Facilitated Nuclear Transport of Calmodulin in Tissue Culture Cells

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Abstract. Calmodulin (CaM) potentiates Ca^{2+}-dependent signaling pathways in both the cytoplasm and nucleus. We have investigated the mechanism of CaM nuclear transport using tissue culture cell microinjection and a permeabilized cell import assay. The inhibition of CaM import by the translocation inhibitor wheat germ agglutinin (WGA) and by chilling, indicates that CaM import is facilitated, but because ATP depletion does not affect CaM import, the mechanism does not appear to be active. Chilling and WGA arrest persist in ATP-depleted cells, indicating that CaM is not retained in the cytoplasm by an ATP-dependent mechanism. In permeabilized cells, both Ca^{2+}-CaM and Ca^{2+}-free CaM are sensitive to extract-dependent WGA and chilling import inhibition. Titration experiments in microinjected and permeabilized cells indicate that a saturable cytosolic factor(s) mediates chilling and WGA arrest.

CALMODULIN (CaM,[1] 16.8 kD) is a ubiquitous Ca^{2+}-binding protein whose primary sequence is perfectly conserved among mammals (Lagacé et al., 1983). CaM belongs to the EF-hand-motif family of Ca^{2+}-binding proteins, including parvalbumin, troponin C, and calbindin (Heizmann and Hunziker, 1991), which contain Ca^{2+}-binding pockets comprised of helix-loop-helix domains. Ca^{2+}-CaM directly activates a number of cytoplasmic target enzymes involved in the regulation of protein phosphorylation (Cohen, 1988; Cohen and Klee, 1988). For example, Ca^{2+}-CaM activates the Ca^{2+}-dependent ATPase (Enyedi et al., 1987) and myosin light chain kinase (Kemp et al., 1987).

In addition to its demonstrated activities in the cytoplasm, CaM target proteins are also present in the nucleus (Harper et al., 1980; Serratosa et al., 1988; Vendrell et al., 1991; Bachs et al., 1992). Cell fractionation studies indicate that the concentration of CaM in nuclei ranges between 1–10 μM, depending upon the cell or tissue type (Bachs et al., 1992). Corneliussen et al. (1994) described a specific nuclear function for CaM. They showed that Ca^{2+}-CaM selectively inhibits transcription by binding to certain transcription factors of the basic helix-loop-helix domain group. Transcription of CaM genes increases during late G1 of the cell cycle, and this leads to elevated cellular CaM levels during S phase (Chafouleas et al., 1982; Sasaki and Hidaka, 1982), which is consistent with a nuclear role for CaM in DNA replication and repair (Bachs et al., 1992).

The nuclear import of proteins proceeds by a series of cytosolic ATP-independent binding reactions, followed by the ATP-dependent accumulation of karyophile in the nucleus (Newmeyer and Forbes, 1988; Richardson et al., 1988; Moore and Blobel, 1992; Forbes, 1992). Import is thought to be initiated by the formation of a targeting complex between a cytoplasmic nuclear localization signal (NLS) receptor(s) and an NLS-containing karyophile (Adam and Gerace, 1991; Garcia-Bustos et al., 1991; Forbes, 1992). In situ competition experiments indicate that, while alternative import pathways may exist for certain U snRNPs (Michaud and Goldfarb, 1992), most nuclear proteins are imported by a predominant NLS-mediated pathway and share the same import receptor(s) (Michaud and Goldfarb, 1993).

A key feature of the nuclear envelope is the presence of ~10 nm diam diffusion channels, presumably located within the 120–150-nm diam annulus of the pore complex (Peters, 1986; Dingwall, 1991; Garcia-Bustos et al., 1991; Forbes, 1992; Hinshaw et al., 1992). In the case of a small karyophile such as histone H1 (21 kD), which is imported by a saturable ATP-dependent mechanism, diffusion through these pores is precluded by the formation of a cytoplasmic complex with an NLS-receptor (Breeuwer and Goldfarb, 1990). The nuclear pores should still be considered as an alternative route for the nucleocytoplasmic exchange of small proteins, and may, in fact, be used for the passive transport of the cAMP-dependent protein kinase catalytic subunit after its dissociation from the anchored regulatory subunit (Harootunian et al., 1993).

Our interest in CaM stems from issues relating to the mechanism of establishing and maintaining its distribution

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1. *Abbreviations used in this paper*: CaM, calmodulin; fl-CaM, FITC-labeled CaM; fl-TI, fluorscein-labeled trypsin inhibitor; GlcNAc, *N*-acetylglucosamine; NLS, nuclear localization signal; TI, trypsin inhibitor; TnC, troponin C.

across the nuclear envelope. CaM represents an unusual class of nuclear protein because of its small size, the lack of an obvious NLS motif (Dingwall and Laskey, 1991), and its presence in significant concentrations on both sides of the nuclear envelope. In this study, we sought to determine whether CaM import occurs by passive diffusion, facilitated diffusion, or by an ATP-dependent mechanism. Several criteria were tested. Chilling serves to distinguish passive transport from facilitated transport (Richardson et al., 1988; Breeuwer and Goldfarb, 1990). Wheat germ agglutinin (WGA), which binds to the *N*-acetylglucosamine (GlcNAc)-containing nucleoporins (Forbes, 1992) at the pore complex (Finlay et al., 1987; Newmeyer and Forbes, 1988; Akey and Goldfarb, 1989), inhibits pore complex-mediated transport (Yoneda et al., 1987; Dabauvalle et al., 1988; Michaud and Goldfarb, 1992), but does not prevent the passive transport of small dextrans or nonnuclear reporter proteins such as lysozyme (14.4 kD) or soybean trypsin inhibitor (21 kD) (Yoneda et al., 1987; Dabauvalle, 1988; Breeuwer and Goldfarb, 1990). ATP depletion, of course, tests for ATP dependence (Newmeyer and Forbes, 1988; Richardson et al., 1988).

Materials and Methods

Proteins

CaM was purified from bovine brain according to Gopalakrishna and Anderson (1982) using Ca^{2+}-dependent phenyl Sepharose affinity chromatography. Soybean trypsin inhibitor (TI) was purchased from Sigma Chem. Co. (St. Louis, MO). Troponin C was purified from material kindly provided by S. Ebashi (University of Tokyo, Japan) with the help of F. Vellani (ETH, Zurich, Switzerland) according to Greaser and Gergeley (1973). Calbindin-9K was a gift from E. Thulin (University of Lund, Sweden). The purification of histone H1 was as described in Breeuwer and Goldfarb (1990). To produce the CaM tryptic peptide containing amino acids 1–106 (P106), CaM was proteolyzed with trypsin (Boehringer Mannheim, Germany) essentially as described (Guerini et al., 1984). P106 was purified by reverse phase HPLC (Applied Biosystems, Foster City, CA) using an analytical Nucleosil C-18 (Macherey and Nagel, Oensingen, Switzerland) 250 × 4 mm, 3 μM, 120A column, or semipreparative Nucleosil C-18 250 × 10, 10 μM, 300A column according to Guerini et al. (1984). The fragment was further purified and desalted by RP-HPLC using 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid, 70% acetonitrile in water, respectively, as buffers. P106 was analyzed by polyacrylamide gel electrophoresis in presence of 6 M urea as described in Guerini et al. (1984).

