

# Evidence for the Regulation of Exocytic Transport by Protein Phosphorylation

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**Abstract.** We investigated the effects of the protein phosphatase inhibitors okadaic acid and microcystin-LR upon transport of newly synthesized proteins through the exocytic pathway. Treatment of CHO cells with 1  $\mu$ M okadaic acid rapidly inhibited movement of a marker protein (vesicular stomatitis virus G protein) from the endoplasmic reticulum to the Golgi compartment. Both okadaic acid and microcystin-LR also inhibited transport in an in vitro assay reconstituting movement to the Golgi compartment, at concentrations equivalent to those required to inhibit phosphatase activity. Inhibition both in vivo and in vitro could be antagonized by protein kinase inhibitors, suggesting that protein phosphorylation was di-

rectly responsible for this effect. An early stage in the transport reaction associated with vesicle formation or targeting was inhibited by protein phosphorylation, which could be reversed by fractions enriched in protein phosphatase 2A. Protein kinase antagonists did not inhibit transport between sequential compartments of the exocytic pathway in vitro, suggesting that protein phosphorylation is not itself required for vesicular transport. During mitosis, vesicular transport is inhibited simultaneous to the activation of maturation-promoting factor. It is proposed that the inhibition caused by okadaic acid and microcystin-LR involves a similar mechanism to that responsible for the mitotic arrest of vesicular transport.

**A**LTHOUGH generally considered a constitutive process (Pfeffer and Rothman, 1987), transport of proteins to the plasma membrane is regulated during cell division (Warren et al., 1983). Using CHO cells infected with a temperature-sensitive mutant of vesicular stomatitis virus (VSV),<sup>1</sup> Featherstone et al. (1985) were able to show that the initial stage of transport (from the ER to the Golgi compartment) was blocked during mitosis. This block, which was coincident with a similar arrest in endocytosis (Berlin et al., 1978; Warren et al., 1984), is believed to be associated with the disassembly and partitioning of single-copy organelles between the two daughter cells (for review see Warren, 1989). At present, the precise mechanisms responsible for the mitotic arrest of vesicular transport are unclear, although the demonstration that endocytic vesicle fusion could be inhibited by p34<sup>cdc2</sup> in vitro (Tuomikowski et al., 1989) has led to the suggestion that one or more protein kinases might be involved.

Protein phosphorylation is the major posttranslational modification performed by eukaryotic cells, and is involved in the regulation of a multitude of cellular processes (for review see Edelman et al., 1987), including the cell cycle (for

reviews see Nurse, 1990; Freeman and Donoghue, 1991). Thus, entry into mitosis is controlled by activation of maturation-promoting factor (MPF), a serine/threonine protein kinase, which is itself regulated by several other serine/threonine and tyrosine kinases (Nurse, 1990). However, the level of phosphorylation of any given protein depends upon the balance between kinase and phosphatase activities acting upon individual sites, and mutations in protein phosphatase genes can also cause defects in the cell cycle (Dooan and Morris, 1989; Booher and Beach, 1989; Kinoshita et al., 1990; Sneddon et al., 1991). Moreover, *INH*, a negative regulator of MPF, was identified as a form of protein phosphatase 2A (Lee et al., 1991).

In contrast to the many protein kinases identified so far, only a limited number of protein phosphatases have been found. Indeed, all soluble serine/threonine phosphatases (PPs) isolated to date belong to one of four subtypes based upon the molecular properties of their respective catalytic subunits (for review see Cohen, 1989). Each subtype may have several family members encoded by alternative splicing or multiple genes, and at least five other genes encoding potential protein phosphatase catalytic subunits have been identified in mammalian and *Drosophila* cDNA libraries (Cohen et al., 1990b). Study of the role of PPs in cellular processes has been greatly facilitated by the recent isolation of two highly selective inhibitors, okadaic acid (OKA) (Bilaljan and Takai, 1988; Haystead et al., 1989) and microcystin-LR (MC) (MacKintosh et al., 1990; Honkanen et al.,

1. *Abbreviations used in this paper:* endo D and endo H, endoglycosidases D and H, respectively; FBS, fetal bovine serum; MC, microcystin-LR; MPF, maturation-promoting factor; OKA, okadaic acid; PP, protein serine/threonine phosphatase; VSV, vesicular stomatitis virus.

1990). Both are extremely potent inhibitors of PP 1 and PP 2A (with  $K_i$  values in the nanomolar range for the isolated catalytic subunits), are considerably less effective towards PP 2B (micromolar  $K_i$  values), and have no effect upon PP 2C. OKA, and to a lesser extent MC, is also membrane permeant and can therefore be used to inhibit PP activity in intact cells (for review see Cohen et al., 1990a). Evidence for the involvement of PP 1 and PP 2A in the regulation of mitosis has also been provided by experiments conducted with OKA (Felix et al., 1989; Yamashita et al., 1990; Zheng et al., 1991).

We now demonstrate that OKA inhibits protein transport within the exocytic pathway in intact CHO cells, and that both OKA and MC inhibit an assay which reconstitutes ER-to-Golgi transport *in vitro*. Inhibition is dependent upon the action of an as yet unidentified protein kinase, but can be reversed by fractions enriched in PP 2A.

## Materials and Methods

### Materials

OKA was obtained from Gibco Laboratories, Grand Island, NY; MC, H-8, and staurosporine were from Calbiochem-Behring Corp., La Jolla, CA;  $^{35}$ S-translabel was from ICN Biomedicals Inc., Costa Mesa, CA; and adenosine 5'-[ $\gamma$ - $^{32}$ P] triphosphate was from Amersham Corp., Arlington Heights, IL. Other reagents, except where indicated, were obtained from Sigma Chemical Co., St. Louis, MO.

### Cells and Viruses

Clone 15B CHO and NRK cells were maintained in monolayer culture in minimal essential medium ( $\alpha$ -MEM) supplemented with penicillin, streptomycin, and 8% fetal bovine serum (FBS) (Gemini Bioproducts Inc., Calabasas, CA), in a 5% CO<sub>2</sub>/95% air atmosphere. VSV (Indiana strain) and the mutant strain tsO45 (Flamand, 1970) were propagated in BHK cells as described elsewhere (Pind et al., 1992).

### Infection and Labeling of Cells

Confluent monolayers of clone 15B or NRK cells were infected with wild-type or tsO45 VSV at a multiplicity of 10–20 plaque-forming units per cell in a gassed incubator at 32°C (Beckers et al., 1987; Schwaninger et al., 1991). Cells 3.5–4.5 h after infection were labeled with  $^{35}$ S-translabel as described previously (Beckers et al., 1987). Briefly, cells infected with wild-type virus were labeled with 200  $\mu$ Ci for 2.5 min at 37°C; and tsO45-infected cells to be subsequently perforated were labeled with 100  $\mu$ Ci for 10 min at 40°C, followed by an additional 2 min in the presence of 0.25 mM unlabeled methionine.

tsO45-infected cells that would not be perforated were labeled with 100  $\mu$ Ci for 3 min at 40°C, followed by an additional 2 min in the presence of 0.25 mM unlabeled methionine and 2.5 mg/ml trypsin with occasional rocking. The medium was then aspirated, the dish was transferred to an ice/water bath, and the cells were washed three times with ice-cold 50 mM Hepes, pH 7.2, 90 mM K acetate containing 100  $\mu$ g/ml soybean trypsin inhibitor (50/90/STI). They were then removed from the dish by repeated pipetting into the same buffer, washed, and resuspended in 50/90/STI at a density of  $8 \times 10^7$  cells/ml. Greater than 95% of cells prepared in this way excluded trypan blue.

### Assay of ER-to-Golgi Transport *In Vitro*

Labeled cells were perforated by the hypotonic swelling and scraping procedure described previously (Beckers et al., 1987; Pind et al., 1992). Semiintact cells ( $\sim 25$ – $30 \mu$ g protein) were incubated at 32°C in an assay mix comprising 37 mM Hepes-KOH, pH 7.2, 78 mM K acetate, 2.5 mM Mg acetate, 5 mM EGTA, 1.8 mM CaCl<sub>2</sub>, 1 mM ATP, 5 mM creatine phosphate, 0.2 IU creatinine phosphokinase (rabbit muscle), and 75  $\mu$ g rat liver cytosol, in a final volume of 40  $\mu$ l (Beckers et al., 1987, 1990). Assays containing perforated NRK cells were supplemented with 1 mM UDP-*N*-acetylglu-

cosamine (Schwaninger et al., 1991). OKA and staurosporine were added from stock solutions in 10% (vol/vol) DMSO, and MC was added from solutions in 10% (vol/vol) methanol. In each case the final concentration of solvent present did not exceed 0.5% (vol/vol).

At the end of the incubation, the perforated cells were collected by brief centrifugation (Eppendorf microfuge, 10 s), incubated with endoglycosidase D (endo D) (15B cells) or endo H (NRK cells), and analyzed by SDS-PAGE and autoradiography, as described previously (Beckers et al., 1987; Schwaninger et al., 1991; Pind et al., 1992). Autoradiographs were quantitated by scanning densitometry (Beckers et al., 1987), and results expressed as the percentage of the total G protein in the endo D-sensitive form (GDs), or having one (G<sub>H1</sub>) or two (G<sub>H2</sub>) endo H-resistant oligosaccharide chains, respectively.

### Surface Immunoprecipitation

Clone 15B CHO cells, radiolabeled and trypsin released as described above, were resuspended in  $\alpha$ -MEM containing 5% FBS ( $1.6 \times 10^6$  cells/ml) and incubated as appropriate. The cells were then transferred to ice, collected by centrifugation, washed once with ice-cold PBS containing 5 mg/ml BSA (PBS/BSA), and resuspended in PBS/BSA containing 80  $\mu$ g/ml anti-VSV G protein (luminal epitope) (clone 8G5; Lefrancois and Lyles, 1982) at a density of  $8 \times 10^6$  cells/ml. After incubation on ice for 2 h with occasional mixing, the cells were collected, washed once with PBS/BSA, and lysed in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% vol/vol Triton X-100, and 1 mM PMSF (TBS/TX). Lysates were clarified by centrifugation (5 min at 16,000 g) and immune complexes recovered from the resulting supernatant with protein A-agarose. After several washes with TBS/TX, immune complexes were eluted with boiling SDS sample buffer and analyzed by SDS-PAGE, autoradiography, and scanning densitometry.

