Mitotic Cytosol Inhibits Invagination of Coated Pits in Broken Mitotic Cells

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Abstract. Receptor-mediated endocytosis is inhibited during mitosis in mammalian cells and earlier work on A431 cells suggested that one of the sites inhibited was the invagination of coated pits (Pypaert, M., J. M. Lucocq, and G. Warren. 1987. Eur. J. Cell Biol. 45: 23–29). To explore this inhibition further, we have reproduced it in broken HeLa cells. Mitotic or interphase cells were broken by freeze-thawing in liquid nitrogen and warmed in the presence of mitotic or interphase cytosol. Using a morphological assay, we found invagination to be inhibited only when mitotic cells were incubated in mitotic cytosol. This inhibition was reversed by diluting the cytosol during the incubation. Reversal was sensitive to okadaic acid, a potent phosphatase inhibitor, showing that phosphorylation was involved in the inhibition of invagination. This was confirmed using purified cdc2 kinase which alone could partially substitute for mitotic cytosol.

Receptors that enter the endocytic pathway cluster in specialized regions of the plasma membrane termed coated pits (Anderson et al., 1976). These are assembled from clathrin subunits (Pearse, 1975), which are linked to the cytoplasmic tail of receptors by adaptor complexes (Pearse and Robinson, 1984; Vigers et al., 1986; Pearse, 1988). Once formed, a coated pit undergoing invagination, a process that turns a shallow coated pit into one that is only held to the plasma membrane by a narrow neck. Membrane scission then releases a coated vesicle into the cytoplasm. In a previous study, we reconstituted these processes in broken A431 cells and identified some of their requirements (Smythe et al., 1989). The most intriguing conclusion was that although the formation of coated pits and their scission required both added cytosol and ATP, invagination required neither. This seemed to indicate that new coated pits were primed to undergo invagination.

Different models have been put forward to explain the mechanism responsible for invagination. The most widely accepted derives from the observation that the clathrin lattice underlaying shallow coated pits comprises mainly hexagons whereas the lattice of more invaginated coated pits contains pentagons as well. The conversion of hexagons into pentagons could drive the increase in curvature of the lattice, causing a shallow pit to become more invaginated (Kanaseki and Kadota, 1969; Heuser and Evans, 1980; Larkin et al., 1986; Heuser, 1989). Another model suggests that a change in curvature cannot occur once a coated pit has been assembled, but that the curvature of the coat is built in as the different subunits assemble to form a coated pit (Pearse and Crowther, 1987). A third possibility, which is a hybrid of these two models, is that only the early steps of invagination involve conversion of hexagons to pentagons. Subsequent addition of new subunits at the periphery would then help to close the neck so that scission could occur. Since these models are necessarily speculative, much information could be gained from studying processes which regulate this process. The only known physiological regulation of invagination occurs during mitosis.

Membrane traffic in mammalian cells is almost completely arrested during mitosis (reviewed by Warren, 1985, 1989). In the case of fluid-phase and receptor-mediated endocytosis, the steps along the pathway which are known to be inhibited include internalization by coated pits (Fawcett, 1965; Berlin et al., 1978; Berlin and Oliver, 1980) as well as the recycling of receptors to the cell surface (Warren et al., 1984; Sager et al., 1984). We have shown in a previous study using A431 cells that all categories of coated pits, from shallow to deeply invaginated, were present in both interphase and mitotic cells (Pypaert et al., 1987). If invagination consists of a series of different steps which convert a shallow coated pit into a deeply invaginated one, then inhibition of one of these steps would cause coated pits which follow the block to disappear and those preceding the block to accumulate. This was the conclusion reached by Fawcett (1965) who showed that only shallow coated pits could be found in dividing erythroblasts. This seemed to indicate that the first step of invagination, which turns a flat lattice into a slightly curved one, was inhibited, but not later steps. In the case of mitotic A431 cells, however, the presence of all categories of coated pits shows that all steps of invagination are inhibited. The simplest interpretation of this result is that the same process is involved at each of these steps, for example.

