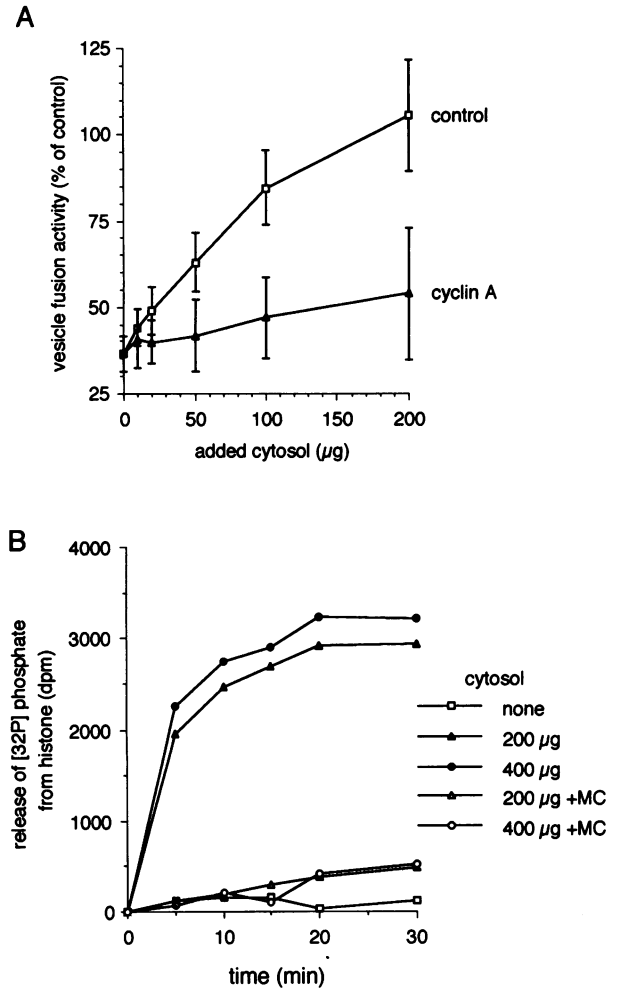

Figure 8. Fusion activity is restored by addition of untreated cytosol. (A) Cytosol (200 μg) was incubated with 1 μg MBP-cyclin A for 1 h, and then staurosporine (sp) and microcystin-LR (mc) were added to final concentrations of 10 and 4 μM, respectively. Membrane fractions were added and the incubations were supplemented with cytosol pretreated for 1 h at 37°C with ATP and control buffer or MBP-cyclin A (1 μg per 200 μg cytosolic protein), as indicated. A control incubation containing staurosporine and microcystin-LR from t = 0 represents 100%. Values are means ± SEM of three experiments, each performed in duplicate. (B) Phosphatase activity of cytosol with or without microcystin-LR (mc; 4 μM) was determined by measuring the release of [^{32}P]phosphate from histones prelabeled with cyclin A-cdc2 kinase. Values are means of duplicates.

brane proteins might also be targets for cdc2 kinase. To address this point we looked at the ability of cytosol to restore activity after both cytosol and membrane fractions were preincubated with the kinase. Donor and acceptor membranes were preincubated separately with MBP-cyclin A and cytosol before addition of the staurosporine/microcystin-LR mix. As expected, fusion was inhibited by ~70% compared with the value when the inhibitors were added at the start of the incubation (Figure 9). However, fusion activity was restored to greater than the control level when control-treated cytosol was added before combining the separate membrane preincubations. In contrast, addition of cytosol pretreated with MBP-cyclin A produced very little additional fusion activity. It is known that at least one cytosolic component of the vesicle fusion apparatus is sensitive to N-ethyl maleimide (NEM) (Braell, 1987; Diaz *et al.*, 1989). However, NEM treatment only marginally affected the ability of cytosol to restore fusion activity to samples pretreated with cyclin A-cdc2 kinase (Figure 9). In contrast to NEM-treated cytosol, mitotic cytosol had limited ability to restore activity to cyclin A-treated samples when compared with equal concentrations of interphase cytosol (Figure 10). The increase in activity provided by mitotic cytosol was only 45% of that provided by interphase cytosol, reflecting the reduced ability of mitotic cytosol to support vesicle fusion under normal conditions.

