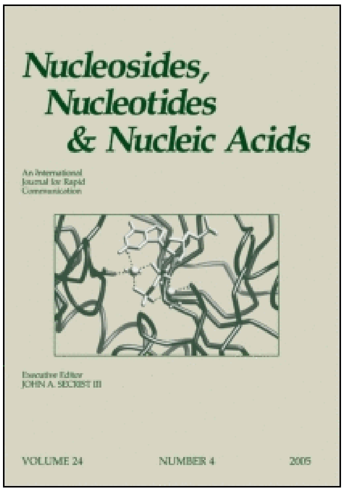

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Trimethylguanosine Nucleoside Inhibits Cross-Linking Between Snurportin 1 and m_3G-CAPPED U1 snRNA

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TRIMETHYLGUANOSINE NUCLEOSIDE INHIBITS CROSS-LINKING BETWEEN SNURPORTIN 1 AND m_3G-CAPPED U1 snRNA

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□ *Macromolecular nuclear import is an energy- and signal-dependent process. The best characterized type of nuclear import consists of proteins carrying the classical NLS that is mediated by the heterodimeric receptor importin α/β. Spliceosomal snRNPs U1, U2, U4, and U5 nuclear import depend both on the 5' terminal m_3G (trimethylguanosine) cap structure of the U snRNA and the Sm core domain. Snurportin 1 recognizes the m_3G-cap structure of m_3G-capped U snRNPs. In this report, we show how a synthesized trimethylguanosine nucleoside affects the binding of Snurportin 1 to m_3G-capped U1 snRNA in a UV-cross-linking assay. The data indicated that TMG nucleoside is an essential component required in the recognition by Snurportin 1, thus suggesting that interaction of Snurportin 1 with U1 snRNA is not strictly dependent on the presence of the whole cap structure, but rather on the presence of the TMG nucleoside structure. These results indicate that the free nucleoside TMG could be a candidate to be an inhibitor of the interaction between Snurportin 1 and U snRNAs. We also show the behavior of free TMG nucleoside in* in vitro *U snRNPs nuclear import.*

Keywords Trimethylguanosine Nucleoside; TMG; m_3GpppG Cap; Snurportin 1; Nuclear Import; U snRNPs; UV Cross-Linking Assay

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INTRODUCTION

The selective transport of proteins and ribonucleoproteins between the nucleus and the cytoplasm of eukaryotic cells is an important activity that integrates the functions of protein synthesis, RNA genesis, and DNA replication. Both protein and nucleoprotein complexes are actively transported through the nuclear pore complex (NPC), embedded in the nuclear envelope, a process characterized by energy and signal dependence, thus indicating that it is mediated by carriers (reviewed in Gorlich and Mattaj[1]).

A major breakthrough in the study of nuclear import was the development of effective *in vitro* systems.[2] Macromolecular import has been well established for proteins that carry a short basic nuclear localization signal (NLS). In a first step, the NLS is recognized by a soluble heterodimeric receptor known as the importin α/β heterodimer. The importin α subunit (60 kDa) functions as an anchor, termed as adapter, between NLS peptide and importin β; importin α binds to the NLS protein; at the same time, importin α binds to the IBB domain of importin β (97 kDa). The NLS-receptor complex is then targeted to the NPC via importin β. This complex is subsequently translocated through the NPC by an energy-dependent mechanism with the help of Ran-GTPase and NTF2.

A second class of NLS is the M9 domain, which is present in a heterogeneous-nuclear hnRNP A1. The M9 domain is enriched in glycine and aromatic residues and can bind directly to its receptor transportin; thus, in this protein import pathway there is no requirement of an adapter (importin α-like) for the binding.[3] Other import signals, like the classical NLS, have been identified. They are composed mainly of basic amino acids and were found in ribosome proteins and histone H1.[4,5] In addition, the U1A and U2B″ import does not follow the NLS model and requires a new mediator of nuclear protein import.[6]