Preparation of Protein Conjugates and Labeling Procedures

Synthesis and conjugation of the SV40 large T antigen NLS peptide P(Lys) (Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Pro-Tyr-Cys) to BSA was performed after Goldfarb et al. (1990). Proteins, 1–2 mg/ml dissolved in 0.5 M sodium carbonate/sodium bicarbonate (carbonate buffer) pH 9.0, were modified at room temperature with FITC or RITC (Fluka AG, Buchs, Switzerland) dissolved in acetone added to a final concentration of 0.5–1 mg/ml. Labeled proteins were purified by Sephadex G25-gel filtration chromatography (Sigma Chemical Co.) in 0.1 M ammonium bicarbonate, lyophilized and stored at −20°C.

Tissue Culture Cell Microinjection

PtK1 cells growing in F12 medium on glass coverslips (Breeuwer and Goldfarb, 1990) were rinsed with PBS and microinjected using either a video-enhanced fully automated AIS-Carl Zeiss injection system equipped with an Eppendorf 5242 microinjector (Carl Zeiss, Oberkochen, Germany) or an Olympus (Lake Success, NY) IMT-2 inverted fluorescence microscope equipped with a Narishige MO-IM2-3 set joystick hydraulic micromanipulator and microinjector. Injector femtotips were purchased from Eppendorf (Hamburg, Germany) or needles were pulled on a Flaming Brown micropipette puller model P80/PC (Sutter Instruments Co., Novato, CA) from 1.0-mm thin walled glass capillaries with filaments (World Precision Instruments, Sarasota, FL). The lyophilized proteins were dissolved in intracellular buffer (100 mM KCl, 11 mM NaCl, 7.2 mM K_2HPO_4, 4.8 mM KH_2PO_4, pH 7.0) and spun at 11,000 *g* for 20 min before microinjection. The concentrations of proteins in the needles are listed in the figure legends. After 30 min incubation at 37°C in F12 medium, the coverslips were exposed to 4% formaldehyde in PBS, rinsed with PBS, and mounted on slides in PBS, 20% glycerol, 0.01 mg/ml DAPI (Fluka) supplemented with DABCO antibleaching agent (Fluka). For energy charge depletion studies, the cells were incubated 30 min before, during, and 30 min after injection in Hank's balanced salt solutions with 10 mM Na-azide (Fluka), 50 mM 2-deoxy-D-glucose (Sigma Chem. Co.), at 37°C. Drug-independent energy depletion was achieved by coinjection of 100 μ/ml apyrase (Sigma Chem. Co.) with the substrates. Chilling studies were performed by cooling the cells on ice 30 min before and 30 min after injection. For WGA inhibition, 2.5 mg/ml WGA (Sigma Chem. Co.) dissolved in intracellular buffer was mixed with the proteins before microinjection and spun for 20′ at 11,000 *g*. *N*-acetylglucosamine (GlcNAc) (Fluka AG) was coinjected at 0.5 M in the needle. Competition experiments were performed as follows. FITC-labeled CaM (fl-CaM, 27 μM) was coinjected with WGA (2.5 mg/ml) and increasing concentrations of unlabeled CaM (50, 100, 300, 500 μM). After 30 min incubation at 37°C in F12 medium, the cells were fixed as described above. Control experiments were performed using unlabeled soybean trypsin inhibitor (300 μM) coinjected with WGA (2.5 mg/ml) and fl-CaM. Fluorescence photomicroscopy was performed on Reichert Polyvar and Olympus microscopes.

Permeabilized Cell Import Assay

Calf brains were obtained fresh from Conti Packing Company Inc. (Rochester, NY), and packed in ice within 1 h of slaughter. Brains were rinsed with cold water to remove excess blood, quartered, and frozen at −80°C. Frozen tissue was thawed in an equal volume of homogenization buffer (20 mM Tris.HCl, pH 7.3, 100 mM KCl, 5 mM EDTA, 2 mM dithiothreitol, 10% vol/vol glycerol, 1 μg/ml each leupeptin, pepstatin, aprotinin, 1 mM PMSF and homogenized for 1 min in a Waring blender. The homogenate was centrifuged in a GSA rotor (Sorvall Instruments, Wilmington, DE) for 25 min at 12,000 rpm (23,430 *g*). The supernatant (~45% of the total volume) was strained through three layers of cheesecloth and centrifuged in a SW41 rotor (Beckman Instruments, Fullerton, CA) for 2 h at 40,000 rpm (272,000 *g*). This extract (CB extract) was dialyzed against buffer A/G (20 mM Tris.HCl, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol, 10% vol/vol glycerol, 1 μg/ml each leupeptin, pepstatin, and aprotinin), aliquoted, and stored at −80°C. A typical protein concentration of the CB extract was 17.8 mg/ml by Bradford assay.

The permeabilized cell import assay was performed using a slight modification of the method of Adam et al. (1990). Briefly, PtK1 cells were cultured on glass coverslips in F12 medium plus 10% FBS (GIBCO BRL, Gaithersburg, MD) under 5% carbon dioxide for 24–48 h (to subconfluency). Cells were chilled on ice for 30 min, rinsed with 1 ml cold buffer A/G, and covered with 1 ml of cold buffer A/G+ 40 μg/ml digitonin for 5 min on ice. The digitonin solution was then aspirated off and the coverslips were rinsed 2× with 1 ml of cold buffer A/G. Coverslips were drained and inverted over 20 μl of reaction mixture for 20 min at either 4°C (chilled) or room temperature (warm, ~23°C). After incubation, coverslips were immersed in 1 ml of PBS + 3.7% wt/vol formaldehyde pH 7.5 for 10 min, rinsed with water, drained, and mounted.

Ca^{2+}-ATPase Assay

Ca^{2+}-ATPase activity was measured by monitoring the release of inorganic phosphate from ATP as described (Lanzetta et al., 1979). Ca^{2+}-ATPase from human erythrocytes was a gift from F. Hofmann (ETH, Zurich, Switzerland). Reactions were incubated for 10 min at 37°C in 20 mM Hepes, pH 7.2, 100 mM KCl, 0.43 mM EGTA, and 0.5 mM $CaCl_2$ to achieve a 15-μM free Ca^{2+}-concentration. For preincubations with WGA and C20W, WGA (0.5 mg/ml) and C20W (0.1 mg/ml) were incubated with CaM (0.1 mg/ml) for 5 min at 37° before the Ca^{2+}-ATPase activity measurements.

Results

CaM Import Is Arrested in Chilled Cells

A hallmark of facilitated nuclear transport is its strong sensi-

tivity to chilling which results in the NLS-dependent accumulation of substrate at the nuclear periphery. In contrast, passive diffusion across the nuclear envelope is relatively insensitive to low temperatures. Thus, small reporter proteins that lack NLSs, such as lysozyme, soybean trypsin inhibitor (TI) and cytochrome c, peptides, and dextrans with Stoke's radii less than the 10-nm diam nuclear pores diffuse into nuclei of chilled cells. The covalent attachment of NLS peptides to cytochrome c leads to its transport arrest in chilled cells because of its association with cytoplasmic targeting factors (Breeuwer and Goldfarb, 1990). Thus, an NLS obligates even a small protein to follow a facilitated transport pathway.

