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Phosphorylation of the Nuclear Transport Machinery Down-regulates Nuclear Protein Import *in Vitro**

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We have examined whether signal-mediated nucleocytoplasmic transport can be regulated by phosphorylation of the nuclear transport machinery. Using digitonin-permeabilized cell assays to measure nuclear import and export, we found that the phosphatase inhibitors okadaic acid and microcystin inhibit transport mediated by the import receptors importin β and transportin, but not by the export receptor CRM1. Several lines of evidence, including the finding that transport inhibition is partially reversed by the broad specificity protein kinase inhibitor staurosporine, indicate that transport inhibition is due to elevated phosphorylation of a component of the nuclear transport machinery. The kinases and phosphatases involved in this regulation are present in the permeabilized cells. A phosphorylation-sensitive component of the nuclear transport machinery also is present in permeabilized cells and is most likely a component of the nuclear pore complex. Substrate binding by the importin α·β complex and the association of the complex with the nucleoporins Nup358/RanBP2 and Nup153 are not affected by phosphatase inhibitors, suggesting that transport inhibition by protein phosphorylation does not involve these steps. These results suggest that cells have mechanisms to negatively regulate entire nuclear transport pathways, thus providing a means to globally control cellular activity through effects on nucleocytoplasmic trafficking.

Nucleocytoplasmic transport is carried out by nuclear pore complexes (NPCs),[1] large supramolecular structures that span the nuclear envelope (1). Whereas small macromolecules (less than ~20–40 kDa) can passively diffuse through aqueous channels that traverse the NPC, larger macromolecules are translocated through the NPC by temperature- and signal-dependent mechanisms (for reviews, see Refs. 2–4). Signal-mediated transport through the NPC is mostly mediated by nucleocytoplasmic shuttling receptors belonging to the importin β/karyopherin β superfamily.

Recent work has pointed to the existence of a substantial diversity in the transport signals that are recognized by different members of the importin β/karyopherin β receptor family. Several nuclear transport signals have been analyzed in detail. The best characterized signal for nuclear protein import (nuclear localization sequence (NLS)) consists of a short stretch of amino acids enriched in basic residues, as exemplified by the "classical" NLS of the SV40 large T-antigen (5). The classical NLS is recognized by the adapter protein importin α_1, which binds to the import receptor importin β. A second well characterized NLS is the "M9" sequence of heterogeneous nuclear ribonucleoprotein A1 (6), which consists of a 38-amino acid stretch that is enriched in aromatic residues and glycine. The M9 sequence binds directly to the transport receptor transportin (7). The best characterized nuclear export sequence is a short leucine-rich amino acid sequence, which was originally found in the human immunodeficiency virus type 1 Rev protein (8) and in the protein kinase inhibitor (9). The leucine-rich nuclear export sequence is recognized by the export receptor CRM1 (10, 11).

Most signal-mediated nucleocytoplasmic transport is regulated by the small GTP-binding protein Ran, which shuttles between the nucleus and cytoplasm. The guanine nucleotide exchange factor for Ran (RanGEF or RCC1) is restricted to the nucleus, whereas the GTPase-activating protein for Ran (RanGAP) and RanBP1, a protein that further accelerates the rate of RanGAP-stimulated GTP hydrolysis by Ran (12, 13), are concentrated in the cytoplasm. As a result of this compartmentalization of Ran regulators, GTP-bound Ran is likely to have a substantially higher concentration in the nucleus than in the cytoplasm. RanGTP directly binds to nuclear transport receptors of the importin β superfamily, but the effects of this binding are different for import and export receptors. Whereas RanGTP promotes the dissociation of cargo from import receptors, it enhances the binding of cargo to export receptors (3). In this fashion, intranuclear RanGTP appears to be important for the loading and unloading of cargo on transport receptors and thus has a key role in determining the directionality of nuclear transport. Additional mechanisms by which RanGTP promotes vectorial nuclear transport are starting to be defined. In importin β-mediated transport, RanGTP is suggested to be important for dissociating the import complex consisting of importins α and β from nucleoporins on the nucleoplasmic side of the NPC in terminal steps of import (14–16). In contrast, the RanGTP that becomes bound to importin β during import complex disassembly in the nucleus and the RanGTP that becomes incorporated into export complexes appear to promote the targeting of these components to the cytoplasmic side of the NPC (17, 18).

The NPC consists of a central ring-spoke structure flanked by fibrils emanating from its nucleoplasmic and cytoplasmic surfaces. In vertebrate cells, the NPC is thought to consist of

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[1] The abbreviations used are: NPCs, nuclear pore complexes; NLS, nuclear localization sequence; GFP, green fluorescent protein; GMP-PNP, guanosine 5′-(β,γ-iminotriphosphate); FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; GST, glutathione *S*-transferase; ATPγS, adenosine 5′-*O*-(thiotriphosphate); PP, protein phosphatase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

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>50 different polypeptides (1). Of the vertebrate nucleoporins that have been molecularly characterized, many have distinctive localizations within the three-dimensional structure of the NPC: some are localized specifically to its cytoplasmic fibrils (Nup358/RanBP2 (19–21) and Nup214/CAN (22)) and others to the nucleoplasmic fibrils (Nup50,[2] Nup98 (24), and Nup153 (25, 26)), and yet other proteins such as the components of the p62 complex occur on both sides of the NPC, near the central gated channel (27). A number of nucleoporins, particularly those containing Phe-Gly repeat motifs, have been shown to directly interact with nuclear import and export receptors of the importin β family (for review, see Ref. 3). *In vivo* and *in vitro* studies have led to the model that nuclear transport receptor·cargo complexes traverse the NPC by stepwise transfer between discrete nucleoporins in different NPC regions (for review, see Ref. 28).

It is well established that cargo-specific regulatory mechanisms can control the nucleocytoplasmic transport of certain proteins in different functional states of the cell. Phosphorylation and dephosphorylation of various transcription factors, which are often sequestered in the cytoplasm of cells, have been shown to activate their nuclear import or export signals (29). For example, dephosphorylation of NFAT (nuclear factor of activated T-cells) by calcineurin exposes an NLS, leading to rapid import of the protein into the nucleus (30), whereas rephosphorylation of NFAT in the nucleus by protein kinase A, glycogen synthase kinase-3, and possibly other kinases triggers its export (31). This allows the localization of individual proteins to be controlled in response to cell signaling events.

In addition to these well described cargo-specific regulatory mechanisms, several studies suggest that the nuclear transport machinery itself can be regulated, thus affecting the transport of a large number of different cargoes in a more global fashion. Feldherr and Akin described a substantial decrease in the rate of signal-mediated import in growth-arrested 3T3 fibroblasts compared with the import rate in proliferating cells (32) and also reported variations of import rates during the cell cycle of 3T3 cells (33). Interestingly, a cytosolic kinase was implicated in the stimulation of nuclear import by simian virus 40-transformed cell extract (34). However, these phenomena have not been characterized in biochemical detail.