### Indirect Immunofluorescence

Clone 15B CHO cells were infected with tsO45 VSV as described above with the exception that after the initial 45-min binding period at 30°C the infection was continued at 40°C for an additional 3 h. Cells were then removed from culture dishes by limited trypsinolysis at 40°C as described above, and washed cells were resuspended in  $\alpha$ -MEM containing 5% FBS ( $1.6 \times 10^6$  cells/ml) and incubated as appropriate. They were then collected by centrifugation, washed twice with ice-cold PBS, and fixed in 2% paraformaldehyde in PBS for 10 min at room temperature. The fixed cells were washed once in PBS and resuspended to a density of  $2 \times 10^6$  cells/ml in PBS; and 200- $\mu$ l aliquots were transferred to round glass coverslips by centrifugation (Cytospin 2; Shandon Southern Products Ltd., Runcorn, UK). They were then processed essentially as described previously (Plutner et al., 1991) co-staining with anti-VSV G protein (clone 8G5) and rabbit anti-mannosidase II (generous gift of M. G. Farquhar, University of California, San Diego) and subsequently with Texas red-conjugated goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR) and FITC-conjugated goat anti-rabbit IgG (Cappel; Organon Teknica Corp., Durham, NC), respectively. Images were viewed with a confocal scanning laser microscope (MRC-600; Bio-Rad Laboratories, Cambridge, MA), and photographed using Kodak TMAX film (Eastman Kodak Co., Rochester, NY).

### Preparation of Cytosol

Adult male Sprague-Dawley rats ( $\sim 250$  g) were anesthetized, the livers exsanguinated by perfusion through the hepatic-portal vein with ice-cold PBS, and excised. Washed livers were finely minced and homogenized in 3 vol of 25 mM Hepes, pH 7.2, 125 mM K acetate (25/125) containing 1 mM PMSF, 1  $\mu$ M pepstatin A, 10  $\mu$ g/ml leupeptin, and 5  $\mu$ g/ml chymostatin with 10 strokes of a tight-fitting dounce (Wheaton "200"; Thomas Sci., Swedesboro, NJ). The homogenate was centrifuged for 10 min at 15,000 rpm (Beckman JA 20 rotor; Beckman Instruments, Inc., Palo Alto, CA) and the resulting supernatant further centrifuged for 90 min at 35,000 rpm (Beckman SW41 rotor). The lipid layer was aspirated and discarded; the remainder of the supernatant was recovered and flash-frozen in liquid N<sub>2</sub>. Aliquots, typically 15–20 mg/ml protein, were stored at  $-80^\circ\text{C}$ .

### Assay of Phosphorylase Phosphatase

Glycogen phosphorylase was the generous gift of T. A. J. Haystead, University of Washington, Seattle, WA. [ $^{32}$ P]Glycogen phosphorylase, inhibitor-2, and the catalytic subunits of PP 1 and PP 2A were prepared essentially as

described by Cohen et al. (1988). For the analysis of phosphorylase phosphatase activity in mock transport assays, 10- $\mu$ l aliquots containing mock-radiolabeled semiintact cells were incubated in 25 mM Tris-HCl, pH 7.4, containing 0.5 mM EDTA, 0.5 mM DTT, 7.5 mM caffeine, and 1.5 mg/ml [ $^{32}$ P]glycogen phosphorylase (final volume 20  $\mu$ l) at 37°C for 1 min. The reaction was terminated by the addition of 100  $\mu$ l 20% (wt/vol) TCA, and the liberated [ $^{32}$ P]phosphate determined by liquid scintillation counting (Beckman LS5000TD). Analysis of phosphorylase phosphatase activity in cytosolic fractions and in membrane extracts was performed as described by Cohen et al. (1988).

## Results

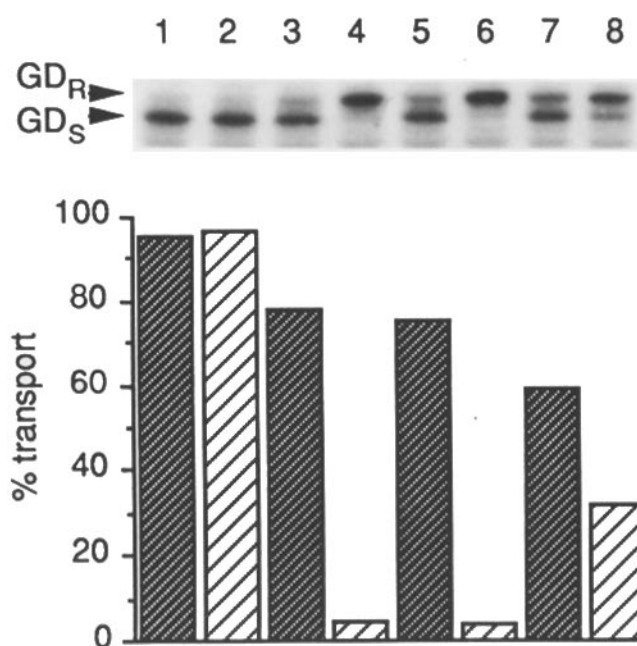
### OKA Inhibits ER-to-Golgi Transport in Intact Cells

To investigate the effect of OKA upon protein transport we infected clone 15B CHO cells with the tsO45 mutant of VSV (Flamand, 1970). At the restrictive temperature (40°C) tsO45 G protein forms aggregates which cannot be exported from the ER. Upon shift to the permissive temperature (32°C) this block is released, and export proceeds at the normal rate. ER-to-Golgi transport can be followed by determining the structure of the N-linked oligosaccharides associated with VSV G protein. Thus, arrival within the *cis*-Golgi compartment is accompanied by the conversion of the oligosaccharides to the Man<sub>5</sub>-GlcNAc<sub>2</sub> form by the resident enzyme  $\alpha$ -1,2-mannosidase I (Tabas and Kornfeld, 1979). Since clone 15B cells are deficient in the enzyme *N*-acetylglucosamine transferase I (GlcNAc Tr I) (Gottlieb et al., 1975), N-linked oligosaccharides present on VSV G protein transported through the *cis*-Golgi compartment and beyond are uniquely sensitive to digestion by endo D (Mizuuchi et al., 1984). The acquisition of sensitivity to endo D can therefore be used as a measure of the arrival of VSV G protein within the Golgi compartment in clone 15B cells.

Addition of OKA directly upon shifting radiolabeled cells to the permissive temperature had no effect (Fig. 1, lanes 1 and 2). In contrast, preincubation of the cells at 40°C for 15 min in the presence of OKA (1  $\mu$ M) abolished transport during a subsequent chase at 32°C (lanes 3 and 4). Inhibition was not affected by preincubation of labeled cells with cycloheximide (100  $\mu$ g/ml), but was partially antagonized by prior incubation with the nonselective protein kinase inhibitor, 2-aminopurine (10 mM), although this reagent itself partially inhibited transport (lanes 5–8). These results suggest that inhibition may involve the activity of a protein kinase, but does not require additional protein synthesis.

To extend these results, two "wild-type" cell lines (CHO and NRK) were treated with OKA under similar conditions. Unlike clone 15B, these cells possess functional GlcNAc Tr I (Gottlieb et al., 1985), which renders N-linked oligosaccharides insensitive to endo D (Mizuuchi et al., 1984), but allows processing to the complex form resistant to endo H (a marker for arrival in the *cis*/*medial*-Golgi compartment (Schwaninger et al., 1991). In both cases, no endo H-resistant VSV G protein could be detected in cells incubated in the presence of OKA (data not shown).

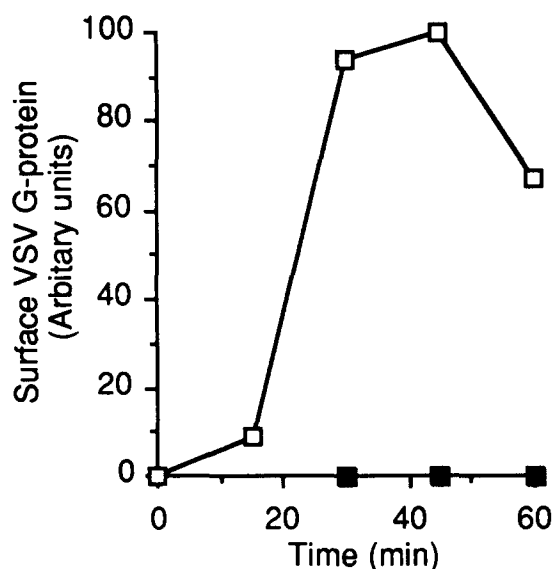
Although no previous reports have suggested that OKA has any effect upon oligosaccharide-processing enzymes, we considered it important to eliminate the possibility that the observed effects resulted from the inhibition of one or more of these enzymes rather than from a direct inhibition of transport. Previous studies have demonstrated that move-



**Figure 1.** OKA inhibits protein transport in intact CHO cells. Clone 15B CHO cells infected with tsO45 VSV were pulse labeled, washed, and suspended in 50/90/STI as described in Materials and Methods. Aliquots ( $4 \times 10^5$  cells) were resuspended in  $\alpha$ -MEM containing 5% FBS ( $1.6 \times 10^6$  cells/ml) (lanes 1–4), or medium containing 100  $\mu$ g/ml cycloheximide (lanes 5 and 6) or 10 mM 2-aminopurine (lanes 7 and 8). The suspensions were incubated on ice (lanes 1 and 2) or at 40°C (lanes 3–8) for 5 min; OKA was added to a final concentration of 1  $\mu$ M (lanes 2, 4, 6, and 8); and incubations were continued for an additional 15 min. The suspensions were then transferred to ice for 15 min and subsequently incubated at 32°C for 45 min. Cells were collected by centrifugation and processed as described in Materials and Methods. The amount of G protein present in the endo D-resistant (GD<sub>R</sub>) and -sensitive (GD<sub>S</sub>) forms was determined by scanning densitometry, and transport assessed as the percentage sensitive to endo D:  $(GD_S/[GD_S + GD_R]) \times 100$ .

ment of VSV G protein to the cell surface is unaffected by inhibition of N-linked oligosaccharide-trimming enzymes (Burke et al., 1984). We therefore examined the effect of OKA upon the surface expression of G protein. As shown in Fig. 2 ( $\square$ ), radiolabeled tsO45 VSV G protein could be detected by surface immunoprecipitation within 30 min of shifting cells to the permissive temperature, and reached a maximum after 45 min; kinetics consistent with those previously described (Pfeiffer et al., 1985). In contrast, treatment of clone 15B CHO cells with OKA completely abolished our ability to detect radiolabeled G protein at the plasma membrane (Fig. 2,  $\blacksquare$ ); but did not influence the ability to precipitate the protein from total cell lysates (data not shown). These results clearly demonstrate that treatment with OKA directly inhibits vesicular trafficking in the exocytic pathway.