Fig. 1 shows the import of fluorescein-labeled proteins, fl-P(Lys)-BSA (*a*), fl-Hl (*b*), fl-CaM (*c*), and fl-TI (*d*) in chilled cells 30 min after microinjection. The import of fl-CaM, like that of fl-P(Lys)-BSA and fl-Hl, was inhibited by chilling and accumulated in the cytoplasm. In contrast, the diffusive import of fl-TI was unaffected by chilling. In a number of chilled cells, fl-CaM accumulated in the perinuclear region (Fig. 1 *c*, see below). The protein concentrations indicated in the legends are those of the injected solution. It is difficult to assess the volume of injected solution, but we estimate that this amounts to no more than 10% of the cell's volume. Usually, then, the intracellular concentration of injected fl-CaM was in the physiological range of 1–10 μM for CaM in cells and tissues (Bachs et al., 1992).

Upon chilling, the bulk of the microtubule network of PtK1 cells depolymerizes (not shown). Under these conditions it is possible that microinjected fl-CaM becomes complexed to the large amount of free tubulin and microtubule-associated proteins. The transport arrest of fl-CaM in chilled cells could be the result of binding or aggregation among fl-CaM and these newly soluble proteins in the cytosol. We ruled out this possibility by showing that the nocodazole-induced depolymerization of microtubules in warm cells did not result in the transport arrest of microinjected fl-CaM (not shown). In fact, we observed by immunofluorescence, as was previously noted (Keith et al., 1983), that the microinjection of mg/ml solutions of CaM into warm PtK1 cells sometimes caused the local depolymerization of microtubules. As an aside, we observed that microinjected fl-CaM associated with the spindle apparatus of mitotic PtK1 cells (not shown). In conclusion, microtubule depolymerization is not sufficient to arrest the transport of microinjected fl-CaM.

CaM Import Is Arrested by WGA

WGA is thought to prevent NLS-mediated import by binding to GlcNAc-containing nucleoporins and inhibiting pore complex function (Forbes, 1992). mAb414 and other monoclonal antibodies that bind subsets of GlcNAc-containing nucleoporins also inhibit nuclear transport. Fig. 2 shows the effect of WGA on the import of microinjected fl-P(Lys)-BSA (*a* and *b*), fl-Hl (*c* and *d*), fl-CaM (*e* and *f*), and fl-TI (*g* and *h*). The import of fl-P(Lys)-BSA (*a*), fl-Hl (*c*), and fl-CaM (*e*) was arrested in the cytoplasm by WGA, although not in the presence of the lectin's ligand *N*-acetylglucosamine (GlcNAc) (*b*, *d*, and *f*, respectively). Only the diffusion-driven import of fl-TI was unaffected by WGA (*g*). The import of fl-P(Lys)-BSA and fl-Hl were also inhibited by the microinjection of mAb414; however, the import of fl-CaM and fl-TI was unaffected by mAb414 (not shown). Neither WGA nor mAb414, an antinucleoporin antibody, had any

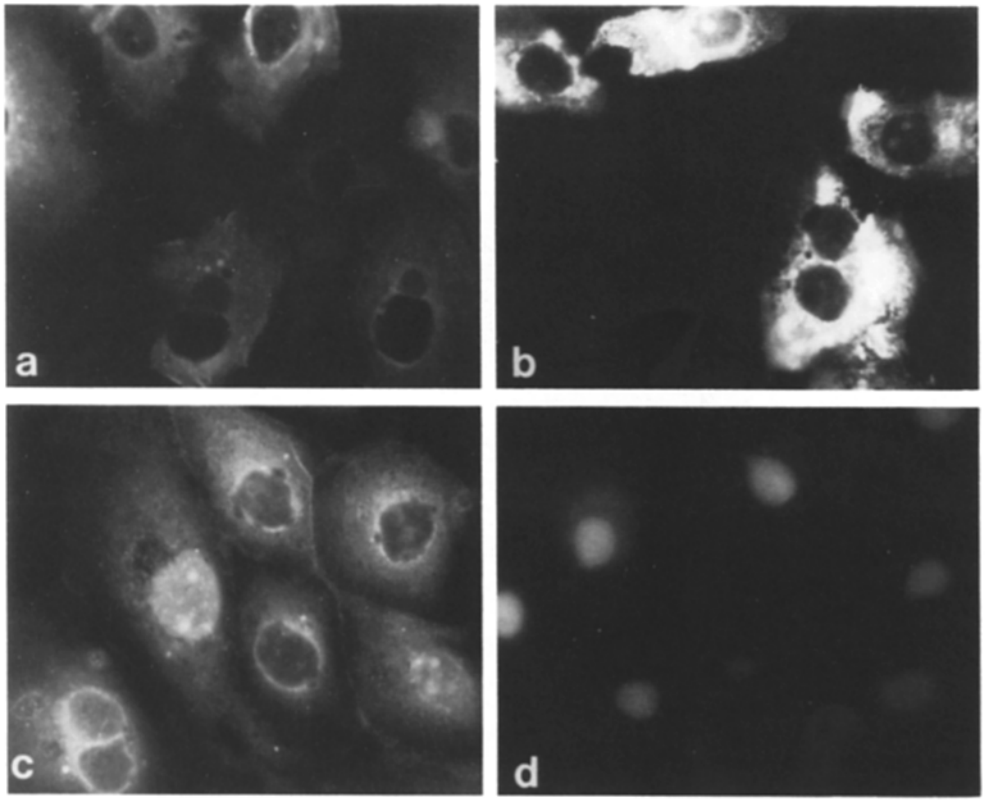


Figure 1. Effect of chilling on protein import. fl-P(Lys)-BSA (22 μM) (*a*), fl-Hl (48 μM) (*b*), fl-CaM (36 μM) (*c*), and fl-TI (48 μM) (*d*) were microinjected into chilled PtK1 cells and incubated on ice for 30 min before fixation.