Regulation of the nuclear transport machinery could involve soluble transport factors and/or proteins of the stationary phase, the nucleoporins. Srp1p, the yeast homologue of importin α, has been shown to be phosphorylated, although phosphorylation did not induce a detectable difference in the affinity of Srp1p for cargo (35). Nucleoporins can be extensively modified by phosphorylation and/or *O*-glycosylation (36), but effects of these modifications on the rate of nuclear transport have not been detected. Since different nucleoporins or different regions of individual nucleoporins are hypothesized to be used for different transport pathways, their modification might have distinct, pathway-specific effects.

To investigate the potential regulation of nuclear transport by protein phosphorylation, we analyzed the effects of protein phosphatase and kinase inhibitors on nuclear transport using digitonin-permeabilized cell transport assays. We show that phosphorylation of a component(s) of the nuclear transport machinery specifically down-regulates the nuclear import pathways mediated by importin β and transportin, but has no apparent effect on the nuclear export pathway mediated by CRM1. The target of this regulation is a component of the permeabilized cells, possibly a nucleoporin, rather than a cytosolic factor. We discuss the possibility that global inhibition of nuclear import (but not export) could provide an efficient means to coordinately regulate the distribution of a large number of nucleocytoplasmic shuttling proteins.

[2] T. Guan, Kehlenbach, R. H., Schirmer, E. C., Kehlenbach, A., Fan, F., Clurman, B. E., Arnheim, N., and Gerace, L., submitted for publication.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells were grown either on plastic dishes in Dulbecco's modified Eagle's medium (GFP-NFAT-transfected cells and cells for import assays on coverslips) or in suspension in Joklik's modified S-minimum essential medium (cells for all other import assays). Both media contained 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. All tissue culture reagents were from Life Technologies, Inc.

Nuclear Transport Assays—Recombinant nuclear transport factors Ran and RanQ69L (37), importins α and β (38), and NTF2 (39) were prepared as described. For some experiments, Ran was loaded with GDP or GMP-PNP (18). Nuclear import substrates (FITC-BSA-NLS and Cy5-GST-M9) were prepared as described (40). FITC-BSA-NLS was used in all reactions unless otherwise indicated. Adherent HeLa cells were grown on coverslips; permeabilized with 30 μg/ml digitonin (Calbiochem) in transport buffer (20 mM Hepes, pH 7.3, 110 mM KOAc, 2 mM $Mg(OAc)_2$, and 1 mM EGTA) containing 2 mM dithiothreitol and 1 μg/ml each leupeptin, pepstatin, and aprotinin; and subjected to nuclear import reactions for 30 min at 30 °C as described (41). Cells were then fixed with 3.7% formaldehyde in phosphate-buffered saline and analyzed by fluorescence microscopy using a Zeiss Axiophot. When suspension cells were used for import assays, the reactions were performed as described (39) and contained 2.5 mg/ml cytosol unless otherwise indicated. Reactions were standardized by assigning a fluorescent value of 100 arbitrary units to a reaction carried out in the absence of phosphatase or kinase inhibitors. For cell cycle analysis, 300 μg/ml RNase was added to the reactions after 25 min, and incubation was continued for 5 min at 30 °C. After washing, the cells were resuspended in 200 μl of transport buffer containing 100 μg/ml RNase and 20 μg/ml propidium iodide (Sigma). Flow cytometry was performed using the FACSCalibur (Becton Dickinson). The propidium iodide signal in the FL3 channel was used to classify populations of cells as G_1, S, or G_2. The FITC signal in the FL1 channel of these populations was used to determine the level of nuclear import. The nuclear export assay using GFP-NFAT-transfected HeLa cells was carried out as described (40). Okadaic acid, microcystin, and staurosporine (all from Calbiochem) were added to transport assays from 1 mM stock solutions in Me_2SO.

In Vitro Binding Experiments—To measure the binding of endogenous importin α·β to GST-NLS (kindly provided by S. Lyman) 8×10^5 digitonin-permeabilized cells were incubated in a final volume of 80 μl of transport buffer with 2.5 mg/ml cytosol and an ATP-regenerating system in the absence or presence of a phosphatase inhibitor. After 15 min at 30 °C, GST-NLS was added to 55 μg/ml, and incubation was continued for 15 min. Cells were then collected by centrifugation, and 4 volumes of transport buffer containing 0.03% Tween 20 and 4 μl of glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) were added to the supernatant. After binding for 2 h at 4 °C, the beads were washed four times with transport buffer containing 0.03% Tween 20, and bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis, followed by immunoblot analysis. Importins α and β were detected using specific antibodies and an enhanced chemiluminescence system (Pierce).

To measure the binding of importin β to nucleoporins, 1.2×10^7 permeabilized cells were incubated in a final volume of 1 ml of transport buffer containing an ATP-regenerating system in the absence or presence of 2 μM okadaic acid. After 15 min at 30 °C, cells were washed with transport buffer containing 50 mM glycerophosphate and then solubilized on ice in 1.4 ml of Nonidet P-40 buffer (1% Nonidet P-40, 50 mM Tris-HCl, pH 8, 5 mM EDTA, 5 mM EGTA, 15 mM $MgCl_2$, 60 mM glycerophosphate, 100 μM KF, 100 μM $NaVO_4$, 2 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, and 300 mM NaCl). One volume of Nonidet P-40 buffer without NaCl was added to the soluble proteins, resulting in a final NaCl concentration of 150 mM. Two μg of S-His-importin β (kindly provided by J. Bednenko), bound to 5 μl of protein S-agarose beads (Novagen), and 20 μg/ml of Ran that had been loaded with either GDP or GMP-PNP (18) were added to 600 μl of solubilized proteins. After the addition of BSA to a final concentration of 2 mg/ml and incubation overnight at 4 °C, the beads were washed five times with Nonidet P-40 buffer containing 150 mM NaCl, and bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis, followed by Western blotting. Nucleoporins were detected using the RL1 antibody (42) and the enhanced chemiluminescence system.