To further investigate the stage at which vesicular trafficking was inhibited, we examined CHO cells infected with tsO45 VSV by indirect immunofluorescence microscopy. After a 15-min incubation at the permissive temperature in the absence of OKA, VSV G protein became concentrated in the perinuclear region and colocalized with the



**Figure 2.** Effect of OKA on surface expression of VSV G protein. Clone 15B CHO cells were infected with tsO45 VSV, radiolabeled, and trypsin released as described in Materials and Methods. Aliquots ( $8 \times 10^5$  cells) were incubated at  $40^\circ\text{C}$  in the presence (■) or absence (□) of OKA ( $1 \mu\text{M}$ ) for 15 min, incubated at  $0^\circ\text{C}$  for an additional 15 min, and then at  $32^\circ\text{C}$  for times as indicated. Radio-labeled G protein as recovered from the plasma membrane by immunoprecipitation as described in Materials and Methods.

Golgi marker protein mannosidase II (Fig. 3, A–D). However, in cells pretreated at the restrictive temperature with OKA and shifted to the permissive temperature in the continued presence of the drug, VSV G protein showed a reticular staining pattern characteristic of retention within the ER (Fig. 3, F and H). Interestingly, OKA-treated cells showed a considerably more diffuse Golgi staining pattern than control cells, suggesting that the drug was inducing fragmentation of this organelle (Fig. 3, E and G). Despite this apparent breakdown, analysis at high magnification confirmed that VSV G protein and mannosidase II failed to colocalize in the OKA-treated cells (Fig. 3, G and H, *small arrows*).

A similar result was obtained in cells incubated at the permissive temperature for 45 min. In this case control cells showed significant peripheral staining of VSV G protein (Fig. 3 I, *broad arrows*), consistent with our ability to detect the protein by surface immunoprecipitation, while the distribution of G protein in OKA-treated cells was essentially unchanged from that observed after 15 min, and did not colocalize with mannosidase II (Fig. 3, M–P).

### MC and OKA Inhibit ER-to-Golgi Transport in Semiintact Cells

To further investigate the molecular basis of the effects of PP

inhibitors on protein trafficking, experiments were conducted with “semiintact cells.” These are a population of cells which have been permeabilized by mechanical shear under conditions which cause the removal of the soluble contents, but which allow the retention of secretory organelles in a functionally intact form. Transport of protein from the ER to the Golgi compartment in semiintact cells is dependent upon the addition of ATP and a high-speed supernatant (“cytosol”) (Beckers et al., 1987, 1990; Schwaninger et al., 1991; for reviews see Balch, 1989, 1990).

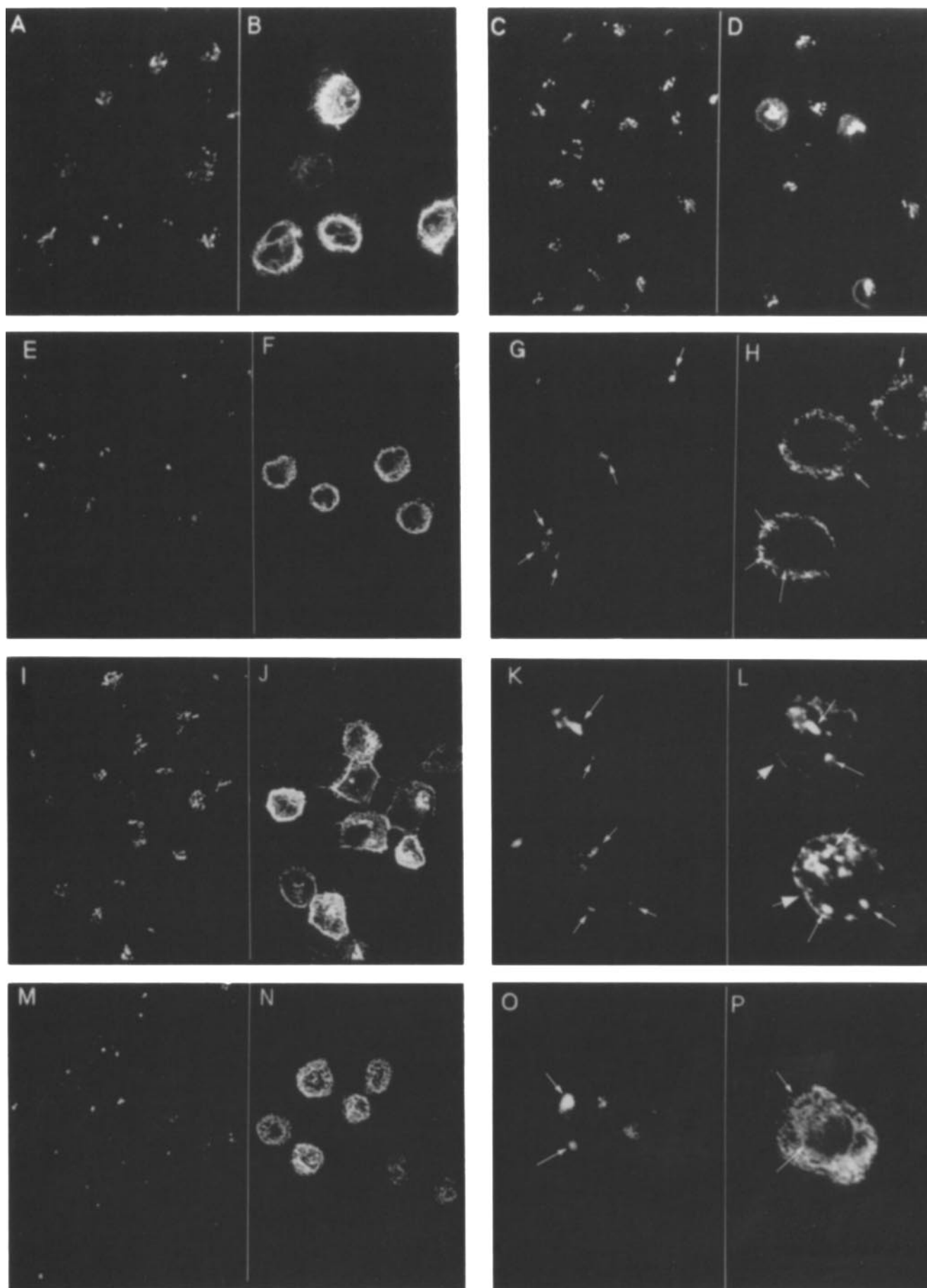
Addition of either MC ( $1.25 \mu\text{M}$ ) or OKA ( $2.5 \mu\text{M}$ ) to the *in vitro* transport assay caused a 70% reduction in the amount of VSV G protein transported to the Golgi compartment in a cytosol-dependent manner (Fig. 4, columns 1–5). To investigate whether the phosphatases involved in this inhibition were present exclusively in either the membrane or cytosolic fractions, semiintact cells and cytosol were pretreated separately with MC on ice, and excess inhibitor removed by pelleting or gel filtration, respectively. Assays were then conducted using combinations of treated and mock-treated components. Little inhibition was observed when the assay contained either mock-treated semiintact cells or cytosol (Fig. 4, columns 6 and 7), whereas inhibition equivalent to that obtained by the direct addition of MC occurred when treated semiintact cells and cytosol were combined (column 8). This result indicates that the PPs whose inhibition by OKA and MC is responsible for the observed arrest in transport are not localized exclusively to the membrane fraction, although as this fraction likely contains some residual cytosolic components we cannot be completely certain that the relevant phosphatase(s) are not exclusively cytosolic.

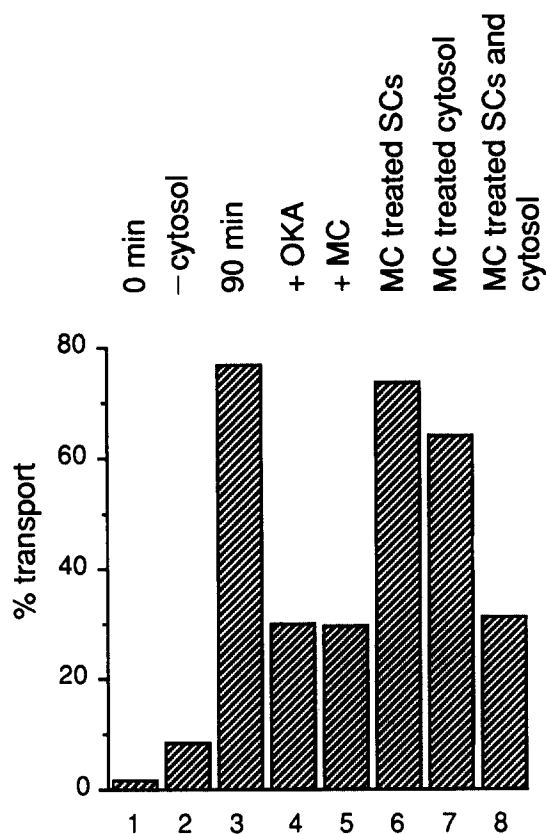
As shown in Fig. 5, both MC and OKA inhibited protein transport in a dose-dependent fashion consistent with their effects on protein phosphatase activity. Half-maximal inhibition of transport was obtained at  $\sim 40 \text{ nM}$  MC and  $150 \text{ nM}$  OKA, respectively (Fig. 5). In other experiments, half-maximal values ranging from 30 to  $100 \text{ nM}$  MC and 100 to  $500 \text{ nM}$  OKA were obtained (data not shown), but in each case the overall relationships were maintained. Thus, (a) no inhibition of transport was observed when  $>50\%$  of the maximal phosphorylase phosphatase activity remained (a measure of PP 1 and PP 2A activity), (b) MC was approximately fourfold more potent than OKA, and (c) maximal inhibition of transport was 60–80% of the cytosol-dependent signal. The variability between experiments probably results from differences between individual preparations of semiintact cells, which typically contained 30–50% of the total phosphorylase phosphatase activity present in the assay.