A third class of import signal was identified in U snRNPs (uridine-rich small nuclear ribonucleoproteins). The U snRNPs are essential components of the splicing machinery assembled in a complex sequence of events in both the nuclear and the cytoplasmic compartments (see reviews in Will and Luhrmann[7,8]). The trimethylguanosine (m_3G) cap is the hallmark of the U snRNAs (U small nuclear RNAs family).[9] Major U snRNPs U1, U2, U4/U6.U5 are essential spliceosomal factors, they are complexes of U snRNAs and a very characteristic set of common and specific polypeptides.[10] The common polypeptides (B′, B, D1, D2, D3, E, F, and G) form a common structure present in all the spliceosomal U snRNPs, the so-called Sm core. This event requires the survival of motor neuron (SMN) protein complex's activity.[8,11–13] With the sole exception of U6 snRNA, which is capped with MeGTP,[14] all the major U snRNAs contain the m_3G cap in human cells. Shortly after polymerase II transcription, U snRNAs are capped with a 7-methylguanosine (m^7G) structure.[15] The newly transcribed U snRNAs are exported to the cytoplasm by binding to an adaptor protein called PHAX.[16] The stable formation of Sm core in the cytoplasm promotes the hypermethylation of the m^7G cap to a 2,2,7-trimethylguanosine-cap (m_3G).[17] Both the Sm binding site domain and the m_3G cap constitute the bipartite nuclear localization signal that targets the U snRNPs to the nucleus.[18,19]

Snurportin 1, a 45-kDa protein, mediates nuclear import of m_3G-capped U snRNPs.[20] Snurportin 1 is thus an adaptor that recognizes the m_3G-cap structure and links U snRNPs to importin β for subsequent transport to the nucleus.[20,21] Once in the nucleus, Snurportin 1 returns to the cytoplasm after each round of import. The re-export of Snurportin 1 is mediated by CRM1.[22] The import of U snRNPs does not need importin α.[23] Furthermore, the depletion of importin α stimulates the nuclear import of U snRNPs, indicating competition between U snRNP nuclear import factors and importin α.[23]

Previously, our group has synthesized high amounts of non-commercial TMG (trimethylguanosine) nucleoside[24] by using a modified synthetic procedure described for nucleosides conjugates.[25] Moreover, we showed that the synthesized TMG product is immunologically identical to the m_3G cap of the U snRNPs. These experiments used antibodies raised against TMG-KLH conjugates. Anti-TMG antibodies precipitated the U snRNAs and this pull-down could be competed with free TMG.[24] Here, we show how TMG nucleoside affects the binding of Snurportin 1 to m_3G-capped U1 snRNA in a new UV-cross-linking assay created based on previous procedures.[20,26] We next attempted to test the effect caused by the addition of TMG nucleoside in an *in vitro* U snRNPs import using Hela digitonin permeabilized cells. Surprisingly, TMG not only did not block U snRNPs import, but in fact stimulated it, when added in concentrations lower than 0.5 mM. When 1 and 2 mM of free TMG were added, no U snRNPs import stimulation was observed. We discuss the aspects involved in the behavior of free TMG in nuclear import and cross-linking assays.

RESULTS AND DISCUSSION

Snuportin1 Binds Only to m_3G Cap in the New Cross-Linking Assay

The purified his-tagged Snurportin 1 has an apparent molecular weight of 47 kDa (Figure 1, lane 1) in 15% SDS-PAGE gel. In this gel, we could observe that Snurportin 1 has been well expressed and purified through nickel-NTA agarose and was suitable for using in further experiments. We have created a new UV-cross-linking assay based on previous publications[20,26] and tested its efficiency in the binding of Snurportin 1 to m^7G and m_3G caps. U1 snRNA was transcribed using radioactive label and capped

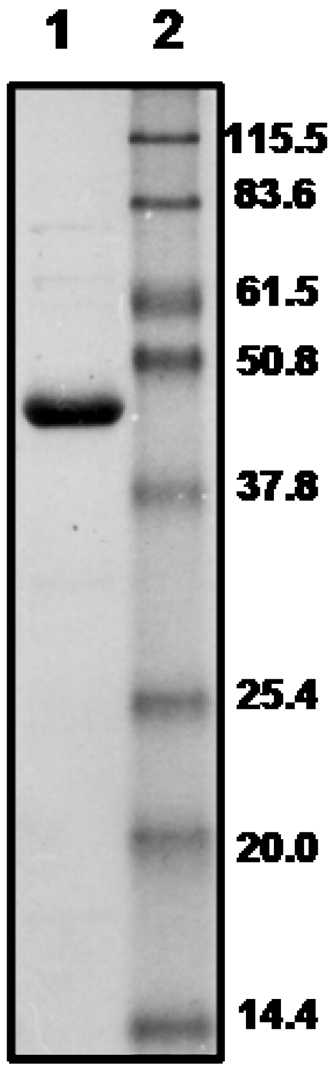

FIGURE 1 Expression and purification of recombinant Snurportin 1. Snurportin 1 was expressed in pQE30 (Qiagen) as his-tagged recombinant protein and purified through nickel-NTA agarose. Snurportin 1 was submitted to 15% SDS-PAGE gel in order to check its integrity and purity. Five micrograms of purified recombinant Snurportin 1 (lane 1); protein molecular weight markers (lane 2). The numbers on the right indicate the molecular weights in kDa. The gel was stained with Coomasie.

with m^7G and m_3G nucleotides and was then mixed with recombinant Snurportin 1.