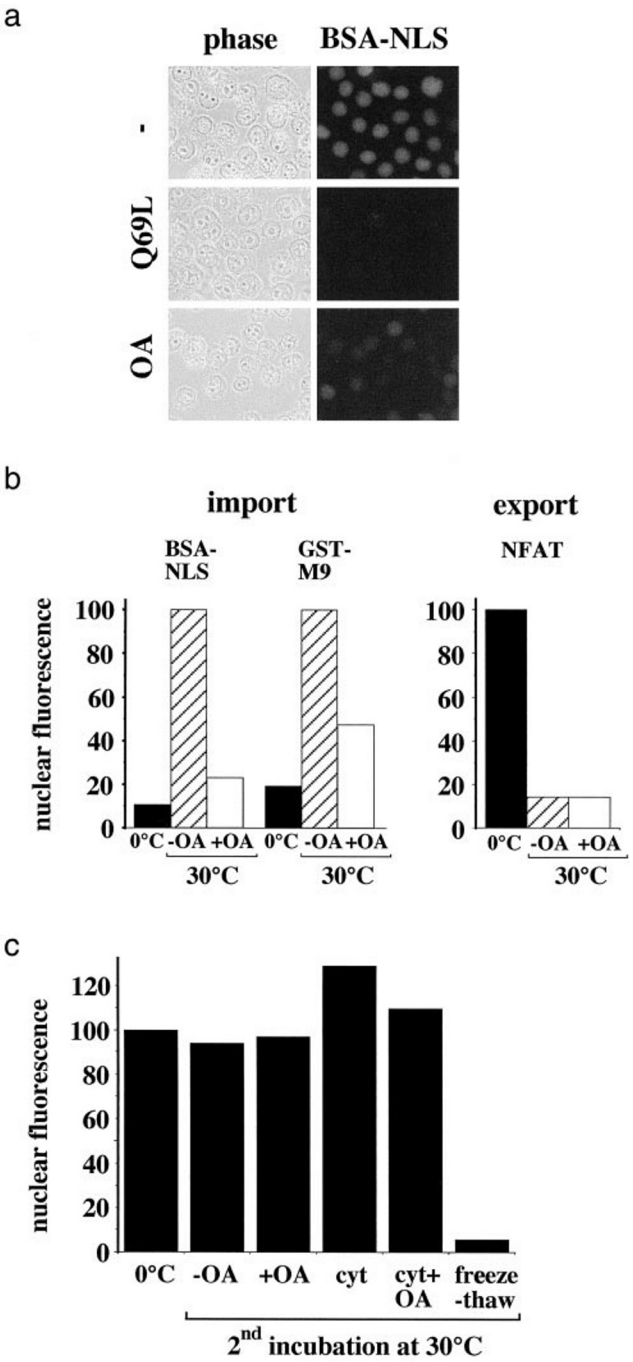

FIG. 1. **Inhibition of nuclear import, but not export, by okadaic acid.** *a*, import reactions were performed on adherent HeLa cells in the presence of cytosol with no further additions (−; *upper panels*) or with the addition of 40 μg/ml RanQ69L (*Q69L*; *middle panels*) or 2 μM okadaic acid (*OA*; *lower panels*). FITC-BSA-NLS was used as the import substrate. Note the different levels of inhibition of nuclear import by okadaic acid in individual cells. *b*, import reactions were performed using suspension HeLa cells with either FITC-BSA-NLS or Cy5-GST-M9 as import substrate. GFP-NFAT-transfected HeLa cells were used for nuclear export reactions (40). 1 μM okadaic acid was added to the reactions as indicated. *c*, cells were preincubated with cytosol, ATP, and Cy5-BSA-NLS to load the nuclei with the import substrate. After washing, cells were kept on ice or incubated at 30 °C for 30 min in the absence or presence of 2 μM okadaic acid (OA) and/or cytosol (*cyt*) as indicated. In one condition (*freeze-thaw*), the preincubated cells were permeabilized by freezing in liquid nitrogen and thawing on ice and subsequently were incubated at 30 °C without cytosol.

RESULTS

Protein Phosphorylation Negatively Regulates Nuclear Import in Vitro—We have investigated the effects of serine/threonine phosphatase inhibitors on nuclear protein import and export to determine whether elevated phosphorylation of components of the nuclear transport machinery can alter the nuclear transport rate. We carried out these studies using *in vitro* nuclear transport assays consisting of digitonin-permeabilized cells reconstituted with exogenous cytosol (39–41). Initially, we examined the nuclear import of a substrate containing a classical basic amino acid-type NLS in adherent HeLa cells. As shown in Fig. 1*a*, a substrate consisting of FITC-labeled BSA coupled to the NLS of the SV40 large T-antigen (BSA-NLS) was efficiently imported into the nucleus in the absence of a phosphatase inhibitor. All cells exhibited a qualitatively similar level of import as judged by visual inspection. RanQ69L, a mutant Ran that cannot hydrolyze its bound GTP and is therefore predominantly in the GTP-bound form (43), strongly inhibited import in all cells, as described previously (44). The phosphatase inhibitor okadaic acid also inhibited import, although the level of inhibition varied from cell to cell: some nuclei exhibited a very low level of substrate accumulation, comparable to that obtained with RanQ69L, whereas others showed an intermediate level of import. No accumulation of the import substrate at the nuclear envelope was observed in samples containing okadaic acid, indicating that there was no stable association of cargo with the nuclear envelope under these conditions. Cell-to-cell variability in the level of import inhibition by okadaic acid was consistently observed in experiments both with HeLa cells and with normal rat kidney cells (data not shown). The observed cell-to-cell heterogeneity in the okadaic acid inhibition in adherent cells parallels the broad, heterogeneous distribution of cell-associated fluorescence seen in the flow cytometry profile of okadaic acid-treated import reactions using suspension cells (see Fig. 3*a*).

We next used flow cytometry (39) to quantitatively analyze inhibition of several different nuclear transport pathways in HeLa cells. In addition to analyzing nuclear import of BSA coupled to the SV40 T-antigen NLS, we also examined nuclear import of GST fused to the M9 NLS of heterogeneous nuclear ribonucleoprotein A1 as well as nuclear export of GFP-NFAT (40), which contains a leucine-rich nuclear export sequence (45). In this experiment, okadaic acid strongly inhibited the nuclear import of the substrate containing the T-antigen NLS (nuclear fluorescence decreased from 100 arbitrary units in the absence of okadaic acid to 23 units in the presence of okadaic acid) and, to a lesser extent, the import of the cargo with the M9 NLS (from 100 to 47 units; Fig. 1*b*). In contrast, nuclear export of GFP-NFAT was not affected by okadaic acid (Fig. 1*b*). Although export of NFAT *in vivo* is stimulated by phosphorylation (31), export of NFAT *in vitro* is largely independent of the phosphorylation state of NFAT under the conditions of our assay (40).