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the conversion of hexagons into pentagons or the addition of new subunits at the periphery, and it is this process that is blocked during mitosis.

To gain more information about the nature of this inhibition, we set about reproducing it in a cell-free system. We have developed a system using broken mitotic HeLa cells incubated in mitotic cytosol and have shown that invagination can be inhibited in a reversible fashion. We have also shown that cyclin B-p34<sup>cdc2</sup> protein kinase complex (cdc2 kinase), purified from starfish oocytes, can partially substitute for mitotic cytosol, indicating that phosphorylation reactions are involved in the inhibitory process.

Materials and Methods

Materials

Okadaic acid was a gift from Prof. P. Cohen, Dundee University, Scotland. It was stored as a 0.1 mM solution in 2% (vol/vol) DMSO at -20°C. Media and supplements used for cell culture were obtained from Flow Laboratories, Rickmansworth, England or Northumbria Biologicals Ltd.,Camlington, England, and tissue culture plastics were from Sterilin Ltd., Feltham, England. Plastic roller bottles (850 cm<sup>2</sup>) were purchased from Becton Dickinson Labware, Lincoln Park, NJ. Unless otherwise specified, all other reagents were obtained from Sigma Chemical Co. or BDH, Poole, England.

Cells

HeLa cells were grown at 37°C in minimal essential medium (MEM) supplemented with 10% (vol/vol) fetal bovine serum, nonessential amino acids, and 100 U/ml each of penicillin and streptomycin in an atmosphere of 5% CO<sub>2</sub>/95% air. Suspension HeLa cells were grown in MEM modified for suspension cultures, at 37°C in an atmosphere of 5% CO<sub>2</sub>/95% air.

Preparation of Mitotic and Interphase (G1) Cells

Roller bottles were seeded at a density of 3-4 x 10<sup>6</sup> cells/cm<sup>2</sup>, gassed with 5% CO<sub>2</sub>/95% air, and grown at 37°C on a rotator at 0.25 rpm. After 24 h the bottles were spun at 200 rpm for 3 min at 37°C to remove debris and clumps, and the medium replaced by fresh medium. After another 24 h, when they were 50-70% confluent, the cells were blocked in S phase by incubation for 16 h in fresh medium containing 5 mM thymidine. The medium was then replaced by medium without thymidine to allow cells to enter and proceed through the G2 phase of the cell cycle. After 7 h, the medium was replaced by medium containing 40 mg/ml nocodazole (Zieve et al., 1980). Cells were incubated in nocodazole for 4 h and the mitotic cells were then detached by rotation at 250 rpm for 3 min (Kliewecz, 1975). Typically, 3 x 10<sup>6</sup> cells were obtained from each roller bottle, of which >97% were mitotic (prometaphase) as determined by staining with bis-benzamide (Hoechst dye 33258; see Berlin et al., 1978). A further 5 min at 37°C, some samples were diluted 20-fold with prewarmed MEM containing 100 U/ml each of penicillin and streptomycin, for several days before being thawed. The experimental results showed no difference between these two methods.

Preparation of Broken Cells

Mitotic and interphase cells were broken by freeze-thawing in liquid nitrogen just before use. They were first washed with KHEM (50 mM KCl, 10 mM EGTA, 50 mM Hepes, 1.92 mM MgCl<sub>2</sub>, adjusted to pH 7.2 with KOH) at 4°C and then split into aliquots of ~10<sup>6</sup> cells. After a second wash in KHEM the cells were centrifuged at 1,000 g for 5 min at 4°C, then resuspended in 20 µl of KHEM, and immersed in liquid nitrogen for ~10 s. Thawing was achieved by shaking the tubes in a 37°C waterbath for ~15 s and then quickly transferring them to ice. In some experiments, the cells were frozen and left in liquid nitrogen for several days before being thawed. Our experimental results showed no difference between these two methods.