DISCUSSION

Previous work, using either semipurified enzymes or extracts from mitotic cells, has demonstrated that endocytic vesicle fusion in vitro is inhibited by cyclin B-

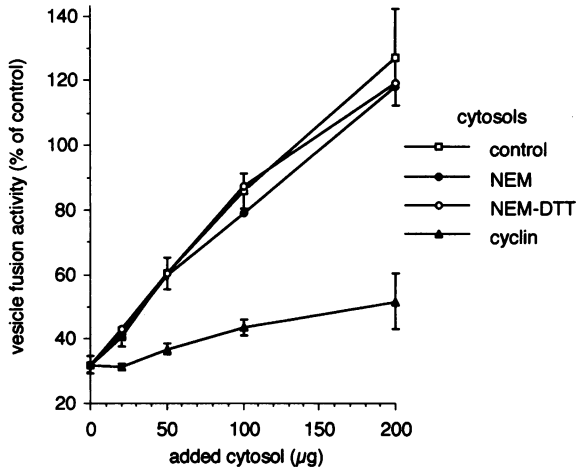

Figure 9. Restorative activity of cytosol is NEM insensitive. Donor and acceptor membranes were treated with 3 mM NEM for 30 min at 4°C, and then 3 mM DTT was added. Membranes were incubated separately with ATP, cytosol (200 μg total, prepared without DTT), and MBP-cyclin A (1 μg total) for 1 h at 37°C. Staurosporine (10 μM) and microcystin-LR (4 μM) were added, and the incubations were combined and continued for a further 2 h to measure fusion activity. Samples were supplemented after addition of inhibitors with the indicated amounts of cytosol. Cytosol treatments are as follows. Control: 1 h at 37°C with an ATP cocktail. NEM: +2 mM NEM, 30 min 4°C, +3 mM DTT quench and then 1 h 37°C with ATP. NEM-DTT: as NEM, but DTT and NEM added together. Cyclin: as NEM-DTT but incubated with MBP-cyclin A (1 μg per 200 μg cytosol) for 1 h at 37°C. A control sample, containing microcystin-LR and staurosporine from t = 0, represents 100% activity. Values are means ± SEM of three experiments or two experiments (NEM, NEM-DTT) each in duplicate.

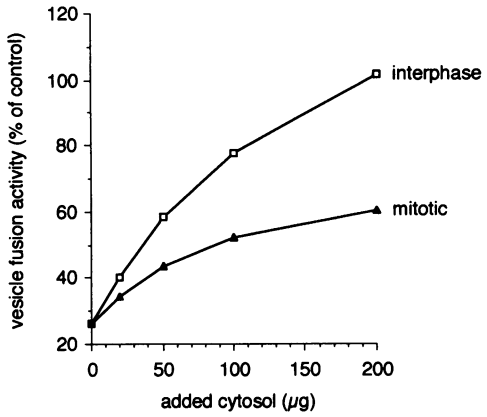

Figure 10. Limited restoration of fusion activity by mitotic cytosol. (A) Donor and acceptor membranes were incubated separately with cytosol (200 μg total) and MBP-cyclin A (1 μg) for 1 h at 37°C, and then staurosporine (10 μM) and microcystin-LR (4 μM) were added. The membranes were combined and supplemented, as indicated, with interphase or mitotic cytosols that had been pretreated for 1 h at 37°C with ATP and staurosporine/microcystin-LR. Values are means of two experiments, each performed in duplicate.