We performed several control experiments to rule out the possibility that the observed decrease in nuclear accumulation of the import substrates in the presence of phosphatase inhibitors resulted from changes in the normal permeability barrier of the nuclear envelope (*i.e.* leakiness). First, the inhibition of nuclear import of BSA-NLS by okadaic acid was fully reversible. When cells were preincubated in the presence or absence of okadaic acid and then washed and subjected to a standard import reaction in the absence of a phosphatase inhibitor, accumulation of the import substrate reached similar levels in both cases, irrespective of the preincubation (data not shown). Second, both in the absence and presence of okadaic acid, nuclear export of GFP-NFAT was strongly inhibited by leptomycin B, a well characterized inhibitor of CRM1-mediated export (10, 11, 46). Third, when nuclei were preloaded with fluorescent import substrate by incubating permeabilized cells at

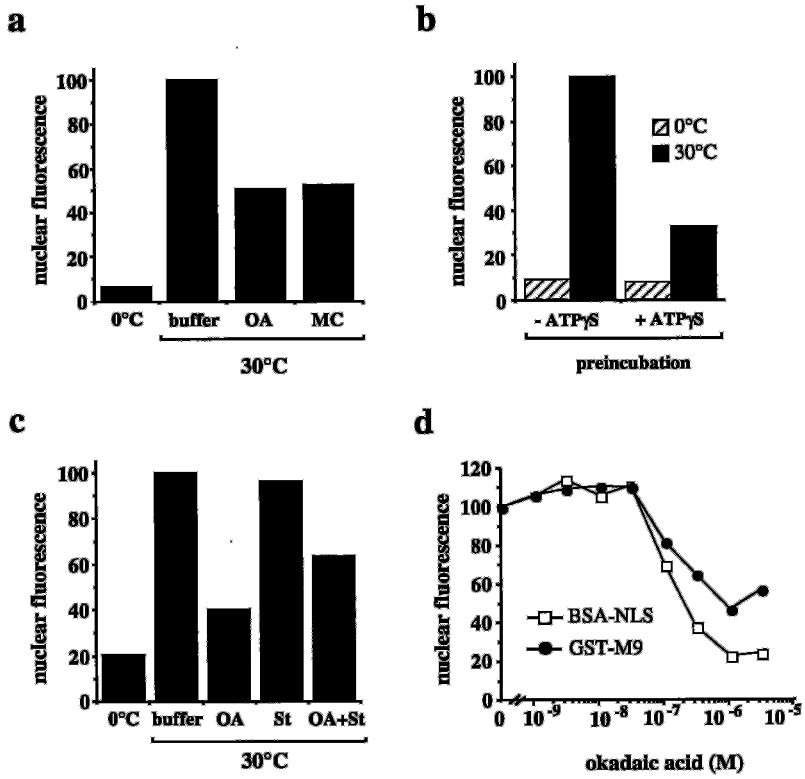

FIG. 2. **Regulation of nuclear import by phosphorylation/dephosphorylation.** *a*, nuclear import reactions were performed with suspension HeLa cells at 0 or 30 °C. Okadaic acid (*OA*; 1 μM) or microcystin (*MC*; 1 μM) was added as indicated. *b*, cells were preincubated with cytosol in the absence or presence of 400 μM ATPγS. Cells were washed and nuclear import reactions were performed at 0 or 30 °C with fresh cytosol in the presence of an ATP-regenerating system. *c*, nuclear import reactions were performed in the absence or presence of 2 μM okadaic acid (*OA*) or 10 μM staurosporine (*St*) as indicated. *d*, nuclear import reactions using BSA-NLS or GST-M9 as substrate were performed with increasing concentrations of okadaic acid as indicated. In *a–d*, all reactions were performed in the presence of cytosol.

30 °C with cytosol in a first import reaction and then incubated again at 30 °C in a mock reaction in buffer, the nuclei retained essentially the same fluorescence, even in the presence of 2 μM okadaic acid (20 times the concentration needed for half-maximal inhibition of import; see below; Fig. 1*c*). When cytosol instead of buffer alone was added to the second incubation, there was a modest increase in the level of fluorescence compared with the level obtained by the first incubation alone (Fig. 1*c*, compare *first* and *fourth bars*). This increase may result from the import of substrate that had not been washed away from the permeabilized cells after the first incubation. When okadaic acid was added together with cytosol in the second incubation, this increase was less pronounced, consistent with inhibition of nuclear import by okadaic acid (Fig. 1*c*, compare *fourth* and *fifth bars*). As a control, when cells were subjected to freeze-thawing to permeabilize the nuclear envelope, the import substrate was almost completely lost from the nuclei (Fig. 1*c*, *sixth bar*). This was not due to gross disruption of the nuclei since they remained morphologically intact as judged by phase-contrast microscopy (data not shown). These experiments clearly demonstrate that the permeability barrier of the nuclear envelope is intact in the presence of okadaic acid. Taken together, these results show that the nuclear import pathways mediated by the transport receptors importin β and transportin are specifically inhibited by the phosphatase inhibitor okadaic acid. In contrast, nuclear protein export mediated by CRM1 does not appear to be affected by okadaic acid.

We also tested whether microcystin, a serine/threonine phosphatase inhibitor that is structurally distinct from okadaic acid, diminishes nuclear import. Fig. 2*a* shows that microcystin inhibited nuclear import of BSA-NLS to a similar level compared with okadaic acid. Microcystin, like okadaic acid, had no detectable effect on nuclear export of GFP-NFAT (data not shown). To investigate whether the effects of the phosphatase inhibitors were due to increased levels of protein phosphorylation, we preincubated permeabilized cells with cytosol and ATPγS to induce thiophosphorylation of normally phosphorylated proteins, prior to carrying out an import reaction. Since the thiophosphate group is a poor substrate for cellular phosphatases (47), proteins quite stably retain this modification, which functionally mimics a normal phosphate group. Such pretreated cells showed substantially reduced nuclear import in a subsequent reaction in the absence of ATPγS compared with control cells (Fig. 2*b*), consistent with a role of protein phosphorylation in the inhibition of nuclear import of BSA-NLS. Nuclear export of GFP-NFAT, on the other hand, was not affected by pretreatment of cells with ATPγS (data not shown). If enhanced protein phosphorylation resulting from treatment of permeabilized cells with okadaic acid or microcystin were responsible for inhibition of nuclear import, one would further expect that transport inhibition by those reagents would be antagonized by inhibitors of protein kinases. The broad spectrum protein kinase inhibitor staurosporine had no effect on nuclear import of BSA-NLS (Fig. 2*c*). However, staurosporine added in conjunction with okadaic acid partially reversed the inhibitory effect of okadaic acid on BSA-NLS import. Staurosporine also partially relieved the okadaic acid-mediated inhibition GST-M9 import, whereas export of GFP-NFAT was not significantly affected (data not shown). For both the importin β- and transportin-mediated import pathways, half-maximal stimulation of import in reactions containing phosphatase inhibitors was observed at concentrations of ~200–300 nM staurosporine. The staurosporine concentration examined in Fig. 2*c* (10 μM) yields the maximum reversal of transport inhibition that can be obtained with this reagent. We were unable to reverse the effect of okadaic acid by including the more specific serine/threonine kinase inhibitors H-89 (which inhibits protein kinase A), bisindolylmaleimide (which inhibits protein kinase C), KN-62 (which targets Ca^{2+}/calmodulin-dependent protein kinase), and roscovitine and butyrolactone (which affect cyclin-dependent kinases) or the general tyrosine kinase inhibitor tyrphostin A25 (data not shown). We conclude from these experiments that microcystin and okadaic acid reduce nuclear import levels by inhibiting a protein phosphatase, thus causing increased phosphorylation of one or more components of the transport machinery. The observation that staurosporine can