Cell breakage was determined by assaying the release of lactate dehydrogenase as described previously (Smythe et al., 1989). Typically, 95% of mitotic cells and 90% of interphase cells were broken by this method.

Preparation of Cytosols

Mitotic and interphase cytosols were prepared from suspension HeLa cells. The cells were grown in 150-cm<sup>2</sup> plastic flasks and, 2 d before an experiment, were transferred to a glass stirrer flask in a final volume of 1 liter, at a density of ~3 x 10<sup>6</sup> cells/ml. After growing for 24 h at 37°C, half of the suspension was spun at 1,000 g for 10 min at room temperature and the cell pellet resuspended in 1 liter of fresh medium containing 100 ng/ml nocodazole. These cells were grown for another 24 h after which time a suspension of mitotic cells was obtained. The mitotic index was typically >95%. The other half of the culture was made up to 1 liter by adding fresh medium and allowed to grow for 24 h. This generated an interphase population at a density of 4-7 x 10<sup>6</sup> cells/ml. Cells were harvested by spinning both mitotic and interphase cell suspensions at 1,000 g for 10 min at room temperature. The cell pellets were resuspended in ice-cold 150 mM KCl, 10 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.2 and left on ice for 10 min. After one wash in KHEM the cells were resuspended in 1 vol of KHEM containing 1 mM DTTP and the following protease inhibitors: 25 µg/ml leupeptin, 25 µg/ml aprotonin, 1 mM benzamid HCl, and 10 µg/ml pepstatin. This protease inhibitor cocktail was stored at ~20°C as a 100-fold concentrate in DMSO. The cells were broken using a ball bearing homogenizer with a 20 µm clearance (Balch et al., 1984). This gave a cell breakage usually in excess of 95%. The homogenate was centrifuged at 360,000 g for 30 min at 4°C and the supernatant rapidly frozen and stored in liquid nitrogen. Before each experiment, the concentration of protease inhibitors in the cytosols was adjusted to 50 µg/ml leupeptin, 50 µg/ml aprotonin, 2 mM benzamid HCl, and 20 µg/ml pepstatin. The protein concentration of the cytosols was 7-10 mg/ml, as determined by the method of Bradford (1976), using BSA as a standard.

The histone kinase activity of each cytosol was determined by incubating 5 µl of cytosol, diluted 1: to 100-fold in KHEM, with 15 µl of reaction mix for 15 min at 37°C. The reaction mix comprised KHEM containing 107 mM β-glycerophosphate, 67 mM NaF, 1.33 mM MgCl<sub>2</sub>, 1.33 mM ATP, 2.67 mg/ml histones type III-S, and 0.25 µCi/µl 32P-ATP (Amersham, Aylesbury, England). The reaction was terminated by spotting 12 µl of each sample onto a grid of Whatman P81 ion exchange chromatography paper. The grids were washed three times over a 45 min-period in 150 mM phosphoric acid, then dipped in 95% (vol/vol) ethanol/H<sub>2</sub>O, and air dried. The radioactivity on each square of the grid was measured using Ready-Solv CP (Beckman Instruments, Inc., Palo Alto, CA) as the scintillation cocktail.

The specific activity of the histone kinase in each cytosol was determined over the linear range of activity and was typically 1-3 nmol/min/mg of cytosolic protein for mitotic cytosols. The activity of interphase cytosols was typically 15-fold lower than this.

The mitotic cytosols prepared by this method were found to be capable of causing nuclear envelope breakdown when incubated with isolated rat liver nuclei following the method of Newport and Spahn (1987) (data not shown).

Purification of cdc2 Kinase

cdc2 kinase (cyclin B-p34<sup>cdc2</sup> complex) was purified to apparent electrophoretic homogeneity from starfish oocytes as described previously (Labbé et al., 1989, with histone kinase activities of 200-500 pmol/min/µl. Aliquots were frozen rapidly in liquid nitrogen and stored at ~80°C. Before each experiment, the kinase was rapidly thawed and diluted in KHEM to give a final activity of ~10 pmol/min/µl, which is equivalent to the activity of the mitotic cdc2 kinase (see legend to Fig. 7). Protease inhibitors were added before the incubation at the same concentration as in mitotic or interphase cytosols.

