Cell-free Fusion of Endocytic Vesicles Is Regulated by Phosphorylation

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Abstract. Okadaic acid and microcystin-LR, both potent inhibitors of protein phosphatases (PP), blocked vesicle fusion in a cell-free system. The effect of okadaic acid was reversed by the purified catalytic subunit of PP2A, but not PP1. Inhibition was gradual, required Mg-ATP, and was reduced by protein kinase inhibitors, indicating that it was mediated via protein phosphorylation. A candidate protein kinase would be cdc2 kinase, which normally is active in mitotic extracts and has been shown to inhibit endocytic vesicle fusion (Tuomikoski, T., M.-A. Felix, M. Dorée, and J. Gruenberg. 1989. *Nature (Lond.)*. 342:942–945). However, it would appear that cdc2 kinase is not responsible for inhibition by okadaic acid. When compared to cytosol prepared from mitotic cells, okadaic acid did not increase cdc2 kinase activity sufficiently to account for the inhibition. In addition, inhibition was maintained when cdc2 protein was depleted from cytosol.

THE pathway of receptor-mediated endocytosis is used by mammalian cells to internalize many macromolecules, including nutrients and polypeptide hormones (Goldstein et al., 1985). The transferrin cycle is one of the most closely studied examples and provides a useful model (Bleil and Bretscher, 1982; Hopkins and Trowbridge, 1983). Halotransferrin binds to cell surface receptors located in clathrin-coated pits, which rapidly invaginate and pinch off to form coated endocytic vesicles. After at least partial removal of the clathrin coat (Rothman and Schmid, 1985), transferrin is delivered to the endosome by a specific membrane fusion event. Iron dissociates from transferrin in the acidic environment of the endosome (Dautry-Varsat et al., 1983; Klausner et al., 1983), and the apoprotein recycles to the cell surface.

Although the transferrin cycle has been defined largely by kinetic and morphological studies on intact cells, understanding of the molecular events that enable the receptor to pass between endocytic compartments requires the reconstitution of transport in cell-free systems. We have described a cell-free assay for the specific fusion of endocytic vesicles (Woodman and Warren, 1988, 1989) and this, together with studies by others using different endocytic markers (Davey et al., 1985; for reviews see Warren et al., 1988; Gruenberg and Howell, 1989), has defined broad requirements for vesicle fusion. Fusion requires ATP and proteins on the vesicle membranes, as well as cytosolic proteins. At least one of these cytosolic proteins is inactivated by the alkylating agent *N*-ethyl maleimide, and some evidence suggests that this is a protein also required for exocytic vesicle fusion (Diaz et al., 1989). In addition, fusion is inhibited by nonhydrolyzable analogues of GTP (Mayorga et al., 1989; Wessling-Resnick and Braell, 1990), indicating a role for GTP-binding proteins (Gorvel et al., 1991). Evidence that endocytic vesicle fusion is regulated by protein phosphorylation was first provided by Tuomikoski et al. (1989), who showed that cell-free fusion was inhibited by partially purified starfish cdc2 kinase. This may be linked to the inhibition of endocytic traffic that occurs during mitosis (Fawcett, 1965; Berlin et al., 1978; Warren et al., 1984; Sager et al., 1984), since cdc2 kinase activity controls the entry of the cell into M-phase (Nurse, 1990).

We have investigated further the regulation of endocytic vesicle fusion by protein phosphorylation. However, rather than add exogenous protein kinases to the fusion assay mix, we have adopted a different approach. Phosphorylation of proteins is generally controlled by a cycle of a kinase and opposing phosphatase (Cohen, 1989). Either activation of the kinase or inhibition of the phosphatase will lead to hyperphosphorylation. We show here that specific phosphatase inhibitors, okadaic acid and microcystin, inhibit endocytic vesicle fusion. Our results show that vesicle fusion in interphase extracts is regulated by phosphorylation, and suggest that a kinase distinct from cdc2 kinase is responsible.

Materials and Methods

Materials

All reagents, unless otherwise specified, were obtained from Sigma Chemical Co. (St. Louis, MO), or BDH Chemicals Ltd. (Poole, UK). Okadaic acid was a gift from Y. Tsukitani (Fujisawa Pharmaceutical Co., Tokyo, Japan) or bought from Moana Bioproducts Inc. (Honolulu, HI). It was stored

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in 10% DMSO at a final concentration of 1 mM and then diluted in water to a 10× concentrate before use. Microcystin-LR was stored in water at −20°C. Sheep antitransferrin antiserum was obtained from the Scottish Antibody Production Unit (Carluke, Scotland). The kinase inhibitor, 6′-dimethylaminopurine (6′-DMAP),[1] was stored at −20°C as a 50-mM stock in water adjusted to pH 7.0 with NaOH. 5′-*p*-Fluorosulfonylbenzoyl adenosine (FSBA) was diluted from a 100-mM stock in DMSO.

Phosphatases

The catalytic subunits of type 1 and type 2A protein phosphatases (PP1 and PP2A) were prepared from rabbit skeletal muscle as described previously (Cohen et al., 1988) and stored in 50% (vol/vol) glycerol at −20°C.

Radiolabeling

Human transferrin was radiolabeled with ^{125}I to a specific activity of ~10^7 cpm/μg exactly as described (Woodman and Warren, 1991).