### MC Inhibits an Early Step in Transport

Analysis of protein transport *in vitro* has revealed an appar-

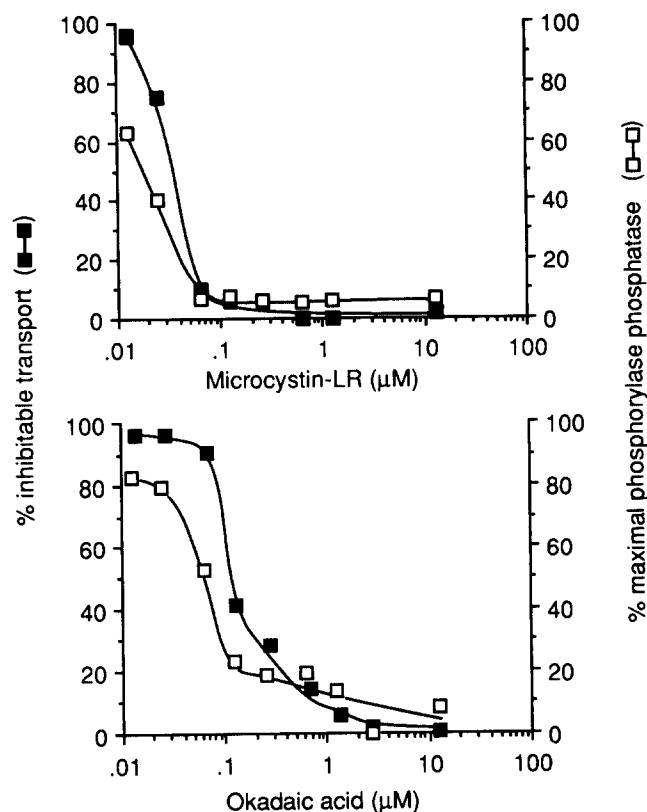
**Figure 3.** OKA blocks export from the endoplasmic reticulum. Clone 15B CHO cells infected with tsO45 VSV were released from culture dishes by limited trypsinolysis. Aliquots ( $4 \times 10^5$  cells) were resuspended in  $\alpha$ -MEM containing 5% FBS ( $1.6 \times 10^6$  cells/ml) in the absence (A–D and I–L) or presence (E–H and M–P) of  $1 \mu\text{M}$  OKA and incubated at  $40^\circ\text{C}$  for 15 min. The suspensions were then transferred to ice for 15 min and subsequently either maintained on ice (A and B) or incubated at  $32^\circ\text{C}$  for 15 min (C–H) or 45 min (I–P). Cells were collected by centrifugation and processed for indirect immunofluorescence as described in Materials and Methods. Golgi compartments were detected by staining with an antisera to mannosidase II (A, C, E, I, K, M, and O) or VSV G protein (B, D, F, H, J, L, N, and P). Images were photographed at low magnification (A–F, I, J, M, and N) or high power (G, H, K, L, O, and P). Small arrows indicate Golgi region and broad arrows the cell surface.





**Figure 4.** MC and OKA inhibit protein transport in semiintact cells (SCs). Clone 15B CHO cells were infected with tsO45 VSV, radiolabeled, and SCs prepared as described in Materials and Methods. Aliquots were assayed in the absence or presence of OKA (2.5  $\mu$ M) or MC (1.25  $\mu$ M), respectively. MC-treated SCs were prepared by resuspending SCs in 50/90 containing 1.25  $\mu$ M MC, incubating on ice for 10 min, and collecting the SCs by centrifugation (3 min at 500 g; 4°C). They were then resuspended in 50/90 without inhibitor. MC-treated cytosol was prepared by incubating cytosol (100  $\mu$ l) with 1  $\mu$ M MC, on ice for 15 min, and centrifuging (5 min at 1,500 g) through a 1-ml column of Biogel P6-DG (Bio-Rad Laboratories, Richmond, CA) equilibrated with 25/125.

ent lag between the budding of carrier vesicles from the ER, and their fusion with the *cis*-Golgi compartment (Beckers et al., 1990; for review see Balch, 1989). Since the results of our immunofluorescence studies suggested that PP inhibition affects the "early" (budding and targeting) steps (Fig. 3), we examined whether this was also the case in vitro. MC was added at various times during the course of a 90-min incubation and the amount of G protein which had become resistant to the action of the inhibitor determined. A time-dependent increase in the amount of VSV G protein resistant to the action of the inhibitor was observed (Fig. 6, ■). Thus, ~60% of the transportable G protein (0.46/0.79) was resistant after 15 min of the incubation (■), a time at which <10% (0.06/0.79) had been transported to the *cis*-Golgi compartment (□). The rate at which VSV G protein became resistant to the action of MC was similar to that observed with other "early" inhibitors such as GTP $\gamma$ S and the anti-rab1b monoclonal 4D3c (Beckers et al., 1990; Plutner et al., 1991), consistent with the hypothesis that the component(s) involved



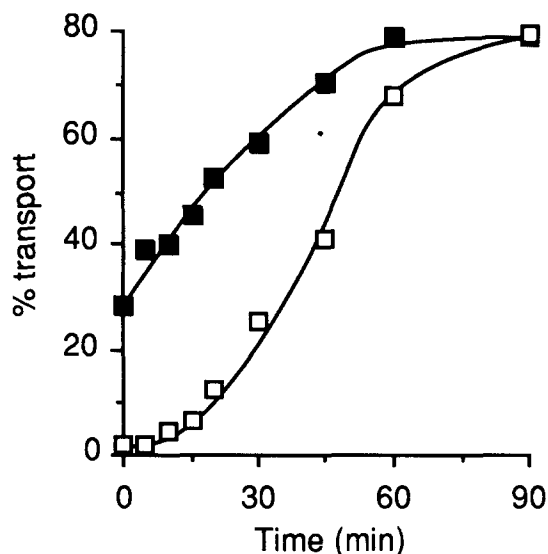
**Figure 5.** Concentration dependence of OKA- and MC-mediated inhibition of protein transport and phosphorylase phosphatase activity in semiintact cells. Clone 15B CHO cells were infected with tsO45 VSV, radiolabeled (■) or mock-labeled (□), and semiintact cells prepared as described in Materials and Methods. Aliquots were assayed for protein transport (■) or phosphorylase phosphatase activity (□) in the presence of MC (top) or OKA (bottom) as described in Materials and Methods. For protein transport, results are expressed as the percentage uninhibited of the maximum inhibitable transport. Maximal transport was 70% and uninhibitable transport 26.9%. Maximal phosphorylase phosphatase activity was 6.5 mU/ml.

are normally dephosphorylated during the initial stages of vesicular transport, and with our observations of the distribution of VSV G protein in OKA-treated cells.

#### ***Inhibition Is Dependent upon ATP Concentration and Can Be Prevented by Protein Kinase Antagonists***

The extent of phosphorylation of any substrate depends upon the balance between the competing action of protein kinases and phosphatases. Consequently, although PP inhibitors act directly to prevent dephosphorylation, in most instances they indirectly cause hyperphosphorylation due to the continued action of protein kinases (Cohen et al., 1990a). The partial antagonism between OKA and 2-aminopurine observed in intact cells (Fig. 1) is consistent with the hypothesis that the mechanism of transport inhibition involves increased protein phosphorylation, rather than decreased dephosphorylation per se. We therefore decided to investigate the potential role of protein kinases in protein trafficking.

Analysis of ER-to-Golgi transport in vitro is routinely conducted in a buffer containing 1 mM ATP. However, in the

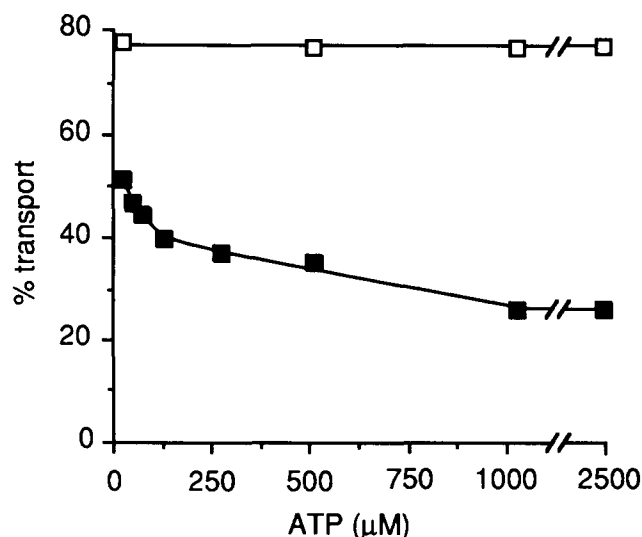


**Figure 6.** MC inhibits an early stage in vesicular transport. Semi-intact cells prepared as described above were assayed at 32°C. At various times during the incubation, individual aliquots were either transferred to ice (□), or MC was added to a final concentration of 1.25  $\mu$ M, and the incubation continued for the remainder of a total of 90 min (■).

presence of a regenerating system, <20  $\mu$ M is actually required for a maximal signal (Beckers, C. J. M., and W. E. Balch, unpublished observation). Although protein kinases typically have  $K_m$  values for ATP in the region of 10  $\mu$ M and might therefore be expected to be almost maximally active under these conditions we examined whether altering the nucleotide concentration influenced the degree of inhibition observed.

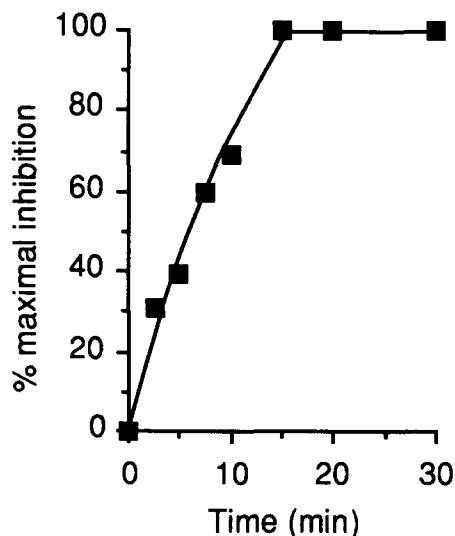
In these experiments the basal ATP concentration was  $\sim$ 25  $\mu$ M. Increasing the concentration of MgATP between 25  $\mu$ M and 2.5 mM did not affect the amount of G protein transported to the Golgi compartment in control incubations (Fig. 7, □). In contrast, MC-mediated inhibition of ER-to-Golgi transport increased from  $\sim$ 40% of the cytosol-dependent signal at 25  $\mu$ M ATP to >75% at 2.5 mM (Fig. 7, ■). This result confirmed the direct involvement of one or more protein kinases in the inhibitory process, with the overall reaction having an apparent  $K_m$  for ATP of  $\sim$ 30  $\mu$ M.

To further investigate the mechanism responsible for the inhibition of transport, the effects of selected protein kinase antagonists were examined. In the presence of a 1-mM ATP-regenerating system, 2-aminopurine (5 mM), H-7 (0.8 mM), and H-8 (1 mM) did not affect transport in control assays. However, these inhibitors antagonized MC- or OKA-mediated inhibition by 40, 50, and 95%, respectively (data not shown). Inhibition of transport by PP inhibitors could also be antagonized by staurosporine (2.5  $\mu$ M). Accordingly, we used this reagent to analyze the temporal aspects of MC-mediated inhibition. As shown in Fig. 8, a time-dependent loss in the ability of staurosporine to prevent inhibition was observed, with 50% maximal inhibition occurring when the addition of MC and staurosporine were separated by  $\sim$ 5.5 min. A similar result was obtained using H-8 (data not shown).