After UV irradiation and RNAse 1 treatment, the samples were subjected to SDS-PAGE and the protein was visualized by autoradiography. Snurportin 1 clearly bound to the m_3G cap as indicated by the signal observed (Figure 2, lane 2); however, Snurportin 1 did not bind to m^7G cap (Figure 2, lane 1), thereby indicating the efficiency of our optimized assay. Furthermore, we

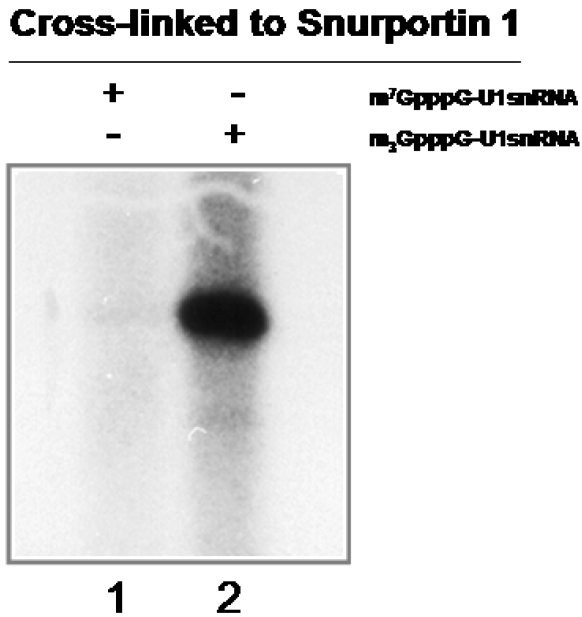

FIGURE 2 UV cross-linking assay. U1 snRNA was transcribed with both m^7GpppG and m_3GpppG caps in an assay with only ^{32}UTP (no cold UTP). To the cross-linking assay, 1.5 μg of Snurportin 1 was incubated with radio-labeled U1 snRNAs on ice in a total volume of 10 μL for 15 min. Both mixtures were irradiated with 254 nm UV Sylvania G8T5 for 5 min at a distance of 2 cm. The cross-linked Snurportin 1 was analyzed on a 15% SDS-PAGE gel. Snurportin 1 cross-linked to m^7GpppG-U1 snRNA (lane 1) and to m_3GpppG-U1 snRNA (lane 2).

tested whether the nucleoside synthesized here, i.e. the TMG nucleoside, is able to compete with the binding of Snurportin 1 to the m_3GpppG-capped U1 snRNA, as the free m_3GpppG cap does.

Trimethylguanosine Nucleoside (TMG) Inhibits Cross-Linking between Snurportin 1 and m_3G-Capped U1 snRNA

Free m_3GpppG cap can compete with the binding of Snurportin 1 to m_3GpppG-capped U1 snRNA. In Figures 3a and b, we observe the competition with 1.0 (Figure 3a, lane 2), 0.1 (Figure 3b, lane 2) and 0.01 mM (Figure 3b, lane 3) of m_3GpppG cap. By comparing the disappearance of Snurportin 1 cross-linked bands (Figure 3a, lane 2; Figure 3b lane 2) with the cross-linked snurportin 1 band (Figures 3a and b, lane 1) we could conclude that 1 and 0.1 mM m_3GpppG cap produced a total inhibition of binding. These data verify that the binding of Snurportin 1 to m_3GpppG cap, in the cross-linking assay as created here, has the same specificity of m_3G cap oligo (m_3GpppAmpUmpA) observed previously.[20] TMG was synthesized from $m_{2,2}$G[27] by methylation of position 7 of the ring with dimethyl sulphate.[24] Surprisingly, free TMG nucleoside was also able to