Cells

A431 cells were maintained in DME supplemented with 10% (vol/vol) FCS, 100 U/ml of both penicillin and streptomycin. Suspension HeLa cells were grown in spinner flasks in MEM modified for suspension cultures (Gibco Laboratories, Paisley, Scotland) and supplemented with 10% (vol/vol) FCS, 100 U/ml of both penicillin and streptomycin, and nonessential amino acids. Mitotic HeLa cells were prepared by growing cells in the presence of the microtubule inhibitor nocodazole, which blocks the formation of the mitotic spindle (Zieve et al., 1980). HeLa cells were resuspended in fresh growth medium containing 0.1 μg/ml nocodazole (Boehringer Mannheim-GmbH, Mannheim, Germany) to a concentration of 3×10^5 cells per ml. After 24 h cells were harvested by centrifugation. Mitotic indices were assessed by bis-benzamide (HOECHST dye 33258; Sigma Chemical Co., St. Louis, MO) staining of chromosomes; typically, a mitotic index of >95% was achieved. Mitotic indices of unsynchronized cultures were ~4%.

Membrane Preparations

Donor and acceptor endocytic vesicles were prepared from cells to which either ^{125}I-transferrin or antitransferrin antibody was bound at 4°C, and then internalized for 5 min at 37°C. Full details for the internalization of ligands and preparation of membrane fractions are provided elsewhere (Woodman and Warren, 1989, 1991).

Cytosol Preparations

Preparation of cytosol from A431 cells was as described elsewhere (Woodman and Warren, 1991). Cytosols were desalted on Biogel P6 columns (Bio-Rad Laboratories, Cambridge, MA) into Hepes buffer (HB; 140 mM sucrose, 70 mM potassium acetate, 20 mM Hepes, pH 7.2) containing 1 mM DTT. Desalting was monitored using [^{3}H]ATP (Amersham International, Amersham, UK) and showed that ~90% of ATP was removed. HeLa cytosol was prepared from parallel cultures of unsynchronized and mitotic cells. Cells (~3×10^8 mitotic, 6×10^8 interphase, in 1 liter of growth medium) were sedimented, resuspended in 150 mM KCl, 10 mM triethanolamine, pH 7.2, and left on ice for 10 min. After one wash in mitotic buffer (MB; 50 mM KCl, 10 mM EGTA, 1.92 mM $MgCl_2$, 50 mM Hepes, pH 7.2) cells were resuspended in twice the volume of MB 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 as for membrane preparations and the extracts were centrifuged at 400,000 g_{av} for 30 min in a TL100 bench-top ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). Protein concentrations were typically 8–10 mg/ml (HeLa) and 5–7 mg/ml (A431). Cytosol was frozen and stored in liquid nitrogen.

Fusion Assays

Assays for endocytic vesicle fusion were carried out essentially as described (Woodman and Warren, 1989), with the following modifications. For a standard assay, cytosol (100 μg) was made up to 20 μl with HB (A431) or MB (HeLa) and added to 4 μl of an ATP-regenerating cocktail. Okadaic acid, microcystin-LR, or control buffers (4 μl) were added and, unless otherwise stated, incubated for 30 min 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 then incubated for a further 2 h at 37°C. Samples were diluted in ice-cold immunoprecipitation buffer (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] SDS, 1% [wt/vol] sodium deoxycholate, 0.1% BSA) and processed exactly as described (Woodman and Warren, 1989). Each experiment included a control sample, containing an ATP-depleting cocktail of 50 mM glucose and 50 IU hexokinase (4 μl). All results are means of duplicate determinations.

Histone Kinase Assays

Cytosols were assayed for histone kinase activity following the method described by Labbé et al. (1989) with the following modifications. Cytosol (5 μl at 1.0 mg/ml in the appropriate buffer: HB for A431 cytosol, MB for HeLa cytosol; at this concentration, all extracts catalyzed incorporation of phosphate from [γ-^{32}P]ATP into histones linearly over the time course of the assay) was incubated with 15 μl of assay mix (containing 50 μg histone type IIIs, 1 mM Mg-ATP, and 2 μCi [γ-^{32}P]ATP [Amersham International; 5,000 Ci/mmol]) for 15 min at 37°C; then labeled histones were precipitated onto P81 phosphocellulose paper and washed extensively in 150 mM H_3PO_4 to remove unincorporated radioactivity. Control samples without histone were assayed and histone-dependent incorporation of [^{32}P]phosphate was calculated by subtraction. SDS-PAGE analysis confirmed that >90% of radioactivity was incorporated into histone (not shown).

Immunodepletion

Cdc2 kinase protein was depleted from A431 cytosol using a monoclonal antibody raised against *Xenopus* cdc2, which cross-reacted with the mammalian protein. Hybridoma supernatant (40 ml) was passed several times over a 0.4-ml protein A–Sepharose column. The column was washed extensively in HB and unpacked. The beads were spun down and excess buffer was removed with a syringe needle. Cytosol (300 μl) and an ATP-regenerating cocktail (30 μl) were added, and the mixture was incubated with occasional mixing for 90 min at room temperature before pelleting the beads. A control cytosol sample was incubated with untreated protein A–Sepharose beads equilibrated in HB.

