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Cyclin A-mediated inhibition of intra-Golgi transport requires p34^{cdc2}

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An in vitro assay was used to study the role of p34^{cdc2} in cyclin A-mediated vesicular transport inhibition. It was shown that the S-phase kinase p33^{cdk2} reduced the effect of cyclin A on transport assays performed with sHeLa cytosol, even though histone kinase was strongly activated. Also, transport with FT210 cytosol (which is temperature-sensitive for p34^{cdc2}) was inhibited by cyclin A only at the permissive temperature. However, the phosphatase inhibitor microcystin inhibited transport without any requirement for p34^{cdc2} activity. These results show that transport is inhibited by cyclin A via p34^{cdc2}, and also by another kinase, possibly downstream of p34^{cdc2}.

p34^{cdc2}; p33^{cdk2}; Cyclin A; Microcystin; Intra-Golgi transport

1. INTRODUCTION

During mitosis, vesicular transport is inhibited at almost every stage so far examined (reviewed in [1]), including ER-Golgi [2] and intra-Golgi [3] transport, and the formation [4] and recycling [5] of endocytic vesicles in vivo. In vitro, the fusion of endocytic vesicles is inhibited, via both cyclin A- and cyclin B-dependent mechanisms [6,7] . It has also been found in vivo and in vitro that both exocytic and endocytic transport are inhibited by phosphatase inhibitors, without apparent involvement of mitotic kinase [8–10].

Entry into mitosis requires activation of the key kinase p34^{cdc2} [11]. The archetypal mitotic kinase MPF [12] comprises p34^{cdc2} and cyclin B, which is synthesised and destroyed each cell cycle [13]. This complex is not activated until the G2-M boundary, due to regulatory phosphorylation (reviewed in [14]), but p34^{cdc2} also forms a complex with cyclin A which is not regulated by inhibitory phosphorylation [15]. The cyclin A and B complexes with p34^{cdc2} have different kinetics of activation [16], different locations [17] and probably different targets [18] in the cell . While some proteins are known to be modified directly by p34^{cdc2} (for example the endocytic pathway GTP binding protein rab 4 [19] or nuclear lamins [20,21]), it is not presently clear whether p34^{cdc2} acts as a mitotic 'workhorse' [22] or as a master controller activating a variety of downstream pathways. The onset of S-phase is controlled, like the onset of mitosis, by a cyclin-kinase complex. The major S-phase kinase is p33^{cdk2} associated with cyclin A [23], and its activity is regulated by inhibitory phosphorylation [24].

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We previously studied the mitotic regulation of an in vitro assay of intra-Golgi transport. The assay reconstitutes the vesicle-mediated transport of the vesicular stomatitis virus glycoprotein (VSV-G) between successive cisternae of the Golgi stack [25,26]. A Golgi-enriched 'donor' fraction is prepared from a VSV-infected mutant CHO cell line (clone 15B) which lacks the enzyme *N*-acetyl-glucosaminyltransferase I (NAGT I) normally present in the *medial* cisternae of the stack, and incubated with an 'acceptor' Golgi fraction prepared from uninfected wild-type cells. G protein transfer from the *cis* compartment of the donor stacks to the NAGT I-containing *medial* cisternae of the acceptor stacks leads to incorporation of [^{3}H]GlcNAc (from UDP-[^{3}H]GlcNAc) into the G protein. The process requires ATP and cytosolic factors which we supplied in sHeLa cytosol. The membranes used were 'KCl-washed' [27] and therefore dependent on the cytosol for provision of certain transport components.

We found that the assay was inhibited by cytosol with high mitotic activity (measured as histone kinase activity), either isolated from mitotic cells, or generated by treatment of interphase cytosol with cyclin A [28]. Transport inhibition was dependent on kinase activation, since it could be blocked by the kinase inhibitor staurosporine. It was not possible, however, to prove that p34^{cdc2}, rather than p33^{cdk2}, was responsible for the transport inhibition.

This paper addresses this problem using recombinant p33^{cdk2}, and cytosol from a cell line with a temperature-sensitive mutation in p34^{cdc2}. The results confirm that the mitotic p34^{cdc2} kinase is the direct target of cyclin A in transport inhibition. It also demonstrates that transport can be inhibited by a non-histone kinase, perhaps one downstream of the mitotic kinase.