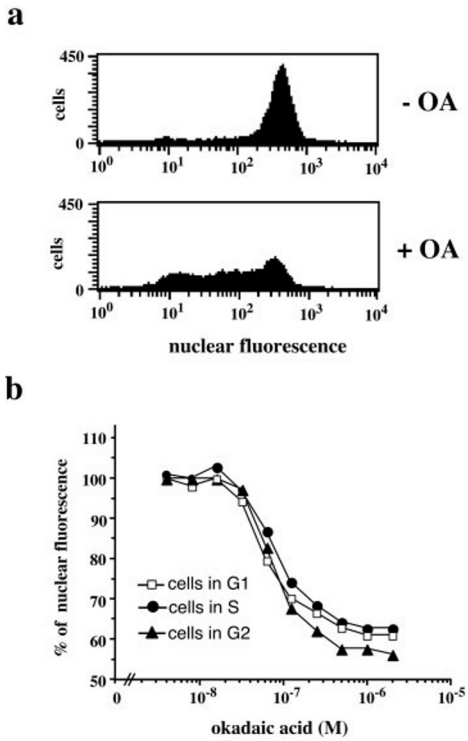

FIG. 3. *a*, cell-to-cell variation in sensitivity to okadaic acid. Nuclear import reactions with FITC-BSA-NLS were performed with suspension HeLa cells and cytosol in the absence or presence of 1 μM okadaic acid (*OA*). The flow cytometry profiles of the import reactions are shown. *b*, cell cycle stage dependence of nuclear import. Import reactions were performed with suspension HeLa cells and cytosol in the presence of increasing concentrations of okadaic acid with FITC-BSA-NLS as import substrate. Cells were stained with propidium iodide after the import reaction, and flow cytometry was used to identify cells in the G_1, S, and G_2 phases of the cell cycle and to quantify the level of import. Import is expressed as the percentage of nuclear fluorescence in a control reaction without okadaic acid to normalize for the somewhat higher absolute level of fluorescence in S and G_2 cells compared with G_1 cells, resulting from the greater number of NPCs in S and G_2 cells.

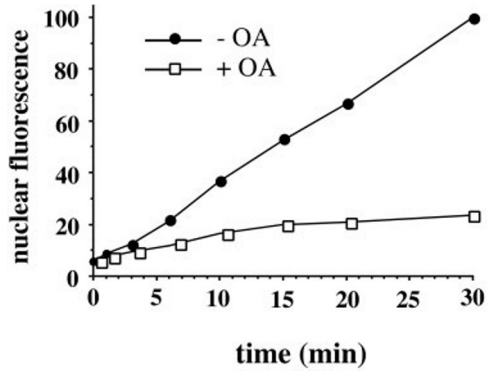

FIG. 4. **Comparison of the kinetics of nuclear import in the presence and absence of okadaic acid.** Nuclear import reactions with suspension HeLa cells and FITC-BSA-NLS were performed in the presence or absence of 1 μM okadaic acid (*OA*) for the indicated periods of time, and import was quantified by flow cytometry.

only partially reverse these inhibitory effects suggests that at least two kinases (one staurosporine-insensitive) are responsible for the inhibitory phosphorylation.

Microcystin, which is equally effective in inhibiting the two major cellular phosphatases protein phosphatase 1 (PP1) and protein phosphatase 2 (PP2A), inhibited nuclear import of BSA-NLS half-maximally at a concentration of ~50 nM, consistent with an effect on either PP1 or PP2A. Okadaic acid can be used to discriminate between these two phosphatases. PP1 is half-maximally inhibited by okadaic acid at concentrations between 20 and 315 nM, whereas PP2 is inhibited at subnanomolar concentrations (48). We therefore investigated the concentration dependence of inhibition of nuclear import by okadaic acid for the two substrates, BSA-NLS and GST-M9. The data in Fig. 2*d* show that both import pathways are equally sensitive to okadaic acid, with half-maximal inhibition at ~100 nM. This points to an involvement of a PP1-type activity in the inhibition of nuclear import, rather than a PP2A activity. PP1 is distributed throughout the cell, including the nucleus (49), a localization consistent with an effect of the phosphatase on either a cytosolic or nuclear factor. Calcineurin (PP2B), the protein phosphatase that is involved in the shuttling of NFAT (50), is not affected by okadaic acid or microcystin at the concentrations used in these experiments.

As noted above, we observed cell-to-cell differences in the level of inhibition of nuclear import by phosphatase inhibitors (see Fig. 1*a*). After a nuclear import reaction in the presence of cytosol, a single peak of fluorescence was typically observed by flow cytometry (406 arbitrary units of fluorescence at the peak with a mean of 364 units; Fig. 3*a*, *upper panel*). When okadaic acid was included in the reaction (Fig. 3*a*, *lower panel*), the distribution of cells was much broader, ranging from the fluorescence level characteristic of import reactions that had been incubated on ice (~20 units; data not shown) to almost the level of fluorescence of cells in a standard, untreated import reaction. In this experiment, the mean fluorescence in the presence of okadaic acid was 156 units.

To determine whether this cell-to-cell variation in sensitivity to okadaic acid was related to the cell cycle position of individual cells, we compared nuclear import of BSA-NLS and import inhibition by okadaic acid in G_1, S, and G_2 phase cells. To this end, the cells were incubated with the DNA stain propidium iodide after the import reaction, allowing us to classify them into G_1, S, and G_2 stages in the subsequent flow cytometric analysis according to their DNA content (51). Fig. 3*b* shows that there was no detectable difference in the sensitivity of the HeLa cell population in G_1, S, or G_2 to okadaic acid. Moreover, 50% of maximum inhibition in all three populations was observed at ~100 nM okadaic acid, the same value determined for the asynchronous population of cells (compare with Fig. 2*d*). The same results were obtained when normal rat kidney cells were used in the assay instead of HeLa cells (data not shown). Thus, the cell-to-cell differences in import inhibition in a HeLa population are not due to differential sensitivity of cells in different cell cycle phases to okadaic acid. The physiological basis of the cell-to-cell variation in sensitivity of nuclear import to okadaic acid remains to be determined. However, it should be noted that we also observed an experiment-to-experiment variation in the average level of nuclear import inhibition of a population of cells by okadaic acid, ranging from 40 to 75% (compare Figs. 1*b* and 3*b*). In experiments with strong inhibition of nuclear import by okadaic acid, the distribution of cell-associated fluorescence was more uniform, unlike the broad distribution in Fig. 3*a* (data not shown).

