

# The mechanism of inhibition of Ran-dependent nuclear transport by cellular ATP depletion

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Ran-dependent nuclear transport requires a nuclear pool of RanGTP both for the assembly of export complexes and the disassembly of import complexes. Accordingly, in order for these processes to proceed, Ran-dependent nuclear import and export assays *in vitro* require the addition of GTP to produce RanGTP. Notably, no ATP requirement can be detected for these transport processes *in vitro*. But *in vivo*, when cells are depleted of ATP by the addition of sodium azide and 2-deoxyglucose to block ATP production by oxidative phosphorylation and glycolysis,

respectively, Ran-dependent nuclear import and export are rapidly inhibited. This raised the question of whether there is an ATP requirement for these nuclear transport pathways in an intact cell that has remained undetected *in vitro*. Here we report that the free (but not total) GTP concentration rapidly drops to an undetectable level upon ATP depletion as does the availability of RanGTP. Our conclusion is that the inhibition of Ran-dependent nuclear transport observed upon ATP depletion *in vivo* results from a shortage of RanGTP rather than the inhibition of some ATP-dependent process.

## Introduction

That carrier-dependent nuclear transport of cargo across the nuclear pore complex (NPC)\* requires energy is well established, but how is this energy used? Of the ~30 proteins that comprise the yeast NPC, none of them resemble an ATPase or GTPase, making it unlikely that the NPC itself has to hydrolyze nucleoside triphosphates (NTPs) to power transport (Rout et al., 2000). Consistent with this, movement of a carrier (with or without bound cargo) from one side of the NPC to the other does not require energy (Kose et al., 1997; Yokoya et al., 1999; Ribbeck and Gorlich, 2001) and is believed to occur by repeated binding and release of the carrier to sequential NPC proteins in a process called “facilitated diffusion” (for review see Talcott and Moore, 1999). In contrast, however, the signal-mediated accumulation of import cargo in the nucleus or export cargo in the cytoplasm does require energy, and we now know that in many cases, at least

part of the energy requirement can be linked to the small GTPase Ran (Moore, 1998).

The majority of nuclear carriers belong to the karyopherin- $\beta$  (Kap- $\beta$ ) superfamily, and the one diagnostic feature of carriers in this family is that they all bind Ran, but critically only when Ran is in the GTP- rather than the GDP-bound form (Nakielnny and Dreyfuss, 1999). Nuclear transport complexes containing a Kap- $\beta$  carrier use the concentration of RanGTP, which varies widely between the cytoplasm and nucleus, as a positional cue to regulate their assembly and disassembly (Gorlich et al., 1996; Izaurralde et al., 1997). The concentration of RanGTP is kept low in the cytoplasm and high inside the nucleus by localizing the Ran GTPase activating protein (GAP) to the cytoplasm and the Ran guanine nucleotide exchange factor (GEF) (RCC1) to the nucleus.

Crucially, having bound RanGTP has opposite effects on import versus export carriers on their ability to simultaneously bind cargo (Gorlich et al., 1996). Kap- $\beta$  carriers that function in import will only bind their cargo in the absence of RanGTP (i.e., in the cytoplasm) and the subsequent binding of RanGTP inside the nucleus triggers the release of cargo. Thus, import of a classical nuclear localization sequence (cNLS)-containing cargo mediated by Kap- $\beta$ 1 and its adaptor Kap- $\alpha$  requires both nuclear Ran and GTP, however, GTP hydrolysis by Ran is not required for entry of the cargo into the nuclear interior (Schwoebel et al., 1998; Englmeier et al., 1999). Instead, nuclear RanGTP is required for the disassembly of an incoming transport complex and the release of cargo triggered by the interaction of Kap- $\beta$ 1

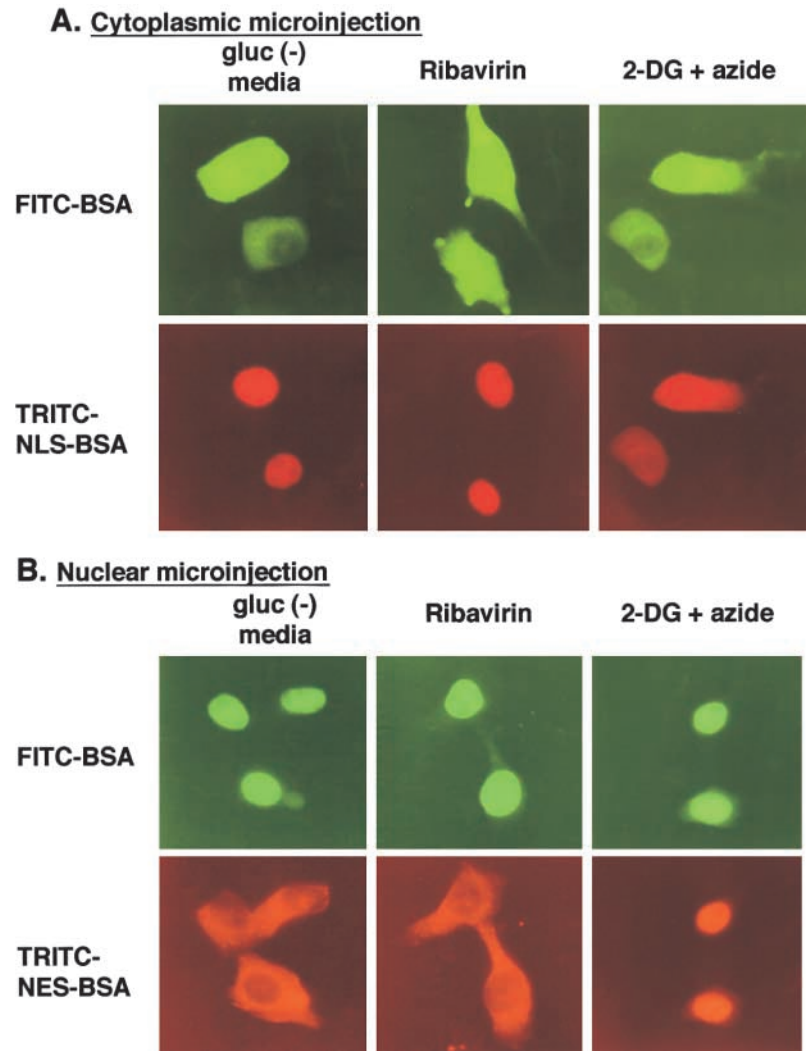
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\*Abbreviations used in this paper: CAS, cellular apoptosis susceptibility; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; GST, glutathione-S-transferase; Kap- $\beta$ , karyopherin- $\beta$ ; NEM, *N*-ethylmaleimide; NES, nuclear export sequence; cNLS, classical nuclear localization sequence; NPC, nuclear pore complex; NTP, nucleoside triphosphate; RanBD, Ran binding domain; RT, room temperature.

Key words: Ran; nuclear; transport; ATP; ribavirin

**Figure 1. ATP depletion of cells with 2-deoxyglucose/Na azide inhibits both nuclear import and export, whereas GTP depletion with ribavirin does not.** HeLa cells were incubated in  $\text{gluc}^-$  media (2 h),  $\text{gluc}^-$  media containing ribavirin (2 h), or  $\text{gluc}^-$  media containing 2-deoxyglucose/azide (20 min). Cells were injected in the cytoplasm (A) with FITC-BSA marker (1.25 mg/ml) and the nuclear import reporter TRITC-NLS-BSA (1.3 mg/ml), or in the nucleus (B) with the marker and the nuclear export reporter TRITC-NES-BSA (4.5 mg/ml). After injection, the cells were incubated for an additional 30 min in the indicated medium followed by fixation and fluorescence microscopy.



with RanGTP (Kutay et al., 1997b). GTP hydrolysis by Ran in this import pathway occurs only when the RanGTP-Kap- $\beta$ 1 complex recycles back through the NPC and encounters the RanGAP in the cytoplasm. Thus, GTP hydrolysis by Ran during Ran-dependent nuclear import is required for recycling transport carriers after import, rather than for powering the movement of an import cargo through the NPC into the nucleus. Export carriers exhibit the reverse behavior, requiring bound RanGTP to simultaneously bind their nuclear export sequence (NES)-containing cargo with high affinity. Upon contact of the export complex with the RanGAP in the cytoplasm, RanGTP hydrolyzes its bound GTP to become RanGDP. As a result of this GTP hydrolysis, both RanGDP and cargo are released by the carrier, terminating export.