2. MATERIALS AND METHODS

2.1. *Materials*

Cyclin A and $p33^{cdk2}$ were prepared from bacterial lysates (as described in [28,29]). Cell culture media were bought from Gibco BRL, with the exception of RPMI, which was made in-house. Microcystin and staurosporine were purchased from Calbiochem. Borosilicate glass assay tubes were obtained from Samco, and [^{3}H]UDP-*N*-acetylglucosamine from New England Nuclear. Monoclonal anti-VSV-G antibody was affinity-purified from the culture supernatant of the 8G5 cell line, a kind gift of Drs. Lefrançois and Lyles [30]. Polyclonal rabbit anti-mouse IgG was purchased from Dakopatts. Glassfibre filters 934-AH and P81 paper were obtained from Whatman. [γ-^{32}P]ATP was purchased from Amersham.

2.2. *Cells*

The CHO, 15B CHO and suspension HeLa cells were maintained as described in [28]. FM3A and FT210 cells were maintained at 32°C in RPMI with 2% bicarbonate, supplemented with 10% foetal calf serum, 200 mM glutamine, 1% penicillin/streptomycin and 1% non-essential amino acids.

2.3. *Cytosols and membranes*

All membranes used in the work in this paper were prepared as in [28]. Golgi fractions were salt-washed as described [27] except that they were washed in a final concentration of 1 M KCl for 10 min on ice. sHeLa cytosol was prepared as described, and FM3A and FT210 cytosols were prepared in the same way, using ~1 × 10^9 cells per preparation.

2.4. *Transport assays*

Transport assays were performed basically as in Balch [25], with modifications for the pretreatment of cytosol and the use of salt-washed Golgi. For assays using sHeLa cytosol, assay premixtures were prepared containing transport buffer and ATP-regenerating system (25 mM HEPES-KOH, pH 7.0, 25 mM KCl, 2.5 mM MgOAc, 1 mM DTT, 4 μM palmitoyl coenzyme A, 1 mM ATP, 1 mM UTP, 2 mM creatine phosphate, 10 μg/ml rabbit muscle creatine kinase) and exogenous proteins or equivalent buffers as appropriate. Cytosol (50 μg) was added to this and the whole preincubated for 20 min at 37°C. Then the reactions were removed to ice, and the salt-washed membranes added. The reactions were incubated for 10 min at room temperature, and then at 30°C for 2 h. During the first 30 min of the transport reactions, 2 μl samples were withdrawn for histone kinase assay of the cytosol.

For assays using cytosols from the murine cell lines FM3A and FT210, premixtures were made containing transport buffer and ATP-regenerating system. The cytosol (20 μg) was added to this, and the mixture was preincubated for 20 min at either 32°C or 39°C. Then, on ice, recombinant cyclin A or drugs (or equivalent buffer) were added, and this mixture was incubated for 20 min at 32°C. Then the reactions were removed to ice, and the salt-washed membranes added. The assays were incubated for 10 min at room temperature, and then at 30°C for 2 h. During the first 30 min of the transport reactions, 5 μl samples were withdrawn for histone kinase assay of the cytosol.

Histone kinase assays were according to Félix et al. [31]. For sHeLa cytosol, 2 μl aliquots (containing ~2 μg cytosol) were removed from transport assays during the first 30 min of the transport reaction, and assayed for 10 min at 37°C in the presence of histones and [γ-^{32}P]ATP. For mouse cytosols, 5 μl samples (containing ~2 μg cytosol) were assayed for 20 min at 32°C. Under these conditions, the calculated specific activity of sHeLa cytosol did not markedly differ from that at 37°C.

In all cases, the results shown are typical examples of assays performed under the conditions described with various preparations of membranes and cytosol.

3. RESULTS

3.1. *The effect of the S-phase kinase, $p33^{cdk2}$, on the transport assay*

$p33^{cdk2}$ is closely related to $p34^{cdc2}$. It requires association with a cyclin for its activity and, while $p34^{cdc2}$ is associated with cyclins A and B in vivo, $p33^{cdk2}$ is associated with cyclins A, C, D and E [24,32,33]. Therefore, exogenous $p33^{cdk2}$ should be capable of competing with endogenous $p34^{cdc2}$ for cyclin A, but would not be expected to mediate in vitro transport inhibition.

sHeLa cytosol was treated with cyclin A in the presence or absence of 1 μg $p33^{cdk2}$, and then tested for both histone kinase activity and support of the transport reaction (Fig. 1).