Conditions of Incubation

Broken cell suspensions were diluted 25-fold with ice-cold KHEM and centrifuged at 1,000 g for 5 min at 4°C. Each pellet was resuspended in 30 µl of KHEM, KHEM or KHEM containing cdc2 kinase, and 3 µl of an ATP-regenerating system containing 26.7 mM MgATP, pH 7.0, 53.5 mM creatine phosphate, and 0.38 mg/ml creatine phosphokinase. The samples were incubated for 5 or 10 min at 0 or 37°C, chilled on ice for 5 min, and then fixed for electron microscopy as described below. After the first incubation at 37°C, some samples were diluted 20-fold with prewarmed KHEM and incubated for another 5 min at 37°C. To other samples a one-tenth volume of an ATP-depleting system (50 U/ml hexokinase in 100 mM glucose) was added and the cells incubated for a further 5 min at 37°C.

In some experiments okadaic acid was added to the cytosol or KHEM both before and after the 20-fold dilution, at a final concentration of 1 µM.

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Preparation of the Samples for Electron Microscopy

The cells were fixed in 0.5% (wt/vol) glutaraldehyde (Fluka BioChemika, Glossop, England) in 0.1 M cacodylate buffer, pH 7.4, for 1 h at room temperature. They were then washed three times in the same buffer and centrifuged at 1,000 g for 10 min. The pellets were postfixed in 1% (wt/vol) osmium tetroxide (Taab Laboratories Equipment Ltd., Aldermaston, England) in 0.1 M cacodylate buffer, pH 7.4, for 30 min. After dehydration in a graded series of solutions of ethanol, the pellets were embedded in Epon 812 resin (Taab Laboratories Equipment Ltd.). Serial sections were cut and mounted on carbon/formvar coated grids. The average section thickness was 35 nm. The sections were stained for 15 min with a 2% (wt/vol) solution of uranyl acetate in 50% (vol/vol) ethanol and for 5 min with a solution of lead citrate according to the method of Reynolds (1963). They were viewed in a Philips CM10 electron microscope.

Quantitative Morphological Analysis

Sampling Protocol. Coated pits and vesicles were sampled using a modification of the method we described previously (Smythe et al., 1989). Disectors (Sterio, 1984) were placed across the sections systematically with a random start. Each disector consisted of three consecutive sections. Coated pits and coated vesicles were sampled only if they were present in the first two sections and had disappeared in the third (the "look-up"). Coated pits were photographed at 28,500×, and printed at a 10-fold higher magnification. Each field used to count coated pits and vesicles was photographed at a magnification of 6,600× and then printed at a final magnification of 16,720×. These fields were used to estimate the numbers of coated pits and vesicles per mm of plasma membrane (linear densities).

Measurement of Coated Pits. For each coated pit sampled, the section through the middle of the pit was identified from serial sections and used to estimate the degree of invagination using a modification of the method described previously (Pypaert et al., 1987). For each coated pit the length of plasma membrane (L) and the diameter of the neck (l) were measured by counting the number of intersections with a single square lattice grid with a distance of 5 mm between lines (see Weibel, 1979). The results were expressed as 1−(l/L), so that a flat coated pit would have a value of 0 while of plasma membrane (L) and the diameter of the neck (l) were measured.

Measurement of Plasma Membrane. The length of plasma membrane was estimated from micrographs by counting the number of intersections of the membrane with the lines of a single square lattice grid with a distance of 4 cm between lines.