Western Blot Analysis

To assay for cdc2 protein or cyclin B, cytosol samples were run on 10% polyacrylamide gels and transferred to nitrocellulose according to the method of Towbin et al. (1979). Immunoblots using the monoclonal anti-cdc2 antibody were visualized by incubating with ^{125}I-protein A (1 μg/ml; 10^7 cpm/μg) and exposing to X-Omat AR-5 film (Eastman Kodak Co., Rochester, NY). Blots were quantitated by counting each band for radioactivity and subtracting a background value. Immunoblots using polyclonal rabbit antisera were visualized with a horseradish peroxidase–conjugated goat anti-rabbit antiserum (diluted 1:10^4), followed by treatment with the enhanced chemiluminescence detection system (Amersham International) and exposure to X-Omat AR-5 film.

Protein Assays

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

Results

Cell-free Fusion of Endocytic Vesicles Is Blocked by Phosphatase Inhibitors

Fusion of endocytic vesicles was monitored by an assay described previously (Woodman and Warren, 1988, 1989). Donor endocytic vesicles, isolated from cells containing internalized ^{125}I-transferrin, were mixed with acceptor endocytic vesicles carrying internalized antitransferrin antibodies. In the presence of Mg-ATP and a cytosol fraction, vesicle fusion occurred, and precipitation of the resulting radiolabeled

1. *Abbreviations used in this paper*: 6′-DMAP, 6′-dimethylaminopurine; FSBA, 5′-*p*-fluorosulfonylbenzoyl adenosine; HB, Hepes buffer; MB, mitotic buffer; PP1, PP2A, PP2B; type 1, 2A, and 2B protein phosphatases, respectively.

immunecomplex on *Staphylococcus aureus* cells gave an indirect measure of fusion. All results are expressed as the ATP-dependent immunoprecipitation of ^{125}I-transferrin, after subtracting the signal obtained from incubations depleted of ATP by hexokinase and glucose. For all experiments described below the ATP-independent signal was <2% of that obtained from a complete incubation.

Okadaic acid and microcystin-LR, both potent inhibitors of PP1 and PP2A (Bialojan and Takai, 1988; Haystead et al., 1989; MacKintosh et al., 1990), inhibited vesicle fusion markedly (Fig. 1). The maximum inhibition (90%) and IC_{50} (~0.15 μM) of the two compounds were similar, suggesting that they acted on the same site. Okadaic acid, when added to 1 μM at the end of the incubation at 37°C, did not reduce the immunoprecipitation of ^{125}I-transferrin (not shown) indicating that vesicle fusion itself was blocked, rather than the generation of the signal. The ATP/(ADP + AMP) ratio, measured by the method of Scott et al. (1977), remained at >20:1 during the course of the incubation, confirming that okadaic acid did not affect the ATP-regenerating cocktail.

The IC_{50} for the inhibition of fusion by okadaic acid and microcystin-LR appears at first inconsistent with the much higher affinities of the two inhibitors for the catalytic subunits of PP1 and PP2A (Cohen et al., 1989; MacKintosh et al., 1990). Okadaic acid has a K_i for PP1 of ~10 nM, and for PP2A of <0.1 nM, while those of microcystin-LR are 0.06 and <0.01 nM, respectively. It seemed likely that the apparent IC_{50}'s were determined by the concentration of phosphatase, rather than the affinity of each inhibitor, and this was confirmed when vesicle fusion was titrated against okadaic acid at two cytosol concentrations (Fig. 2). At 4.0 mg/ml cytosol, half-maximal inhibition was achieved at ~0.17 μM okadaic acid. A 10-fold decrease in cytosol concentration resulted in a 7-fold decrease in the IC_{50} of okadaic acid. This close correlation also suggests that the majority of the relevant phosphatase(s) are cytosolic.

Okadaic acid acts rapidly, yet inhibition by okadaic acid was not immediate (Fig. 3 *A*). This is seen more easily if the data are replotted to show the extent of inhibition at each

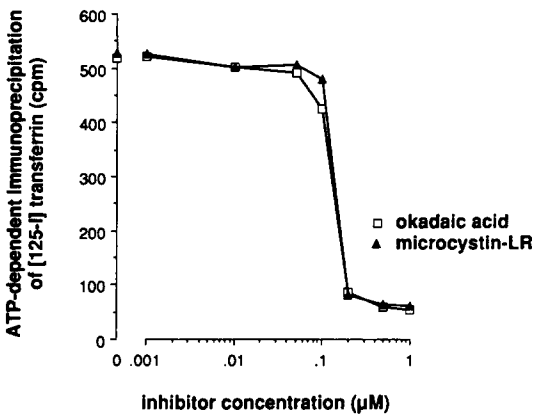


Figure 1. Phosphatase inhibitors block cell-free fusion of endocytic vesicles. A431 cytosol was preincubated with 1 mM Mg-ATP and okadaic or microcystin-LR for 30 min at 37°C. Membranes and unlabeled transferrin were added and the incubation was continued for a further 2 h before the extent of vesicle fusion was determined. Concentrations refer to those in the final assay mix.