In vitro, neither Kap- $\beta$ 1-mediated import of cNLS-containing cargo nor Crm1-mediated export of leucine-rich NES-containing cargo are inhibited by the addition of a nonhydrolyzable ATP analogue (Schwoebel et al., 1998; Englmeier et al., 1999). In vivo, however, ATP depletion of cells by metabolic poisons that block oxidative phosphorylation and/or glycolysis causes a rapid shutdown of cNLS-mediated import (Richardson et al., 1988; Shulga et al., 1996). We wanted to determine by what mechanism ATP deple-

tion inhibits Ran-dependent nuclear transport in vivo, particularly to determine whether there is an ATP-dependent step in these transport pathways in vivo that has remained undetected in vitro. Here we report that the in vivo inhibition of Ran-dependent nuclear transport observed upon ATP depletion is likely caused by depletion of cellular RanGTP resulting from a lack of free GTP, rather than inhibition of an ATP-dependent step required for transport.

## Results

### Nuclear transport is inhibited in 2-deoxyglucose/azide-but not ribavirin-treated cells

Because the metabolisms of adenine and guanine nucleotides are tightly linked in vivo (Hershfield and Seegmiller, 1976; Detimary et al., 1997; Kondo et al., 2000), we hypothesized that a simple explanation for the inhibition of Ran-dependent nuclear import in vivo by ATP depletion might be that this treatment also results in depletion of GTP, preventing the generation of RanGTP. To test this hypothesis, the Ran-dependent nuclear transport capability of cells "ATP depleted" with 2-deoxyglucose and sodium azide was compared with that of cells "GTP depleted" with ribavirin. Azide inhibits the production of ATP by oxidative phosphorylation,

whereas 2-deoxyglucose inhibits ATP production by glycolysis. Ribavirin is an inhibitor of IMP dehydrogenase (the rate limiting enzyme in de novo GTP biosynthesis) and ribavirin treatment is known to decrease intracellular GTP levels (Finch et al., 1993; Yalowitz and Jayaram, 2000).

After treatment with either ribavirin or 2-deoxyglucose/azide in  $\text{gluc}^-$  medium, the labeled import substrate TRITC-NLS-BSA (containing the NLS of the SV40 T antigen) was injected into the cytoplasm of HeLa cells to monitor import. In other cells, the labeled export substrate TRITC-NES-BSA (containing the Rev NES) was injected into the nucleus to monitor export. As a control, cells were injected after incubation in  $\text{gluc}^-$  media. As expected from previous reports (Richardson et al., 1988; Shulga et al., 1996), TRITC-NLS-BSA was imported into the nuclei of control cells, but not imported into the nuclei of cells treated with 2-deoxyglucose/azide (Fig. 1 A). Likewise, nuclear injected TRITC-NES-BSA was exported from the nuclei of control cells, but not cells treated with 2-deoxyglucose/azide. Unexpectedly, however, treatment with ribavirin to lower GTP levels affected neither the nuclear import of TRITC-NLS-BSA nor the export of TRITC-NES-BSA (Fig. 1).

#### Total nucleotide levels in ATP- and GTP-depleted cells

To compare the nucleotide levels in cells after these treatments, nucleotides were isolated from control and treated cells and quantitated by FPLC (Kremmer et al., 1989). We found that treatment with 2-deoxyglucose/azide in  $\text{gluc}^-$  medium for 1 h decreased total cellular ATP to  $\sim 40\%$  of the level in cells incubated in just  $\text{gluc}^-$  medium (8.22 vs. 3.27 nmoles/ $10^6$  cells; Table I). In trial experiments, when FCS was omitted from the medium during treatment, 2-deoxyglucose/azide treatment for 1 h resulted in much lower ATP levels ( $<10\%$  of normal; unpublished data). We found however that the cells were in such bad shape after such severe ATP depletion that they were very difficult to microinject (unpublished data). We found that addition of FCS to the medium during treatment made the cells more amenable to microinjection and did not prevent the inhibition of nuclear transport by 2-deoxyglucose/azide.

As hypothesized, ATP depletion with 2-deoxyglucose/azide also caused a decrease in the total GTP content from 2.58 to 1.85 nmoles/ $10^6$  cells (72% of control levels). GTP depletion with ribavirin, however, decreased the GTP content almost twice as much to 36% of control levels (0.87 nmoles/ $10^6$  cells) without significantly affecting the ATP concentration (8.42 vs. 8.32 nmoles/ $10^6$  cells). Yet, as shown in Fig. 2, these GTP-depleted cells are still transport competent unlike the ATP-depleted cells, indicating that inhibition of nuclear transport in 2-deoxyglucose/azide-treated

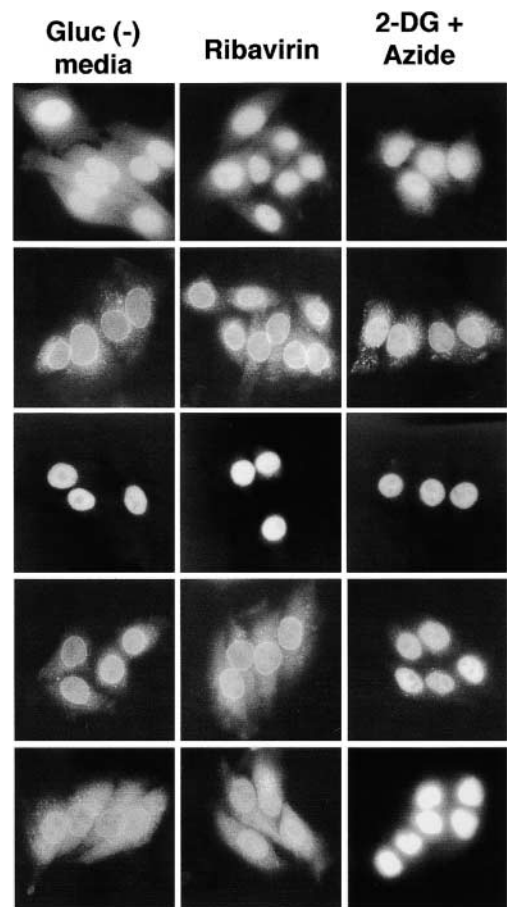


Figure 2. **The cellular localization of nuclear transport factors after 2-deoxyglucose/sodium azide or ribavirin treatment.** Cells were treated with control  $\text{gluc}^-$  media for 2 h,  $\text{gluc}^-$  media containing ribavirin for 2 h, or  $\text{gluc}^-$  media containing 2-deoxyglucose/sodium azide for 1 h. The cells were fixed and probed with antibodies against the indicated nuclear transport factor, followed by fluorescently labeled second antibody. Note that Kap- $\alpha$  accumulates in the nuclei of 2-deoxyglucose/azide-treated cells, whereas the distribution of the other transport factors remains essentially unchanged.

cells is not the result of a decrease in the levels of total GTP. Note that although other cell types can contain higher ATP/GTP ratios than we found here (Franklin and Twose, 1977; Lee et al., 1985), our measured ATP/GTP ratios of 3.2–3.5 in control cells are nearly identical to those previously determined in HeLa cells (Finch et al., 1993).

Ran has a 10-fold higher affinity for GDP than GTP and although the RanGEF drastically increases the rate of nucleotide loading, it does not affect the relative affinity of Ran for GTP and GDP (Klebe et al., 1995). Other factors may modulate

Table I. **Measurement of total nucleotide levels in HeLa cells after various treatments**

	ATP	ADP	GTP	GDP	ATP/ADP	GTP/GDP
Glucose $^-$ media, 1 h	8.22 $\pm$ 0.75	0.75 $\pm$ 0.17	2.58 $\pm$ 0.23	0.29 $\pm$ 0.07	11.0	8.9
Glucose $^-$ media, 2 h	8.32 $\pm$ 0.59	0.69 $\pm$ 0.05	2.4 $\pm$ 0.2	0.21 $\pm$ 0.03	12.1	11.4
Ribavirin, 2 h	8.42 $\pm$ 0.43	0.69 $\pm$ 0.01	0.87 $\pm$ 0.01	0.17 $\pm$ 0.02	12.2	5.1
2-deoxyglucose + sodium azide, 1 h	3.27 $\pm$ 0.60	0.53 $\pm$ 0.13	1.85 $\pm$ 0.29	0.27 $\pm$ 0.07	6.2	6.9

Values are expressed as nmoles nucleotide/ $10^6$  cells. Nucleotides were measured in total cellular extracts by FPLC as described in the Materials and methods. Each value represents the mean  $\pm$  SD of three independent experiments.

this process in vivo but, based on our current knowledge, the ratio of RanGTP versus RanGDP generated in a loading reaction is dependent on the ratio of free GTP to GDP in the immediate environment. Because of this, we then measured the total GDP (and ADP) levels to determine whether an altered GTP/GDP ratio rather than the total concentration of GTP in the 2-deoxyglucose/azide-treated cells might be responsible for the observed transport inhibition. Treatment with 2-deoxyglucose/azide did result in a decrease in the GTP/GDP ratio from 8.3 to 6.9. However, treatment with ribavirin affected the GTP/GDP ratio more severely, dropping it to 5.1. Thus, both the total GTP level and GTP/GDP ratio are lower after ribavirin treatment than 2-deoxyglucose/azide treatment (Table I), and yet ribavirin-treated cells remain transport competent, whereas 2-deoxyglucose/azide treated cells do not. Therefore, it is not the decrease in total GTP or the GTP/GDP ratio after 2-deoxyglucose/azide treatment that is responsible for the observed nuclear transport inhibition.