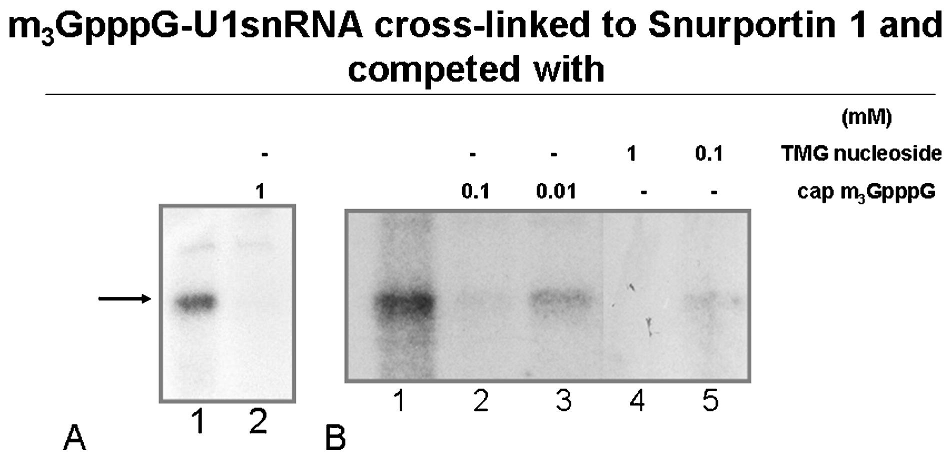

FIGURE 3 Snurportin 1 binding to m3GpppG cap is inhibited in the presence of free TMG nucleoside. U1 snRNA was transcribed with m3GpppG cap in an assay without UTP cold. To the cross-linking assay, 1.5 μg of Snurportin 1 was incubated with radio-labeled U1 snRNAs on ice in a total volume of 10 μL for 15 min. Both mixtures were irradiated with 254 nm UV Sylvania G8T5 for 5 min at a distance of 2 cm. In competition assays, Snurportin 1 was mixed for 30 min on ice with either m3GpppG cap or free TMG nucleoside prior to UV irradiation. The cross-linked Snurportin 1 was analyzed on a 12% SDS-PAGE gel. (A) Snurportin 1 cross-linked to m3GpppG-U1snRNA (lane 1, see arrow) competed with 1 mM m3GpppG cap (lane 2). (B) Snurportin 1 cross-linked to m3GpppG-U1snRNA (lane 1) competed with 0.1 (lane 2) and 0.01 (lane 3) mM m3GpppG cap, or with 1 (lane 4) and 0.1 (lane 5) mM TMG nucleoside.

inhibit the binding between Snurportin 1 and m3G cap in UV-cross-linking assays when used in 1 and 0.1 mM as a competitor (Figure 3, lanes 4 and 5), although it could be anticipated from the crystal structure of 2,2,7-trimethyl-GpppG bound to Snurportin 1 (cap-binding fragment) published very recently.[28] In the present assay, only in the presence of 1 mM TMG a total inhibition of binding was obtained. In fact, TMG nucleoside has a decreased strength of interaction with Snurportin 1 of approximately one order of magnitude. This effect may be due to the presence of the whole nucleotide structure of m3GpppG.

We also observed that $m_{2,2}$G (2,2-dimethylguanosine) nucleoside was unable to inhibit the UV-cross-linking between Snurportin 1 and m3GpppG-capped U1 snRNA (not shown), confirming that methylation in position 7 of the guanosine ring is essential in this respect.[20,24] Therefore, these data strongly indicate that TMG nucleoside is an essential component required in the recognition by Snurportin 1, thus suggesting that interaction of Snurportin 1 with U1 snRNA is not strictly dependent on the presence of whole cap structure, but rather on the presence of TMG nucleoside structure. However, whether artificial constructs made with TMG nucleoside can exhibit the same binding avidity with native Snurportin 1 is still an open question. Moreover, the next step will be to characterize the binding properties of TMG nucleoside in *in vitro* nuclear import assays.

U snRNP Import Is Stimulated by Trimethylguanosine Nucleoside (TMG) in Concentrations under 0.5 mM

It is known that U snRNP import is effectively blocked by trimethylguanosine (m_3G) cap analog $m^{2,2,7}$GTP but it is not affected by the monomethylguanosine cap analog m^7GTP.[23] Both effects may be seen in Figure 4, Panels B, C, and D, respectively. The nuclear fluorescence seen in Figure 4, Panel A, due to nuclear import of fluorescent U snRNPs, has decreased in Panel B, by adding cap m_3GpppG. On the other hand, Panels C and D the show that nuclear fluorescence is the same as control (Panel A), thus demonstrating that the adding of m^7GpppG cap has not affected the import rate of fluorescent U snRNPs into the nucleus.

In this direction we tested whether our compound, the free TMG, could alter an *in vitro* U snRNPs import. We verified that at concentrations higher than 0.5 mM there is U snRNP import inhibition, mimicking the effect observed in the presence of m_3GpppG cap analog but, surprisingly, under this concentration we observed a stimulation of U snRNPs import.