**Figure 7.** ATP dependence of MC-mediated inhibition. Semi-intact cells prepared as described in Materials and Methods were assayed in buffers containing ATP-regenerating systems buffered at 0–2.5 mM ATP and in the absence (□) or presence (■) of MC (1.25  $\mu$ M).

Both staurosporine and H-8 are potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases, but will also inhibit other protein kinases (for review see Ruegg and Burgess, 1989). Experiments designed to directly investigate the potential role of protein kinase C in the inhibitory process demonstrated that MC-mediated inhibition occurred in the absence of  $Ca^{2+}$ . Thus, inhibition occurred when incubations with MC were conducted in the presence of EGTA, and staurosporine was added simultaneous to the addition of  $Ca^{2+}$  to relieve the EGTA block of



**Figure 8.** Kinetics of MC-mediated inhibition of protein transport. Semi-intact cells prepared as described in Materials and Methods were assayed in the presence of MC (1.25  $\mu$ M). At times indicated, staurosporine was added to a final concentration of 2.5  $\mu$ M, and the incubation continued for a total of 90 min. Results are expressed as the percentage of maximal inhibition.

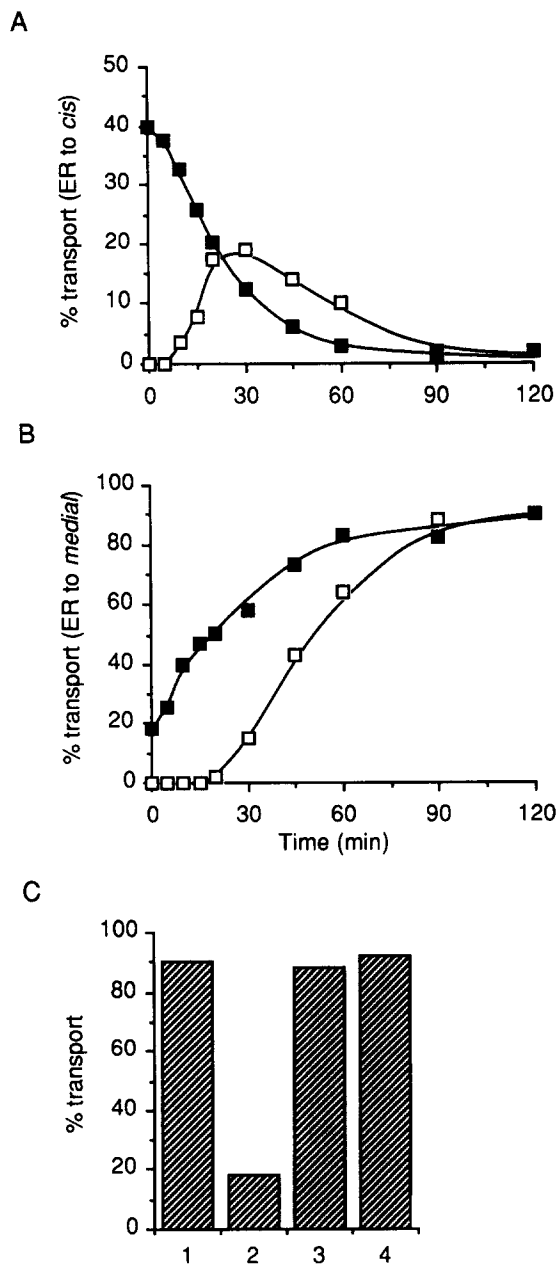
transport (data not shown). In addition, inhibitory peptides specific for protein kinase C, cAMP-dependent protein kinases, and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II did not prevent MC-mediated inhibition when added to 100  $\mu\text{M}$ , concentrations equivalent to those capable of completely inhibiting the target enzymes in microinjection studies (for review see Hardie, 1988). Together these data argue against the involvement of protein kinase C, cAMP-dependent protein kinases, or  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II in the modulation of protein trafficking, although the possibility that an isozyme of protein kinase C that is insensitive to both  $\text{Ca}^{2+}$  and the autoinhibitory peptide is involved cannot be excluded.

### *cis-to-medial Intra-Golgi Transport Is Inhibited by MC but Unaffected by Staurosporine*

If transport between adjacent compartments of the exocytic pathway involved the cyclic phosphorylation and dephosphorylation of particular components, it would be expected that inhibition of either phosphorylation or dephosphorylation would arrest transport. Thus, the ability to antagonize MC- and OKA-mediated inhibition of transport by nonselective protein kinase inhibitors suggested that the components involved in vesicular transport are not ordinarily phosphorylated during individual cycles of budding and fusion. However, this possibility could not be completely eliminated, given the inhibitory effect of 2-aminopurine upon intact cells (Fig. 1) and the rapid entry of VSV G protein into a MC- and OKA-resistant state in vitro (Fig. 6).

To directly investigate the potential role of protein phosphorylation in vesicular transport in the absence of PP inhibitors we took advantage of our recent success in extending the in vitro ER-to-Golgi transport assay to encompass the sequential movement of VSV G protein to the *medial* cisternae (Schwaninger et al., 1991). Since this involves two temporally distinct rounds of budding and fusion, we examined whether differences existed between the effects of protein phosphatase and protein kinase inhibitors on the two individual reactions. NRK cells were infected with wild-type VSV, and transport to the *cis* and *medial* compartments was determined by the appearance of the  $G_{H1}$  and  $G_{H2}$  forms of G protein, respectively (Schwaninger et al., 1991). Inclusion of MC in the incubation medium inhibited transport from the ER to the *cis*-Golgi compartment ( $G_{H1} + G_{H2}$ ) by  $\sim 30\%$ , with the majority of the transported VSV G protein present in the  $G_{H1}$  form (Fig. 9, A and B; ■). This result indicates that MC inhibits transport both to and from the *cis*-Golgi compartment, and is similar to that obtained with GTP $\gamma$ S, another "early" inhibitor which blocks both transport events (Schwaninger et al., 1991; and data not shown). Moreover, consistent with the inhibition of an early step in both ER-to-*cis* and *cis*-to-*medial* Golgi transport, addition of MC at later times during the incubation resulted in the rapid accumulation of inhibitor-resistant VSV G protein, and a coordinate decrease in the ratio of  $G_{H1}$  to  $G_{H2}$  (Fig. 9, A and B).

Under the above conditions, MC-mediated inhibition was also antagonized by staurosporine, which itself did not influence control incubations (Fig. 9, C). The observation that transport was not inhibited when MC and staurosporine were both present argues against MC-mediated inhibition in the absence of staurosporine being an artefact due to the inhibition of any of the oligosaccharide-processing enzymes



**Figure 9.** Effects of MC and staurosporine on intra-Golgi transport. Semiintact cells were prepared from NRK cells infected with wild-type VSV as described in Materials and Methods, and assayed in the presence of UDP-*N*-acetylglucosamine. (A and B) At the times indicated aliquots were transferred to ice (□), or MC was added to a final concentration of 1.25  $\mu\text{M}$  and the incubation continued for the remainder of the 120-min time course (■). The semiintact cells were then collected and processed with endo H as described previously (Schwaninger et al., 1991). Results are expressed as the percentage of VSV G protein in the  $G_{H1}$  (A) or  $G_{H2}$  (B) form, respectively. (C) Semiintact cells were assayed for a total of 120 min in assay mix alone (1), or assay mix containing 1.25  $\mu\text{M}$  MC (2), 1.25  $\mu\text{M}$  MC + 2.5  $\mu\text{M}$  staurosporine (3), or 2.5  $\mu\text{M}$  staurosporine (4), respectively. Results are expressed as the percentage of G protein present as the  $G_{H2}$  form.

responsible for creating resistance to endo H, and is consistent with our hypothesis that a similar inhibitory mechanism is involved in 15B CHO and NRK cells. Both ER-to-*cis* and *cis*-to-*medial* transport were also inhibited by MC and OKA



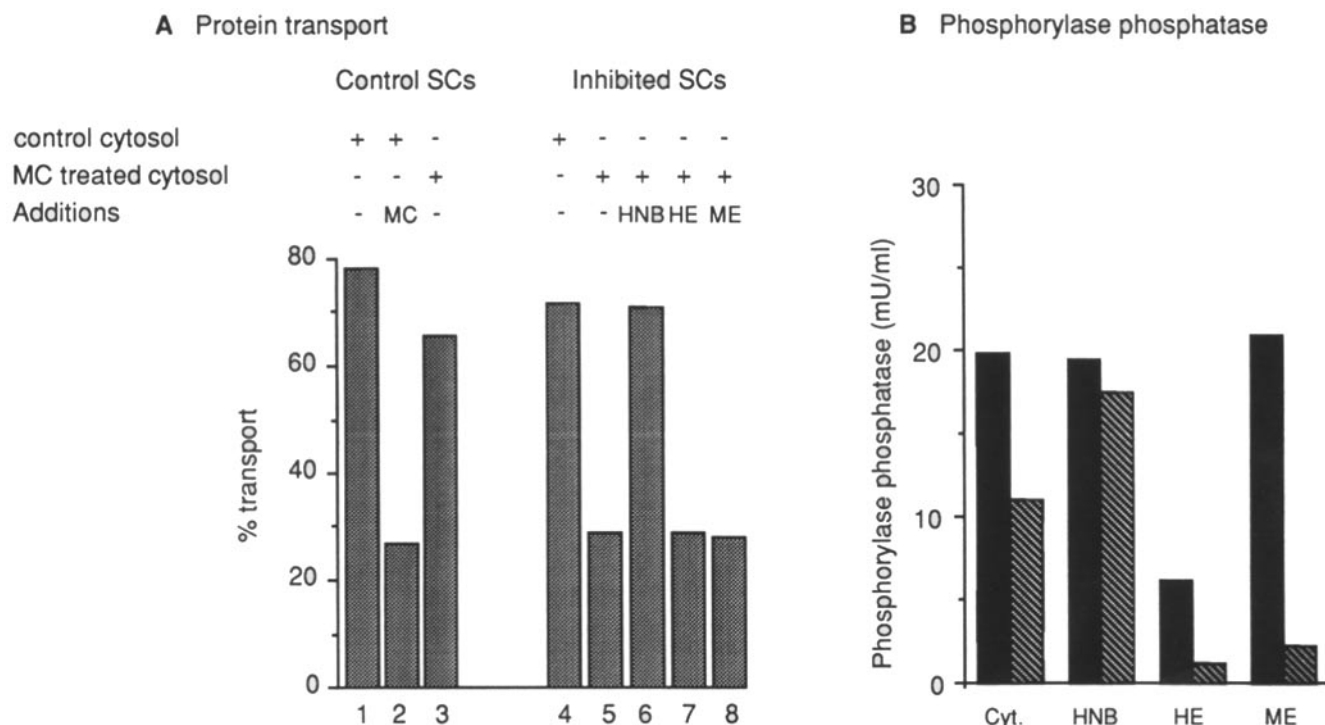
in semiintact wild-type CHO cells (data not shown). In addition, since the protein kinase antagonist did not inhibit transport in either of two sequential rounds of budding and fusion, these results are consistent with the hypothesis that protein phosphorylation causes an arrest in transport; and that it is not ordinarily part of the mechanism by which vesicles move between adjacent compartments. However, the possibilities that transport ordinarily involves a protein kinase resistant to inhibition by the antagonists used and that a distinct enzyme is responsible for the inhibition of transport cannot be excluded.