The Mono-Q and Source 15Q FPLC columns used here for the quantitation of nucleotides retain and fractionate all mono-, di-, and triphosphate nucleotides (Hartwick and Brown, 1975; Kremmer et al., 1989). After 2-deoxyglucose/azide treatment, we did not see a corresponding increase in any of the nucleotides (such as AMP) that might possibly represent breakdown or interconversion products of ATP (unpublished data). This is consistent with a previous report that a decrease in cellular ATP stimulated by anoxia correlates with a rise at time points similar to those observed here in the metabolic breakdown products of ATP, such as adenosine, that are not retained on our FPLC columns (Grune and Siems, 1993).

### 2-deoxyglucose/azide treatment results in a buildup of Kap- $\alpha$ in the nucleus

To explore other possibilities for the 2-deoxyglucose/azide inhibition of nuclear transport, ribavirin- and 2-deoxyglucose/azide-treated cells were fixed and examined by indirect immunofluorescence microscopy to determine whether these treatments result in a change in the intracellular localization of proteins required for nuclear transport. We did not observe any significant changes in the localization of Ran, the RanGAP, or the RanGEF (RCC1) after either treatment (Fig. 2). Note that cells treated with 2-deoxyglucose/azide tend to round up somewhat, resulting in a decrease in the apparent cytoplasmic volume. This morphological change can result in a slightly altered appearance (see for example the Ran localization) without actually changing the nucleocytoplasmic distribution of the transport factors (Fig. 2). Kap- $\beta$ 1 in some experiments appeared to slightly concentrate in the nucleus after 2-deoxyglucose/azide treatment, but this change was neither dramatic nor consistent. Kap- $\alpha$ , in contrast, was found to dramatically shift its localization exclusively to the nuclei of 2-deoxyglucose/azide-treated cells. Normally, Kap- $\alpha$  mainly appears either cytosolic or bound at the nuclear envelope as can be seen in the ribavirin- and gluc<sup>-</sup>-treated cells (Fig. 2).

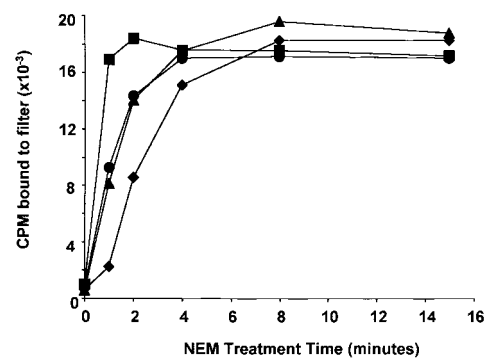
During cNLS-mediated import, Kap- $\alpha$  crosses the NPC into the nucleus in a complex with cNLS-containing cargo and Kap- $\beta$ 1 (Gorlich et al., 1995). Upon contact with RanGTP in the nucleoplasm, this complex disassembles and Kap- $\alpha$  is then recycled back to the cytoplasm by the CAS

(cellular apoptosis susceptibility) protein carrier (Kutay et al., 1997a). CAS requires bound RanGTP to bind Kap- $\alpha$  with high affinity, thus both the import of Kap- $\alpha$  complexed with cargo and Kap- $\beta$ 1 and the export of Kap- $\alpha$  mediated by CAS require RanGTP (Melchior et al., 1993; Moore and Blobel, 1993; Kutay et al., 1997a). Accumulation of both the *Saccharomyces cerevisiae* homologue of Kap- $\alpha$  (Srp1p) and vertebrate Kap- $\alpha$  in the nucleus has been observed previously in cells predicted to have a low concentration of nuclear RanGTP. Examples of this are cells expressing a mutant Ran that cannot be converted to the GTP-bound form, or when the Ran GEF is inactivated (Koepp et al., 1996; Tachibana et al., 2000). We wondered therefore whether the cells upon 2-deoxyglucose/azide treatment might be becoming deficient in RanGTP, thus explaining their transport defect. Accordingly, we devised a method for directly measuring cellular RanGTP to see how this was affected by 2-deoxyglucose/azide treatment.

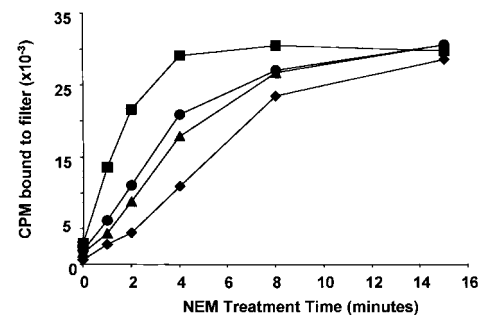
### Measurement of in vivo RanGTP levels

The percentage of cellular Ran in the GTP-bound form at steady-state has never been measured, primarily for technical

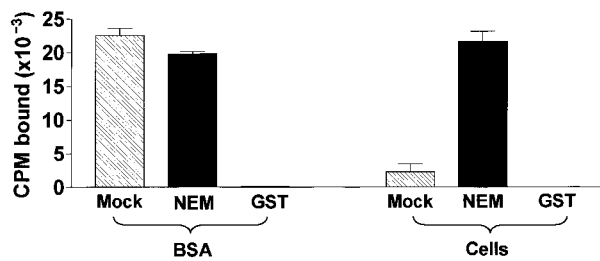
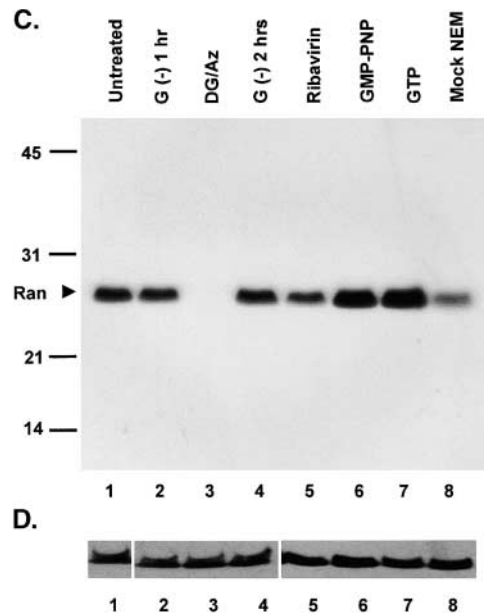
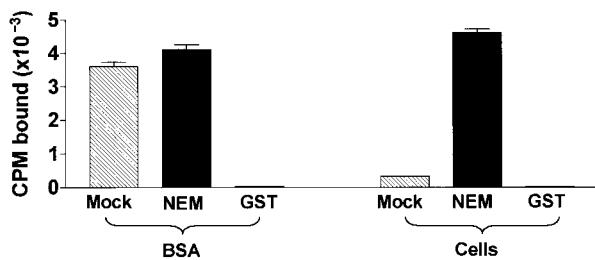
#### A. RanGEF activity in lysate



#### B. RanGAP activity in lysate



**Figure 3. NEM treatment of HeLa cellular lysates inhibits both RanGEF and RanGAP activity.** Cellular lysates were prepared as described in the Materials and methods and treated with either (◆) 10, (▲) 20, (●) 40, or (■) 60 mM NEM for the indicated period of time, quenched with excess DTT, and measured as follows: (A) measurement of RanGEF activity in HeLa cell extracts after NEM treatment; (B) measurement of RanGAP activity in HeLa cell extracts after NEM treatment. Both RanGAP and GEF activity were measured as described in the Materials and methods.