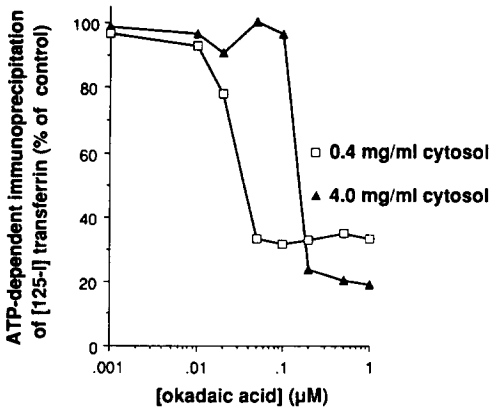
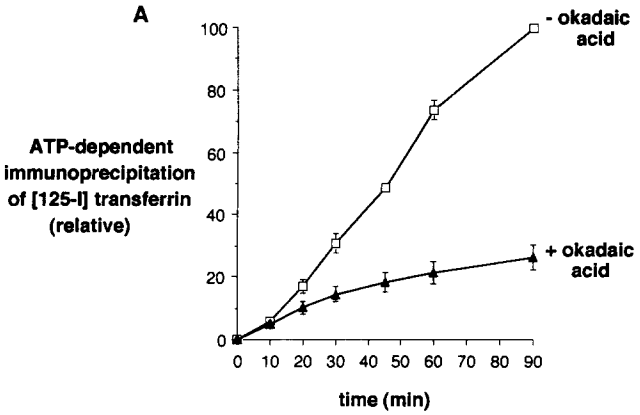


Figure 2. Titer of okadaic acid depends on cytosol concentration. Vesicle fusion was assayed at different concentrations of okadaic acid using either 0.4 or 4.0 mg/ml A431 cytosol. The concentration of both okadaic acid and cytosol refers to the concentration in the final assay mix. For clarity the results are normalized, and activity in the absence of okadaic acid is expressed as 100% in each case (the extent of fusion at 0.4 mg/ml cytosol was 23% of that at 4.0 mg/ml cytosol).



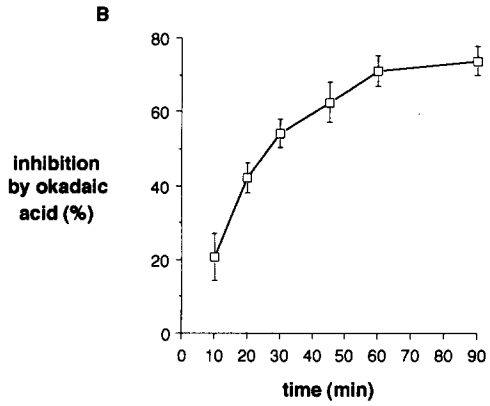


Figure 3. Inhibition by okadaic acid is time dependent. (*A*) Complete fusion mixes were incubated for varying times at 37°C with or without 1 μM okadaic acid, and the extent of vesicle fusion was determined. Values are the average ± SEM from three separate experiments, each normalized (control incubation at 90 min = 100). (*B*) The data are expressed as the percent inhibition by okadaic acid at each time point. All samples were counted for 30 min.

time point (Fig. 3 *B*). Inhibition was clearly time dependent, with a $t_{1/2}$ for inhibition of ~15–20 min. Furthermore, the extent of inhibition was increased by preincubating cytosol with okadaic acid and Mg-ATP at 37°C before addition of membranes. In a typical experiment over 2 h, inhibition was increased from 73 to 84%. For this reason, unless otherwise indicated, experiments included a 30-min preincubation of cytosol with phosphatase inhibitors.

Inhibition by Okadaic Acid Is Reversed by PP2A But Not by PP1

To determine which phosphatase is involved in the regulation of vesicle fusion we attempted to reverse the inhibition by adding purified catalytic subunits of PP1 or PP2A. 0.2 μM okadaic acid was added to cytosol, and then preincubated with Mg-ATP for 30 min at 37°C. Catalytic subunits of PP1 or PP2A were added just before addition of membranes. Addition of ~0.2 μM PP2A increased activity above that seen in the absence of inhibitor (Fig. 4). PP1 did not restore fusion, even when added at five times the concentration of okadaic acid. A control experiment showed that the PP1 preparation was active (not shown). The stimulation of fusion by PP2A in the absence of okadaic acid (31%) was typical of that seen in several experiments.

PP2A could restore vesicle fusion after a complete assay mix had been incubated with okadaic acid (Fig. 5), indicating that okadaic acid did not inhibit fusion simply by inactivating the membranes. After 40 min, fusion was inhibited by 85%. A 1.5-fold molar excess of PP2A was added and, after a lag, the rate of fusion rose to a level similar to that of the untreated control, although the final extent of fusion did not reach that found in the control incubation. Inhibition could also be partially reversed by diluting the assay mix 10-fold into fresh cytosol, indicating that endogenous phosphatases were capable of restoring activity (not shown).