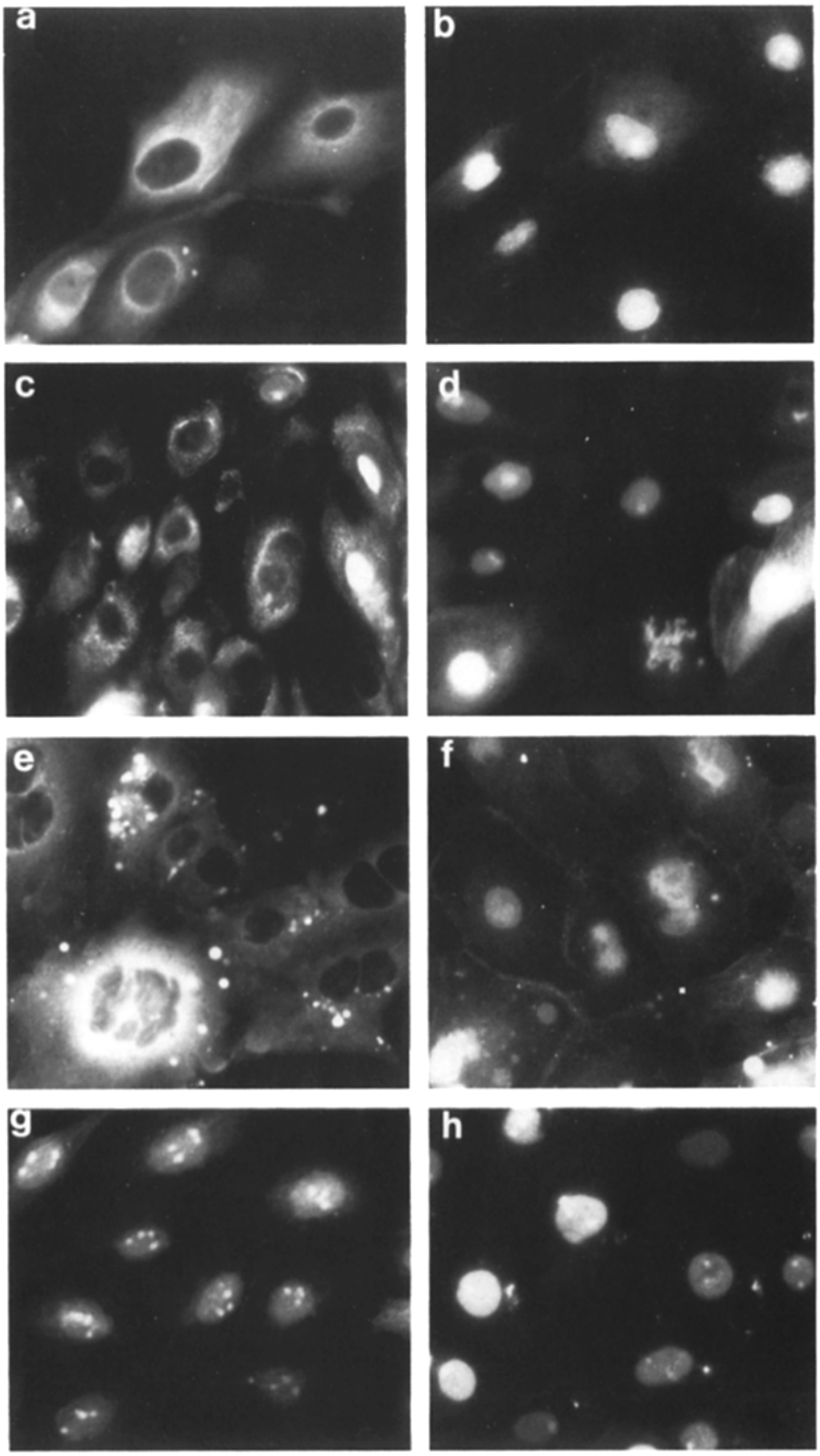


Figure 2. Effect of WGA on protein import. fl-P(Lys)-BSA (11 μM) (*a* and *b*), fl-H1 (24 μM) (*c* and *d*), fl-CaM (30 μM) (*e* and *f*), and fl-TI (24 μM) (*g* and *h*) were coinjected with either 2.5 mg/ml WGA (*a*, *c*, *e*, and *g*) or 2.5 mg/ml WGA plus 0.5 M GlcNAc (*b*, *d*, *f*, and *h*). Injected cells were incubated at room temperature for 30 min.

effect on the nuclear accumulation of fl-lysozyme (Ju, Y., unpublished results). In separate experiments, we tested the effects of the lectin from *Datura stramonium* (Jimson weed), which has a similar specificity to WGA, and con A, which does not bind to exposed nucleoporins. Whereas 5 mg/ml Jimson weed lectin inhibited both fl-P(Lys)-BSA and fl-CaM import, 5 mg/ml con A had no effect on the import of either protein (Ju, Y., unpublished results).

Though CaM is not covalently modified with GlcNAc residues, WGA might, in any case, bind to fl-CaM and indirectly inhibit import. We tested this possibility in two ways. First, we examined the effect of WGA on the CaM-dependent activation of the plasma membrane Ca^{2+}-ATPase (Carafoli, 1991). The activity of the ATPase was stimulated 2.3-fold by CaM (see Materials and Methods for conditions). In the presence of WGA, CaM still stimulated the ATPase 2.1-fold. In contrast, the synthetic peptide C20W, which is known to bind CaM with high affinity ($K_d \sim 10^{-8}$) (Vorherr et al., 1990), inhibited the CaM stimulation of the ATPase (1.04-fold effect). Second, we tried to show the formation of WGA-CaM complexes by affinity chromatography. CaM was passed over a WGA-Sepharose column and WGA was passed over a CaM-Sepharose column, both in the presence and absence of Ca^{2+}. In each case, WGA and CaM quantitatively eluted from these columns in the void volume, indicating no interaction between the proteins (not shown).

Effect of WGA on Import of Troponin C, Calbindin, and CaM P106 Peptide

We tested the specificity of WGA inhibition by examining its effect on the nuclear accumulation of two small EF-hand Ca^{2+}-binding proteins (Heizmann, 1991), microinjected fl-troponin C (TnC, 17 kD), and fl-calbindin (9 kD). TnC has the same dumbbell structure as CaM and has a similar amino acid sequence. Calbindin has only one globular domain containing two high affinity EF-hand Ca^{2+}-binding sites. P106 is a CaM tryptic fragment that contains the amino terminal 106 residues of the 148-amino acid mammalian CaM sequence. Although P106 contains the amino terminal Ca^{2+}-binding domain, the central helical domain, and half of the carboxy terminal Ca^{2+}-binding domain, it does not activate a number of CaM-dependent enzymes (Guerini et al., 1984; Newton et al., 1984). Fig. 3 shows that after microinjection, fl-TnC (*a*), fl-calbindin (*c*), and P106 (*e*) accumulated in nuclei. WGA inhibited the import of fl-TnC (*b*), but not that of either fl-calbindin (*d*) or P106 (*f*). These data indicate that the import of TnC, like its close relative CaM, can be mediated, whereas calbindin and P106 import proceeds by passive diffusion, indicating that the carboxy terminal 42 amino acids of CaM are required for mediated import.

CaM Import Is Not Affected by ATP Depletion

Fig. 4 *A* shows that the depletion of ATP from cells by metabolic poisons blocked the import of fl-P(Lys)-BSA (*a*) and fl-histone H1 (*b*), but have no effect on the import of fl-CaM (*c*) or fl-TI (*d*). Similarly, Fig. 4 *B* shows that comicroinjected 100 U/ml apyrase (which converts ATP to ADP and AMP) inhibited the nuclear import of fl-P(Lys)-BSA (*b*) but had no effect on the nuclear accumulation of fl-CaM (*a*). The injection of apyrase at concentrations as high as 250 U/ml had no inhibitory effect on fl-CaM import. Though we cannot rule out that enough ATP or other NTPs remain in the cells after these treatments to drive active fl-CaM import, we conclude that fl-CaM localization is distinguished by occurring in cells containing ATP concentrations that are insufficient to support import of fl-P(Lys)-BSA and fl-H1.