**A. RanBD pulldown of Ran[ $\alpha$ - $^{32}$ P]GTP****B. RanBD pulldown of Ran[ $\gamma$ - $^{32}$ P]GTP****Figure 4. Isolation of RanGTP with a RanBD fused to GST.**

(A and B) NEM treatment of cellular lysate allows the recovery of RanGTP. Either cellular lysates or a solution of BSA were spiked with recombinant Ran loaded with either (A) [ $\alpha$ - $^{32}$ P]GTP or (B)

[ $\gamma$ - $^{32}$ P]GTP before pulldown of RanGTP with GST-RanBD. The radiolabeled Ran and NEM were added together with 2 mM GDP- $\beta$ S upon cell lysis as described in the Materials and methods. Samples were either NEM treated to abolish RanGAP and GEF activity (100 mM NEM for 20 min) before quenching with 200 mM DTT, or mock treated in which the order of addition of NEM and DTT was reversed. After gel filtration to remove excess NEM and DTT, RanGTP was isolated in a pulldown with GST-RanBD and glutathione-agarose. In the indicated samples, GST was substituted for the GST-RanBD; these samples were mock treated. Shown are the CPM of radiolabeled Ran associated with the GST-RanBD glutathione-agarose beads after washing. (C) No free RanGTP can be detected in HeLa cells treated with 2-deoxyglucose/azide. HeLa cells were either untreated (lanes 1 and 6–8), incubated in  $\text{gluc}^-$  media for either 1 (lane 2) or 2 h (lane 4), incubated with 2-deoxyglucose/azide in  $\text{gluc}^-$  media for 1 h (lane 3), or incubated with ribavirin in  $\text{gluc}^-$  media for 2 h (lane 5). After treatment, cells were scraped in buffer A containing 0.1% Triton X-100 and were either NEM treated as above to abolish RanGAP and GEF activity (lanes 1–7) or mock treated (lane 8). The unlabeled nucleotides added upon cellular lysis were 2 mM GDP- $\beta$ S (lanes 1–5 and 8), GMP-PNP (lane 6), or GTP (lane 7). After NEM or mock treatment, RanGTP was isolated from the soluble cytosolic extract by incubation with GST-RanBD and glutathione-agarose. Bound protein was solubilized with SDS-PAGE sample buffer, separated on a 15% gel, transferred to nitrocellulose, and immunoblotted with an anti-Ran antibody. Note in particular the absence of detectable RanGTP in cells treated with 2-deoxyglucose/azide. (D) Immunoblot of total cell extracts shows that the amount of total cellular Ran doesn't change after these treatments.

reasons. A major problem with measuring the intracellular concentration of RanGTP is that upon cell lysis and preparation of a cellular extract, Ran and its GAP and GEF become mixed together instead of remaining spatially separate. In particular, the mixing of RanGTP with the RanGAP results in very (artificially) low levels of RanGTP that can be immunoprecipitated from cellular extracts even when the extracts are kept cold (Lounsbury et al., 1996). Thus, we needed some way of inhibiting RanGAP and RanGEF activity in cellular lysates to accurately measure the amount of RanGTP that was present in the cells before lysis. In experiments not shown, we found that the Ran GAP and GEF activities in the cellular lysates as measured with exogenously added Ran were not altered after 2-deoxyglucose/azide treatment (unpublished data). This result indicated that 2-deoxyglucose/azide treatment does not affect RanGTP production by altering the activity of either of these enzymes by, for example, inhibiting an ATP-dependent posttranslational modification.

We reported previously that the RanGEF activity of RCC1 is inhibited by pretreatment of RCC1 with the alkylating agent *N*-ethylmaleimide (NEM) (Schwoebel et al., 1998), and here we report that the RanGAP activity is also inhibited by treatment with NEM. We found that both

RanGAP and RanGEF activities in cellular lysates were inhibited by NEM in a time- and dose-dependent fashion (Fig. 3, A and B). In every case, excess DTT was added after NEM treatment to quench the activity of NEM before assay. Note that despite the NEM sensitivity of the Ran GEF and GAP activities as shown here, Ran itself is known to be unaffected by NEM treatment (Moore and Blobel, 1992).

Having in hand a treatment to substantially reduce RanGAP and RanGEF activity in cellular lysates, we used this NEM treatment in conjunction with a new assay that we devised to measure available RanGTP present in a cellular lysate. This assay utilizes the Ran binding domain (RanBD) of RanBP1, which only binds RanGTP and not RanGDP (Beddow et al., 1995). After cell lysis and NEM treatment, the extract is passed over a desalting column to remove NEM and DTT. A glutathione-S-transferase (GST) pull-down is then performed with GST-RanBD, and bound Ran is determined by immunoblotting with an anti-Ran antibody. Because only Ran in the GTP-bound form binds the RanBD, any Ran isolated in this pulldown must be in the GTP-bound form. Furthermore, only RanGTP not already complexed with RanBP1 or any other protein that binds to this region of RanGTP will be isolated in this pulldown.

To confirm that a 20-min incubation with 100 mM NEM inhibits RanGAP and RanGEF activity sufficiently in cellular extracts such that RanGTP can be quantitatively recovered in a pull-down with GST-RanBD, [ $\alpha$ - $^{32}$ P]GTP- or [ $\gamma$ - $^{32}$ P]GTP-loaded recombinant Ran was added (together with NEM) to the lysate immediately upon cell lysis. As a control for the efficiency of recovery of radiolabeled RanGTP in the absence of the RanGAP and RanGEF and endogenous unlabeled RanGTP, a BSA solution was substituted for cellular extract in some samples. As shown in Fig. 4, A and B, exogenously added [ $^{32}$ P]GTP-Ran can be recovered by the GST-RanBD from cellular extracts to the same extent as from a BSA solution with, but not without, NEM treatment of the extract. A negligible amount of counts was recovered when GST was substituted for GST-RanBD. As the radiolabeled RanGTP and NEM were added together immediately upon cell lysis, these experiments confirmed that this NEM treatment was capable of preserving RanGTP present in a cellular extract after cell lysis.

Shown in Fig. 4 C are the amounts of free RanGTP that can be isolated from extracts of 2-deoxyglucose/azide- and ribavirin-treated cells. In untreated cells (lane 1), or cells incubated in  $\text{gluc}^-$  media for 1 (lane 2) or 2 h (lane 4), substantial amounts of free RanGTP could be detected in the GST-RanBD eluate. That NEM treatment is inhibiting the GAP and GEF activity normally present in cellular extracts can be seen by comparing the amount of Ran in lane 1 (NEM-inactivated extract from control cells) with the amount in lane 8 (mock-inactivated extract from control cells). The decreased RanGTP in lane 8 is presumably due to GAP or GEF activity in the lysate. In all of these samples except those in lanes 6 and 7 (and in the experiment shown in Fig. 3), an excess of GDP $\beta$ S (together with the NEM) was added upon cell lysis. This was to ensure that if any nucleotide exchange occurred on Ran before inactivation of the RanGEF, the Ran would be loaded into the GDP- and not the GTP-bound form, thus avoiding an overestimation of the cellular RanGTP. When GTP (lane 7) or GMP-PNP (lane 6) were added in place of GDP $\beta$ S, a slight increase in isolated RanGTP could be detected, indicating a slight amount of residual RanGEF activity that was not detected in the experiment shown in Fig. 3. We concluded that this NEM treatment was inhibiting the GAP and GEF activity in the lysate as much as was technically possible, and that this treatment would allow us to obtain a good estimate of the relative levels of free RanGTP present in the cell after these various treatments. The most significant result from this experiment was that after 1 h of treatment with 2-deoxyglucose/azide, no RanGTP could be detected in the lysate (compare lane 3 with lanes 1 and 2). Immunoblotting with an anti-Ran antibody of the different extracts before incubation with the RanBD indicated that the total cellular Ran content was unchanged after these different treatments (Fig. 4 D), indicating that only free RanGTP was lost, and not total Ran. Ribavirin treatment (2 h) resulted in a slight decrease in the available RanGTP (Fig. 4 C, compare lanes 4 and 5), but the amount remaining was still significantly higher than in the 2-deoxyglucose/azide-treated cells.