#### ***Inhibition Can Be Reversed by Fractions Enriched in Protein Phosphatase 2A***

The results described above suggested that one or more of the components involved in vesicular transport must be maintained in the dephosphorylated state, and that either PP 1 or PP 2A is responsible. Although both OKA and MC show higher affinity for PP 2A than PP 1 (Bialojan and Takai, 1988; Haystead et al., 1989; MacKintosh et al., 1990; Honkanen et al., 1990), we could not be certain that the results described in Fig. 5 implicated either enzyme, at least in part because of the indirect nature of transport inhibition. To determine whether PP 1 or PP 2A alone was required to maintain the dephosphorylated state, we examined whether MC-mediated inhibition could be reversed.

As protein phosphorylation appears necessary to generate the inhibitory state, semiintact clone 15B CHO cells were incubated with cytosol and MC at 32°C for 20 min to allow maximal inhibition to occur (Fig. 8). They were then pelleted, resuspended in a second mix without MC, and incubated for an additional 90 min. As shown in Fig. 10A, no inhibition was observed when the second mix contained untreated cytosol (columns 1 and 4). In contrast, inhibition equivalent to that obtained in the continuous presence of MC was observed when the final incubation mix contained cytosol preincubated with MC (columns 2 and 5). These results were not influenced by the presence or absence of protein kinase antagonists in the second incubation mix, indicating that protein(s) in the untreated cytosol had reversed the inhibition which had occurred during the preincubation.

Addition of the purified catalytic subunits of PP 1 and PP 2A to MC-inhibited semiintact cells in the presence of MC-treated cytosol did not reverse the inhibition (data not shown), although in control experiments limited phosphorylase phosphatase activity could be detected after incubation under these conditions. The catalytic subunits do not normally exist in a free state, and the presence of the regulatory subunits may influence activity towards particular substrates (Sola et al., 1991). We therefore prepared fractions enriched in the holoenzymes of the two PPs. Analysis of the rat liver cytosol used in the reversal assay indicated that it contained



**Figure 10.** Reversal of MC inhibition. (A) Semiintact clone 15B cells were assayed directly from ice (control SCs), or preincubated for 20 min at 32°C in assay mix containing 1.25  $\mu$ M MC, pelleted, and resuspended in 50/90 (Inhibited SCs). Aliquots of inhibited SCs were subsequently incubated for an additional 90 min in the presence of control or MC-treated cytosols as indicated, augmented with the heparin-agarose nonbinding fraction of cytosol (HNB), heparin-agarose eluate (HE), or CHO cell membrane extract (ME) ( $\sim$ 1 mU/ml phosphorylase phosphatase activity), respectively. MC-treated cytosol was prepared by incubating aliquots (100  $\mu$ l) made 1 mM in MgATP and 1  $\mu$ M MC at 32°C for 10 min, and then centrifuging through Biogel P6-DG equilibrated in 25/125 containing 100  $\mu$ M MgATP. (B) HNB, HE, and ME fractions were prepared essentially as described by Pondaven and Cohen (1987) using rat liver cytosol and CHO 15B cell homogenates, respectively, and centrifuged through Biogel P6-DG columns equilibrated with 25/125 containing 0.25 mM Na mercaptoethanesulphonic acid, to remove buffer constituents inhibitory to the transport assay. Phosphorylase phosphatase activity was measured in the absence (■) or presence (▨) of inhibitor-2 as described in Materials and Methods.

~20 mU phosphorylase phosphatase per ml of which 30–40% was type 1 (inhibited by inhibitor-2; Cohen, 1989; Fig. 10 B; and data not shown). We fractionated this cytosol by heparin-agarose chromatography to resolve PP 1 and PP 2A (Erdodi et al., 1985; Pondaven and Cohen, 1987). As expected, in the presence of 0.1 M KCl PP 2A was eluted in the unbound fraction, whereas PP 1 bound to the column and was eluted by 0.5 M KCl (Fig. 10, B). Subsequent analysis indicated that MC-mediated inhibition was reversed by the heparin-agarose unbound fraction, but not by equivalent activities of the bound and eluted fraction (Fig. 10, columns 6 and 7).

In a second approach, a 0.5-M KCl extract of CHO post-nuclear membranes was prepared (Pondaven and Cohen, 1987). This contained ~22 mU phosphorylase phosphatase/ml, of which ~90% was inhibitable with inhibitor-2 (type 1). As shown in Fig. 10 (column 8), the CHO cell extract, like the heparin-agarose bound fraction of cytosol (column 7), was unable to reverse MC-mediated inhibition under the assay conditions used.

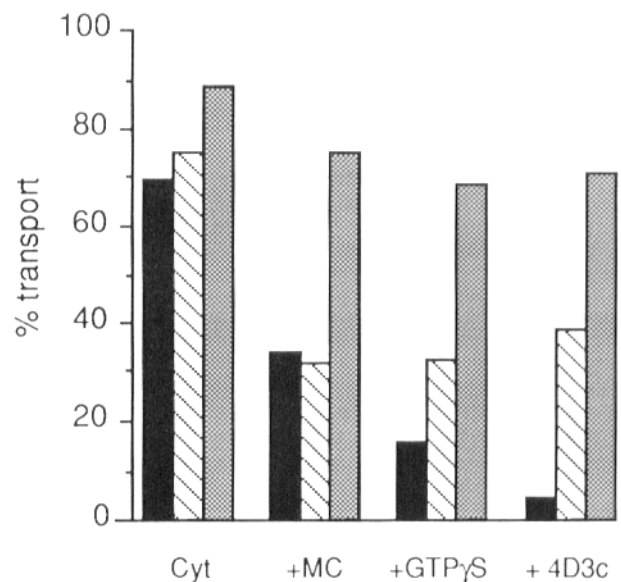
In additional experiments, gel filtration chromatography of unfractionated cytosol revealed a major peak of reversal activity of 100–180 kD, and reversal activity was associated with the major peaks of casein phosphatase activity when cytosol was fractionated by anion exchange chromatography (data not shown). These data are consistent with the distribution of the PP 2A holoenzymes, and together they suggest that inhibition of PP 2A is primarily responsible for causing inhibition of protein transport.

#### MC-inhibited Semiintact Cells Remain Sensitive to Other "Early" Transport Inhibitors

The morphological and biochemical data presented above indicate that treatment of cells with phosphatase inhibitors blocks exocytic transport at an early step presumably related to priming or budding of the donor compartment. Our ability to reverse this inhibition using fractions enriched in PP 2A allowed us to ask whether the inhibited membranes remained sensitive to other kinetically "early" inhibitors such as GTP $\gamma$ S and the inhibitory monoclonal antibodies to rab1b, or had progressed beyond any of these stages. Semiintact CHO 15B cells were incubated on ice or at 32°C in the presence or absence of MC (1.25  $\mu$ M) for 30 min and collected by centrifugation. They were then resuspended in fresh assay mixtures containing untreated cytosol and selected inhibitors. As shown in Fig. 11, cells incubated at 32°C in the absence of MC were almost completely resistant to subsequent inhibition by MC, GTP $\gamma$ S, or monoclonal 4D3c (stippled columns). In contrast, virtually no additional VSV G protein to that which had escaped the initial inhibition by MC was transported to the Golgi compartment when reversal was conducted in the presence of MC, GTP $\gamma$ S, or 4D3c (shaded columns).

#### Discussion

The results described above demonstrate that transport of newly synthesized proteins from the ER to the Golgi compartment is inhibited by exposure to inhibitors of protein phosphatases. However, treatment of cells with OKA produces marked hyperphosphorylation (Haystead et al., 1989;



**Figure 11.** Inhibited semiintact cells remain sensitive to early transport inhibitors. Semiintact clone 15B cells were either maintained on ice (■), or incubated at 32°C in assay mixture with (▨) or without (▩) 1.25  $\mu$ M MC for 30 min, collected by centrifugation, and resuspended in 50/90. Cell aliquots were then added to fresh assay mixtures containing MC (1.25  $\mu$ M), GTP $\gamma$ S (10  $\mu$ M), or monoclonal 4D3c (2.5  $\mu$ g), respectively, and either directly incubated at 32°C for 90 min (control, MC, and GTP $\gamma$ S) or preincubated at 0°C for 90 min to allow antibody binding and subsequently at 32°C for an additional 90 min (4D3c). ER-to-Golgi transport was assessed as described in Materials and Methods.

Cohen et al., 1990a; Zheng et al., 1991). At least three pieces of evidence support the conclusion that protein phosphorylation, rather than a lack of protein dephosphorylation per se, is actually responsible for the inhibition of transport observed in the present study: (1) inhibition of transport in intact cells was antagonized by the nonspecific protein kinase inhibitor, 2-aminopurine; (2) MC-mediated inhibition of semiintact cells was sensitive to the concentration of ATP, and (3) inhibition in vitro could be antagonized by simultaneous treatment with protein kinase inhibitors such as staurosporine and H-8.

At present the identity of the protein kinase(s) responsible for the inhibition remains unclear. Since the mitotic arrest of vesicular transport appears temporally related to the activation of MPF (Featherstone et al., 1985; Nurse, 1990), this kinase is obviously an attractive candidate. However, the results obtained do not support this hypothesis. For example, MPF kinase activity is absent during interphase and requires cyclin synthesis for activation (for review see Nurse, 1990), whereas inhibition of transport in intact cells was not prevented by protein synthesis inhibitors. In addition, the rat liver cytosol used in the present study had no detectable histone H1 kinase activity (Smythe, C., and H. W. Davidson, unpublished observation), and did not acquire such activity when incubated with semiintact cells and MC under conditions which inhibited protein transport (data not shown). Similarly, cytosol prepared from nocodazole-arrested HeLa cells, containing active MPF both before and after incuba-

tion, supported transport to the same extent as interphase cytosol (data not shown).