Figure 4 also shows an U snRNPs *in vitro* import assay in the presence of free TMG. Surprisingly, TMG not only did not block U snRNPs import, but in fact stimulated it (Figure 4, compare F with control E) when used with the molarity of 0.5 mM. Nevertheless, when 1 and 2 mM free TMG were added (Figure 4, G and H, respectively) no U snRNPs import stimulation was observed. In conclusion, when TMG is added in low concentrations (under 0.5 mM, not shown), it stimulates U snRNPs nuclear import. These experiments have been repeated several times, including various TMG concentrations, both the stimulation and inhibitory effects observed were the same.

U snRNP Import Is Blocked by Trimethylguanosine Nucleoside (TMG) in Concentrations of 1 mM

A fluorescent BSA conjugated with a peptide containing the reverse NLS sequence ("SLN") was also used to check the veracity of the effect caused by free TMG in a U snRNPs nuclear import assay. As the SLN signal is not recognized by the protein import pathway, it was used to discard the possibility of a passive diffusion of the conjugated. When TMG is used at a final concentration of 2 mM it seems to be toxic to the cells as shown when it was assayed with BSA-SLN, leading to a passive diffusion of fluorescent conjugate into the nucleus (Figure 5, panel D). This effect was monitored by fluorescence in the nucleus (Figure 5, panel D compared with the control A). BSA-SLN in *in vitro* import assays either alone (Figure 5, panel A) or with the addition of 1 mM (Figure 5, panel C) or 0.5 mM (Figure 5, panel B) of TMG nucleoside did not lead to increased nuclear fluorescence. A slight fluorescence can be seen around the nuclear membrane. These experiments

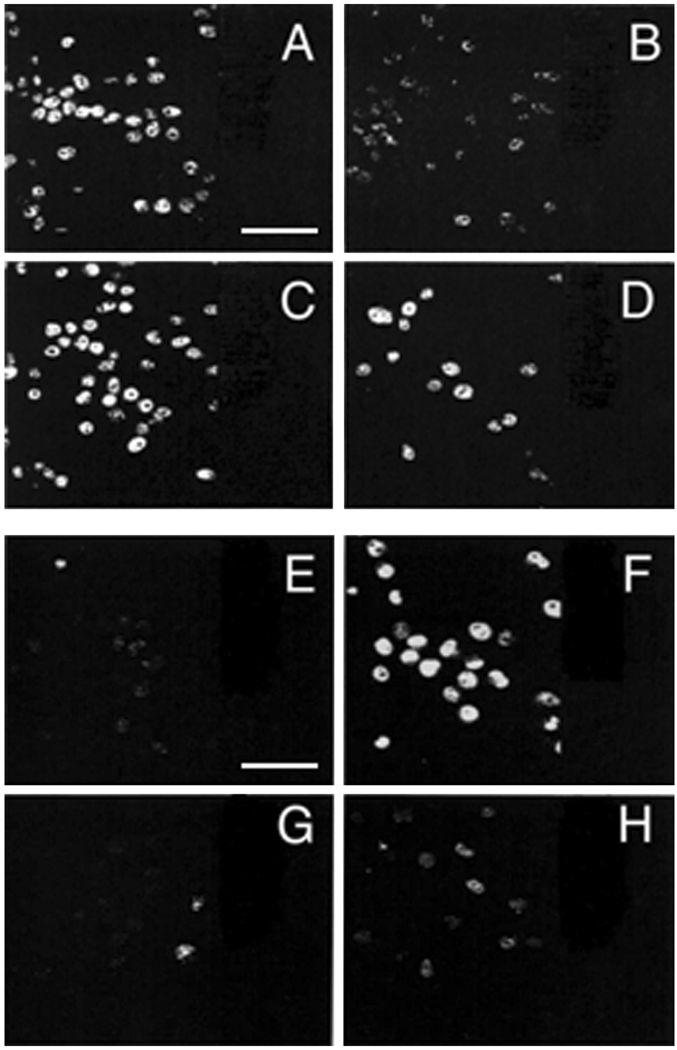

FIGURE 4 U snRNPs import *in vitro* assay in the presence of TMG nucleoside. Digitonin-permeabilized HeLa cells were incubated with purified and fluorescently labeled U snRNPs for 90 min, at room temperature, in the presence of *Xenopus* egg extracts importin α depleted, nucleoplasmin core (NPc), GTP, ATP, and an ATP regeneration system (see Material and Methods). (A) and (E) U snRNPs import reaction control in the absence of free nucleosides or nucleotides; (B) U snRNPs import in the presence of 2 mM m_3GpppG cap; (C) and (D) U snRNPs import in the presence of 2 mM m^7GpppG; (F)–(H) U snRNPs import in the presence of 0.5, 1, and 2 mM of TMG nucleoside, respectively. Magnification bars represent 20 μm. ABCD and EFGH represent different assays.

confirm that both the stimulation of import of U snRNPs in the presence of 0.5 mM TMG free nucleoside, and the blocking of U snRNPs import in the presence of 1 mM TMG is due to an active import of the karyophile.