### **Buildup of Kap- $\alpha$ in the nucleus of 2-deoxyglucose/azide-treated cells occurs concomitantly with a loss of RanGTP**

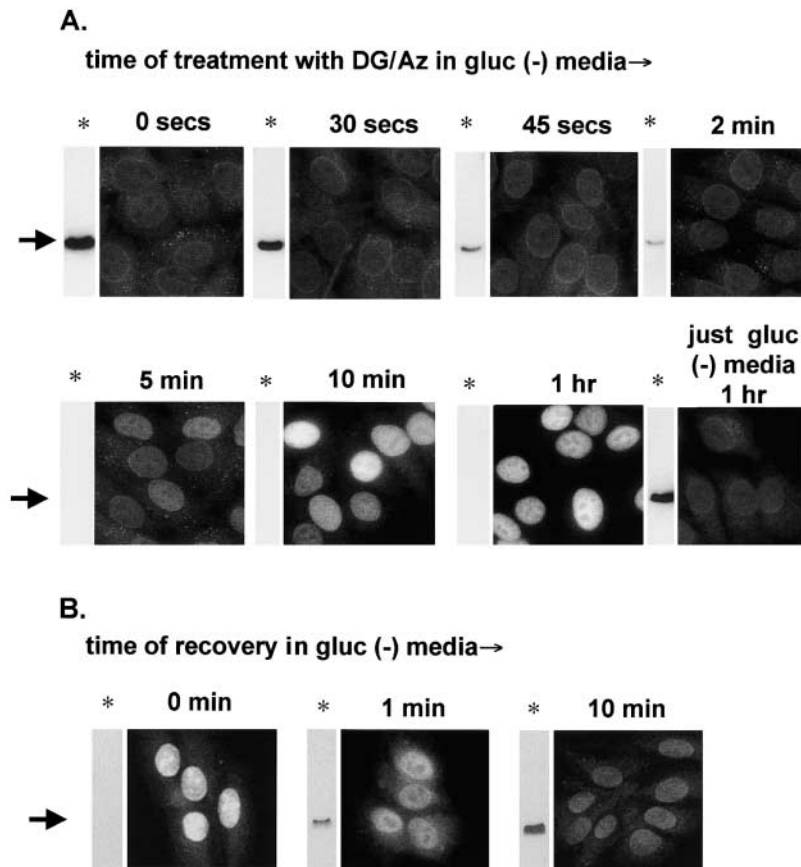
Now that we had established that 2-deoxyglucose/azide treatment for 1 h drops the available RanGTP to undetectable levels, we wanted to see whether the disappearance of RanGTP upon 2-deoxyglucose/azide treatment correlated in time with the cessation of transport. A time course examination of 2-deoxyglucose/azide treatment was performed, and at every time point, cells were either fixed and the localization of Kap- $\alpha$  was determined by immunofluorescence microscopy, or lysed and measured for available RanGTP as described above (Fig. 5). We found that the amount of available RanGTP in the lysate detectable in our assay dropped very quickly after the addition of 2-deoxyglucose/azide, with a noticeable decrease in available RanGTP observable just 30 s after addition. The amount of available RanGTP continued to decrease after 45 s and 2 min of treatment, and after 5 min of treatment, available RanGTP was no longer detectable. Available RanGTP remained undetectable by this assay from 5 min to 1 h of treatment. Buildup of Kap- $\alpha$  in the nucleus was slightly apparent after 2 min of treatment, more noticeable at 5 min, and very striking after 10 min, with the cells at this time point exhibiting some heterogeneity in their amount of Kap- $\alpha$  nuclear accumulation. Between 10 min and 1 h of treatment, there was a decrease in this heterogeneity, with every cell exhibiting very strong Kap- $\alpha$  nuclear accumulation after 1 h of treatment.

We also found that the reappearance of RanGTP during recovery from 2-deoxyglucose/azide treatment occurs concomitantly with the movement of Kap- $\alpha$  out of the nucleus (Fig. 5 B). Deoxyglucose/azide-treated cells allowed to recover for only 1 min in  $\text{gluc}^-$  media had a detectable amount of RanGTP and decreased Kap- $\alpha$  signal in the nucleus. After 10 min of recovery, the concentration of available RanGTP was almost back to normal as was the cellular distribution of Kap- $\alpha$ . Thus, the decrease in free RanGTP precedes the nuclear accumulation of Kap- $\alpha$  upon treatment with 2-deoxyglucose/azide, and the reappearance of free RanGTP during recovery from this treatment coincides with movement of Kap- $\alpha$  out of the nucleus.

### **The effects on free (rather than total) GTP levels by 2-deoxyglucose/azide and ribavirin treatment**

As inactivation of the RanGEF resulting in a lack of RanGTP is known to result in a nuclear buildup of Kap- $\alpha$  (Tachibana et al., 2000), we reasoned that the lack of RanGTP in 2-deoxyglucose/azide-treated cells (Figs. 4 and 5) was likely to be triggering the same event. What was unclear from our data however, is why free RanGTP was absent in the 2-deoxyglucose/azide-treated cells (Fig. 4), when these cells had significantly higher levels of GTP than ribavirin-treated cells (Table I). We decided to measure the free rather than the total GTP concentration, reasoning that it was the pool of free GTP rather than total GTP (free plus protein bound) that would be used to load Ran to form RanGTP.

To measure the concentration of free GTP, immediately after lysis we briefly microfuged the sample, and then



**Figure 5. A time course of 2-deoxyglucose/azide treatment and recovery comparing the availability of RanGTP with the nuclear buildup of Kap- $\alpha$ .**

For each time point in A and B, the cellular localization of Kap- $\alpha$  as determined by indirect immunofluorescence microscopy (performed as in Fig. 2) was compared with the RanGTP from NEM-treated cellular extracts that bound to the RanBD (performed as in Fig. 4 C, lanes 2 and 3, arrow). (A) HeLa cells were incubated for the indicated time at 37°C in gluc<sup>-</sup> media containing 2-deoxyglucose/azide. (B) After treatment in 2-deoxyglucose/azide for 1 h, the cells were washed once in prewarmed gluc<sup>-</sup> media, and then transferred to prewarmed gluc<sup>-</sup> media and incubated at 37°C for the indicated time. To emphasize the heterogeneity in Kap- $\alpha$  nuclear accumulation at longer time points, the same relatively short camera exposure time was used for all of these micrographs.

passed the supernatant through a filter (10 kD mol wt cut-off) to remove free nucleotide from that which was bound to proteins over 10 kD. This filtered extract was then measured for its GTP and ATP content. We found that the ATP from control cells that passed through the filter (i.e., free ATP) (4.48 nmoles/10<sup>6</sup> cells) was 55% of the cellular total (8.22 nmoles/10<sup>6</sup> cells) (Table II). Free GTP from control cells (0.93 nmoles/10<sup>6</sup> cells) represented 36% of the total (2.58 nmoles/10<sup>6</sup> cells). Notably, the amount of free GTP present in control cells (0.93 nmoles/10<sup>6</sup> cells) decreases after ribavirin treatment to 0.3 nmoles/10<sup>6</sup> cells, but drops even further to an undetectable level after 2-deoxyglucose/azide treatment (<0.1 nmoles/10<sup>6</sup> cells). Note that the latter value is actually an overestimation, as free GTP and ATP in extracts from 2-deoxyglucose/azide-treated cells were undetectable by FPLC and the values in Table II therefore represent the minimum we can detect by FPLC

rather than the actual nucleotide present in the samples. Even when twice the normal sample volume (containing free nucleotide) was loaded from 2-deoxyglucose/azide-treated cells, there still was not a perceptible peak corresponding to GTP or ATP on the FPLC trace (unpublished data). Thus 2-deoxyglucose/azide treatment has a greater effect on free GTP levels than GTP depletion with ribavirin. We believe that the absence of free GTP in the 2-deoxyglucose/azide-treated cells inhibits loading of Ran with GTP, and this lack of free GTP is likely to be the causative factor in the inhibition of Ran-dependent nuclear transport by ATP depletion.