cdc2 kinase (Tuomikoski *et al.*, 1989; Woodman *et al.*, 1992). This study investigates whether cyclin A-cdc2 can also regulate vesicle fusion in vitro. In this article we have controlled the activity of cyclin A-cdc2 kinase by utilizing a reagent that activates latent protein present in interphase cytosol. Solomon *et al.* (1990) showed that a recombinant cyclin B fusion protein could activate cdc2 kinase in interphase extracts of *Xenopus* eggs. We constructed and purified soluble fusion proteins containing all or part of bovine cyclin A. One protein contained the IgG-binding domain of protein A and the C-terminal 356 residues of bovine cyclin A. Loss of the first 76 residues of the cyclin A removed the "destruction domain" that is required for cell cycle–specific proteolysis of the protein (Glotzer *et al.*, 1991) but did not impinge on the kinase activation domain, which starts at about residue 161 (Kobayashi *et al.*, 1992). The other protein contained all but the first 24 amino acids of bovine cyclin A fused to MBP. Both fusion proteins produced a stable and titratable increase in histone kinase activity, which we ascribe to activation of cdc2 kinase. The two proteins inhibited endocytic vesicle fusion at approximately the same concentration.

An important consideration is the amount of cyclin A required to elicit a response. Unfortunately, we have been unable to activate cyclin B-cdc2 kinase in interphase mammalian extracts by addition of recombinant cyclin B, so that direct comparison of the efficacy of cyclin A- and B-kinases has not been possible. However, our results indicate that inhibition of fusion by cyclin A cannot be attributed simply to the use of inappropriate amounts of cyclin. Although the titer of cyclin varied slightly between batches, in all cases maximal inhibition was observed using ≤0.2 μM cyclin A. This concentration is lower than that of cyclin B used in a similar study (Thomas *et al.*, 1992) and compares both with the concentration of cdc2 found in the cell (see Desai *et al.*, 1992) and with levels of cyclin A required to induce meiosis (Roy *et al.*, 1991) or activate cdc2 kinase (Clarke *et al.*, 1992) in *Xenopus*. We also compared cyclin A-activated cytosols with cytosols prepared from mitotic cells that contained elevated levels of cyclin B-cdc2 kinase. Inhibition by cyclin A was accompanied by histone kinase activities two to three times greater than those of cytosols from mitotic cells. However, this difference appears to reflect the relatively low histone kinase activities of our mitotic cytosols, because the increase in kinase activity in mitotic cytosols compared with interphase cytosols was less than the increase in cyclin B levels. Pines and Hunter (1989) showed that cyclin B levels increased 20-fold between G1 and M phases in HeLa cells. It is known that activation of the kinase at G2-M requires both increased cyclin levels and the action of other regulators (Ducommun *et al.*, 1991; Gautier and Maller, 1991; Gould *et al.*, 1991; Kumagai and Dunphy, 1991; Norbury *et al.*, 1991; Clarke *et al.*, 1992), so that the real increase in cyclin B-cdc2 kinase activity within the cell is likely to be far higher than the five- to sevenfold shown by our extracts. This is probably a consequence of the relative instability of histone kinase isolated from mitotic cells (β-glycerophosphate could not be used to stabilize the kinase, because it inhibited, to some extent, the fusion reaction), coupled with the evident resistance of the mitotic-specific inhibition of fusion to removal of cdc2 kinase activity. Hence, during the course of cytosol preparation and dilution into the assay mix, histone kinase activity would be expected to fall substantially, whereas the inability of these cytosols to support vesicle fusion would remain unaffected. An additional reason for the discrepancy between mitotic and cyclin A-activated extracts might be that cyclin A activates other histone kinase activities (for example cdk2) that are unlikely to play a role in cyclin A-mediated inhibition of fusion, so that only part of the histone kinase activity would contribute to the inhibition we observe.

We established that cyclin A inhibited vesicle fusion via a cdc2-like kinase in two ways. First, the kinase inhibitor staurosporine (Tamaoki *et al.*, 1986), which has been shown to inhibit *Xenopus* cdc2 kinase (Gotoh *et al.*, 1991), blocked at the same concentration both histone kinase activity and the inhibition of vesicle fusion. Second, a fusion protein containing a deletion mutant of cyclin A, unable to activate cdc2 kinase, did not inhibit vesicle fusion.