Evidence that MPF may not be solely responsible for the inhibition of other membrane-related events associated with the entry into mitosis has come from the results of two recent studies. In the first, treatment of *Xenopus* egg extracts with OKA caused dissociation of nuclear membranes from chromatin and could be antagonized by protein kinase inhibitors (Pfaller et al., 1991). However, dissociation could not be induced by purified MPF. Secondly, despite the observations of Tuomikowski et al. (1989), MPF does not appear to be responsible for the OKA-mediated inhibition of endocytic vesicle fusion in vitro (Woodman et al., 1992).

Several other protein kinases have been implicated in the control of events associated with the cell cycle, including protein kinase C, cAMP-dependent protein kinases, and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (Levin et al., 1990; Lamb et al., 1991; Baitinger et al., 1990). MC-mediated inhibition of transport was not prevented by specific peptide inhibitors of these enzymes. Inhibition by staurosporine has been considered diagnostic of the involvement of protein kinase C (Tamaoki et al., 1986) but this inhibitor acts at the ATP-binding site which is highly homologous to those of other serine-threonine-specific kinases (Tamaoki et al., 1986; Edelman et al., 1987) and will inhibit a variety of other protein kinases in vitro (Ruegg and Burgess, 1989). Moreover, the involvement of protein kinase C or  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II in the inhibition of ER-to-Golgi transport is unlikely since inhibition occurred in the absence of  $\text{Ca}^{2+}$ . The identification of the kinase responsible for inhibiting transport is currently under investigation.

In contrast to our uncertainty regarding the identity of the protein kinase(s) involved in the inhibition of vesicular transport, we are more confident that PP 2A is the enzyme responsible for preventing the inhibition during interphase. The reversal of MC-mediated inhibition by untreated cytosol, but not by MC-treated cytosol, under conditions where components other than PPs were not limiting, suggests that a soluble rather than a membrane-associated enzyme is involved. This conclusion does not conflict with the observation that treatment of both soluble and membrane components of the assay were required for maximal inhibition, since our semi-intact preparations generally contain some residual soluble components (Beckers et al., 1987). Typically, the cells comprised 30–50% of the total phosphatase activity present in the assay (including both type 1 and 2A). When PP 1 and PP 2A were separated by heparin affinity chromatography (Erdodi et al., 1985; Pondaven and Cohen, 1987) reversal of inhibition was observed using the unbound fraction, but not with the bound and eluted material. This result is consistent with the distribution of type 2A activity. Furthermore, proteins of an apparent molecular weight equivalent to that of type 2A phosphatase holoenzymes, and coeluting with these enzymes after anion-exchange chromatography (Cohen, 1989), were able to relieve the block in transport. However, we were unable to reverse inhibition using the purified catalytic subunits of either phosphatase type 1 or 2A. Sola et al. (1991) demonstrated that PP 2A holoenzymes showed a significantly higher ratio of histone H1 phosphatase (phosphorylated by  $\text{p34}^{\text{cdc}2}$ ) to phosphorylase phosphatase activity than did the

free catalytic subunits. Thus, although we do not believe that  $\text{p34}^{\text{cdc}2}$  is directly responsible for the phosphorylation events in the present study, we think it likely that our failure to demonstrate reversal by the catalytic subunit of PP 2A reflects a failure to add sufficient functional enzymatic activity rather than an absolute requirement for the regulatory subunits. Nevertheless, we cannot completely eliminate the possibility that a PP with properties highly similar to, but distinct from, type 2A is actually responsible.

At present we also cannot completely eliminate the possibilities that PP 1 is able to reverse the inhibition, if added at sufficiently high concentrations, or that a factor necessary for PP 1 to affect reversal is lost during the 0.5 M KCl treatments inherent in our preparations of this enzyme. However, on balance we believe it likely that PP 2A is the protein phosphatase that is primarily responsible for maintaining protein transport in an uninhibited state.

PP 2A has been implicated in the regulation of other events which occur during the onset of mitosis, including the dissociation of nuclear membranes from chromatin (Pfaller et al., 1991) and the inhibition of endocytic vesicle fusion (Woodman et al., 1992). PP 2A has also been identified as a negative regulator of the activation of MPF (Felix et al., 1989; Kinoshita et al., 1990; Lee et al., 1991); its inhibition may underlie the induction of a mitosis-like state by OKA (Yamashita et al., 1990; Zheng et al., 1991).

The results obtained in this study suggest that an early event in transport, presumably related to the formation or budding of vesicles, is inhibited by protein phosphorylation; consistent with the inhibition of export observed during metaphase (Featherstone et al., 1985). Thus, VSV G protein retained an "ER-like" distribution in OKA-treated CHO cells incubated at the permissive temperature, transport rapidly became resistant to the addition of MC or OKA in vitro, while inhibited semiintact cells rescued by cytosol fractions enriched in PP 2A remained sensitive to other "early" inhibitors (e.g.,  $\text{GTP}\gamma\text{S}$ ; Beckers et al., 1990) at incubation times when control cells were almost completely resistant. This inhibition could not be attributed to a failure to dissolve tsO45 G protein aggregates after incubation at the restrictive temperature since this is largely completed during the incubations on ice in the preparation of the semiintact cells (Balch, W. E., unpublished observation). In addition, inhibition was also observed in cells infected with wild-type virus, and movement from *cis* to *medial* Golgi compartments was also blocked.

In contrast to the complete inhibition of transport observed in vivo, a relatively large proportion of VSV G protein was transported to the *cis*-Golgi compartment in the continuous presence of MC or OKA in vitro (typically 30–40% of the cytosol-dependent signal in semiintact cells infected with tsO45 and up to 70% in those infected with wild-type virus). This signal was not merely the result of a general slowing of transport since no additional movement was observed when incubations in the presence of MC were prolonged for up to 4 h (our unpublished observation). The existence of a significant fraction of VSV G protein which is resistant to inhibition from ice in vitro is consistent with vesicle formation being the stage at which transport is arrested, especially given the apparent indirect nature of the inhibitory mechanism. Thus, in order to completely inhibit transport in intact cells, it was necessary to treat them with OKA for 15 min

under conditions where the marker protein (tsO45 G protein) was retained within the ER. However, in contrast to the reversible phenotype observed with intact cells, shifting semiintact cells to 40°C in vitro causes an irreversible conformational change in tsO45 G protein which prevents subsequent biochemical analysis of transport (Balch, W. E., unpublished observation). Consequently, it was not possible to preincubate semiintact cells using the standard assay in the presence of ATP (to allow phosphorylation to occur) under conditions which would prevent export, precluding the establishment of a completely inhibited state in vitro. Conditions are currently being developed to address this problem.

The observation that MC-mediated inhibition could not be reversed by MC-treated cytosol suggests that relief of the inhibition involves the dephosphorylation of a component associated with the cell membranes at the end of the preincubation. At present many of the components required for vesicular transport remain poorly characterized, making identification of the protein(s) which become phosphorylated a technically difficult task. One intriguing possibility is that they might include members of the *rab* gene family of low molecular weight GTP-binding proteins (Touchot et al., 1987), which have been implicated in several stages of vesicular transport (for review see Balch, 1990). Recently, it was shown that two members of this family, rab 1p (implicated in ER-to-Golgi transport; Plutner et al., 1991) and rab 4p (localized to early endocytic vesicles; van der Sluijs et al., 1991), were hyperphosphorylated during mitosis (Bailey et al., 1991). However, this study also showed that these proteins were substrates in vitro of p34<sup>cdc2</sup> and that their phosphorylation could not be detected in interphase extracts. This result appears to conflict with the results of the present study which suggest that p34<sup>cdc2</sup> is not responsible for the inhibition of transport. At present the evidence that p34<sup>cdc2</sup> is responsible for the phosphorylation of rab 1p observed in mitotic cells remains indirect, and the possibility that it is a substrate for protein kinases other than MPF cannot be excluded. However, it is also possible that modification of rab 1p is not directly related to the inhibition of transport. The functional significance of the phosphorylation of rab 1p remains to be established.

Our results strongly suggest that MPF is not directly responsible for the inhibition of vesicular transport that we observe. However, we believe it likely that transport inhibition and the activation of MPF are related events. For example, a single kinase might be involved in the regulation of both processes, transport inhibition in mitosis could result from a shift in the balance of phosphorylation/dephosphorylation as a consequence of MPF activation and be mimicked by OKA and MC, or a protein kinase which is ordinarily a substrate of MPF might become prematurely activated in the presence of OKA and MC. Alternatively, inhibition of PPs might lead to the activation of a kinase which phosphorylates protein(s) involved in vesicular transport which are also substrates of MPF during mitosis. Determination of which, if any, of these hypotheses is correct and the establishment of the relationship between the effects observed in vivo and in vitro must await the identification of the relevant enzyme(s) and substrate(s), problems which we are currently addressing.

We thank T. A. J. Haystead (University of Washington), M. G. Farquhar (University of California, San Diego), and H. Schulman (Stanford University), for the gift of reagents. We are also grateful to G. Warren (Imperial Cancer Research Fund, London) for communicating results prior to publication; C. Smythe (University of California, San Diego) and T. Hunter (Salk Institute, La Jolla) for helpful discussions; and S. Pind for many useful comments during the preparation of the manuscript.

H. W. Davidson is an SERC/NATO Research Fellow. This work was supported by grants GM 33301, GM 42336, and CA 27489 from the National Institutes of Health (to W. E. Balch), and by the G. Harold and Leila Y. Mathers Charitable Foundation.

Received for publication 11 November 1991 and in revised form 16 December 1991.

**Note Added in Proof.** After submission of this work we became aware of a study reporting the OKA-mediated inhibition of intracellular transport in intact HeLa cells (J. Lucocq, G. Warren, and J. Pryde. 1991. *J. Cell Sci.* 100:753-759).