Fig. 1a

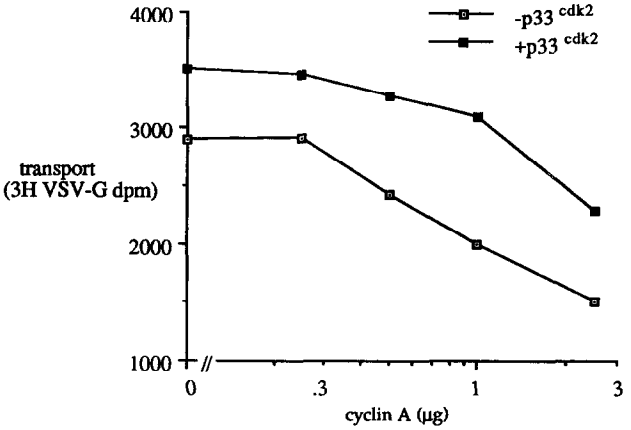


Fig. 1b

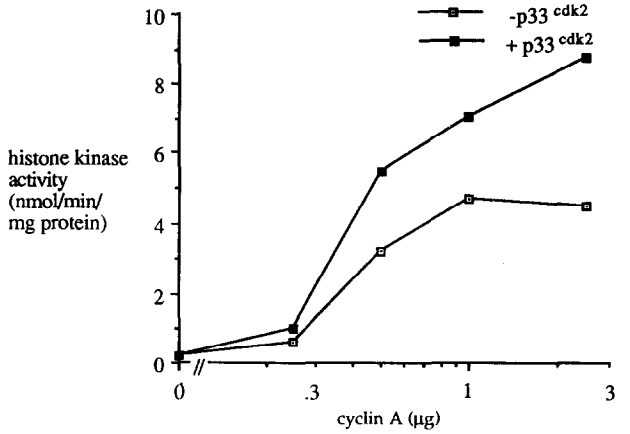


Fig. 1. Reduction in cyclin A-mediated transport inhibition by $p33^{cdk2}$. Recombinant cyclin A was added to the sHeLa cytosol (50 μg) preincubation in varying quantities (or an equivalent of buffer). The titration was performed in the absence (open squares) or presence (closed squares) of 1 μg of $p33^{cdk2}$. Transport and histone kinase assays were performed as described in section 2. (a) Shows the resultant transport activity and (b) shows the histone kinase activity of the cytosols.

In the absence of cyclin A, $p33^{cdk2}$ had no effect on transport, or occasionally increased the transport signal while, in the presence of cyclin A, $p33^{cdk2}$ apparently reduced the level of cyclin available for transport inhibition (Fig. 1a). The histone kinase activity of the cytosol was consistently elevated in the presence of $p33^{cdk2}$ (Fig. 1b). Therefore, elevation of histone kinase activity could not in itself be responsible for inhibition of the transport assay by cyclin A.

To see whether the effect of $p33^{cdk2}$ was linked with its level in the assay, the protein was titrated into transport assays in the presence of a fixed quantity (1 μg) of cyclin A. Fig. 2 shows that the level of transport was increased by increasing levels of $p33^{cdk2}$, though histone kinase activity was concurrently elevated to a high level. It should be noted that, in the absence of cyclin A, $p33^{cdk2}$ for the most part had no effect on the transport or the histone kinase activity of the cytosol, which suggests that very little of the endogenous cyclin in the cytosol was capable of activating the exogenous kinase.

3.2. *Ablation of $p34^{cdc2}$ in the FT210 cell line*

Experiments were performed with cytosol isolated from the murine cell line FT210. This line, a derivative of FM3A, is temperature-sensitive for $p34^{cdc2}$ [34,35]. It behaves like the parental line at 32°C, but at 39°C its $p34^{cdc2}$ protein is inactivated, and the cells become incapable of undergoing mitosis. Cytosol was isolated from these cells in exactly the same way as from sHeLa cells, and supported transport with considerably higher specific activity than sHeLa preparations. An experiment was performed in which the mouse cytosols were pretreated at either permissive or restrictive temperatures, and then treated with cyclin A. The effect of this treatment is shown in Fig. 3.

Cyclin A caused transport inhibition with elevation of histone kinase activity in FM3A cytosol, after incubation at both 32°C and 39°C (Figs. 3a,b). In contrast, FT210 cytosol supported transport inhibition after incubation at 32°C, but could not be inhibited by cyclin A after 39°C incubation (Fig. 3c). Likewise, cyclin A caused histone kinase activation after treatment at the permissive, but not the restrictive temperature (Fig. 3d). This showed that functional $p34^{cdc2}$ was required for cyclin A-mediated histone kinase activation and transport inhibition.