It is possible that facilitated import of CaM is dependent on the ATP-dependent formation of a large or nondiffusible complex in the cytoplasm. If this were the case, CaM would be free to diffuse into the nucleus under conditions of ATP-depletion and might, therefore, escape the inhibitory effects of chilling and WGA. However, this is not the case. Fig. 5 shows that ATP depletion did not effect the WGA-mediated transport arrest of fl-CaM (*b*) or fl-P(lys)-BSA (*c*). The chilling arrest of fl-CaM import was also observed in ATP-depleted cells (not shown). We conclude, therefore, that the import of fl-CaM in warm, ATP-depleted cells remains a facilitated process and is not due to the diffusion of fl-CaM into the nucleus after its release from an ATP-dependent cytoplasmic complex.

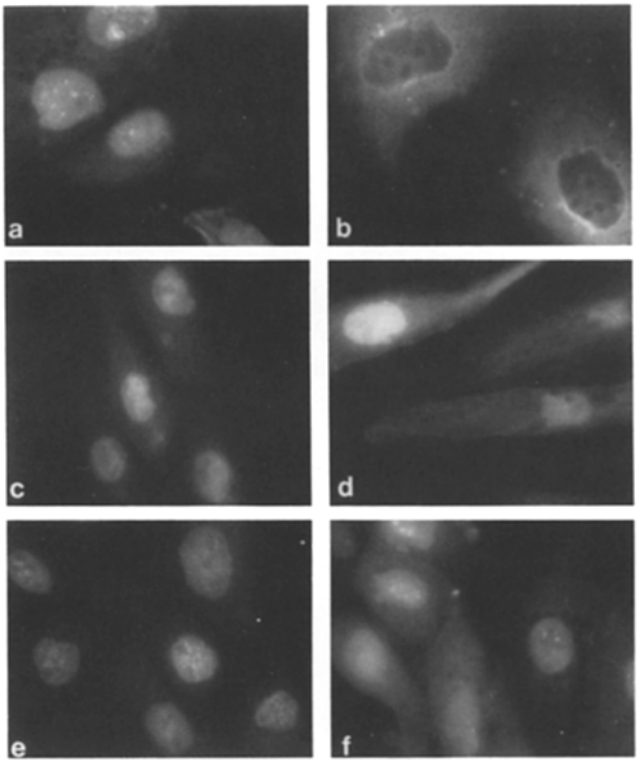


Figure 3. Effect of WGA on import of CaM-related proteins. fl-troponin C (*a* and *b*), fl-calbindin (*c* and *d*), and fl-CaM P106 peptide (*e* and *f*) were microinjected into warm PtK1 cells (*a*, *c*, and *e*) or coinjected with 2.5 mg/ml WGA (*b*, *d*, and *f*). Injected cells were incubated at room temperature for 30 min.

Chilling and WGA Inhibition of fl-CaM Import Is Titratable

If CaM import is facilitated, then its import should be saturable. Saturation kinetics for the import of small proteins like CaM import are difficult to observe because, as was shown for histone H1 (Breeuwer and Goldfarb, 1990), once the receptor-mediated import mechanism is saturated, passive import via the 10-nm nuclear pores provides an alternative pathway. However, the saturation of the CaM import apparatus should result in a loss of sensitivity to chilling and WGA arrest.

As an indicator of receptor saturation we tested whether or not WGA and chilling arrest were overcome by microinjecting high concentrations of CaM. Fig. 6 shows that the WGA-mediated arrest of fl-CaM import were overcome by increasing the concentration of CaM in the needle from 25