TMG has an unstable imidazole ring, which is a property of this product, and the imidazole degradation does not depend on the product's synthesis

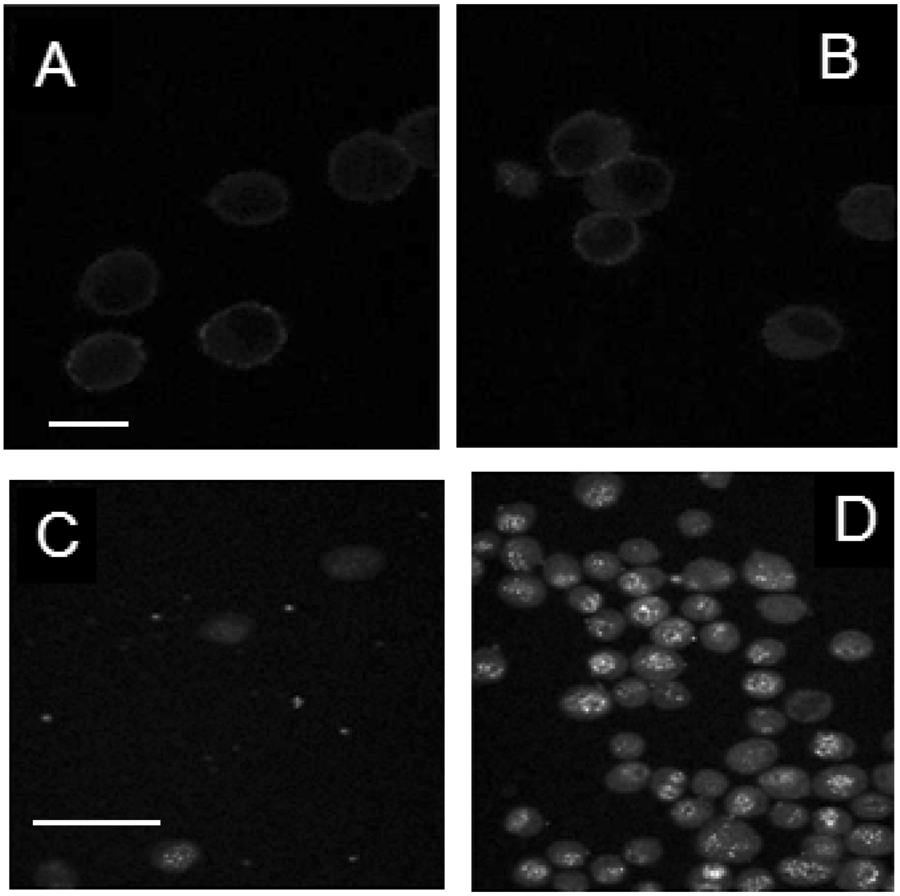

FIGURE 5 BSA-SLN import *in vitro* assay in the presence of TMG nucleoside. Digitonin-permeabilized HeLa cells were incubated with fluorescently labeled BSA-SLN for 10 min, at room temperature, in the presence of *Xenopus* egg extracts, nucleoplasmin core (NPc), GTP, ATP, and an ATP regeneration system (see Material and Methods). (A) BSA-SLN import in the absence of TMG (control); (B)–(D) BSA-SLN Import in the presence 0.5, 1, and 2 mM of free TMG, respectively. Magnifications bars for (A) and (B) represent 10 μm; for (C) and (D), 20 μm.

procedure. The degradation of the imidazole ring is due to two different reactions: depurination, a reaction catalyzed by acids, and the opening of the imidazole ring, by susceptibility to nucleophilic attack in basic pH. Both reactions are favored by the methylation at position 7. Some amount of the products obtained from the imidazole degradation may be present in our preparations; they could be toxic to the nuclear membrane, and the stimulation effect could be simply due to a passive diffusion through broken nuclear membranes. The results observed in the presence of 2 mM TMG indicate that the imidazole degradation is affecting the nuclear membrane, thus leading to a passive nuclear diffusion of BSA-SLN. Nevertheless, the results observed in the presence of 0.5 and 1 mM TMG compared with the negative control BSA-SLN indicated that the minor imidazole degradation products were not affecting the membrane at the concentration range; thus, BSA-SLN is not obtained in the nucleus by passive diffusion in presence of several concentrations of TMG nucleoside. The inhibition effect indicates that TMG at concentrations higher than 0.5 mM really mimics the U snRNPs cap and inhibits the active transport. The stimulation effect under 0.5 mM is so far not easily explained; we can suggest that TMG would have some effect on the molecular attraction and concentration of other factors, including those involved in import, a role already suggested for other protein–protein interaction systems. [29–33]

Future experiments will undoubtedly help to elucidate the roles of TMG nucleoside in the molecular mechanisms behind these processes.