3.3. *Effect of microcystin on FT210 cytosol*

It was notable that transport inhibition required a much smaller elevation of histone kinase activity in FT210 cytosol than in sHeLa cytosol (compare Figs. 1 and 3). It had been observed that okadaic acid and microcystin, inhibitors of phosphatases-1 and -2A [36,37] were incapable of activating sHeLa cytosol or inhibiting transport under transport conditions ([28] and DM unpublished data). When microcystin was added to FT210 cytosol, transport was inhibited (Fig. 4a). At the same time, the histone kinase activity of the cytosol was elevated, though about 50% less than it would be for similar transport inhibition by cyclin A (data not shown). The effect of microcystin was the same after both 32°C and 39°C preincubations, which showed that the effect did not require activity of $p34^{cdc2}$. However, the effect did require kinase activity, since simultaneous addition of microcystin and staurosporine (a general kinase inhibitor; see [38]) to cytosol completely blocked transport inhibition and the small elevation of histone kinase activity (Fig. 4b and data not shown). Okadaic acid had very similar effects upon transport (data not shown).

4. DISCUSSION

$p33^{cdk2}$ alleviated the cyclin A-dependent inhibition of

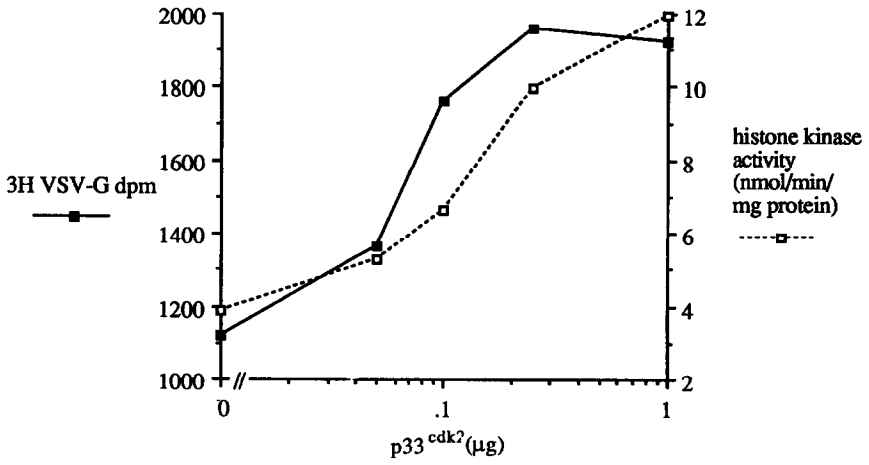
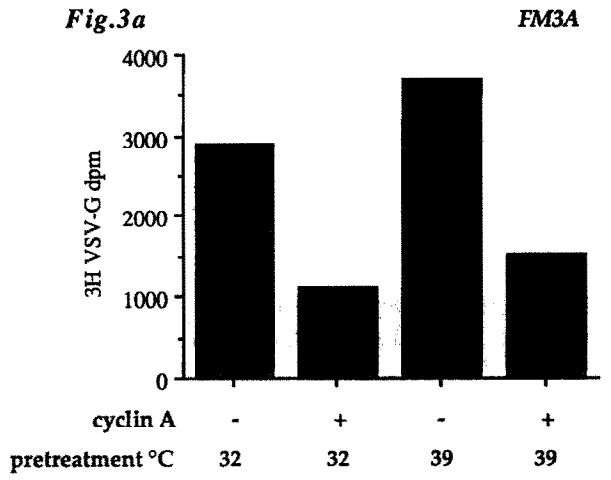
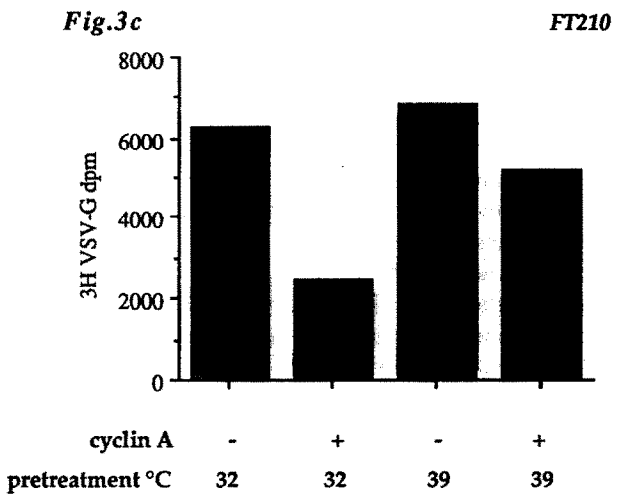
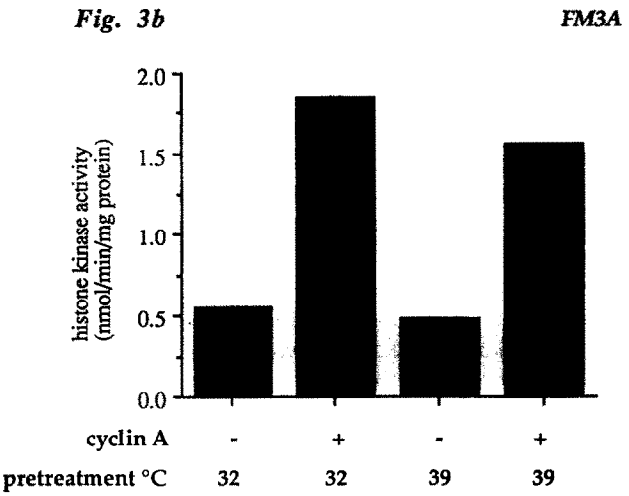