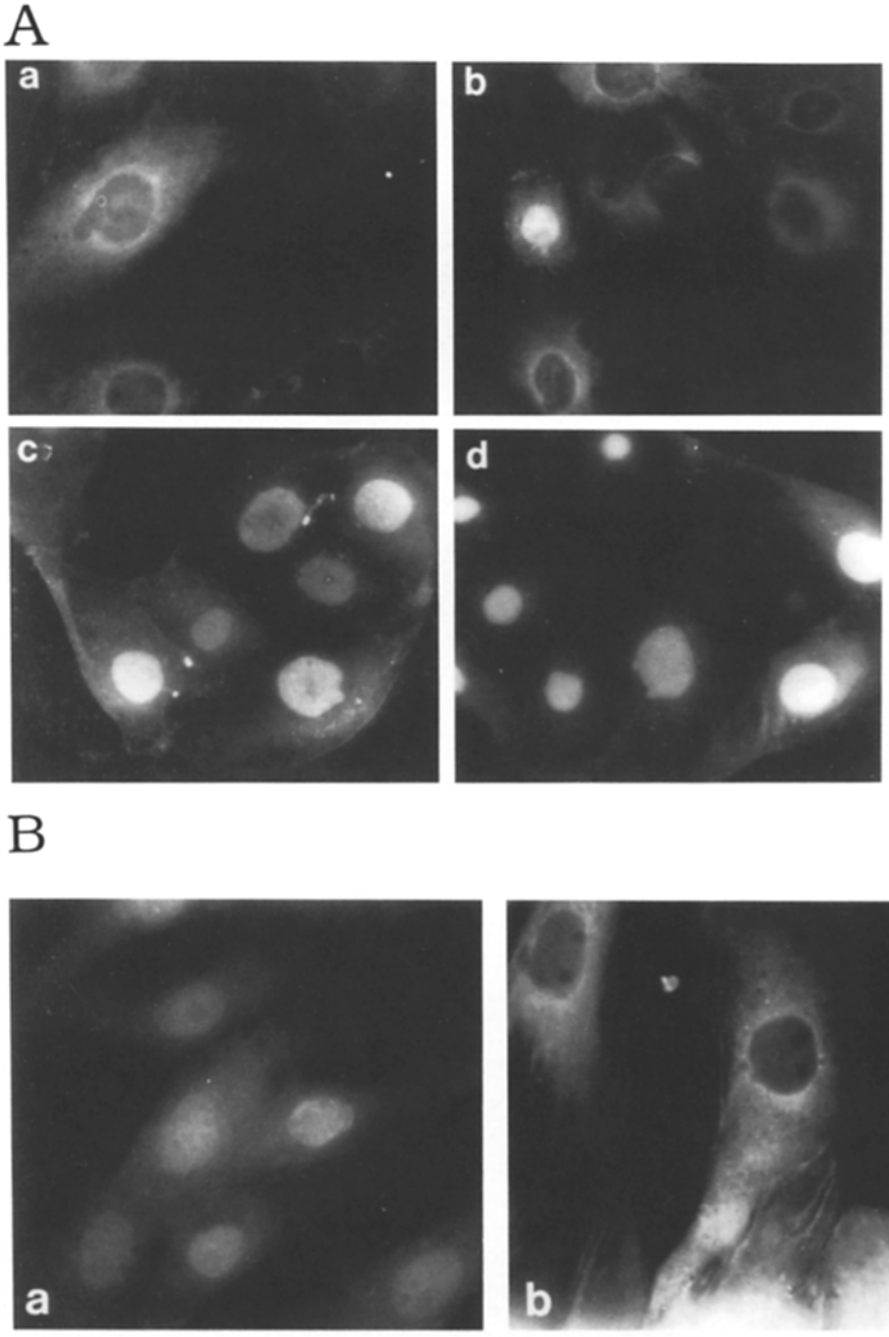


Figure 4. Effect of ATP depletion on protein import. (*A*) PtK1 cells were incubated in the drug mix (10 mM sodium azide, 6 mM 2-D-d-glucose, 1 mM iodoacetamide in Hank's balance buffer) for 10 min before injection. fl-P(Lys)-BSA (22 μM) (*a*), fl-H1 (48 μM) (*b*), fl-CaM (22 μM) (*c*), and fl-TI (48 μM) (*d*) were injected into the drug-treated cells. The cells were then incubated in drug mix for 30 min before fixation. (*B*) Cells were coinjected with apyrase (100 μ/ml) and either fl-CaM (*a*) or fl-P(Lys)-BSA (*b*), incubated at room temperature for 30 min and fixed.

μM (*b*) to 300 μM (*d*). Similarly, the chilling arrest observed when cells were injected with 25 μM CaM (*e*) was overcome by the injection of 300 μM CaM (*f*). The coinjection of 300 μM soybean trypsin inhibitor, a protein of approximately the same size (21 kD) and isoelectric point (4.5) as CaM, together with fl-CaM had no effect on WGA arrest (*c*), indicating that the effect of excess CaM on fl-CaM WGA arrest was specific. Similarly, coinjection of 300 μM BSA had no effect on WGA or chilling arrest (not shown). The titration of transport arrest by excess CaM occurred over approximately the same range for both chilling and WGA treatments. For example, at CaM concentrations between 100–200 μM, only partial chilling or WGA-mediated transport arrest was observed (not shown). Chilling arrest was, however, more sensitive than WGA-mediated arrest to 75 μM CaM. At this concentration chilling arrest was unaffected while WGA-mediated arrest was overcome in many of the cells. This discrepancy may be due to the fact that while the cells were prechilled before CaM injection, WGA and CaM were microinjected at the same time. In these cells, some CaM may have entered the nucleus before the WGA had the opportunity to fully arrest import.

Chilling and WGA Arrest of fl-CaM Import Can Be Reconstituted in Permeabilized Cells

We used a calf brain extract-dependent, digitonin-permeabilized tissue culture cell nuclear import assay based on the

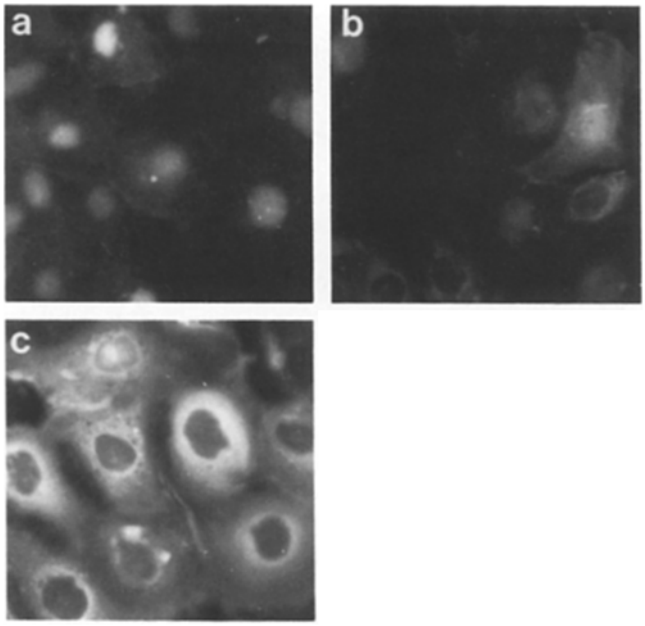


Figure 5. Effect of ATP depletion on WGA inhibition of protein import. fl-CaM (30 μM) was injected into warm ATP-depleted cells (*a*) or coinjected with 2.5 mg/ml of WGA into warm ATP-depleted cells (*b*). As a control for ATP depletion, fl-P(Lys)BSA (22 mM) was injected into ATP-depleted cells (*c*). See Fig. 4 *A* legend for ATP-depletion conditions.

one described by Adam and Gerace (1991) to gain better control over the concentration of import substrates and reagents such as WGA. Fig. 7 shows a double immunofluorescence labeling study using comicroinjected rhodamine-labeled "rh-P(Lys)-BSA" (*left panels*) and fl-CaM (*right panels*). Both rh-P(Lys)-BSA and fl-CaM accumulated in nuclei of warm, extract supplemented cells (*a* and *b*), and were excluded from nuclei of WGA-treated (*c* and *d*) and chilled cells (*e* and *f*). As also observed in microinjection experiments (Fig. 4, *A* and *B*), the import of rh-P(Lys)-BSA (*g* and *i*), but not fl-CaM (*h* and *j*), was sensitive to ATP depletion. The standard buffer conditions of the permeabilized cell import assay contains 1 mM EGTA, so that most of the CaM was Ca^{2+} free. We repeated these studies under buffer conditions that maintained CaM in the Ca^{2+} form and obtained the same results (not shown). Thus both Ca^{2+}-CaM and Ca^{2+}-free CaM are sensitive to chilling and WGA arrest.

Titration of Chilling Arrest in Permeabilized Cells

Microinjection experiments indicated that both chilling and WGA-mediated arrest could be overcome by saturating the capacity of the cytoplasm to complex CaM. Using chilled permeabilized cells supplemented with calf brain extract, the concentration of CaM needed to overcome chilling arrest was determined. Fig. 8 shows that the extract became saturated at ~7.5 μM fl-CaM (*d*). The chilling arrest of fl-CaM at concentrations of 2.5 and 5 μM (*b* and *c*) was reversible upon warming (not shown). Under these conditions, fl-CaM binds strongly to what appears to be intermediate filaments (also apparent in Fig. 7 *f*). That these filaments were not acting as the fl-CaM titratable factor is shown in panel *a*, where, in the absence of calf brain extract, 2.5 μM fl-CaM accumulated in nuclei, presumably by passive diffusion.