MATERIALS AND METHODS

The m^7G-cap analogue was purchased from New England Biolabs (UK). m_3GpppG was synthesized as described elsewhere.[34,35] Radio-labeled nucleotide triphosphates were obtained from Amersham Pharmacia Biotech (Spain). The RNAse inhibitor was obtained from Invitrogen (Karlsruhe, Germany) and the SP6 polymerase used for the pHU1 transcription was from Boehringer Mannheim (Mannheim, Germany). Recombinant Snurportin 1 was a kind gift from Dr. Martin Hetzer. All nuclear import procedures can also be found in Dingwall and Palacios.[36]

Recombinant Snurportin 1 Expression and Purification

Snurportin 1 was cloned into the BamHI-XmaI sites of pQE30 (Qiagen, Hilden, Germany), expressed with an NH_2-terminal His tag and purified on nickel-NTA agarose (Qiagen, Hilden, Germany) followed by dialysis in 20 mM Hepes-KOH, pH 7.5, 200 mM NaCl, 2 mM magnesium acetate, and 250 mM sucrose.

TMG Nucleoside Synthesis

TMG nucleoside synthesis was performed as follows: the TMG nucleoside precursor was $m^{2,2}$G, which was synthesized as described previously.[27] The synthesis of TMG nucleoside used in the cross-linking assays was performed essentially as described previously.[24]

UV-Cross-Linking Assay

The following technique was created based on previous citations.[1,22] pHU1A[37] was transcribed with m_3G and m^7G caps in a transcription assay where α^{32}UTP (and no cold UTP) was used. The transcripts were purified through centri spin 10 (Princenton) columns equilibrated with

Roeder 200 mM KCl (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 200 mM KCl, 500 mM DTT, 500 mM PMSF, 5% glycerol) buffer. To visualize the binding between Snurportin 1 and m_3G by UV-cross-linking, 1 pmol of newly transcribed m_3GpppG-capped U1 snRNA (250–350 × 10^4 counts per min) was incubated for 15 min on ice with 1.5 μg of recombinant Snurportin 1 in a total volume of 10 μL. Reaction mixtures were irradiated at 254 nm with a Sylvania G8T5 germicidal UV lamp for 5 min at a distance of 2 cm and then placed on ice for 2 min. To each reaction, 1 μL RNAse A (3 mg/mL) was added and the samples were incubated for 30 min at 37°C. In competition assays, recombinant Snurportin 1 was mixed with 0.1 and 1 mM m_3G cap or TMG nucleoside on ice for 30 min prior to UV-irradiation. Cross-linked proteins were separated on 12.5 or 15% SDS-PAGE gels and visualized by autoradiography (Film KODAK BioMax MS in an intensify Kodak Biomax MS screen).

Preparation of Fluorescent Karyophiles

U snRNPs were purified from HeLa nuclear extracts by using an immunoaffinity column anti-trimethylguanosine (anti-m_3G) as described by Bach et al.[38] U snRNPs were concentrated to 1 mg/mL with Filtron 30K (Pall Filtron, Dreieich, Germany). In the last step of concentration, U snRNPs were resuspended in 100 mM borate buffer, pH 7.5. FLUOS (Boehringer Mannheim, Mannheim, Germany) was prepared fresh in DMSO (dimethylsulfoxide) and added to U snRNPs in 100-fold molar excess. The reaction proceeded for 45 min at room temperature with rotation in the darkness and was stopped by adding Tris to 10 mM, pH 7.5. The labeled U snRNPs were separated from the free dye by loading the sample in a Bioget p60 (BioRad Laboratories, Richmond, CA) column previously equilibrated with 1 X transport buffer-TB-(20 mM Hepes-KOH, pH 7.3, 110 mM KOAc, 5 mM NaAc, 2 mM MgAc, 1.0 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 1 μg/mL aprotinin; 1 μg/mL pepstatin A, 1 μg/mL leupeptin, 5% v/v sucrose). Fluorescent U snRNPs were repurified again in an anti-m_3G column and eluted using 15 mM 7-methylguanosine in TB.