Recent studies have shown that cyclin A associates not only with cdc2 but also with a novel kinase, cdk2 (p33) (Fang and Newport, 1991; Devoto *et al.*, 1992), that is active at the G1/S boundary in the cell cycle. Although cdk2 associates with other S phase–specific cyclins (Elledge and Spottswood, 1991), cyclin A plays

a role in this transition, as well as at G2/M (Pagano *et al.*, 1992). It is unlikely that cyclin A inhibits vesicle fusion via cdk2, because cell-cycle specific inhibition of membrane transport is restricted to mitosis. However, to show beyond doubt that cdc2 kinase mediates cyclin A–dependent inhibition, we made use of the cell line FT210, which contains a temperature-sensitive lesion of cdc2 that prevents entry into mitosis at the restrictive temperature (Th'ng *et al.*, 1990). Cytosols from the mutant cell line had reduced capacity to mediate inhibition by cyclin A compared with wild-type cells. Although a temperature dependence of this effect was present, a partial phenotype was evident even at the permissive temperature. This is consistent with the reduced levels of cdc2 protein found in FT210 cells even when grown at 32°C (Th'ng *et al.*, 1990) and with the increased doubling time of FT210 cells at 32°C, compared with the parent cell line. It is interesting to note that specific inhibition by cyclin A using the wild-type murine cell was restricted to ~50%. Lower levels of cyclin A inhibited fusion in extracts from three human cell lines (HeLa, K562, and A431) by up to 80%. This may suggest some species specificity in the action of the cyclin A fusion proteins.

As outlined in the INTRODUCTION, the exact roles of cyclin A- and B-kinases in vivo remain unclear. The present work demonstrates that cyclin A, as well as cyclin B, elicits a mitosis-specific functional change in vitro, though further work is required to show that the two kinases act by the same mechanism. This finding has important implications for the control of membrane transport as mammalian cells enter mitosis; assignment of each kinase to a particular role in vivo cannot be determined by biochemical specificity alone but must be influenced by other factors. Chief among these would be the spatial and temporal organization of the active kinase complexes during the G2-M transition. Perhaps closer examination of both the location and state of activation of each kinase during early prophase, when endocytosis is first blocked (Berlin *et al.*, 1978; Warren *et al.*, 1984), might provide an answer to this problem.

Further examples of comparisons of actions of cyclin A- and B-kinases in vitro are limited. However, one study using *Xenopus* extracts shows cyclin-specific effects on microtubule dynamics (Verde *et al.*, 1992). Cyclin A also inhibits transport of vesicular stomatitis virus (VSV) G protein between compartments of the Golgi complex in vitro in a kinase-dependent manner (Stuart *et al.*, 1993). This study and ours are at variance with the report of Thomas *et al.* (1992), who find that cyclin B, but not cyclin A, blocks endocytic vesicle fusion in *Xenopus* extracts. One might attribute such a difference to differences in the assay systems. In particular, the study by Thomas *et al.* (1992) used activated *Xenopus* egg cytosols that would contain very low levels of endogenous cyclins. In contrast, the interphase extracts that we have used contained more substantial levels of cyclin B. Could the inhibition ascribed to cyclin A-cdc2 really be caused by endogenous cyclin B-cdc2, itself activated by cyclin A-cdc2? This seems unlikely, given that levels of cyclin B in interphase extracts are low compared with those in mitotic extracts and that we found little evidence for activation of any downstream kinase by cyclin A. Furthermore, depletion of endogenous cyclin B by synchronization of cells in G1 phase did not alter the extent of inhibition by cyclin A. It is also possible that differences between the two studies could be attributed to the inability of mammalian cyclins to activate *Xenopus* kinases in a manner that leads to inhibition of vesicle fusion. More likely, they may reflect a difference in pathways downstream from cyclin A-cdc2 kinase in *Xenopus* and mammalian extracts or even differences between the way that membrane traffic is controlled during cell division in the two systems. Several such differences are apparent in vivo; endoplasmic reticulum–Golgi transport is blocked in mammalian cells (Featherstone *et al.*, 1985) but not in maturing *Xenopus* oocytes (Ceriotti and Colman, 1989; Kanki and Newport, 1991), whereas the inverse is found for trans-Golgi–cell surface transport (Kreiner and Moore, 1990; Leaf *et al.*, 1990). It is not known whether the endocytic pathway is blocked in maturing oocytes. Given that the target for cyclin A-cdc2 kinase is likely to be cytosolic (see below), the choice of cytosolic extract may have a bearing on the pattern of inhibition.