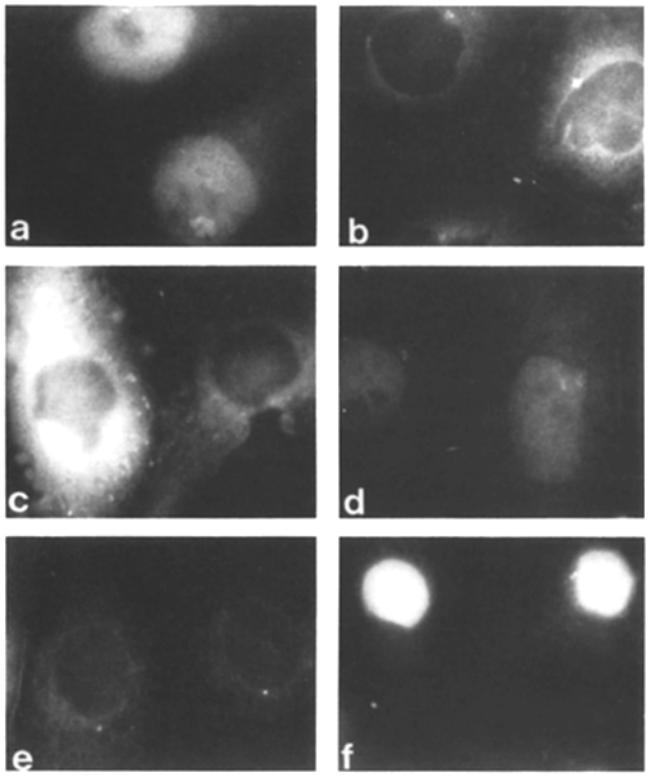
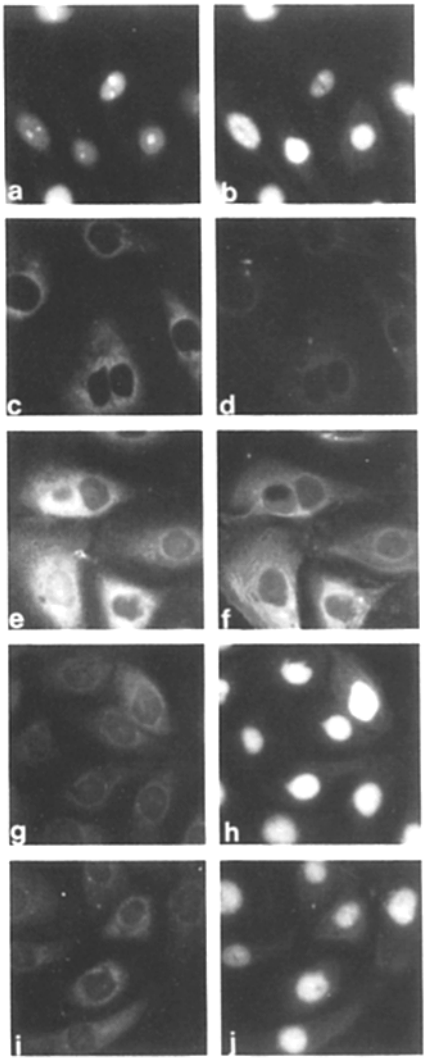


Figure 6. Titration of chilling and WGA arrest in microinjected cells. Cells were microinjected with 25 μM fl-CaM in warm cells (*a*), +WGA (*b*), +WGA +300 μM trypsin inhibitor (*c*), +WGA +300 μM CaM (*d*), or 25 μM fl-CaM in chilled cells (*e*), +300 μM CaM (*f*). WGA concentration was 1.8 mg/ml.

Discussion

Because CaM is small and lacks an apparent NLS motif it is possible that it might cross the nuclear envelope by passive diffusion through the ~10-nm-diam nuclear pores. However, the present data indicate that CaM import occurs by facilitated diffusion, a receptor-mediated mechanism. These experiments do not provide direct insight into the nature of the putative receptor or its location in the cytoplasm. For example, we have yet to determine if the CaM import pathway employs the same NLS-receptor(s) that mediate the cell's predominant import pathway (Michaud and Goldfarb, 1993). The signal that specifies the CaM import pathway could reside either on CaM itself or on a factor that binds CaM in the cytoplasm. If, in fact, CaM import occurs by facilitated diffusion, then it follows that the steady state nucleocytoplasmic distribution of CaM would be determined by the affinity, concentration, and localization of CaM target proteins in the nucleus and cytoplasm. The reversible sensitivity of CaM import to chilling distinguishes it from the chilling insensitive import of microinjected, fluorescence-labeled cAMP-dependent protein kinase catalytic subunit (Harootunian et al., 1993).

The nuclear transport of macromolecules has been repeatedly shown, and is commonly accepted to be an ATP-dependent process (Forbes, 1992). The ATP independence of CaM import provides evidence that ATP hydrolysis may not be an obligatory step along the general pathway of medi-



ated nuclear transport. Alternatively, CaM may be a special case that is imported by a distinct ATP-independent carrier apparatus. Finally, it is possible that the treatments we used to deplete cells of ATP were insufficient to completely remove all ATP. Thus, we cannot rule out the possibility that CaM import requires ATP at significantly lower concentrations than is required for the import of previously characterized karyophiles, both large and small.

The experiments that most strongly suggest that CaM import is facilitated are the chilling and WGA-sensitivity studies. In both cases we often observed nuclear envelope rim staining of fl-CaM in transport-arrested cells. One caveat is the possibility that free Ca^{2+} levels in the cytosol increase when the cells are cooled or injected with WGA, perhaps due to increased leakage across the plasma membrane. If the artifactual increase of cytosolic Ca^{2+} occurred, then CaM might become activated to bind targets in the cytoplasm (Gough and Taylor, 1993), thereby restricting its diffusion. However, we found that the in vitro transport arrest of CaM by chilling and WGA was insensitive to Ca^{2+}.

CaM import is unusual in being insensitive to the anti-nucleoporin antibody mAb414. This result is difficult to rationalize, in part because we do not understand how WGA, mAb414, and other anti-nucleoporin antibodies (Featherstone et al., 1988) inhibit nuclear transport. For example, we cannot explain why the import of some karyophiles is significantly more sensitive to WGA inhibition than others

Figure 7. Cell-free reconstitution of chilling, WGA, and ATP-depletion treatments. Permeabilized PtK1 cells supplemented with 8.9 mg/ml CB extract in A/G buffer were incubated with a mixture of rh-P(Lys)-BSA (5 μM) (*a*, *c*, *e*, *g*, and *i*) and fl-CaM (5 μM) (*b*, *d*, *f*, *h*, and *j*). Dual images of the same cells were obtained using rhodamine and fluorescein filters. Warm cells (*a* and *b*); chilled cells (*c* and *d*); WGA (1.8 mg/ml)-treated cells (*e* and *f*); dialyzed extract without added ATP (*g* and *h*); complete extract treated with apyrase (100 μ/ml) (*i* and *j*).