Results

Invagination of Coated Pits in Broken Mitotic Cells

Preliminary experiments in intact cells showed no difference in the distribution of coated pits between mitotic and interphase HeLa cells (data not shown). This led us to conclude that, as in A431 cells, invagination is inhibited during mitosis in HeLa cells. We then tried to reproduce this inhibition in broken cells. The method chosen for breaking mitotic cells was freeze–thawing in liquid nitrogen. The electron micrographs in Fig. 1 show mitotic HeLa cells after breaking by this method. Most of the cells had the light appearance typical of broken cells (Fig. 1 a; see also Smythe et al., 1989). At low magnification the overall cyto-architecture did not seem greatly altered. However, examination at higher magnification revealed the presence of numerous holes in the plasma membrane (Fig. 1 b). More importantly, the morphology of coated pits seemed unaffected by the freeze–thawing treatment (see also Fig. 3). Mitotic cells were broken and incubated in the presence of mitotic or interphase cytosols prepared from suspension HeLa cells. Incubation was carried out in the presence of an ATP-regenerating system for 5 min at either 0 or 37°C. Coated pits were sampled at the electron microscope, measured, and put into five categories, with the flatter coated pits being included in category 1 and the more invaginated coated pits in category 5. The results are shown in Fig. 2 a. If the broken cells were incubated at 37°C in the presence of mitotic cytosol, the distribution of coated pits did not change when compared to cells that were incubated at 0°C. In contrast, if interphase cytosol was used, there was a marked shift towards more invaginated coated pits at 37°C. Because only one round of internalization via coated pits occurs in broken cells (Smythe et al., 1989), a shift towards more invaginated coated pits was expected. The absence of such a shift when cells were incubated in mitotic cytosol at 37°C indicates that invagination was inhibited. The difference between mitotic and interphase cytosols was not due to the absence of nocodazole in interphase cytosol, since addition of nocodazole before the incubation did not inhibit invagination (data not shown).

To simplify the presentation of the data for this and following experiments, coated pits were put into two rather than five categories. The two categories, described in a previous report (Smythe et al., 1989), were shallow and deeply invaginated coated pits. Shallow coated pits were defined as having neck diameters greater than one-fifth of the length of the coated plasma membrane (corresponding to categories 1−4 in Fig. 2 a), and deeply invaginated coated pits as having neck diameters equal to or smaller than one-fifth of the length of the membrane (corresponding to category 5). This classification was chosen because it gave the most sensitive measure of the invagination process: at 0°C > 80% of the coated pits were shallow but after an incubation at 37°C in interphase cytosol or buffer >80% became deeply invaginated (Smythe et al., 1989).

When the data from Fig. 2 a were reclassified, we found that ~80% of the coated pits remained shallow after an incubation at 0°C with either of the cytosols (Fig. 2b). After a 5-min incubation at 37°C in mitotic cytosol, the distribution did not change. With interphase cytosol, however, >80% became deeply invaginated. We can conclude that invagination is inhibited in broken mitotic HeLa cells, but only in the presence of mitotic cytosol. Shallow coated pits in mitotic cells incubated in mitotic cytosol for 5 min at 37°C are shown in Fig. 3 (c and d). There were no morphological differences between these and coated pits in cells kept at 0°C (Fig. 3, a and b).

In a parallel set of experiments, a population of interphase cells was derived from mitotic cells by removing the nocodazole and allowing the mitotic cells to proceed to GI. They were then broken and incubated with either mitotic or interphase cytosol. Mitotic cytosol did not inhibit invagination in these cells (Fig. 4).

The Inhibition of Invagination by Mitotic Cytosol Is Reversible

To test the reversibility of the inhibition described above, some broken cells were incubated for 5 min at 37°C with mi-
Figure 1. Morphology of broken mitotic HeLa cells. (a) Mitotic HeLa cells broken by freeze-thawing are shown at low magnification. Broken mitotic cells shown at higher magnification. The large arrowhead points to a hole in the plasma membrane of one of the cells. Coated pits are indicated by small arrowheads and the rough endoplasmic reticulum by arrows. M, mitochondria; C, chromosome. Bars: (a) 5 μm; (b) 1 μm.