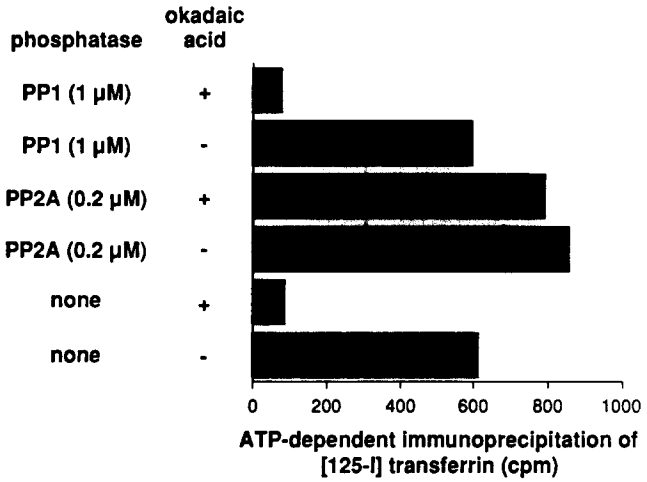


Figure 4. Inhibition is reversed by PP2A but not PP1. A431 cytosol was incubated with Mg-ATP, with or without 0.2 μM okadaic acid, for 30 min at 37°C. Samples were returned to ice and catalytic subunits of PP1 or PP2A were added as indicated. After addition of membranes, samples were incubated for a further 2 h at 37°C. Concentrations refer to those in the final assay mix.

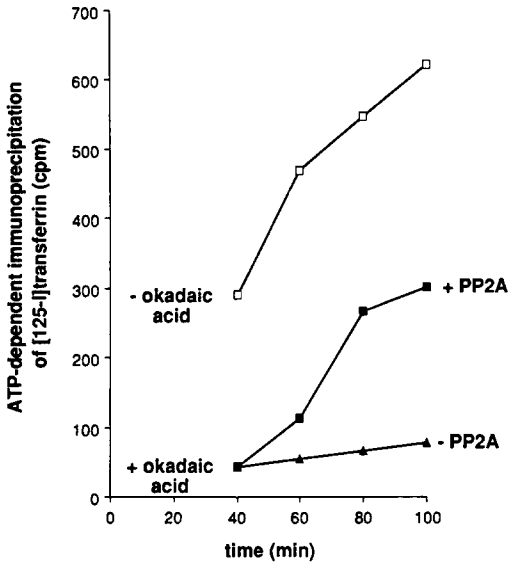


Figure 5. Inhibition by okadaic acid can be partially reversed by PP2A after incubation of a complete assay mix. A431 cytosol was preincubated with Mg-ATP, with or without okadaic acid (0.2 μM); then membranes were added and the incubations continued for 40 min. PP2A (0.3 μM) was added to one set of samples and the incubations were continued as indicated.

Inhibition by Okadaic Acid Is Mediated by a Protein Kinase

The slow inhibition of vesicle fusion by okadaic acid suggested that inhibition required the action of a protein kinase, leading to hyperphosphorylation of a component of the fusion reaction normally dephosphorylated by PP2A. Two findings support this. Firstly, okadaic acid inhibited fusion only in the presence of Mg-ATP (Fig. 6). Although incubations normally included 1 mM Mg-ATP, added to desalted cytosol, vesicle fusion occurred even at 20 μM added Mg-ATP. However, okadaic acid did not inhibit fusion below 100

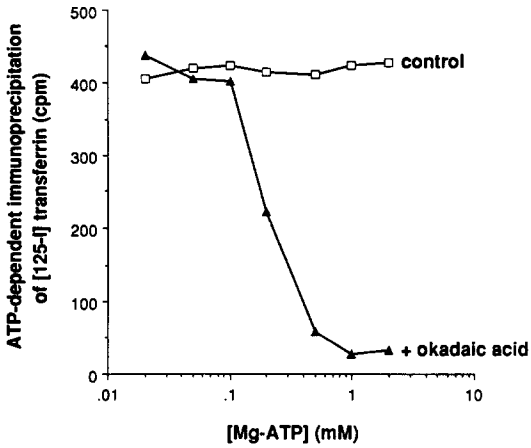


Figure 6. Inhibition by okadaic acid requires Mg-ATP. A431 cytosol was incubated for 30 min at 37°C with or without okadaic acid and the indicated concentration of Mg-ATP. Membranes were added with sufficient Mg-ATP to maintain the Mg-ATP concentration, and the incubation was continued for 2 h. The final concentration of okadaic acid was 1 μM.

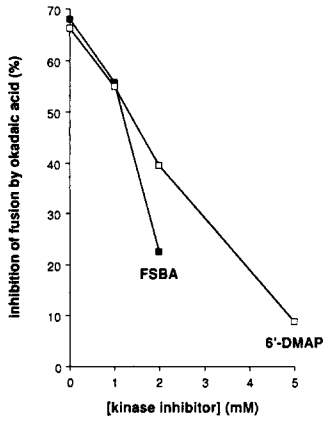


Figure 7. Protein kinase inhibitors reduce inhibition by okadaic acid. A431 cytosol was incubated with 50 μM Mg-ATP and kinase inhibitors as indicated for 15 min at 37°C. Mg-ATP was added to 1 mM, followed by okadaic acid (to 1 μM) or carrier DMSO (to 0.001%). After a further 15 min at 37°C, membranes and transferrin were added and the incubation was continued for 2 h. Inhibition by okadaic acid was calculated at each concentration of kinase inhibitor. FSBA did not affect control incubations at 2 mM. 6′-DMAP inhibited fusion by 15% at 5 mM. Inhibitor concentrations refer to those during the preincubation.