We have used two-stage incubations to identify the location of targets for cyclin A-cdc2 kinase action. Addition of staurosporine and the phosphatase inhibitor microcystin-LR after preincubation of the cytosolic fraction with cyclin A led to almost maximal inhibition of fusion, even though the membrane fractions were never exposed to active kinase. The simplest explanation for this result is that at least some of the targets for cyclin A-cdc2 kinase are cytosolic components of the fusion apparatus. Similarly, addition of staurosporine to cytosol prepared from mitotic cells did not restore fusion activity to levels supported by interphase cytosol, suggesting that at least some mitotic-specific modifications are to cytosolic components. In contrast to cyclin-activated extracts, staurosporine-resistant inhibition by mitotic extracts did not require phosphatase inhibitors. It is possible that components of the fusion apparatus modified by cdc2 kinase no longer partition in the cytosol fraction and so are absent from cytosols derived from mitotic cells. Alternatively, the phosphatase activities required to revert the inhibition by cdc2 kinase may also be modified in mitotic extracts.

We cannot dismiss the possibility that cyclin A-activated or mitotic extracts phosphorylate membrane-bound, rather than cytosolic, components of the fusion apparatus via kinase(s) acting downstream of cdc2 that are insensitive to staurosporine. We have recently demonstrated that endocytic vesicle fusion is regulated by at least one kinase in addition to cdc2 kinase, because

okadaic acid or microcystin inhibit fusion (Woodman *et al.*, 1992). At present, it is unclear whether these kinases normally act downstream or independently of cdc2 kinase. In any event, kinases mediating inhibition by microcystin or okadaic acid are also sensitive to staurosporine, because a combination of staurosporine and microcystin has little effect itself on vesicle fusion.

However, further evidence for phosphorylation of cytosolic component(s) by cdc2 kinase is provided by the finding that untreated cytosol can restore fusion activity to incubations after cyclin A-cdc2 kinase activity has been inhibited. Strikingly, activity is restored even after both membrane fractions have been preincubated with cyclin A–activated extracts. This may indicate that all of the targets for cyclin A-kinase action are found in the cytosol fraction, though it is possible that membrane-bound proteins are also targets for kinase action but are phosphorylated poorly under the conditions that we employed. Our results are not inconsistent with those of Thomas *et al.* (1992), who showed that phosphorylated proteins are found on membrane fractions reisolated from vesicle fusion incubations that included cdc2 kinase. Given their function, it is likely that many cytosolic proteins interact with the vesicle membrane at some stage at least transiently and may be trapped on the membranes upon becoming phosphorylated.

Restoration of fusion activity by addition of untreated cytosol provides a biochemical complementation system to test for potential targets of cyclin A-cdc2 kinase action. Thus, at least one cytosolic component of the fusion apparatus is NEM sensitive (Braell, 1987; Diaz *et al.*, 1989), yet NEM treatment of cytosol has limited effect on its ability to rescue cyclin-treated incubations. Hence, the targets of cyclin A-kinase action are only marginally sensitive to NEM. In contrast, cytosol isolated from mitotic cells has limited ability to rescue cyclin-treated incubations in parallel with its low ability to support fusion itself. This finding suggests that at least one component of the fusion apparatus is modified both in cyclin A-treated cytosol and in cytosol prepared from mitotic cells.

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