#### Entry of Kap- $\alpha$ into the nucleus is also inhibited by 2-deoxyglucose/azide

We had one final question concerning the nuclear accumulation of Kap- $\alpha$  seen upon 2-deoxyglucose/azide treatment

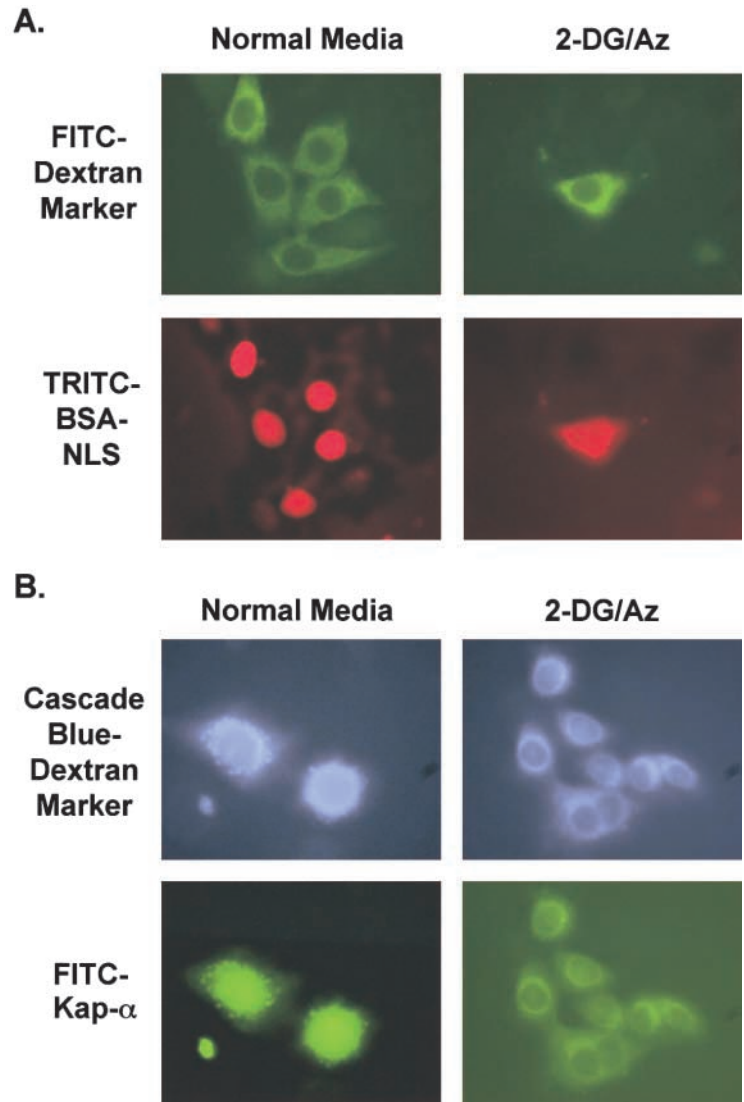
**Table II. Measurement of free nucleotides in HeLa cells after various treatments**

	Free ATP	Total ATP (from Table I)	Free GTP	Total GTP (from Table I)
Glucose <sup>-</sup> media, 1 h	4.48 ± 0.27	8.22 ± 0.75	0.93 ± 0.22	2.58 ± 0.23
2-deoxyglucose + sodium azide, 1 h	<0.1 <sup>a</sup>	3.27 ± 0.60	<0.1 <sup>a</sup>	1.85 ± 0.29
Ribavirin, 2 h	5.93 ± 1.07	8.42 ± 0.43	0.30 ± 0.04	0.87 ± 0.01

Values are expressed as nmoles nucleotide/10<sup>6</sup> cells. Free nucleotide was separated from the total cellular lysate by filtration as described in the Materials and methods. Values for free ATP and GTP represent the mean ± SD of three independent experiments; the values for total ATP and GTP were taken from separate experiments summarized in Table I.

<sup>a</sup>Both the free ATP and GTP from the cells treated with 2-deoxyglucose and sodium azide were essentially undetectable by FPLC, and thus below our lowest standards. The values given for these two measurements therefore indicate the amount of our lowest FPLC standards rather than the exact concentration of nucleotide.

**Figure 6. The entry of microinjected FITC–Kap- $\alpha$  into the nucleus is inhibited by 2-deoxyglucose/azide treatment.** HeLa cells were incubated in either normal media or  $\text{gluc}^-$  media containing 2-deoxyglucose/azide for 20 min. They were then microinjected into the cytoplasm with either (A) TRITC–NLS–BSA (9.1 mg/ml) and a FITC 70-kD dextran marker (6.7 mg/ml) or (B) FITC–Kap- $\alpha$  (1.7 mg/ml) and a Cascade blue 70-kD dextran marker (0.9 mg/ml). After injection (which took  $\sim 10$  min), the cells were incubated for an additional 30 min in the treatment media before fixation.



(Figs. 2 and 5). For Kap- $\alpha$  to accumulate in the nucleus, its nuclear import must continue after its export has stopped. If a lack of RanGTP is solely responsible for this nuclear accumulation of Kap- $\alpha$ , then CAS-mediated export of Kap- $\alpha$  must be more sensitive to RanGTP depletion than the import of the NLS cargo–Kap- $\alpha$ –Kap- $\beta 1$  complex, and there is some evidence that this is in fact the case (Izaurralde et al., 1997; see Discussion). An alternate explanation is that Kap- $\alpha$  can somehow enter, but not exit, nuclei in an energy-independent fashion. There has been one report linking Kap- $\alpha$  to the ubiquitin/proteasome system (Tabb et al., 2000), and other reports that the yeast Kap- $\alpha$  (Srp1) interacts with a yeast NPC protein (Nup2) that is mobile in the NPC (Belanger et al., 1994; Hood et al., 2000; Solsbacher et al., 2000; Dilworth et al., 2001). Because of these reports, we wanted to determine whether Kap- $\alpha$  can possibly enter the nucleus under conditions of energy depletion when the import of cNLS cargo is inhibited.

To test whether Kap- $\alpha$  can still enter the nucleus of energy-depleted cells, we pretreated HeLa cells with either normal media or  $\text{gluc}^-$  media containing 2-deoxyglucose/azide for 20 min. We then microinjected either FITC–Kap- $\alpha$  or

TRITC–NLS–BSA (as a control) into the cytoplasm. After incubation for 30 min in the treatment media, we fixed and observed the cells to see whether these two reporters had entered the nuclear interior (Fig. 6). In untreated cells, cytoplasmically injected FITC–Kap- $\alpha$  could be detected after 30 min in the nuclear interior in addition to the cytoplasm; the FITC–Kap- $\alpha$  is presumably cycling between the cytoplasm and nucleus like the endogenous unlabeled Kap- $\alpha$ . TRITC–NLS–BSA in control cells was exclusively nuclear at this time as in the experiment shown in Fig. 1. In contrast, in cells treated with 2-deoxyglucose/azide, FITC–Kap- $\alpha$  as well as TRITC–NLS–BSA remained exclusively cytoplasmic. This result shows that import of Kap- $\alpha$  into the nucleus is inhibited in energy-depleted cells.

## Discussion

In this study, we examined the mechanism of how cellular ATP depletion inhibits Ran-dependent nuclear transport. First we found that ATP depletion with 2-deoxyglucose/azide inhibited both Ran-dependent import of cNLS cargo and Ran-dependent export of leucine-rich NES cargo but



that GTP depletion with ribavirin inhibited neither (Fig. 1). 2-deoxyglucose/azide (unlike ribavirin) treatment also caused an accumulation of Kap- $\alpha$  inside the nucleus (Fig. 2). Measurement of the total nucleotide levels revealed that after 2-deoxyglucose/azide treatment, the total ATP content decreased to 40% of control, whereas the GTP content only decreased to 72% of control (Table I). In contrast, after ribavirin treatment, the total ATP concentration was unchanged (101% of control), but the total GTP content decreased to 36% of control. Because ribavirin-treated cells remain transport competent whereas 2-deoxyglucose/azide-treated cells do not, these nucleotide measurements did not support our original hypothesis that it is a drop in total GTP levels upon ATP depletion that inhibits transport.

In further experiments, however, we found that 2-deoxyglucose/azide treatment causes the amount of RanGTP available for binding to a GST-RanBD construct to drop to undetectable levels (Fig. 4). We further found that this decrease in RanGTP precedes the nuclear accumulation of Kap- $\alpha$  (Fig. 5). Finally, we found that 2-deoxyglucose/azide treatment resulted in the level of free (unlike total) GTP to drop to undetectable levels. Ribavirin treatment caused a decrease in free GTP to 32% of control (Table II), but this value is substantially higher than the free GTP concentration in 2-deoxyglucose/azide-treated cells. Treatment with ribavirin for longer than 2 h was not tested, but because we did detect a decrease in free RanGTP after 2 h of ribavirin treatment (Fig. 4), it is possible that longer treatment times might eventually cause the RanGTP concentration to drop to a point where Ran-dependent nuclear transport is inhibited.