Fig. 2. Titration of $p33^{cdk2}$ into a transport assay in presence of a fixed quantity (1 μg) of recombinant cyclin A. Cytosols were preincubated with cyclin A and different levels of $p33^{cdk2}$, and then assayed for transport (solid line) and histone kinase (dashed line) activities.







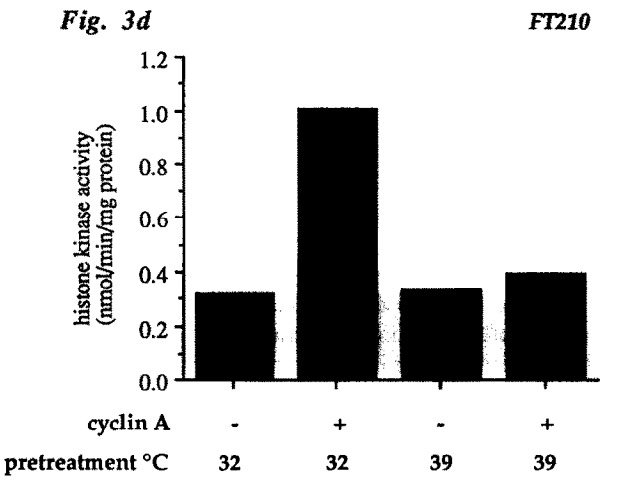


Fig. 3. Effect of cyclin A on FM3A and FT210 cytosols. Cytosol (20 μg) was preincubated in transport buffer for 20 min at 32°C or 39°C. The reactions were removed to ice and treated with either cyclin A (2.5 μg) or equivalent buffer, then incubated for 20 min at 32°C. After this, transport and histone kinase activities were assayed. (a) and (c) Show transport activities and (b) and (d) histone kinase activities.

the transport assay. This result indicated that histone kinase activity was not in itself sufficient for transport inhibition, but that the active kinase is one which competes with $p33^{cdk2}$ for cyclin A. Since $p34^{cdc2}$ and $p33^{cdk2}$ are the only kinases known to associate with cyclin A [23], $p34^{cdc2}$ was by implication the target of cyclin A in inhibition of the assay.

It has not been possible to test the effect of $p34^{cdc2}$ directly in the assay, since recombinant $p34^{cdc2}$ has not yet been successfully activated, nor purified active $p34^{cdc2}$ made available in sufficient quantities to influence the sHeLa system in our hands (D.M., unpublished data). This presumably reflects the fact that $p34^{cdc2}$ complexed with cyclin B is subject to stringent phosphorylation control [39], whereas the level of cyclin A-dependent $p34^{cdc2}$ kinase activity is proportional to the level of cyclin A during the cell cycle [15]. The cyclin A-$p33^{cdk2}$ complex is apparently phosphorylated during G1 in the same way as the cyclin B-$p34^{cdc2}$ complex during interphase [24], but it may well be that the general phosphorylation condition of the cytosol preparations used in this work is conducive to activation of an S-phase kinase, but not an M-phase kinase.