Inhibition of nuclear import by the phosphatase inhibitors could be caused by direct interference with one or more transport steps. Alternatively, the inhibition could be caused by an indirect effect such as a redistribution of receptors or of other shuttling transport factors. To distinguish between these possibilities, we analyzed the kinetics of inhibition of import by okadaic acid. In the case of a direct effect, inhibition should be constant over a broad time range, whereas in the case of an indirect effect, inhibition should increase at later time points. We found that the rate of import of BSA-NLS in the absence of the inhibitor remained constant for at least 30 min and that

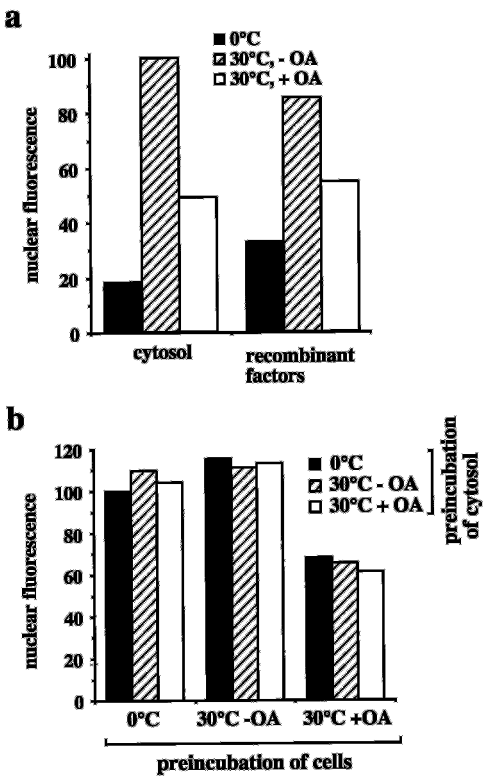

FIG. 5. **Phosphatase inhibitors affect a factor that is associated with the permeabilized cells.** *a*, nuclear import reactions were performed with suspension HeLa cells supplemented with either cytosol or recombinant import factors (12.5 μg/ml Ran, 20 μg/ml importin α, 7.5 μg/ml importin β, and 2.5 μg/ml NTF2). Incubations were at 0 or 30 °C in the absence or presence of 2 μM okadaic acid (*OA*) as indicated. *b*, cells or cytosol was preincubated at 0 °C in the absence of okadaic acid or for 20 min at 37 °C in the absence or presence of 1 μM okadaic acid as indicated. Cells and cytosols were then mixed in all possible combinations, and okadaic acid and staurosporine were added (as appropriate) so that they were present in all samples at final concentrations of 1 and 3 μM, respectively. Samples were then subjected to nuclear import reactions.

import was inhibited at a constant level by okadaic acid throughout the reaction, starting at the earliest time points (*e.g.* after 3 or 6 min; Fig. 4). This argues for a direct effect of the phosphatase inhibitor on the transport machinery. To directly investigate whether import receptor recycling from the nucleus was affected by the phosphatase inhibitors, we incubated permeabilized cells at 30 °C in the presence of cytosol with and without okadaic acid. No differences in the levels of importin β that remained associated with the permeabilized cells after the reaction could be detected by immunoblotting (data not shown). Furthermore, when permeabilized cells were incubated without cytosol, no differences in the levels of importin α or β that were released from the permeabilized cells were detected by immunoblotting whether or not okadaic acid was present during the reaction (data not shown). These results suggest that recycling of these import factors from the nucleus is not affected by phosphatase inhibitors.

Phosphatases, Kinases, and Their Targets Are Associated with the Permeabilized Cells—We next addressed the question of whether the components involved in inhibition of nuclear import (the phosphatases affected by okadaic acid, the kinases antagonized by these phosphatases, and the targets of the kinases in the nuclear transport machinery) are cytosolic or are associated with the permeabilized cells. Initially, we tested if inhibition of the nuclear import of BSA-NLS in permeabilized cells by okadaic acid requires the presence of cytosol, or if the effect is also obtained when reactions are carried out with recombinant import factors instead of cytosol. When transport was carried out in the presence of cytosol (Fig. 5*a*), okadaic acid decreased import of BSA-NLS from 100 fluorescent units in the control reaction to 49 units in the treated cells (with 18 units for the 0 °C control). When import was reconstituted with recombinant import factors, okadaic acid still decreased nuclear import from 85 to 55 units (with 33 units for the 0 °C control). A higher background signal at 0 °C is frequently observed in reactions containing recombinant factors compared with those containing cytosol. This may result from higher levels of nonspecific binding of free substrate and/or substrate·receptor complexes to permeabilized cell components when no blocking proteins from cytosol are present. These results indicate that the phosphatase(s) affected by okadaic acid, as well as the inhibitory kinase(s) antagonized by the phosphatases, are associated with the digitonin-permeabilized cells, at least to a significant degree. Nevertheless, the kinases and phosphatases involved in import inhibition might be present in cytosol as well since we observed stronger inhibition of import by okadaic acid in permeabilized cells reconstituted with cytosol. Here, the level of cell-associated fluorescence at 30 °C compared with the 0 °C background was decreased from 5.6-fold in the absence of okadaic acid to 2.7-fold in the presence of okadaic acid compared with a decrease from 2.6- to 1.7-fold when recombinant factors instead of cytosol were present in the import reaction (Fig. 5*a*).

If both the inhibitory kinases and the phosphorylation-sensitive components of the nuclear transport machinery are associated with the permeabilized cells to a significant level, then inhibitory phosphorylation would be established when the cells alone are pretreated with okadaic acid. These pretreated cells therefore should exhibit transport inhibition in a subsequent import reaction carried out with cytosol if staurosporine is added to block further inhibitory phosphorylation after the initial treatment of cells with okadaic acid. Conversely, if the inhibitory kinases and the phosphorylation-sensitive components of the nuclear transport machinery are both cytosolic transport factors, pretreatment of cytosol alone with okadaic acid should result in inhibitory phosphorylation. This inhibitory state should persist in a subsequent transport reaction carried out with permeabilized cells and staurosporine. To analyze these two possibilities, we preincubated permeabilized cells and HeLa cytosol separately at 0 °C without okadaic acid (as a control) or at 30 °C with or without okadaic acid. After the preincubation, the variously treated cells and cytosols were mixed in all nine possible combinations, and nuclear import reactions were performed. Okadaic acid and staurosporine were added to all reactions to the same final concentration so that the staurosporine-insensitive phosphorylation induced by okadaic acid that reduces nuclear import (Fig. 2*d*) would be present as a constant "background" inhibition in all nine samples. Fig. 5*b* shows that the greatest inhibition of nuclear import was observed when the permeabilized cells had been preincubated at 30 °C in the presence of okadaic acid, irrespective of the initial treatment of the cytosol (compare the last group of bars with the first two). Pretreatment of cytosol with okadaic acid at 30 °C, on the other hand, did not result in inhibition of import compared with cytosol that had been kept at 0 °C or incubated at 30 °C without okadaic acid (compare *black*, *hatched*, and *white bars* in all three groups). It should be noted that in the complete absence of okadaic acid and staurosporine in the reaction, we observed a substantially higher level of fluorescence (207 units), reflecting the lack of inhibition of transport by the staurosporine-insensitive kinase. These data suggest that the components of the nuclear transport machinery inhibited by the staurosporine-sensitive kinase are associated with permeabilized cells and not with cytosol.