Because of these results, we believe it likely that the inhibition of Ran-dependent nuclear transport seen with ATP depletion in fact results from a loss of free GTP needed to produce RanGTP, rather than by an ATP-dependent step in nuclear transport. Because we also found that free ATP also drops to undetectable levels upon 2-deoxyglucose/azide treatment, it remains formally possible that free ATP (in addition to free GTP) is also required for these transport pathways *in vivo*. However, because all of the previously published *in vitro* data is completely consistent with an essential requirement for GTP to make RanGTP, and shows no detectable requirement for ATP or ADP, we favor the interpretation that it is the loss of free GTP rather than free ATP upon 2-deoxyglucose/azide treatment that is the critical event (Schwoebel et al., 1998; Englmeier et al., 1999).

In addition to the experiments shown, we did a number of experiments to rule out other explanations of why 2-deoxyglucose/azide inhibits Ran-dependent nuclear transport. First, we considered the possibility that sodium azide was having a direct effect on transport rather than inhibiting it by lowering nucleotide levels. Azide inhibits oxidative phosphorylation by binding to cytochrome oxidase in the mitochondria, however azide also binds and inhibits other metal-containing proteins such as HRP (Holzwarth et al., 1988). We found, however, that the direct addition of sodium azide at millimolar concentrations to digitonin-permeabilized cells did not have the slightest effect on either the *in vitro* import of TRITC-NLS-BSA or the *in vitro* export of TRITC-NES-BSA (unpublished data). Furthermore, we found that substitution of oligomycin, another

inhibitor of oxidative phosphorylation that works by a different mechanism, gave identical results to sodium azide in terms of Kap- $\alpha$  nuclear buildup (unpublished data). We also found that cytoplasmic microinjection of apyrase, an enzyme that cleaves any tri- or diphosphate nucleotide to its monophosphate derivative, resulted in identical nuclear accumulation of Kap- $\alpha$  (unpublished data).

We considered the possibility that ATP depletion might be inhibiting some ATP-dependent posttranslational modification of a protein involved in nuclear transport, such as an NPC protein or one of the transport factors. With regard to this, we found that neither the RanGEF or RanGAP activities in lysates from 2-deoxyglucose/azide-treated cells were altered when compared with extracts from control or ribavirin-treated cells (unpublished data). It is also possible that the export of large ribonucleoprotein complexes might require an additional energy source, unlike the export of smaller substrates, to power an ATP-dependent helicase for example (Gatfield et al., 2001). We reasoned that the absence of ATP might “jam” the NPC with partially translocated RNPs, preventing the movement of smaller transport complexes. We found, however, that inhibition of RNA synthesis with actinomycin D for several hours before 2-deoxyglucose/azide treatment had no effect on the subsequent nuclear accumulation of Kap- $\alpha$  (unpublished data). We also found that with regard to potential jamming of the NPC, 2-deoxyglucose/azide treatment did not prevent the diffusion of a 25-kD protein through the NPC (unpublished data).

In summary, our results support the conclusion that inhibition of Ran-dependent transport by ATP depletion does not result from inhibition of an ATP-dependent step in these transport pathways. It should be noted that the results reported here do not rule out the possibility that other transport pathways, or the Kap- $\beta$ 1 and Crm1 pathways when transporting much larger cargo than those tested here, might require additional energy sources to facilitate movement across the NPC. However, we believe the inhibition of the pathways studied here directly results from a lack of RanGTP and this lack is caused by a shortage of free GTP needed for the loading of nuclear RanGDP. It is striking that the level of free GTP becomes undetectable after 2-deoxyglucose/azide treatment when the pool of total GTP is still 72% of normal (Table II). One reason for this rapid loss of free GTP seen upon ATP depletion is probably the requirement for ATP in the sequential phosphorylation of GMP to form GDP and GTP (Zalkin and Dixon, 1992). Thus, an absence of ATP should completely inhibit the *de novo* synthesis of GTP. Another key player likely to be involved in regulating relative NTP levels is nucleoside diphosphokinase, which transfers the terminal phosphate from any NTP to the terminus of any NDP (Veron et al., 1994). This could have the effect of depleting GTP pools in parallel with ATP pools in cells under 2-deoxyglucose/azide treatment. Contributing to the rapid loss of free GTP upon ATP depletion is probably the presence of many cellular ATP-using proteins that can also use GTP, e.g., hexokinase (Ferguson et al., 1986). Under normal conditions, these proteins use the most available NTP, which is ATP, but in the absence of free ATP, they can use GTP instead, which would rapidly deplete this pool as well.

If the nuclear accumulation of Kap- $\alpha$  upon energy depletion is caused by a lack of RanGTP, this would have to mean that its CAS-mediated export is more sensitive to RanGTP depletion than its Kap- $\beta$ 1-mediated import. Evidence that Kap- $\alpha$  export is in fact more sensitive to RanGTP depletion than cNLS import was shown by microinjecting radiolabeled proteins or RNAs into *Xenopus* oocytes followed by hand dissection and observing their transport under conditions where nuclear RanGTP is depleted (Izaurralde et al., 1997). This study found that the nuclear export of Kap- $\alpha$  is almost totally inhibited after nuclear injection of 10  $\mu$ M RanGAP (Rna1), but that the import of a cNLS-containing cargo (CBP80) is only slightly reduced by this GAP concentration and requires the nuclear injection of 40  $\mu$ M RanGAP for total inhibition. Thus, these findings support our conclusion that it is the loss of RanGTP that inhibits Ran-dependent nuclear import and export upon ATP depletion rather than the inhibition of some ATP-dependent event.

## Materials and methods

### Materials

Ribavirin was from Sigma-Aldrich. The following antibodies were used: anti-Ran monoclonal antibody from Transduction Laboratories, anti-Ran GAP monoclonal antibody 19C7 ascites fluid (Matunis et al., 1996) donated by M. Matunis (Johns Hopkins University, Baltimore, MD), anti-Kap- $\alpha$  (pendulin) antisera donated by M. Waterman (University of California, Irvine, CA), anti-Kap- $\beta$ 1 monoclonal antibody (Chi et al., 1995) donated by S. Adams (Northwestern University Medical School, Chicago, IL), and anti-RCC1 rabbit antibody (Schwoebel et al., 1998). Rhodamine-conjugated secondary antibodies against mouse and rabbit IgG were from Jackson ImmunoResearch Laboratories. TRITC-NLS-BSA was prepared as previously described (Moore and Blobel, 1992). TRITC-NES-BSA was prepared in the same manner, substituting peptides (CLPPLRLTL) containing the Rev NES (in bold) (Fischer et al., 1995; Wen et al., 1995). Fluorescein and Cascade blue-labeled 70-kD dextrans were from Molecular Probes. FITC-Kap- $\alpha$  was prepared by coupling His-Kap- $\alpha$ 2 (Schwoebel et al., 1998) with a 1:2 molar ratio of fluorescein-5-isothiocyanate (Molecular Probes) according to the manufacturer's instructions. After coupling, FITC-Kap- $\alpha$  was passed over a Nap-5 column equilibrated in 20 mM Hepes-KOH, pH 7.3, 100 mM potassium acetate, 2 mM DTT, snap frozen, and stored at  $-80^{\circ}\text{C}$  in single use aliquots. We found that labeling did not affect the ability of Kap- $\alpha$  to support cNLS-mediated import in permeabilized cells (unpublished data).

### Cell culture and treatments

For energy depletion studies, HeLa cells were washed with PBS, and incubated in glucose-free DME (catalog no. 11966-025; GIBCO BRL) containing penicillin (100 U/ml), streptomycin sulfate (100  $\mu$ g/ml), Hepes (10 mM, pH 7.3), and 10% FCS (hereafter referred to as  $\text{gluc}^-$  media). Where indicated, cells were incubated in  $\text{gluc}^-$  media containing either 10 mM sodium azide and 6 mM 2-deoxy-D-glucose, or 100  $\mu$ M ribavirin for the times indicated in the figure legends. For microinjection, coverslips were rinsed once in PBS, once in  $\text{gluc}^-$  media, and incubated with  $\text{gluc}^-$  media containing 10 mM sodium azide and 6 mM 2-deoxyglucose for 20 min or  $\text{gluc}^-$  media containing 100  $\mu$ M ribavirin for 90 min at  $37^{\circ}\text{C}$  before microinjection. Microinjection was for  $\sim$ 10 min at room temperature (RT) in treatment media, and microinjected cells were then incubated for an additional 30 min at  $37^{\circ}\text{C}$  before fixation.

### Immunofluorescence microscopy

Cells after treatment were placed on ice, washed with ice cold PBS, fixed for 20 min on ice in 3% formaldehyde (diluted from a methanol-free stock) in PBS, rinsed twice with PBS, and permeabilized with 0.1% Triton in PBS for 20 min on ice. Cells were blocked with 10% donkey serum in PBS for 30 min at  $37^{\circ}\text{C}$ , washed two times with PBS, and incubated with primary antibody diluted in blocking serum for 1 h at RT. After washing, cells were incubated with a labeled second antibody in blocking solution for 1 h at RT. After washing, coverslips were mounted and observed as previously described (Schwoebel et al., 1998).