μM Mg-ATP, and inhibited fusion by 94% at 1 mM Mg-ATP. Half-maximal inhibition was achieved at ~200 μM Mg-ATP. Secondly, inhibition by okadaic acid was reduced by the kinase inhibitors FSBA (Colman, 1982) and 6′-DMAP (Verde et al., 1990). Since these inhibitors compete with ATP, cytosol was pretreated with inhibitor for 15 min at 37°C with 50 μM Mg-ATP. Then, okadaic acid and Mg-ATP (1 mM) were added and the incubation was continued for a further 15 min before addition of membranes. 6′-DMAP (5 mM) reduced inhibition from 66 to 9%, and FSBA (2 mM) reduced inhibition from 68 to 23% (Fig. 7). At these concentrations, FSBA did not affect vesicle fusion itself, while 6′-DMAP reduced fusion by only 15%.

Okadaic Acid Does Not Increase cdc2 Kinase Activity Sufficiently to Account for Inhibition of Vesicle Fusion

Endocytic vesicle fusion is inhibited by starfish cyclin B-cdc2 kinase (Tuomikoski et al., 1989) and it was possible that okadaic acid inhibited fusion via this kinase. To investigate this further, we compared in the same experiment both cdc2 kinase activity, and the effect on endocytic vesicle fusion activity of okadaic acid and of cytosol prepared from mitotic cells. Cdc2 kinase activity was defined by the ability to transfer phosphate from [γ-^{32}P]ATP to lysine-rich histones. For each reaction the histone kinase assay was performed under the same conditions as vesicle fusion, without other phosphatase inhibitors, so that the ability to incorporate [γ-^{32}P] from ATP into histones could be compared directly with the inhibition of vesicle fusion. Since it is difficult to generate a preparation of mitotic A431 cells, this experiment used cytosol from mitotic HeLa cells. Interphase and mitotic cytosols were prepared from parallel cultures of cells.

Incubation mixes containing cytosol prepared from mitotic HeLa cells had histone kinase activity 6.3 times higher than those containing interphase HeLa cytosol (Fig. 8 *B*). Levels of histone kinase were 4.8 times higher than interphase levels even at the end of the incubation (not shown). This rise correlated with an increase in the level of cyclin B, a component of active cdc2 kinase (Brizuela et al., 1989), in the cytosol (Fig. 8 *A*). Vesicle fusion was reduced by 51% when cytosol from interphase HeLa cells was substituted by the same amount of cytosol prepared from mitotic cells. To satisfy ourselves that inhibition was not merely a consequence of microtubule disassembly caused by nocodazole treatment of cells used to prepare mitotic cytosol we established, firstly, that nocodazole did not affect fusion when added directly to the assay at 1 μg/ml and, secondly, that cytosol prepared from cells incubated with nocodazole for 4 h instead of 24 h supported fusion as well as interphase cytosol (not shown).

In contrast, a concentration of okadaic acid that inhibited vesicle fusion by 68% using HeLa cytosol gave only a 1.6-fold increase in phosphorylation of histone over the control incubation (Fig. 8 *B*). No additional activation of cdc2 ki-

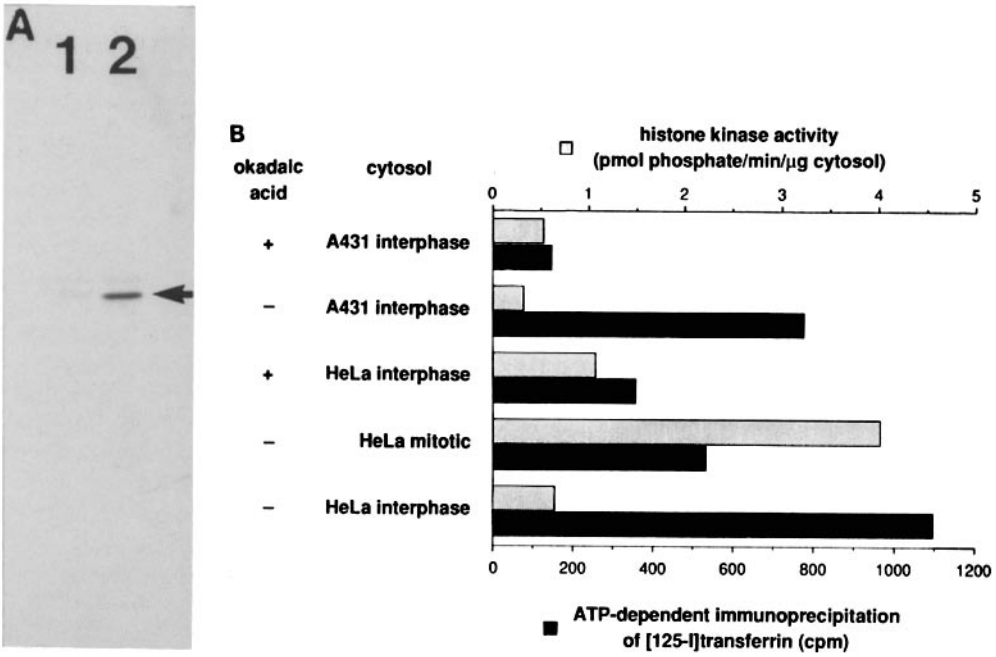


Figure 8. Activation of cdc2 kinase by okadaic acid is not sufficient to account for inhibition of vesicle fusion. (*A*) Cytosol (10 μg) from interphase (lane *1*) or mitotic HeLa cells (lane *2*) was analyzed for cyclin B protein by Western blot. The position of cyclin B (p62) is indicated by the arrow. (*B*) Endocytic vesicle fusion was measured using standard incubations and equal concentrations of cytosol prepared from mitotic HeLa cells, or interphase HeLa and A431 cells treated with or without okadaic acid. Samples (equivalent to 5 μg cytosol) were taken from each incubation mix after addition of membranes, and assayed for histone kinase activity under the same buffer conditions as the fusion assay. All results are means of duplicates.