Figure 2. Invagination in broken mitotic cells in the presence of mitotic or interphase cytosol. Mitotic HeLa cells were broken by freeze-thawing and incubated with either mitotic or interphase cytosol in the presence of an ATP-regenerating system at 0 or 37°C for 5 min, then processed for electron microscopy. Coated pits were sampled at random, measured and each put into one of five (a) or two (b) categories, as defined in the text. Each value is expressed as the mean of three experiments ± standard deviation. (a) The five categories are shown on the left-hand axis. (b) (c) Shallow coated pits; (c) deeply invaginated coated pits.

It was possible that some coated pits could have disappeared during these experiments by detachment of coat subunits from the plasma membrane. This could have interfered with our interpretation of the data, so it was important to compare the linear densities of coated pits for each of the conditions tested, and this is shown in Table I. Although the numbers decreased slightly during the incubation in mitotic cytosol and after the 20-fold dilution in buffer when compared to the cells kept at 0°C, none of these differences were significant when tested using the Student's t test. In interphase cells incubated with interphase cytosol, one might have expected the number of coated pits to decrease significantly as scission occurred. This did not happen but is consistent with...
our observations in broken A431 cells that scission is a rate-limiting step so that deeply invaginated coated pits accumulate, with <25% of them becoming vesicles (Smythe et al., 1989). It is also worth noting that the values in Table I are not significantly different from the values found for intact mitotic and interphase cells (65 ± 22 and 54 ± 48, respectively), indicating that no significant loss of coated pits occurred during the cell breaking and washing steps.

**Reversal Can Be Partially Prevented by Okadaic Acid**

Phosphatases are involved in the exit from mitosis both in vivo (Picard et al., 1989) and in vitro (Félix et al., 1990). We therefore tested the possibility that a phosphatase activity was involved in the reversal of the inhibition described here.

To test this, okadaic acid, a specific inhibitor of protein phosphatase 1 (PP1) and 2A (PP2A) (Cohen et al., 1990), was added to try to block reversal. When cells were incubated with mitotic cytosol and 1 μM okadaic acid for 5 min at 37°C and then diluted 20-fold into buffer containing okadaic acid and incubated for a further 5 min at 37°C, coated pits did not invaginate to the same extent as when okadaic acid was absent during both incubations (Fig. 6). This difference was significant when tested with the Student's *t* test (*p* < 0.05) and corresponds to a 64% inhibition of reversal (see legend to Fig. 6). If okadaic acid was only present during dilution, no effect was observed (data not shown), indicating that a preincubation with okadaic acid was required.

**Figure 3.** Morphology of coated pits in broken mitotic HeLa cells. Mitotic HeLa cells were broken by freeze-thawing and then incubated with mitotic cytosol and ATP for 5 min at 0°C (a and b) or 37°C (c and d), or 5 min at 37°C, followed by dilution in buffer and a further 5 min at 37°C (e and f). Coated pits shown are representative of the types of coated pits found in cells incubated under each of these conditions. Bar, 100 nm.

**Figure 4.** Invagination in broken GI cells in the presence of mitotic or interphase cytosol. GI cells were treated as described in the legend to Fig. 2. Coated pits were measured and put into one of two categories. Each value is expressed as the mean of two experiments ± standard deviation. (○) Shallow coated pits; (●) deeply invaginated coated pits.