### Nucleotide isolation and quantitation

**Total nucleotide.** After treatment, cells were washed with PBS, trypsinized, and the cells were resuspended in ice cold PBS and centrifuged for 5 min at 200 *g*,  $4^{\circ}\text{C}$ . Cells were resuspended in cold PBS, counted, centrifuged for 5 min at 200 *g*,  $4^{\circ}\text{C}$ , and resuspended in 0.7 M HClO<sub>4</sub> (100  $\mu$ l/10<sup>6</sup> cells). Cells were vortexed for 30 s, centrifuged at 3,000 *g* for 20 min at  $4^{\circ}\text{C}$ , and the supernatants were neutralized with solid KHCO<sub>3</sub> (10 mg/10<sup>6</sup> cells) (Kremmer et al., 1989). The precipitates were removed by microcentrifugation twice for 10 min at  $4^{\circ}\text{C}$  each time, and the supernatants were aliquoted and snap frozen in liquid N<sub>2</sub>. Aliquots of these supernatants (each representing 6.5  $\times$  10<sup>5</sup> cells) were used for the measurement of nucleotides. Nucleotides were separated by FPLC and individual nucleotide levels were quantitated by measurement of peak heights (Abs UV<sub>254</sub>) and comparison to standard curves. GTP and ATP were separated on a Mono-Q column with a gradient of 2–55% B (A = H<sub>2</sub>O; B = 1 M ammonium phosphate, pH 7.0; Kremmer et al., 1989). With this protocol, ATP elutes at 48.5% B, and GTP at 52% B. GDP and ADP were separated on a Source 15Q column with a gradient of 0–60% B (A = 7 mM KH<sub>2</sub>PO<sub>4</sub>, pH 4.5; B = 500 mM KH<sub>2</sub>PO<sub>4</sub>, 100 mM KCl, pH 5.0; Hartwick and Brown, 1975). With this protocol, GDP elutes at 23.5% B, and ADP elutes at 26% B.

**Free nucleotide.** After treatment, cells were washed with cold PBS and scraped in cold buffer A (20 mM Hepes, pH 7.3, 120 mM NaCl, 2 mM magnesium acetate, 1 mM DTT). The scrapes from three 100-mm culture dishes were pooled and sonicated. An aliquot of the lysate was snap frozen in liquid nitrogen and saved for Bradford analysis. The cell number was calculated from the protein values compared with the previously determined protein content of a known number of HeLa cells. The remaining lysate was centrifuged in a Microcon centrifugal filter device with a 10,000 mol wt cutoff (Millipore) at 12,000 *g* for 10 min at  $4^{\circ}\text{C}$ . The resulting filtrate was snap frozen in liquid nitrogen. The GTP and ATP content of the filtrate was measured by FPLC as described above.

### NEM treatment and measurement of free RanGTP

**Preparation of extracts and initial NEM experiments.** All steps including incubation with NEM were performed on ice in a cold room using ice cold solutions unless otherwise stated. HeLa cells were washed once with PBS, and then the cells were scraped using a rubber policeman into buffer A containing 0.1% Triton X-100. The lysate was vortexed for 15 s and NEM at different concentrations was added to aliquots of the lysate. Aliquots were removed at different time points and a twofold molar excess of DTT over the NEM in that particular sample was added to quench the NEM activity.

**RanGAP and RanGEF assays.** Before assay, each NEM-treated lysate (quenched with DTT) to be assayed for GAP or GEF activity was gel filtered on a Nap-5 column equilibrated in 20 mM Hepes, pH 7.3, 120 mM NaCl, 5 mM magnesium acetate, 0.05% Tween, and 5 mM DTT. Human recombinant untagged Ran (3  $\mu$ g) was loaded with radiolabeled nucleotide for 2 h on ice in 20 mM Hepes, pH 7.3, 120 mM potassium acetate, 1 mM DTT, 10 mM EDTA, 1 mg/ml BSA, and either 5.5 pmol of 9,000 Ci/mmol [ $\gamma$ -<sup>32</sup>P]GTP (NEN Life Science Products) or 3.3 pmol of [ $\alpha$ -<sup>32</sup>P]GDP prepared as previously described (Schwoebel et al., 1998). The loading reaction was quenched by the addition of MgCl<sub>2</sub> to 40 mM. The loaded Ran was microfuged for 10 min at  $4^{\circ}\text{C}$  and exchanged into buffer B (20 mM Hepes, pH 7.3, 120 mM potassium acetate, 5 mM MgCl<sub>2</sub>, 1 mM DTT) by passage over a NAP-5 desalting column. For the GAP assay, 40,000 CPM of GTP loaded Ran was added to each sample, vortexed, and placed at  $30^{\circ}\text{C}$ . The reaction was stopped after 15 min by the addition of 5 ml ice cold buffer B. The sample was vacuum filtered onto 0.45- $\mu$ m nitrocellulose filters (Schleicher & Schuell), and the filters were washed with 12 ml cold buffer B. The filters were air dried, placed in scintillation fluid, and counted. The GEF assay was performed in the same manner using 20,000 CPM of [ $\alpha$ -<sup>32</sup>P] GDP-loaded Ran per sample.

**RanBD1 pulldowns of Ran[ $\alpha$ -<sup>32</sup>P]GTP and Ran[ $\gamma$ -<sup>32</sup>P]GTP.** Human recombinant untagged Ran (3  $\mu$ g) was loaded with radiolabeled nucleotide for 2 h on ice in 20 mM Hepes, pH 7.3, 120 mM potassium acetate, 1 mM DTT, 10 mM EDTA, 1 mg/ml BSA, and either 34 pmol of 9,000 Ci/mmol [ $\gamma$ -<sup>32</sup>P]GTP or 38.8 pmol of 3,000 Ci/mmol [ $\alpha$ -<sup>32</sup>P]GTP (both from NEN Life Science Products). The loading reaction was quenched by the addition of MgCl<sub>2</sub> to 40 mM, and the loaded Ran was exchanged into buffer B by passage over a NAP-5 column. HeLa cells grown to 70% confluence in 100-mm dishes were placed on ice in the cold room, washed in PBS, drained, and scraped in buffer B containing 0.1% Tween-20, 2.8 mM GDP- $\beta$ S, and 140 mM NEM (except mock-treated samples, which contained 280 mM DTT). Aliquots of the radiolabeled Ran were added immediately to these cell lysates. In some samples, BSA (10 mg/ml in buffer B) was used in place of cellular extract. After dilution, the final concentrations were 100 mM NEM and 2 mM nucleotide. The samples were incubated on ice for 20 min, vortexed, and centrifuged at 13,000 *g* for 10 min at  $4^{\circ}\text{C}$ . The superna-

tants were brought to 200 mM DTT (from a 2 M stock) and applied to a NAP-5 column equilibrated in buffer A. GST–RanBD or GST was added to the NAP-5 eluates and the samples were rotated end over end for 30 min at 4°C. Glutathione-agarose beads were added and the incubation continued for an additional 30 min at 4°C. Approximately 7 µg of GST–RanBD or GST and 40 µl of a 20% slurry of glutathione-agarose beads were added per milligram of cellular extract. After incubation, the beads were sedimented by centrifugation at 1,000 g for 3 min at 4°C. The supernatants were discarded and the pellets washed four times in buffer A. Beads were resuspended in SDS-PAGE sample buffer and aliquots of the resulting supernatant were placed in scintillation fluid and counted.

**RanBD1 pulldowns of RanGTP from treated cells.** After receiving the indicated treatment, HeLa cells grown to 70% confluence in 100-mM dishes were placed on ice in the cold room, washed in PBS, drained, and scraped in buffer B containing 0.1% Tween-20, 2.8 mM nucleotide (GDP-βS except where indicated), and 140 mM NEM (except mock-treated samples, which contained 280 mM DTT). After dilution, the final concentrations were 100 mM NEM and 2 mM nucleotide. After incubation for 20 min on ice, the samples were quenched with DTT and processed for isolation of free RanGTP with the GST–RanBD and glutathione-agarose as described above. Beads were resuspended in SDS-PAGE sample buffer, and after SDS-PAGE, the samples were transferred to nitrocellulose and immunoblotted with an anti-Ran antibody.

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