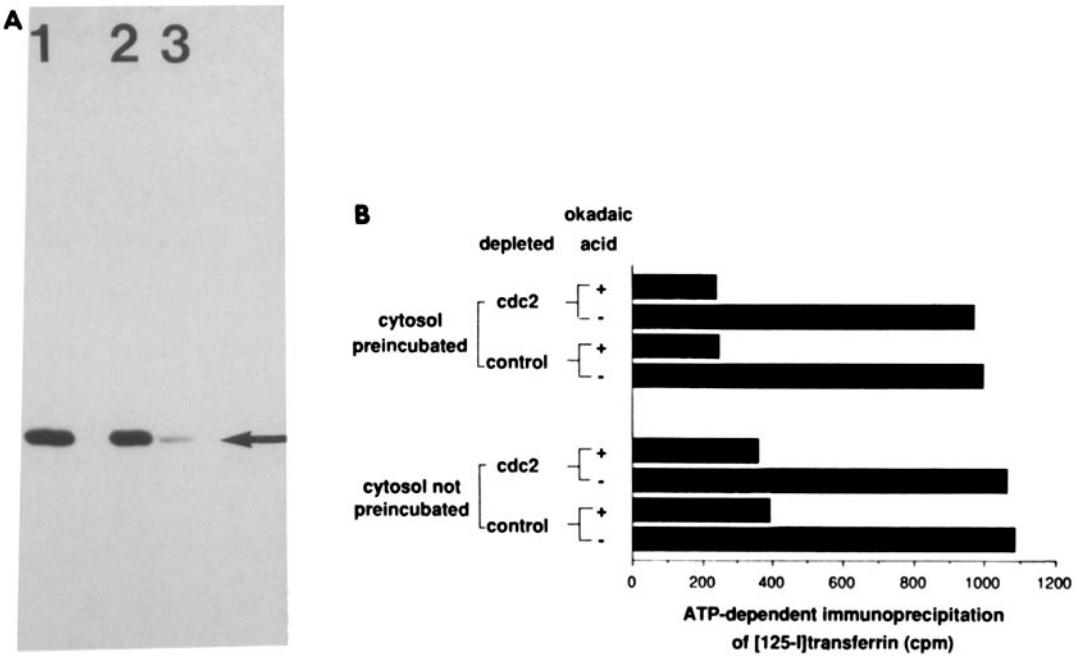


Figure 9. Depletion of cdc2 kinase does not affect inhibition by okadaic acid. (*A*) 20 μg untreated A431 cytosol (lane *1*) and cytosol treated with either control (lane *2*), or anti-cdc2 (lane *3*) protein A–Sepharose beads, were analyzed by Western blotting using a polyclonal antiserum to show the relative concentration of cdc2 kinase protein in each. The position of cdc2 (p34) is indicated by the arrow. (*B*) Fusion assays were performed using control or anti-cdc2 depleted cytosols in the presence or absence of 1 μM okadaic acid. Results are shown from standard incubations, and from incubations where the 30-min preincubation of cytosol was omitted.

nase by okadaic acid occurred during the course of the fusion incubation (not shown).

These data suggested that activation of cdc2 kinase could not account for the inhibition of fusion by okadaic acid in HeLa cytosol. To be sure that cytosol from A431 cells behaved similarly, we also examined the ability of okadaic acid to increase cdc2 kinase activity in this system. Okadaic acid at 1 μM inhibited vesicle fusion by 81%, yet increased histone phosphorylation by only 1.6-fold (Fig. 8 *B*).

Okadaic Acid Inhibits Fusion when Cytosol Is Depleted of cdc2 Kinase Protein

Further evidence that okadaic acid inhibited endocytic vesicle fusion independently of cdc2 kinase was provided by depleting cytosol of cdc2 protein using monoclonal antibodies bound to Sepharose beads. Depletion was monitored by Western blot analysis using a polyclonal antiserum raised to a peptide from the COOH terminus of human cdc2 (Fig. 9 *A*). In addition, quantitative immunoblotting using the monoclonal antibody and ^{125}I-protein A showed that 78% of cdc2 protein was removed. When control and depleted cytosols were assayed for fusion activity, with or without preincubation of cytosol, it was found that okadaic acid inhibited fusion in cdc2-depleted cytosol to the same extent as fusion in control cytosol (Fig. 9 *B*).

Discussion

We have demonstrated that fusion of endocytic vesicles in a cell-free system is inhibited markedly by two reagents, okadaic acid, a C44 polyketal fatty acid, and microcystin-LR, a cyclic heptapeptide. These structurally diverse reagents show remarkable specificity for the catalytic subunits of PP1 and PP2A, with a much lower affinity for type 2B protein phosphatases (Bialojan and Takai, 1988; MacKintosh et al., 1990). Since they produce virtually identical inhibition profiles we conclude that protein phosphatases play a key role in regulating vesicle fusion. The effect of okadaic acid can be reversed, either by addition of excess phosphatase or by dilution into fresh cytosol, which indicates that we are not observing a spurious inactivation of membranes. Inhibition is completely prevented by inclusion of the purified catalytic subunit of PP2A, but not PP1. While we cannot exclude the possibility that the catalytic subunit of PP1 remains inactive because of the absence of essential regulatory component(s), it appears likely that PP2A rather than PP1 is critical for regulation of vesicle fusion.