**Figure 5.** Effect of dilution on invagination in broken mitotic cells. Mitotic cells were broken by freeze-thawing and incubated for up to 10 min at 0 or 37°C. After 5 min at 37°C, some of the cells were diluted 20-fold into prewarmed buffer and incubated for a further 5 min at 37°C. Each value is expressed as the mean of three experiments ± standard deviation. (○) Shallow coated pits; (●) deeply invaginated coated pits.
Table I. Density of Coated Pits on Broken Mitotic and Interphase HeLa Cells

| Condition of incubation | Number of coated pits per millimeter of plasma membrane |
|-------------------------|--------------------------------------------------------|
| Mitotic cells           |                                                         |
| Mitotic cytosol, 5 min, 0°C | 78 ± 24*                                              |
| Mitotic cytosol, 5 min, 37°C | 47 ± 24*                                             |
| Mitotic cytosol, 10 min, 37°C | 57 ± 10*                                             |
| Mitotic cytosol, 5 min, 37°C + 20-fold dilution, 5 min, 37°C | 38 ± 27* |
| Interphase cytosol, 5 min, 0°C | 74 ± 31§                                             |
| Interphase cytosol, 5 min, 37°C | 68 ± 20†                                              |
| Interphase cells         |                                                         |
| Mitotic cytosol, 5 min, 0°C | 74 ± 31§                                             |
| Mitotic cytosol, 5 min, 37°C | 49 ± 28†                                              |
| Interphase cytosol, 5 min, 0°C | 65 ± 8†                                               |
| Interphase cytosol, 5 min, 37°C | 50 ± 36‡                                              |

Values represent means ± standard deviation of three (mitotic cells) or two (interphase cells) experiments.

*, †, ‡, §: groups of values that were compared together using the Student's t test. All differences were nonsignificant.

If interphase cytosol was substituted for the mitotic cytosol, or if G1 cells were used instead of mitotic cells, okadaic acid had no effect on invagination after dilution (data not shown).

Purified cdc2 Kinase Partially Inhibits Invagination

The partial prevention of reversal by a specific phosphatase inhibitor strongly suggests that phosphorylation is involved in inhibiting invagination. The obvious candidate kinase is cdc2 kinase, a key regulator of intracellular events leading to cell division (Nurse, 1990). To test the effect of the kinase on invagination, broken mitotic cells were incubated at 37°C in mitotic cytosol or buffer containing cdc2 kinase, at a similar level of histone kinase activity as mitotic cytosol (see legend to Fig. 7). Incubation with mitotic cytosol at 37°C prevented invagination, whereas buffer alone did not (Fig. 7 a). If cdc2 kinase was included in the buffer, >50% of the coated pits remained shallow. This corresponds to a 58% inhibition of invagination, or about two-thirds of the inhibition observed with mitotic cytosol (see legend to Fig. 7). The extent of inhibition could not be increased by doubling the concentration of cdc2 kinase (data not shown). This shows that the kinase activity was not limiting.

Inhibition by cdc2 kinase was also reversible. Reversal was accomplished by removing ATP during the incubation using an ATP-depleting system in excess of the ATP-regenerating system present during the initial incubation. Cells were incubated for 5 min at 37°C in the presence of mitotic cytosol or cdc2 kinase, then a one-tenth volume of the ATP-depleting system (hexokinase and glucose), or buffer, was added and the cells were re-incubated for 5 min at 37°C. The data are shown in Fig. 7 b. When mitotic cytosol was used, the hexokinase-glucose treatment caused complete reversal of the inhibition, as was observed with a 20-fold dilution of the cytosol (compare with Figs. 5 and 6). If the hexokinase-glucose treatment caused 75% of the coated pits to become deeply invaginated, indicating that the inhibition by cdc2 kinase was reversible.

Discussion

Invagination of coated pits is inhibited during mitosis (Py-
paert et al., 1987), and here we have shown that this inhibition can be reproduced in broken mitotic HeLa cells. Mitotic but not interphase cytosol was able to prevent invagination during incubation at 37°C (see Fig. 2), and this inhibition was reversible if the cytosol was diluted during the incubation (see Fig. 5). This indicates that a factor present in the mitotic cytosol was responsible for the inhibition and had to be present throughout the incubation. A surprising observation was that mitotic cytosol was not able to inhibit invagination in broken interphase cells. This might simply be explained by the fact that invagination both in vivo (Helenius et al., 1980) and in vitro (our own unpublished observations) is very rapid and probably takes less than 30 sec. This may be too short a time for mitotic cytosol to convert membranes to the mitotic state.