## References

- Bailly, E., M. McCaffrey, N. Touchot, A. Zahraoui, B. Goud, and M. Bornens. 1991. Phosphorylation of two small GTP-binding proteins of the Rab family by p34<sup>cdc2</sup>. *Nature (Lond.)* 350:715-718.
- Baittinger, C., J. Alderton, M. Poenie, H. Schulman, and R. A. Steinhardt. 1990. Multifunctional Ca<sup>2+</sup>/calmodulin-dependent protein kinase is necessary for nuclear envelope breakdown. *J. Cell Biol.* 111:1763-1773.
- Balch, W. E. 1989. Biochemistry of interorganelle transport. *J. Biol. Chem.* 264:16965-16968.
- Balch, W. E. 1990. Small GTP-binding proteins in vesicular transport. *Trends Biochem. Sci.* 15:473-477.
- Beckers, C. J. M., D. S. Keller, and W. E. Balch. 1987. Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell* 50:523-534.
- Beckers, C. J. M., H. Plutner, H. W. Davidson, and W. E. Balch. 1990. Sequential intermediates in the transport of protein between the endoplasmic reticulum and the Golgi. *J. Biol. Chem.* 265:18298-18310.
- Berlin, R. D., J. M. Oliver, and R. J. Walter. 1978. Surface functions during mitosis I: phagocytosis, pinocytosis and mobility of surface-bound ConA. *Cell* 15:327-341.
- Bialojan, C., and A. Takai. 1988. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. *Biochem. J.* 256:283-290.
- Boother, R., and D. Beach. 1989. Involvement of a type 1 protein phosphatase encoded by *bws1* in fission yeast mitotic control. *Cell* 57:1009-1016.
- Burke, B., K. Matlin, E. Bause, G. Legler, N. Peyrieras, and H. Ploegh. 1984. Inhibition of N-linked oligosaccharide trimming does not interfere with surface expression of certain integral membrane proteins. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:551-556.
- Cohen, P. 1989. The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* 58:453-508.
- Cohen, P., S. Alemany, B. A. Hemmings, T. J. Resink, P. Stralfors, and H. Y. Lim Tung. 1988. Protein phosphatase-1 and protein phosphatase-2A from rabbit skeletal muscle. *Methods Enzymol.* 159:390-408.
- Cohen, P., C. F. B. Holmes, and Y. Tsukitani. 1990a. Okadaic acid: a new probe for the study of cellular regulation. *Trends Biochem. Sci.* 15:98-102.
- Cohen, P. T. W., N. D. Brewis, V. Hughes, and D. J. Mann. 1990b. Protein serine/threonine phosphatases; an expanding family. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 268:355-359.
- Doonan, J. H., and N. R. Morris. 1989. The *bimG* gene of *Aspergillus nidulans*, required for completion of anaphase, encodes a homolog of mammalian phosphoprotein phosphatase 1. *Cell* 57:987-996.
- Edelman, A. M., D. K. Blumenthal, and E. G. Krebs. 1987. Protein serine/threonine kinases. *Annu. Rev. Biochem.* 56:567-614.
- Erdodi, F., C. Csontos, G. Bot, and P. Gergely. 1985. Separation of rabbit liver latent and spontaneously active phosphorylase phosphatases by chromatography on heparin-Sepharose. *Biochem. Biophys. Res. Commun.* 128:705-712.
- Featherstone, C., G. Griffiths, and G. Warren. 1985. Newly synthesized G protein of vesicular stomatitis virus is not transported to the Golgi complex in mitotic cells. *J. Cell Biol.* 101:2036-2046.
- Felix, M.-A., P. Cohen, and E. Karsenti. 1989. Cdc H1 kinase is negatively regulated by a type 2A phosphatase in the *Xenopus* early embryonic cell cycle: evidence from the effects of okadaic acid. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:675-683.
- Flamand, A. 1970. Etude genetique du virus de la stomatite vesiculaire: classement de mutants thermosensibles spontanés en groupes de complementation. *J. Gen. Virol.* 8:187-195.
- Freeman, R. S., and D. J. Donoghue. 1991. Protein kinases and protooncogenes: biochemical regulators of the eukaryotic cell cycle. *Biochemistry* 30:2293-2302.
- Gottlieb, C., J. Baenziger, and S. Kornfeld. 1975. Deficient uridine diphosphate-N-acetylglucosamine:glycoprotein N-acetylglucosaminyltransferase activity in a clone of Chinese hamster ovary cells with altered surface glycoproteins. *J. Biol. Chem.* 250:3303-3309.

- Hardie, D. G. 1988. Pseudosubstrates turn off protein kinases. *Nature (Lond.)*. 335:592-593.
- Haystead, T. A. J., A. T. R. Sim, D. Carling, R. C. Honnor, Y. Tsukitani, P. Cohen, and D. G. Hardie. 1989. Effects of the tumour promoter okadaic acid on intracellular protein phosphorylation and metabolism. *Nature (Lond.)*. 337:78-81.
- Honkanen, R. E., J. Zwiller, R. E. Moore, S. L. Daily, B. S. Khatra, M. Dukelow, and A. L. Boynton. 1990. Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases. *J. Biol. Chem.* 265:19401-19404.
- Kinoshita, N., H. Ohkura, and M. Yanagida. 1990. Distinct, essential roles of type 1 and 2A protein phosphatases in the control of the fission yeast cell division cycle. *Cell*. 63:405-415.
- Lamb, N. J. C., J.-C. Cavadore, J.-C. Labbe, R. A. Maurer, and A. Fernandez. 1991. Inhibition of cAMP-dependent protein kinase plays a key role in the induction of mitosis and nuclear envelope breakdown in mammalian cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1523-1533.
- Lee, T. H., M. J. Solomon, M. C. Mumby, and M. W. Kirschner. 1991. INH, a negative regulator of MPF, is a form of protein phosphatase 2A. *Cell*. 64:415-423.
- Lefrancois, L., and D. S. Lyles. 1982. The interaction of antibody with the major surface glycoprotein of vesicular stomatitis virus. I. Analysis of neutralizing epitopes with monoclonal antibodies. *Virology*. 121:157-167.
- Levin, D. E., F. O. Fields, R. Kunisawa, J. M. Bishop, and J. Thorner. 1990. A candidate protein kinase C gene, PKC1, is required for the *S. cerevisiae* cell cycle. *Cell*. 62:213-224.
- MacKintosh, C., K. A. Beattie, S. Klumpp, P. Cohen, and G. A. Codd. 1990. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 264:187-192.
- Mizuochi, T., J. Amano, and A. Kobata. 1984. New evidence of the substrate specificity of endo- $\beta$ -N-acetylglucosaminidase D. *J. Biochem. (Tokyo)*. 95:1209-1213.
- Nurse, P. 1990. Universal control mechanism regulating onset of M-phase. *Nature (Lond.)*. 344:503-508.
- Pfaller, R., C. Smythe, and J. W. Newport. 1991. Assembly/disassembly of the nuclear envelope membrane: cell-cycle-dependent binding of nuclear membrane vesicles to chromatin in vitro. *Cell*. 65:209-217.
- Pfeffer, S. R., and J. E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* 56:829-852.
- Pfeiffer, S., S. D. Fuller, and K. Simons. 1985. Intracellular sorting and basolateral appearance of the G protein of vesicular stomatitis virus in Madin-Darby canine kidney cells. *J. Cell Biol.* 101:470-476.
- Pind, S., H. W. Davidson, R. Schwaninger, C. J. M. Beckers, H. Plutner, S. L. Schmid, and W. E. Balch. 1992. Preparation of semi-intact (perfused) cells for the study of vesicular trafficking in vitro. *Methods Enzymol.* In press.
- Plutner, H., A. D. Cox, S. Pind, R. Khosravi-Far, J. R. Bourne, R. Schwaninger, C. J. Der, and W. E. Balch. 1991. Rab 1b regulates vesicular transport between the endoplasmic reticulum and successive Golgi compartments. *J. Cell Biol.* 115:31-43.
- Pondaven, P., and P. Cohen. 1987. Identification of protein phosphatases-1 and 2A and inhibitor-2 in oocytes of the starfish *Asterias rubens* and *Marthasterias glacialis*. *Eur. J. Biochem.* 167:135-140.
- Ruegg, U. T., and G. M. Burgess. 1989. Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. *Trends Pharmacol. Sci.* 10:218-220.
- Schwaninger, R., C. J. M. Beckers, and W. E. Balch. 1991. Sequential transport of protein between the endoplasmic reticulum and successive Golgi compartments in semi-intact cells. *J. Biol. Chem.* 266:13055-13063.
- Sneddon, A. A., P. T. W. Cohen, and M. J. R. Stark. 1991. *Saccharomyces cerevisiae* protein phosphatase 2A performs an essential cellular function and is encoded by two genes. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:4339-4346.
- Sola, M. M., T. Langan, and P. Cohen. 1991. p34cdc2 phosphorylation sites in histone H1 are dephosphorylated by protein phosphatase 2A<sub>1</sub>. *Biochim. Biophys. Acta*. 1094:211-216.
- Tabas, I., and S. Kornfeld. 1979. Purification and characterization of a rat liver Golgi  $\alpha$ -mannosidase capable of processing asparagine-linked oligosaccharides. *J. Biol. Chem.* 254:11655-11663.
- Tamaoki, T., H. Nomoto, I. Takahashi, Y. Kato, H. Morimoto, and E. Tomita. 1986. Staurosporine, a potent inhibitor of phospholipid/Ca<sup>++</sup> dependent protein kinase. *Biochem. Biophys. Res. Commun.* 135:397-402.
- Touchot, N., P. Chardin, and A. Tavittian. 1987. Four additional members of the *ras* gene superfamily isolated by an oligonucleotide strategy: molecular cloning of YPT-related cDNAs from a rat brain library. *Proc. Natl. Acad. Sci. USA*. 84:8210-8214.
- Tuomikowski, T., M.-A. Felix, M. Doree, and J. Gruenberg. 1989. Inhibition of endocytic vesicle fusion in vitro by the cell-cycle control protein kinase cdc2. *Nature (Lond.)*. 342:942-945.
- van der Sluijs, P., M. Hull, A. Zahraoui, A. Tavittian, B. Goud, and I. Mellman. 1991. The small GTP-binding protein rab4 is associated with early endosomes. *Proc. Natl. Acad. Sci. USA*. 88:6313-6317.
- Warren, G. 1989. Mitosis and membranes. *Nature (Lond.)*. 342:857-858.
- Warren, G., C. Featherstone, G. Griffiths, and B. Burke. 1983. Newly synthesized G protein of vesicular stomatitis virus is not transported to the cell surface during mitosis. *J. Cell Biol.* 97:1623-1628.
- Warren, G., J. Davoust, and A. Cockcroft. 1984. Recycling of transferrin receptors in A431 cells is inhibited during mitosis. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2217-2225.
- Woodman, P. G., D. I. Mundy, P. Cohen, and G. Warren. 1992. Cell-free fusion of endocytic vesicles is regulated by phosphorylation. *J. Cell Biol.* 116:331-338.
- Yamashita, K., H. Yasuda, J. Pines, K. Yasumoto, H. Nishitani, M. Ohtsubo, T. Hunter, T. Sugimura, and T. Nishimoto. 1990. Okadaic acid, a potent inhibitor of type 1 and type 2A protein phosphatases, activates cdc2/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:4331-4338.
- Zheng, B., C. F. Woo, and J. F. Kuo. 1991. Mitotic arrest and enhanced nuclear protein phosphorylation in human leukemia K562 cells by okadaic acid, a potent protein phosphatase inhibitor and tumor promoter. *J. Biol. Chem.* 266:10031-10034.