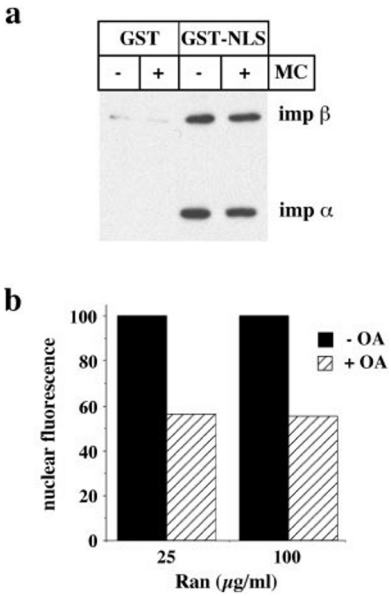

FIG. 6. **A phosphatase inhibitor does not affect the activity of the soluble import factors importin α, importin β, and Ran.** *a*, shown is the binding of importins α (*imp α*) and β (*imp β*) to GST or GST-NLS after incubation of cells with cytosol and GST or GST-NLS in the absence (−) or presence (+) of 5 μM microcystin (*MC*). *b*, nuclear import reactions with BSA-NLS as substrate were performed with suspension HeLa cells supplemented with cytosol in the absence (*black bars*) or presence (*hatched bars*) of 2 μM okadaic acid (*OA*) and either 25 or 100 μg/ml recombinant Ran. Samples with 100 μg/ml Ran had a slightly higher absolute value of fluorescence and were independently normalized to 100 units in the absence of okadaic acid.

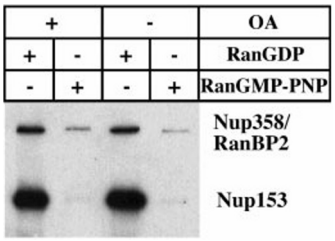

FIG. 7. **Binding of importin β to Nup153 and Nup358/RanBP2 is not inhibited by okadaic acid.** Nonidet P-40 lysates obtained from digitonin-permeabilized HeLa cells that had been preincubated in the absence (−) or presence (+) of 2 μM okadaic acid (*OA*) were incubated with importin β coupled to protein S-agarose beads. RanGMP-PNP (or RanGDP as a control) was added to 20 μg/ml as indicated. Bound proteins were analyzed by immunoblotting using the RL1 antibody (42) to detect *O*-glycosylated nucleoporins.

In a complementary approach, we preincubated permeabilized cells in the presence of ATPγS without cytosol to stably thiophosphorylate target proteins and to render them insensitive to phosphatase action. After washing, these pretreated cells exhibited a substantially reduced rate of nuclear import in a subsequent transport reaction with cytosol and an ATP-regenerating system compared with control cells (data not shown). Again, this indicates that both the phosphorylated target protein(s) of the nuclear transport machinery and the inhibitory kinase(s) are present in the permeabilized cells. Nevertheless, when cytosol was included in the preincubation with ATPγS (see Fig. 2*b*), the difference between the treated reactions and the control reactions was more pronounced, suggesting that cytosolic kinases further enhance the inhibition of nuclear import. In summary, these data indicate that at least a substantial component of the nuclear transport machinery that is targeted by the inhibitory kinase is found in the permeabilized cells. It remains possible that some targets of the inhibitory kinases are nucleocytoplasmic shuttling factors.

Analysis of Specific Steps and Components in Nuclear Import for Effects of Phosphatase Inhibitors—We next investigated whether any well defined steps and components involved in nuclear import are affected by the phosphatase inhibitors. Although the NLS peptide of the SV40 large T-antigen does not contain amino acids that can be phosphorylated, the formation of an import complex could also be affected by phosphorylation of an import receptor. We therefore analyzed whether the binding of a classical NLS-containing substrate to importin α·β is altered in transport assays containing a phosphatase inhibitor. When permeabilized cells together with cytosol and the substrate GST-NLS were incubated with or without microcystin and the binding of the cytosolic substrate·importin α·β complex to glutathione-Sepharose subsequently was examined, no changes in the binding of endogenous importins α and β to the import substrates were evident (Fig. 6*a*). Thus, for the importin β pathway, neither the receptor complex nor the import substrate appears to be affected by phosphorylation in a way that would alter their interaction.

Changes in the concentration of intranuclear RanGTP could conceivably lead to inhibition of nuclear import since intranuclear RanGTP is suggested to be involved in the dissociation of the import complex consisting of importins α and β and cargo at the nucleoplasmic side of the NPC and in the release of importin β from nucleoporins (for review, see Ref. 3). However, nuclear export *in vitro* is strongly dependent on exogenous Ran (40), in part because Ran is largely lost from the digitonin-permeabilized cells and because RanGTP is required for the formation of the export complex in the nucleus (10) and is also involved in targeting of the export complex to the cytoplasmic side of the NPC (18). We consider it unlikely that the intranuclear concentration of RanGTP is perturbed in the presence of phosphatase inhibitors because export of GFP-NFAT was not affected by okadaic acid (see Fig. 1*b*). This suggests that phosphatase inhibitors do not significantly alter the intranuclear concentration of RanGTP. To examine this further, we tested if adding increasing concentrations of Ran, which in part should be converted to RanGTP in the nucleus, would abolish the inhibitory effect of okadaic acid on import of BSA-NLS. In agreement with our observations on nuclear export, okadaic acid resulted in the same level of inhibition of import in reactions containing either 25 or 100 μg/ml exogenous Ran (Fig. 6*b*).

The binding of nuclear transport receptors to nucleoporins is central to their translocation through the NPC (3). Since inhibitory kinases affect permeabilized cell components (see above), it is very plausible that phosphorylation of nucleoporins is responsible for inhibition of nuclear import. Previous work has shown that nucleoporins are phosphorylated under certain conditions, particularly in mitotic cells (36, 52). In an *in vitro* phosphorylation experiment involving incubation of permeabilized cells with [γ^{32}-P]ATP, we observed phosphate incorporation into the nucleoporins immunoprecipitated by the RL2 antibody, which binds to a number of *O*-linked glycoproteins of the NPC (Ref. 42; data not shown). Including okadaic acid in the incubation with [γ^{32}-P]ATP only modestly increased the level of phosphorylation of some of the nucleoporins (data not shown).