What do our observations tell us about the nature of this inhibition? The fact that it is reversed in a manner sensitive to okadaic acid indicates that a phosphorylation–dephosphorylation cycle is involved. Inhibition would be caused by a kinase activity in the mitotic cytosol, and reversed by a phosphatase. Since dilution up to 1,000-fold still allows reversal (unpublished observations), the phosphatase would have to remain bound to the membrane or coated pits after dilution. This is consistent with the observation that at least one of the phosphatases known to be sensitive to okadaic acid, PPI, can be found associated with membranes (Cohen, 1989). Okadaic acid was not, however, completely effective in preventing reversal so another phosphatase could be involved in this process as well. A good candidate would be pp50 phosphatase, which has been found in association with isolated coated vesicles (Pauloin and Jollès, 1986), though not as yet in coated pits.

The involvement of phosphorylation in the inhibition of invagination is consistent with our observations that ATP depletion causes reversal and that purified cdc2 kinase is nearly as efficient as mitotic cytosol in inhibiting invagination. Recent studies have shown that cdc2 kinase is the key regulator of intracellular events that lead to cell division (reviewed by Nurse, 1990). The involvement of the kinase in the inhibition of fusion of endocytic vesicles in vitro (Tuomikoski et al., 1989) and of invagination (this study) correlates well with the observation that, in macrophages and A431 cells, endocytic uptake is inhibited at the beginning of prophase (Berlin et al., 1978; Warren et al., 1984), when the kinase is known to be activated, and only resumes at the anaphase–telophase transition, when the kinase becomes inactivated.

Since cdc2 kinase alone does not fully substitute for mitotic cytosol in preventing invagination, even at levels of histone kinase activity higher than in mitotic cytosol, other cytosolic factors, perhaps kinases, could be involved. There is, however, the possibility of species incompatibility, since the cdc2 kinase was purified from starfish and the cells were of human origin. Either way, it is important to note that our data do not prove that cdc2 kinase acts directly on coated pits. It may be acting via other enzymes that remain bound to coated pits following breakage and washing. The kinase could also be working via enzymes which are part of the clathrin coat itself, since previous reports have shown the presence of kinase activities in isolated coated vesicles (Pauloin et al., 1982; Bar-Zvi and Branton, 1986). If such enzymes are involved, one would have to assume that they become rapidly inactivated when the cdc2 kinase is removed, in order to explain the rapid reversal of inhibition that follows dilution of the mitotic cytosol.

What could be the targets for these enzymes on clathrin coated pits? Nearly every single constituent of coated pits and coated vesicles has been shown to be phosphorylated in vivo or in vitro. They include clathrin heavy chains (Martin-Perez et al., 1989) and light chains (Usami et al., 1985; Schook and Puszkin, 1985), as well as the 100- and 50-kD adaptins (Keen and Black, 1986; Bar-Zvi et al., 1988). A good candidate for the target would be the adaptor complex. Invagination is accompanied by congregation of adaptor complexes within the coat. Phosphorylation could prevent this, for example by electrostatic repulsion. This would result in "freezing" coated pits throughout mitosis, as we have observed both in vivo and in vitro. Another possible target for phosphorylation could be the putative enzyme which converts hexagons into pentagons within the clathrin lattice (see Pypaert et al., 1987). If, however, as mentioned earlier, curvature of coated pits cannot be increased once a coated pit has assembled, but is created as the coat is being built (Pearse and Crowther, 1987), then phosphorylation of one of the coat components could prevent their incorporation at the periphery of a coated pit.

Our finding that purified cdc2 kinase can effectively inhibit invagination should make it much easier to identify the enzymes involved in the mitotic inhibition of invagination, and their targets within the coated pit. This should help us to understand better the mechanism underlying the formation of coated vesicles from the plasma membrane during endocytosis.

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