Our results are consistent with regulation of vesicle fusion by phosphorylation of key component(s) of the fusion reaction, where the degree of phosphorylation is controlled by a system of opposing protein kinase and phosphatase. Inhibition of PP2A would result in hyperphosphorylation and inactivation of these component(s). This interpretation would also explain the finding that addition of the catalytic subunit of PP2A actually stimulates vesicle fusion above control levels. Evidence for the kinase is provided by the observations that, firstly, okadaic acid does not inhibit at low Mg-ATP concentrations and, secondly, that protein kinase inhibitors reduce the inhibition caused by okadaic acid. We cannot yet exclude the possibility that okadaic acid also activates the kinase to further increase phosphorylation.

It was previously reported that fusion of endocytic vesicles in *Xenopus* cytosols was inhibited by starfish cyclin B-cdc2 kinase (Tuomikoski et al., 1989). Our data suggest that

okadaic acid inhibits vesicle fusion via a protein kinase that must be present, or activated by okadaic acid, in interphase extracts. This would seem to exclude any role for cdc2 kinase, which is active only in mitotic cell extracts. However, we were concerned that a small proportion of cells in an unsynchronized population are mitotic, resulting in low but significant cdc2 kinase activity in these extracts. One might argue that with phosphatase activity abolished this kinase activity would be sufficient to cause phosphorylation of a fusion component. Furthermore, okadaic acid can promote spontaneous and premature activation of cdc2 kinase in cycling extracts from *Xenopus* eggs by inhibiting PP2A (Felix et al., 1990) and causes a transient increase in cdc2 kinase activity in BHK cells blocked in S phase (Yamashita et al., 1990).

For these reasons we have compared the abilities of okadaic acid and cdc2 kinase to inhibit fusion and phosphorylate histones. Histones can be used as a model for the fusion component that is inactivated by phosphorylation, since they are phosphorylated by cdc2 kinase and dephosphorylated by PP1 and PP2A, and by cytosol in a manner prevented by okadaic acid (Lee et al., 1990; Woodman, P. G., and G. Warren, unpublished observations). We have shown that cytosol prepared from cells blocked in mitosis by nocodazole contains elevated levels of cyclin B and cdc2 kinase activity, consistent with the observation of Whitfield et al. (1990). Furthermore, mitotic cytosol supports vesicle fusion poorly, when compared with interphase cytosol. However, when okadaic acid and mitotic cytosol were compared in the same experiment for their effects on vesicle fusion and histone phosphorylation, okadaic acid was found to promote approximately four-fold less histone phosphorylation than mitotic cytosol, yet inhibited fusion to a greater degree. This demonstrates that okadaic acid does not activate cdc2 kinase significantly in extracts from unsynchronized cells and moreover suggests that there is insufficient cdc2 kinase activity in these extracts to account for the inhibition of fusion when phosphatases are blocked. Furthermore, depletion of cdc2 kinase does not substantially affect the inhibition by okadaic acid. Treatment of interphase cytosols with an anti-cdc2 antibody removed more than 78% of the cdc2 protein but did not reduce the inhibition of fusion by okadaic acid. Together, these results suggest that okadaic acid inhibits fusion via a kinase other than cdc2 kinase, though proof of this will require isolation of the putative kinase.

The additional kinase could work downstream from cdc2 kinase to provide indirect regulation of vesicle fusion during mitosis. Alternatively, vesicle fusion might be regulated during interphase by protein phosphorylation. Okadaic acid also inhibits fluid-phase endocytosis in intact interphase HeLa cells (Lucocq, J., G. Warren, and J. Pryde. *J. Cell Sci.* In press.). There is evidence that phosphorylation of some receptors determines their distribution within the cell. For example, phosphorylation of the EGF receptor follows ligand binding, and may cause receptor down regulation (Whiteley and Glaser, 1986). There is little evidence to date that the endocytic pathway as a whole is regulated. However, the idea that cells have the ability to regulate the volume of endocytic transport according to their growth requirements, or in response to external stimuli, is attractive. For cycling receptors, regulation of fusion could be used as a mechanism of controlling the number of receptors expressed on the cell surface, which would regulate the uptake of certain ligands.

We wish to thank Dr. Rosemary Stuart for help and advice during the course of this study, and for critical reading of the manuscript. We thank Jörg Adamczewski and Tim Hunt, Imperial Cancer Research Fund Clare Hall Laboratories, for the gift of the monoclonal antibody to cdc2 protein; Clare McGowan and Larry Gerace, Research Institute of Scripps Clinic, San Diego, for the anti-cdc2 and anti-cyclin B sera; and Professor G. Codd, Dundee University, for the gift of microcystin-LR. We also thank Regina Kieckbusch and Ewen Souter for some HeLa cytosol preparations.

Received for publication 17 May 1991 and in revised form 4 October 1991.

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