BSA was cross-linked to a non-functional peptide with the reverse NLS sequence (SLN) (15–20 peptides per molecule) as described for BSA-NLS.[39] Conjugation to the carrier protein was obtained through the N-terminal cysteine residue and the glycine residues serve as a flexible hinge or spacer to separate the peptide from the protein itself. BSA-NLS conjugate was also labeled with FLUOS following the manufacturer's instructions.

Preparation of Permeabilized Cells

The cells were cultivated in a Dulbecco's modified Eagle's medium (Gibco), supplemented with 10% fetal calf serum and penicillin/

streptomycin (Gibco-BRL) at 37°C, 5% CO_2, and transferred to 10 plastic flasks (125 m^2). They were grown to approximately 70% confluence and harvested using 5 mL of a non-enzymatic cell dissociation solution (Sigma) for 10 min at 37°C. The cells were detached from the walls and then transferred to a 50-mL Falcon tube. The subsequent steps were carried out on ice. The cells were washed 3 times with ice-cold permeabilization buffer-PB (50 mM Hepes-KOH, pH 7.3, 50 mM KOAc, 8 mM $MgCl_2$, 2 mM EGTA) and resuspended to a cell density of 1–5 × 10^6 cells/mL in ice-cold PB. Digitonin (Fluka, Taufkirchen, Germany) was then added to 50 μg/ml and the cells were left on ice for exactly 5 min. Afterwards, the cells were washed three times with PB and finally resuspended at a density of 5–10 × 10^6 cells/mL in TB that contained 5% DMSO. The cells were then aliquoted into cold Eppendorf tubes and the tubes were frozen slowly in a Styrofoam box for 1 h at −20°C and then stored at −80°C.

Nuclear Import *In Vitro* Assay

Both U snRNPs and BSA-SLN *in vitro* import reactions have always contained 1 μL of a 5 mg/mL nucleoplasmin core (NPc), obtained as described elsewhere,[40] 1 μL of a 10 × energy-regenerating system (the final concentration in the assay must be 0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 50 μg/mL creatine phosphokinase), 1 μL of digitonin-permeabilized cells (1 × 10^4 cells), TB 1 X (from 5 mL transport buffer plus 5 μL DTT 1 M, 50 μL Trasylol 500000 KIE Bayer, 5 μL pepstatin 1 mg/mL, 5 μL PMSF 1 M, 1 μL leupeptin 5 mg/mL, 250 mM sucrose) with or without TMG nucleoside m_3G or m^7G caps.

BSA-SLN import was performed in a final volume of 10 μL always containing 2 μL of *Xenopus* egg extract[41] previously spun at 13,000 rpm for 10 min and 1 μL of a 1/10 dilution of BSA-SLN in TB (at a final concentration of 70 μg/mL). First, the BSA-SLN was premixed with the *Xenopus* extract and the nucleoside or nucleotides and incubated on ice for 5 min. Then, a mixture was prepared with the cells and the NPc and then added to the first premix. To start the import reaction, the energy mix was added and the reaction proceeded for 10 min, at room temperature. For U snRNPs import, each reaction was performed in 15–17 μL and also contained 6 μL of importin α–depleted *Xenopus* extract (described below), 0.5 μL of tRNA (to a final concentration of 0.2 mg/mL), and 5 μL of fluorescent U snRNPs (to a final concentration of 20 μg/mL). Initially, U snRNPs were premixed with *Xenopus* extract and then the nucleoside or nucleotides and the mixture were left on ice for 15 min. Then a mixture was prepared with tRNA, NPc, and digitonin-permeabilized cells and added to the Eppendorfs containing the first premix. In order to start the reaction, the energy mix was added and the reaction was proceeded for 60–90 min at room temperature, in the dark.

Both reactions were stopped by fixing with 200 μL of 8% (w/v) paraformaldehyde on ice together with 200 μL DAPI dye (330 ng/μL) in TB. The fixed reaction was layered over 700 μL of 30% sucrose in a tube containing a poly(lysine)-coated coverslip (MW 30,000–70,000) at 1 mg/mL in water. After centrifugation (3,000 rpm, 15 min) the coverslip was recovered, washed with PBS, and mounted on top of a drop of Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and viewed directly. Images acquisition and the quantification of nuclear fluorescence were performed as described previously.[36,39]

Xenopus Extracts

The *Xenopus* extract[41] used in U snRNPs import was importin α depleted by passing through an immunoaffinity column with an antibody against an N-terminal peptide from *Xenopus* importin α as described previously.[23]

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