The binding of importin β to two nucleoporins, Nup358/RanBP2 and Nup153, is readily detectable *in vitro* (16, 17). These proteins have been suggested to represent early and late binding sites, respectively, for the import complex at the NPC (16, 19). To directly examine whether okadaic acid affects the ability of importin β to bind to these proteins, we incubated permeabilized cells in the presence or absence of okadaic acid.

The cells were then solubilized, and cell extracts containing nucleoporins were incubated with importin β coupled to protein S-agarose beads in the presence of RanGDP or RanGMP-PNP as a specificity control (see below). As shown in Fig. 7, solubilized Nup358/RanBP2 and Nup153 bound to importin β to the same degree, irrespective of the presence of okadaic acid during the initial incubation of the permeabilized cells. Moreover, we found that with or without okadaic acid pretreatment, the binding of Nup153 to importin β was completely abolished by including RanGMP-PNP in the incubation, and the binding of importin β to Nup358/RanBP2 was strongly diminished. When coprecipitated proteins were analyzed on silver-stained gels, we detected no differences in the banding pattern in the absence or presence of okadaic acid (data not shown). These results are consistent with previous work that suggested that RanGTP releases importin β from Nup153 (53) and also with studies demonstrating that high concentrations of RanGTP inhibit the binding of importin β to Nup358/RanBP2 (17). We also investigated the co-immunoprecipitation of nucleoporins with endogenous importin β from cell lysates prepared from permeabilized cells. No difference in coprecipitating Nup153 or Nup358/RanBP2 could be detected when the permeabilized cells had been incubated in the absence of cytosol with or without okadaic acid (data not shown).

Taken together, our results suggest that phosphatase inhibitors do not interfere with the interaction of importin β with Nup358/RanBP2 or Nup153, which are presumed to mediate early and late steps of nuclear import at the NPC, respectively. It remains possible that phosphatase inhibitors block the movement of importin β and transportin complexes through the NPC by inhibiting their association with other nucleoporins at intermediate transport steps and/or by inhibiting the transfer of the import complex between different nucleoporins. Since these reactions have yet to be defined, we currently lack the methodology to directly test this hypothesis.

DISCUSSION

Here, we provide evidence that phosphorylation of a component(s) of the nuclear transport machinery strongly down-regulates nuclear import mediated by importin β and transportin, yet has no apparent effect on CRM1-mediated nuclear export. The amount of substrate bound to importin α·β in transport assays is not changed in the presence of a phosphatase inhibitor. Therefore, the effects we have described in our study do not appear to be due to phosphorylation of the nuclear import substrate itself, which is a well recognized mechanism for regulating the activity of nuclear import and export signals.

We found that the protein kinase inhibitor staurosporine partially reverses the inhibitory effect of protein phosphatase inhibitors on nuclear import. Since the stimulatory effect of the kinase inhibitor is observed only in the presence of the phosphatase inhibitor, we assume that under normal conditions in the *in vitro* transport assays, dephosphorylation of the relevant components of the transport machinery predominates over phosphorylation. Nevertheless, the kinases that can mediate this down-regulation of nuclear import clearly are present in cycling HeLa cells, even if their activity is not dominant under standard assay conditions. This argues that cells possess the capacity to down-regulate their nucleocytoplasmic transport machinery in response to appropriate physiological situations (see below). The observation that staurosporine only partially reverses the effect of okadaic acid suggests the involvement of at least two inhibitory kinases, one of them sensitive and the other insensitive to staurosporine.

Several lines of evidence indicate that the kinases and phosphatases responsible for the inhibition in our assay, as well as the components of the nuclear transport machinery that are inhibited by protein phosphorylation, are at least in large part permeabilized cell constituents rather than cytosolic transport factors. First, a stably inhibited state could be created by preincubating permeabilized cells with ATPγS or okadaic acid. Since the inhibitory effect of preincubating cells with ATPγS was more pronounced when cytosol was present during the preincubation, it appears possible that a fraction of the kinases is either cytosolic or shuttles between the nucleus and the cytoplasm. Second, our studies suggested that okadaic acid was not creating a situation in which Ran was limiting in the import system and indicated that the import receptors retained the ability to bind cargo. We believe that the most likely target of protein phosphorylation in permeabilized cells that is responsible for inhibition of nuclear import is a component of the NPC. Although we were unable to detect any effect of phosphatase inhibitors on the binding of importin β to Nup358/RanBP2 and Nup153, it is plausible that phosphorylation inhibits the binding of the import receptors to other uncharacterized nucleoporins required for import or that it alters the transfer of the import complex between different nucleoporins. Our data are consistent with the possibility that phosphorylation involves components of the import machinery used by multiple receptor pathways, although it also is possible that phosphorylation affects multiple targets, each specialized for a single import pathway.

When this manuscript was in preparation, Czubryt *et al.* (54) reported that activation of the mitogen-activated protein kinase ERK2 by H_2O_2 in aortic vascular smooth muscle cells inhibited nuclear import of a synthetic substrate containing a classical NLS. The effects described in our study clearly differ from the ones described by these authors, as the MEK1 inhibitor PD98059, which completely blocked the inhibitory effect of H_2O_2 on nuclear import in their study, was unable to antagonize the inhibition by okadaic acid in our *in vitro* import assay,[3] in contrast to staurosporine.

Interestingly, the effects described in our study result in the down-regulation of at least two major nuclear import pathways, but not in the activity of a major nuclear export pathway. This selective inhibition of nuclear import could result in the coordinate redistribution of a host of nucleocytoplasmic shuttling proteins to the cytoplasm such as transcription and DNA replication factors. We propose that this type of transport regulation, which may occur during growth arrest (32) or other states, could be important for mediating general changes in nuclear activity.

The results of this study show that elevated phosphorylation of one or more components of the nuclear transport machinery, which is induced by okadaic acid or microcystin *in vitro*, can inhibit nuclear import in HeLa cells. Other reports have suggested that certain types of phosphorylation may enhance nuclear import in some cell types. Feldherr and Akin (34) described an increase in nuclear import of a synthetic substrate resulting from the activity of a cytosolic kinase. Similarly, Mishra and Parnaik (23) reported that treatment of permeabilized cells with alkaline phosphatase inhibited nuclear import *in vitro*, an effect that could be antagonized by incubating the phosphatase-treated cells with cytosol enriched in protein kinase C. Collectively, our data together with that from previous studies (23, 34, 54) point to a situation in which different kinases can either positively or negatively regulate the activity of the nuclear import machinery. The identification of the specific target proteins in the nuclear import machinery that are affected by these kinases will be required to further investigate this aspect of nuclear transport regulation.

[3] R. H. Kehlenbach and L. Gerace, unpublished observations.

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