The role of $p34^{cdc2}$ was supported by the effect of its ablation on the transport assay in FT210 cytosol. In the absence of $p34^{cdc2}$, cyclin A had no effect on the transport or the histone kinase activity of the mutant cytosol. Cyclin A caused a maximum of 2–4-fold elevation of histone kinase activity, thus inhibition of the transport assay requires lower histone kinase activity in FT210

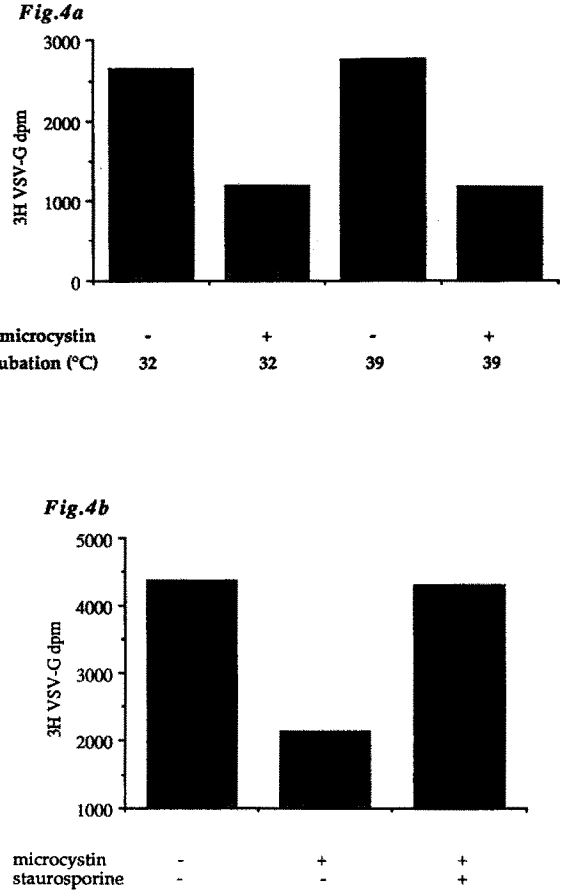


Fig. 4. Effect of the phosphatase inhibitor microcystin on FM3A and FT210 cytosols. Cytosol was preincubated in transport buffer for 20 min at either 32°C or 39°C. The assays were next preincubated for 20 min at 32°C with or without 1 μM microcystin and staurosporine, and then (a) transport and (b) histone kinase activities were assayed.

cytosol than in sHeLa cytosol. This difference between the cell lines may reflect a difference in their levels of $p34^{cdc2}$ or its accessory proteins.

Transport in the presence of FT210 cytosol was inhibited by microcystin, an inhibitor of phosphatases-1 and -2A, while histone kinase activity was not significantly elevated. This showed that transport could be regulated by another, non-histone kinase either downstream of $p34^{cdc2}$ or independent of it. Staurosporine had no direct effect on transport, but could block microcystin-mediated inhibition. This indicated that microcystin was not altering activity of a kinase required for interphase transport, but rather causing activation of a kinase capable of inhibiting it.

Phosphatase inhibitors appear to act on vertebrate systems on two levels. Okadaic acid has been shown to cause maturation of *Xenopus* oocytes [40] and activation of oocyte extracts [41], and pseudomitotic effects with histone kinase activation of BHK cells [42]. It has been shown to cause activation of $p34^{cdc2}$ by blocking inhibition of the cdc25 phosphatase [43] or by promoting inactivation of wee1 kinase [44]. However, in HeLa cells okadaic acid inhibits transport and causes pseudomitotic Golgi vesiculation without elevation of histone kinase activity [8]. Likewise, in vitro exocytic [9] and endocytic [10] assays were inhibited by okadaic acid and microcystin, via inhibition of PP2A, without involvement of $p34^{cdc2}$. It has been suggested that *Xenopus* oocytes and hamster cell lines differ from the okadaic acid-refractory systems in cell-cycle controls; also, *Xenopus* oocytes possess stored cyclins, and BHK cells are able to manufacture them under cell-cycle blocks, whereas human cell lines did not accumulate cyclin B [45]. Nevertheless, cell lines not advanced into mitosis by okadaic acid may possess other kinases susceptible to intervention by phosphatase inhibitors.

It appears that, while high mitotic kinase activity is responsible for the inhibition of intra-Golgi transport, $p34^{cdc2}$ might not act directly on the system, but rather use an intermediate kinase. Since this appears to be the case in a number of other transport assay systems, it is possible that a single kinase mechanism will be found to be responsible for inhibition of all these vesicular transport events.

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