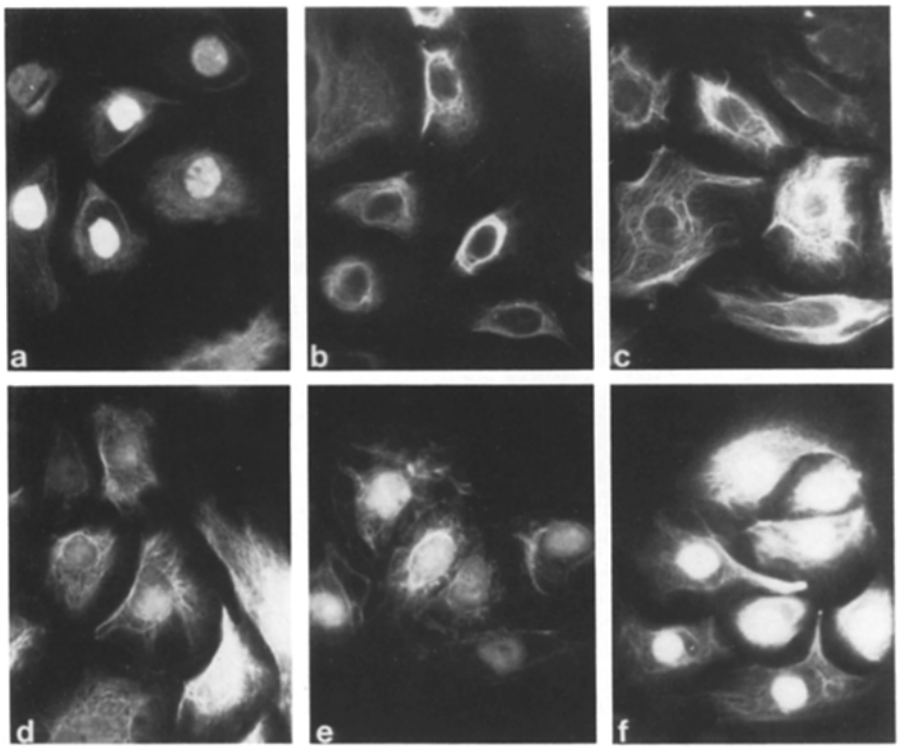


Figure 8. Titration of chilling arrest in permeabilized cells. Permeabilized PtK1 cells were incubated on ice in the absence (*a*) or presence of 8.9 mg/ml CB extract in A/G buffer (*b–f*) plus fl-CaM at 2.5 μM (*a* and *b*), 5.0 μM (*c*), 7.5 μM (*d*), 10 μM (*e*), and 20 μM (*f*).

(Fischer et al., 1991; Michaud and Goldfarb, 1992). Unfortunately, not many different transport substrates have been tested for sensitivity to both WGA and anti-nucleoporin antibodies, probably because the antibodies have not until recently been commercially available. One potentially significant difference between WGA and mAb414 is the capacity of WGA to bind a broader spectrum of GlcNAc-containing nucleoporins than mAb414. We should note that Wong et al. (1991) found that the association of CaM with isolated nuclei was insensitive to WGA. These experiments were performed with isolated nuclei in the absence of cytosolic extract and thus are consistent with our in vitro results which show that cytosolic extract is required for transport arrest by chilling (Fig. 8) and WGA (not shown).

What are the mechanistic implications of facilitated CaM import? Foremost, this model predicts that access of CaM to the diffusion channel(s) in the pore complex is restricted. The pore complex-associated diffusion channels are poorly understood. While they exclude larger dextrans and proteins, precedent for the controlled diffusion of ions through the pores comes from several reports which indicate that free nuclear and cytoplasmic Ca^{2+} levels can be regulated independently (see Al-Mohanna et al., 1994). These phenomena imbue the nuclear pore with enigmatic properties that are not explained by current models for nuclear pore complex function (Dingwall, 1991). It is these uncertainties which in part caused us not to discard outright the unusual characteristics of CaM import as artifact. In the case of small NLS-containing nuclear proteins such as histone H1, access to the nuclear pore is restricted by the formation of targeting complexes which are too large to diffuse through the pores or, alternatively, are diffusionally restricted by binding to cytoplasmic elements (Breeuwer and Goldfarb, 1990).

Insight into the mobility of CaM within the cell has come from fluorescence anisotropy studies in tissue culture cells (Gough and Taylor, 1993). In cells with low free Ca^{2+}, microinjected fl-CaM was mostly free to tumble (low anisotropy), although its translation was restricted by the crowded cytoplasm. In cells stimulated to produce transiently higher cytosolic and nuclear Ca^{2+} levels, the anisotropy of fl-CaM increased, indicating the formation of CaM-target enzyme complexes. Under all conditions, the anisotropy of fl-CaM in the nucleus was relatively greater than in the cytoplasm. In a previous in situ study, using a Ca^{2+}-sensitive fluorescent CaM conjugate, the temporal formation of Ca^{2+}-CaM from apo-CaM was correlated with the appearance of free cytosolic Ca^{2+} (Hahn et al., 1992). These studies indicate that apo-CaM is free to diffuse in the cytosol and that Ca^{2+}-CaM, due to its binding to targets, is less mobile. Two basic mechanisms could explain how free apo-CaM is unable to diffuse into nuclei of chilled or WGA-injected cells. First, it is possible that apo-CaM is complexed with relatively small cellular factor(s) or protein(s) and this increases its effective radius enough to preclude passive diffusion through the nuclear pores. Second, the perinuclear region may contain a "filtering" apparatus that binds CaM locally, restricting its access to the pore complex. Here, CaM would have to run a "gauntlet" of perinuclear-binding sites in order to diffuse across the nuclear envelope. Consistent with this, electron microscopy has revealed the mass clustering of karyophilic gold particles on filaments extending into the cytoplasm from the pore complex (Scheer et al., 1988; Richardson et al., 1988; Forbes, 1992). The first model requires that intracellular CaM should always be bound by a cytoplasmic factor. The second model allows for CaM to diffuse about freely except in the vicinity of the pore complexes. The titration experiments using permeabilized cells indicates that at least one component of the arresting apparatus is present in soluble cytosolic extracts.

Chilling, WGA, and ATP-depletion sensitivity are commonly accepted as criteria for mediated nuclear transport. CaM import is unique in that it is insensitive to ATP depletion. This finding has led us to reevaluate the dogma that nuclear transport is necessarily ATP dependent (see Paine, 1993). The notion that the pore complex-associated transporter mechanism requires the input of free energy is consistent with two common observations: (*a*) After the ATP-independent targeting of proteins to the nuclear envelope, ATP is required for nuclear accumulation, and (*b*) the high degree of observed nuclear accumulation for most karyophilic proteins is consistent with transport against a karyophile activity gradient. There is, however, no direct evidence that vectorial translocation is coupled to ATP hydrolysis. ATP hydrolysis may be required before translocation for the decomposition of a stable targeting complex (Goldfarb, 1992), for movement of karyophiles along fibrils that lead to the pore complex (see Forbes, 1992), or for conformational changes that create access to the translocation mechanism. Although karyophiles accumulate in the nucleus, there is little evidence that the transporter mechanism can pump against a concentration gradient of diffusable substrate. The nuclear accumulation of nonphysiological macromolecules such as soybean trypsin inhibitor and lysozyme (Breeuwer and Goldfarb, 1990) is testament to the capacity of the nucleus to accumulate both acidic and basic proteins after their passive diffusion through the nuclear pores. The nonphysiological nuclear accumulation of these proteins is insensitive to WGA, chilling, and ATP depletion.

The results presented here raise specific questions about the free energy requirements of facilitated nuclear transport and whether there are one or more independent translocation mechanisms. Key to these issues is the nature of the factors that mediate CaM chilling and WGA arrest. Future studies will focus on the fractionation and reconstitution of mediated CaM import in permeabilized cells.

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